A Global Metabolic Perspective on Aluminum Toxicity in Human Astrocytes: Implications for Neurological Disorders

by

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Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Biomolecular Sciences

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Abstract:

Aluminum (Al) has been associated with a variety of neurological disorders such as senile plaques, Alzheimer's disease, Parkinson's disease, and encephalopathies. The molecular mechanisms that facilitate Al to be involved in these diseases have yet to be fully unravelled. Using astrocytes as a model cerebral cellular system, we have identified biochemical modules that are altered by Al toxicity. The tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) were found to be severely impeded as a consequence of Al toxicity. The inability of the cell to generate ATP in an oxidative fashion led to a dearth of energy available for cellular processes. Consequently, Al-stressed astrocytes were unable to form a filamentous actin cytoskeleton. Loss of the actin cytoskeleton due to Al challenge resulted in abnormal "globular" cellular morphology. Similar observations were reported in astrocytes subjected to hydrogen peroxide (H₂O₂), a natural reactive oxygen species (ROS).

Investigation into the effects of Al and ROS on mitochondrial metabolism, helped identify a mitochondrial isoform of lactate dehydrogenase (LDH), a discovery with major implications on the brain energy budget. This LDH, under non-stressed conditions, was found to contribute to oxidative energy production. However, under Al and ROS insult it took a more prominent role as an attenuator of ROS stress through the amplified generation of the antioxidant pyruvate. The reaction of pyruvate with ROS lead to a build-up of acetate, which was fixed into lipid stores within Al and ROS-treated astrocytes. In addition, the Al and ROS-induced perturbation of the mitochondria rendered the astrocytes incapable of β-oxidation.
of fatty acids. This phenomenon was exacerbated by a diminished capability of the Al and ROS-stressed astrocytes to produce L-carnitine. The loss in L-carnitine production promoted the rerouting of the antioxidant α-ketoglutarate (KG) toward ROS sequestration in the Al and ROS-treated astrocytes. In fact, these astrocytes became oil-generating moieties under the influence of the Al and ROS-toxicity.

The reduced capacity of astrocytes to produce energy, the loss of the actin-cytoskeletal network, the shift in metabolic networks, and the accumulation of lipids are all factors that contribute to astrocyte dysfunction. Since astrocytes are integral to the optimal functioning of the brain, these biological events may be contributing factors linking Al to neurological disorders. Hence, this global metabolic view on Al toxicity that is fuelled by a disruption of iron (Fe) homeostasis and the promotion of an oxidative environment, provides intriguing molecular insights on how this trivalent metal may contribute to neurological diseases and reveals some potential therapeutic cues on mitigating these abnormalities.
Acknowledgements:

I would like to extend my gratitude and deepest thanks to a great number of people who made my PhD endeavour possible. First and foremost my supervisor and mentor Dr. Appanna. His words of wisdom and his philosophies on science and life have been and will be prized assets in my life. The opportunity to do science with Dr. Appanna was a once in a lifetime chance that I will forever cherish.

I would also like to thank Dr. Ryan Mailloux. Everything I’ve accomplished can be linked to his guidance or inspiration. Ryan, along with Dr. Appanna opened my mind to a unique way of thinking about science. I am grateful not only for his help with my PhD, but for having the opportunity to know him.

Many thanks to my supervisory committee Dr. Siemann and Dr. Omri. Their input and ideas helped shape and improve my thesis research. I feel privileged to have been a student of theirs and thoroughly enjoyed every opportunity I had to learn from them. I would also like to give my personal thanks to Dr. Bazinet (Department of Nutritional Sciences, University of Toronto) for his insight and help to make this thesis come to fruition.

I would also like to acknowledge the humbling experience of my interactions with Dr. Chris Exley (Keele University) and Dr. Walter Lukiw (Louisiana State University) at both the 8th (Prague, Czech Republic) and 9th (Niagara Falls, Canada) Keele meetings on Aluminum I attended. It is amazing to see people who are so devoted to their science and it is also inspiring to have your work accepted by experts in your scope of research.
Past and present members of the Appanna research group, I would like to thank for the many-many good times that have been had. They have made the most difficult days easier to bear with their continual inspiration and levity. My family and friends, you do not know how important you have been to me not only through my thesis, but also in my life. They've helped me through many difficult times and I will not forget that. Thank you for being patient, understanding, and supportive throughout my studies.

Lastly, I would like to thank Johanna Delongchamp for all her help over the years as a confidant and a board to which I could bounce any and all ideas off of with complete support and free of ridicule. Her wisdom and intelligence has helped guide me through these last 5 years and I feel lucky to have her around.
Contributing Publications:


Table of Contents:

Page #

Abstract.......................................................................................................................III
Acknowledgements.................................................................................................V

Section 1: Introduction..............................................................................................1

1.1 - Cellular Physiology and Biochemistry of the Brain...........................................1
   1.1.1 - Structural Role of Astrocytes.................................................................2
   1.1.2 - Astrocytes in Brain Energy Dynamics....................................................3
   1.1.3 - Astrocytes and Neurotransmitter Clearance.........................................6
   1.1.4 - Astrocytes, Lipids, and Cholesterol........................................................8

1.2 - Aspects of Astrocyte Biochemistry.................................................................10
   1.2.1 - The Cytoskeleton of Astrocytes............................................................10
   1.2.2 - Astrocytes and Energy Production.......................................................12
   1.2.3 - Lipid Metabolism and Astrocytes.........................................................23
   1.2.4 - Metabolism in the Nucleus..................................................................31

1.3 - Brain Metabolism and Disease.......................................................................31

1.4 - Environmental Pollutants and Neurological Disorders.................................32

1.5 - The Blood-Brain Barrier and Protection from Xenobiotics............................32

1.6 - Metal Homeostasis and Toxicity in the Brain...............................................33

1.7 - The Generation and Effects of ROS...............................................................36

1.8 - Antioxidative Defense Systems...................................................................42

1.9 - Aluminum, a Suspected Neurotoxin...............................................................45

Thesis Objectives.....................................................................................................51

Section 2: Materials and Methodology....................................................................53

2.1 - Reagent List..................................................................................................53

2.2 - Equipment List.............................................................................................55

2.3 - Culturing of Astrocytes and Hepatocytes.....................................................57
   2.3.1 - Astrocytes (CCF-STTG1).....................................................................57
   2.3.2 - Hepatocytes (HepG2)..........................................................................57
   2.3.3 - Culture Initiation and Passaging.............................................................57

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
2.3.4 - Cell Storage Procedure .................................................. 59

2.4 - Cell treatment and Viability ............................................... 60

2.5 - Metabolite Supplementation and Recovery Experiments ........ 61

2.6 - Fluorescence Microscopic Techniques ................................ 62
  2.6.1 - Intracellular Aluminum and the Morin Stain .................. 62
  2.6.2 - Detection of Intracellular ROS .................................. 63
  2.6.3 - Visualization of the Cytoskeleton of CCF-STTG1 cells ..... 64
  2.6.4 - Immunofluorescence Studies to Localize and Quantify Intracellular Proteins ................................................. 65
  2.6.5 - Detection of Intracellular Lipid Droplets ..................... 67
  2.6.6 - Visualization of Glycogen Granules ............................ 68

2.7 - Cellular Isolation and Subcellular Fractionation .................. 68

2.8 - Measuring Oxidized Products in Al and ROS Treated Cells ....... 71
  2.8.1 - Oxidized Proteins ................................................. 71
  2.8.2 - Oxidized Lipids .................................................. 72

2.9 - HPLC Studies .................................................................... 73
  2.9.1 - Spent Fluid Analysis .............................................. 73
  2.9.2 - HPLC Analysis of the CFE, Mitochondria, Nucleus, and Cytoplasm ................................................................. 74
  2.9.3 - Mitochondrial Reactions .......................................... 75
  2.9.4 - Special HPLC Procedures for L-Carnitine and Palmitate .. 76

2.10 - 13C-NMR Analysis of Lactate Consumption .......................... 77

2.11 - Native Polyacrylamide Gel Electrophoresis (PAGE) Studies .... 78
  2.11.1 - Preparing BN-PAGE Gels ...................................... 78
  2.11.2 - Native PAGE buffers ........................................... 80
  2.11.3 - In-gel Activity Staining ......................................... 80
  2.11.4 - NAD(P)H-generating Enzymes (LDH, NAD(P)-ICDH, KGDH, ME, and MDH) ................................................. 82

  2.11.5 - FAD-reducing Enzymes (SDH) ................................ 85
  2.11.6 - Cytochrome C Oxidase Activity ............................... 86
  2.11.7 - ATP-generating Reactions (Enzyme-linked Assays) ...... 86
  2.11.8 - PDH and BN-Agarose Gel Electrophoresis .................. 88
  2.11.9 - Lipogenic Enzymes (CL, ACS, and ACC) .................... 88
  2.11.10 - Enzymes Involved in L-Carnitine Biosynthesis (BBADH and BBDOX) ...................................................... 90
  2.11.11 - Enzymes Involved in Glutamate Homeostasis (GDH, GluS, and GlnS) .................................................. 91
2.12 - Oxygen Consumption Assays.................................92

2.13 - SDS-PAGE and Immunoblotting..........................92
  2.13.1 - SDS-PAGE..............................................92
  2.13.2 - SDS PAGE Buffers....................................93
  2.13.3 - Two-dimensional (2D) SDS-PAGE.....................94
  2.13.4 - Immunoblotting Techniques..........................94
  2.13.5 - Immunoblot Detection Methods.......................97
  2.13.6 - Immunoblotting Buffers..............................98

2.14 - In-cell Immunoblotting....................................98

2.15 - Quantitative PCR for LDHA-like 6B.....................99

2.16- Statistical Analysis......................................99

Section 3: Results......................................................100

  3.1 - Preliminary Observations................................100
  3.2 - Al and Oxidant Induced Loss of Cytoskeletal Architecture..108
  3.3 - Astrocytes and the Lactate Conundrum.....................126
  3.4 - The Involvement of mLDH in Al and ROS Stress Attenuation..137
  3.5 - Disruption of L-carnitine Metabolism and Lipid Accumulation...151
  3.6 - The Impact of Al and ROS on Other Functions of Astrocytes...162
  3.7 - Nuclear Metabolism under Al and ROS Stress...............166

Section 4: Discussion..................................................168

  4.1 - Al Induces ROS and Altered Astrocytic Metabolism...........170
  4.2 - Al and Oxidant Induced Loss of Cytoskeletal Architecture....173
  4.3 - Astrocytes and the Lactate Conundrum......................179
  4.4 - The Involvement of mLDH in Al and ROS Stress Attenuation....184
  4.5 - Disruption of L-carnitine Metabolism and Lipid Accumulation.193
  4.6 - The Impact of Al and ROS on Other Functions of Astrocytes...199
  4.7 - Nuclear Metabolism in Astrocytes..........................203

Section 5: Conclusion....................................................206

Section 6: Literature Cited..........................................209

Section 7: Appendix.....................................................228
List of Figures:

**Section 1: Introduction**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Scaffolding in the brain</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Metabolic support of neurons by astrocytes</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>The glutamate-glutamine cycle</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Actin cytoskeleton dynamics</td>
<td>10</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Glycogen metabolism in astrocytes</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Conversion of glucose into lactate</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Lactate oxidation by the neural mitochondria</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Ketone body metabolism in astrocytes</td>
<td>19</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Acetate metabolism in astrocytes</td>
<td>21</td>
</tr>
<tr>
<td>Figure 1.10</td>
<td>Cholesterol synthesis in astrocytes</td>
<td>25</td>
</tr>
<tr>
<td>Figure 1.11</td>
<td>Fatty acid synthesis in astrocytes</td>
<td>26</td>
</tr>
<tr>
<td>Figure 1.12</td>
<td>L-Carnitine biosynthesis</td>
<td>28</td>
</tr>
<tr>
<td>Figure 1.13</td>
<td>Fatty acid transport for β-oxidation</td>
<td>29</td>
</tr>
<tr>
<td>Figure 1.14</td>
<td>The generation of free radicals from molecular oxygen</td>
<td>37</td>
</tr>
<tr>
<td>Figure 1.15</td>
<td>ROS production from the ETC</td>
<td>38</td>
</tr>
<tr>
<td>Figure 1.16</td>
<td>Redox active metals catalyze ROS production</td>
<td>39</td>
</tr>
<tr>
<td>Figure 1.17</td>
<td>Lipid Peroxidation by ROS</td>
<td>40</td>
</tr>
<tr>
<td>Figure 1.18</td>
<td>The ROS defense mechanisms elaborated by biological systems</td>
<td>45</td>
</tr>
<tr>
<td>Figure 1.19</td>
<td>The sources and effects of Al in biological systems</td>
<td>47</td>
</tr>
<tr>
<td>Figure 1.20</td>
<td>Al inhibits oxidative energy production</td>
<td>48</td>
</tr>
<tr>
<td>Figure 1.21</td>
<td>Summary of results from Mailloux <em>et al</em> in Al-treated HepG2 cells</td>
<td>49</td>
</tr>
</tbody>
</table>
Section 2: Materials and Methodology

Figure 2.1 – Synopsis of cell culturing.................................................................59
Figure 2.2 – Overview of cell treatments and recovery experiments.................61
Figure 2.3 – A diagram of the preparation for immunofluorescence microscopy...67
Figure 2.4 – Cellular and Sub-cellular isolation procedure...............................70
Figure 2.5 – The in-gel detection of NAD(P) dependent enzymes.....................83
Figure 2.6 - The in-gel detection of FAD-dependent enzymes............................85
Figure 2.7 – Enzyme linked in-gel detection of ATP producing enzymes.............87
Figure 2.8 – Enzyme linked in-gel detection of ATP-CL....................................89
Figure 2.9 – Overview of the immunoblotting procedure....................................95

Section 3: Results

Figure 3.1 – Growth profile of astrocytes treated with 0.1mM Al over a 24h period.........................................................................................................................101
Figure 3.2 – Viable cell growth of astroglial cells treated with H₂O₂ for varying time periods.................................................................................................................102
Figure 3.3 – Morin stain for Al in astroglioma cells...........................................103
Figure 3.4 – Al and H₂O₂ induced ROS...............................................................104
Figure 3.5 – Metabolite profile of the spent fluid from astrocytes.....................106
Figure 3.6 – Protein profile of the spent fluid from astrocytes............................107
Figure 3.7 – Phase contrast microscopic studies of human astrocytoma cells.......108
Figure 3.8 – Fluorescence microscopic studies of human astrocytoma cells.........110
Figure 3.9 - Microscopic studies of human astrocytoma cells................................111
Figure 3.10 - Energy status of stressed human astrocytoma cells.......................112
Figure 3.11 – Mitochondrial membrane potential in astrocytes.........................112
Figure 3.12 – Oxygen consumption assay of astroglial mitochondria.........................115
Figure 3.13 – Activity and expression of CK in astrocytoma cells under Al insult.116
Figure 3.14 - CK and the actin cytoskeleton in astrocytes...........................................118
Figure 3.15 - Analysis of profilin-2 levels.................................................................119
Figure 3.16 - Al-induced cytoskeletal dysregulation and profilin-2 expression........120
Figure 3.17 - Al-evoked ROS in the astrocytoma cell line........................................121
Figure 3.18 - Anti-oxidant treatment and the recovery of cytoskeletal architecture and energy status in human astrocytes (CCF-STTG1) .........................123
Figure 3.19 - A molecular link between Al toxicity and morphological perturbation in human astrocytoma cells.................................................................125
Figure 3.20 - Lactate utilization by an astrocytic cell line...........................................126
Figure 3.21 - Lactate consumption by mitochondria derived from this astrocytic cell line....................................................................................................................127
Figure 3.22 - Lactate as a source of mitochondrial energy in an astrocytic cell line .....128
Figure 3.23 - Oxidative metabolism of lactate in astrocytic mitochondria.............130
Figure 3.24 - Lactate promotes aerobic respiration in astrocytic mitochondria....131
Figure 3.25 - Mitochondrial lactate metabolism..........................................................132
Figure 3.26 - BN PAGE analyses of LDH in an astrocytic cell line.........................133
Figure 3.27 - 2D immunoblot analysis of LDH.............................................................134
Figure 3.28 - Lactate dehydrogenase localization in an astrocytic cell line..........135
Figure 3.29 - Localization of LDH in the mitochondria............................................136
Figure 3.30 - The discovery of a mitochondrial LDH................................................137
Figure 3.31 – Monocarboxylate profile of astrocytic spent fluid............................138
Figure 3.32 - Monocarboxylate levels in the CFE.......................................................138
Section 4: Discussion

Figure 4.1 – A global view of the effects of Al on astrocyte physiology.................. 169
Figure 4.2 – Cellular dysfunction under Al and ROS stress................................. 172
Figure 4.3 – Al and ROS induced interference with actin polymerization............. 178
Figure 4.4 – The versatile role of a mitochondrial LDH in human astrocytes....... 182
Figure 4.5 – Mitochondrial LDH is involved in attenuating ROS and lipid production............................................................................................... 193
Figure 4.6 – The effects of Al and ROs on L-carnitine metabolism.................... 198
Figure 4.7 – Glu is shifted toward KG production and not neurotransmitter recycling.................................................................................................. 201
Figure 4.8 – A hypothetical metabolic module in the nucleus of astrocytes........ 204

Section 5: Conclusion

Figure 5.1 – A global perspective on Al-toxicity in Astrocytes: Implications for neurological disorders............................................................. 208

Section 7: Appendix

Appendix 7.1 – The glycolytic pathway................................................................. 228
Appendix 7.2 - The tricarboxylic acid cycle.................................................... 229
Appendix 7.3 – The electron transport chain................................................. 230
Appendix 7.4 – HPLC chromatograms for common metabolites.................. 231
Appendix 7.5 – cDNA Sequence for LDHA-like 6B........................................ 232
Appendix 7.6 - Viable cell count of CCF-STTG1 cells................................. 233
Appendix 7.7 - Non-viable cell count for CCF-STTG1 cells......................... 234
Appendix 7.8 – Caspase 3 cleavage in astrocytes.......................................... 235
Appendix 7.9 – In-gel activity stain for SDH.................................................... 236
Appendix 7.10 - Activity of LDH in HepG2 cytoplasm and mitochondria
Appendix 7.12 - Activity of PDH in HepG2 mitochondria
Appendix 7.13 - Fluorescence microscopy of triglyceride levels in HepG2 cells
Appendix 7.14 - KG and Succinate levels in HepG2 cells
Appendix 7.15 - Carnitine homeostasis in HepG2 cells
Appendix 7.16 - Expression levels of BDFOX in HepG2 cells
Appendix 7.17 - Palmitate metabolism in Al treated HepG2 cells
List of Tables:

Section 2: Materials and Methodology

Table 2.1 – Antibody incubation for immunofluorescence microscopy..........................55
Table 2.2 – List of reagents required to pour a 4-16% linear gradient BN gel.................78
Table 2.3 – Brief overview of enzymes measured using BN-PAGE technique.............82
Table 2.4 – A List of the Antibodies Employed for Immunoblot Analysis.................89

Section 3: Results

Table 3.1 – Oxidized lipids and proteins profile in CCF-STTG1 cells.........................105
Table 3.2 – relative transcript levels of LDHA like 6B (a mitochondrial isoenzyme of LDH) ...........................................................................................................................................142
Table 3.3 – Lactate consumption and monocarboxylate production in astrocytic mitochondria..................................................................................................................143
Table 3.4 - In-gel activity analysis for lipogenic enzymes.............................................161
Table 3.5 – Metabolite levels of Al and ROS treated astrocyte nucleus.......................166

Section 7: Appendix

Appendix 7.11 – Lactate consumption and monocarboxylate production in HepG2 mitochondria.................................................................238
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu M )</td>
<td>Micromolar</td>
</tr>
<tr>
<td>( \mu L )</td>
<td>Microlitre</td>
</tr>
<tr>
<td>( \mu g )</td>
<td>Microgram</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>ACN</td>
<td>Aconitase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acetyl-CoA synthase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminum (III)</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance Units</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BBADH</td>
<td>( \gamma )-Butyrobetaine Aldehyde Dehydrogenase</td>
</tr>
<tr>
<td>BBDOX</td>
<td>Butyrobetaine Dioxygenase</td>
</tr>
<tr>
<td>BN</td>
<td>Blue Native</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>Blue Native Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeters</td>
</tr>
<tr>
<td>CFE</td>
<td>Cell Free Extract</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
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<td>Cl</td>
<td>Citrate Lyase</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeters</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>Cr</td>
<td>Creatine</td>
</tr>
<tr>
<td>CSB</td>
<td>Cell Storage Buffer</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine III</td>
</tr>
<tr>
<td>Cyt C Ox</td>
<td>Cytochrome C Oxidase (complex IV)</td>
</tr>
<tr>
<td>DCFDA</td>
<td>Dichlorodihydrofluorescein Diacetate</td>
</tr>
<tr>
<td>DCIP</td>
<td>Dichloroindophenol</td>
</tr>
<tr>
<td>ddH2O</td>
<td>deionized and distilled water</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenyl Hydrazine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide (oxidized)</td>
</tr>
<tr>
<td>FADH2</td>
<td>Flavin Adenine Dinucleotide (reduced)</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FUM</td>
<td>Fumarase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate Dehydrogenase</td>
</tr>
</tbody>
</table>
PMS.................................Phenazine Methosulfate
PTB.................................Protein Transfer Buffer
qPCR...............................quantitative Polymerase Chain Reaction
ROS.................................Reactive Oxygen Species
sec.................................Seconds
SDH.................................Succinate Dehydrogenase
SDS-PAGE..........................Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD.................................Superoxide dismutase
TBARS..............................Thiobarbituric Acid Reactive Species
TBEA.................................Trypan Blue Exclusion Assay
TBS.................................Tris-Buffered Saline
TCA.................................Tricarboxylic Acid
TTBS.................................Tween-20 Tris-Buffered Saline
TEMED..............................N,N,N,N-tetramethylethylenediamine
v/v..................................volume/volume
VDAC...............................Voltage-Dependent Anion Channel (mitochondrial marker)
VitC.................................Vitamin C (ascorbic acid)
w/v..................................weight/volume
Section 1: Introduction

1.1 - Cellular Physiology and Biochemistry of the Brain

The brain is the central processing unit of the human body, but it is the least understood of all the organ systems. Its complexity stems from having multiple functional subsystems which include sensory functions, motor functions, and regulatory systems (1). This is an oversimplification, as these systems can be further divided and are spread regionally throughout the brain. Indeed, one could spend a lifetime writing about the CNS. Interestingly, the brain only accounts for 2% of the body’s mass, however, it consumes 18-20% of its energy reserves (1, 2). Another tenant about the brain is that neural tissues are very oxidative like the muscles, heart, and liver (1). In addition to consuming a great deal of the body’s energy resources, the brain consumes 20-50% of the available arterial blood O₂, depending on activity and demand (3, 4). The neuronal processes that utilize all these energy resources include neurotransmitter production/recycling, vesicular trafficking, and maintaining the ion gradient of the neuron for neurotransmission (1, 5).

One aspect of the brain that makes it interesting from a cellular standpoint is the heavy reliance of the functional unit of the brain (the neurons), on supportive tissues (glial cells). Neuroglia consists of microglia, oligodendrocytes, ependymal cells, NG2 cells, and astrocytes. Microglia act in the brain as resident macrophages, playing an immunity role (1). Oligodendrocytes serve to form myelin to sheath and insulate neurons (5). Ependymal cells line the cavities of the CNS and form cerebral spinal fluid, which they then circulate with their cilia (1). NG2 cells are the most
recently discovered cell type in the brain and are termed NG2 due to the proteoglycan which is present on their cell surface (6). NG2 cells are multipotent and appear to have the ability to differentiate into oligodendrocytes, astrocytes, and even neurons (7). Astrocytes serve more of a “star” role in the brain, where they physically and chemically interact with the neurons (8).

1.1.1 – Structural Role of Astrocytes:

Astrocytes outnumber neurons in the CNS by a factor of 10:1 (8). Astrocytes form the scaffolding of the brain by interacting both with the vascular endothelia (the blood supply) and with the neurons (9). This interaction occurs through their astrocytic end-feet or filopodia. The filopodia also act to insulate the synaptic cleft (space between the post-synaptic and pre-synaptic neuron) by wrapping around it and isolating it (10) (Figure 1.1).

**Figure 1.1 – Scaffolding in the brain.** Astrocytes form the structure of the brain by interacting with numerous neurons and the cerebral blood supply using their astrocytic filopodia. Astrocytes also use their filopodia to insulate the synaptic cleft.
The scaffolding effect of the astrocytes serves to ensure that neurotransmission goes unabated at the synaptic cleft, by isolating it from any exogenous chemicals. The interaction of the astrocytic filopodia with the vascular endothelia guarantees that much needed nutrients are brought into the brain and unwanted chemicals are kept out. Thus, astrocytes are a key component of the BBB.

1.1.2  – Astrocytes in Brain Energy Dynamics:

D-glucose was once thought to be the main energy source of the brain. Although, the brain does utilize a great deal of glucose, under circumstances of increased energy requirements (intense neurotransmission) the neurons can supplement their diet with other carbon sources such as lactate, acetate, and ketone bodies (2, 5, 11, 12). In addition, the brain keeps a store of glycogen within the astrocytes that can be mobilized when nutrients are in demand or during hypoglycemia (13). All these alternative energy sources give the brain options to support cellular functions and neurotransmission (Figure 1.2).
Figure 1.2 – Metabolic support of neurons by astrocytes. The carbon sources for the brain enter through the blood supply. These carbon sources are glucose, lactate, acetate, and ketone bodies (acetoacetate and β-hydroxybutyrate). Glucose and lactate are taken up directly by astrocytes and neurons. Glucose undergoes glycolysis to yield pyruvate. Glucose in astrocytes can be stored as glycogen to be used during times of intensified energy demand. Pyruvate is used to make ATP through the TCA cycle. In astrocytes, pyruvate is redirected to lactate and given to the neurons during low energy status, through the astrocyte-neuronal lactate shuttle (ANLS). The astrocyte derived lactate is shunted toward pyruvate to support ATP production. Ketone bodies and acetate are taken up by the astrocytes and fixed into acetyl-CoA, which can enter the TCA to produce energy or yield pyruvate. This pyruvate is used to make lactate that may be donated to the neurons via the ANLS or stored as glycogen if nutrients are plentiful.

The energy demands of neurons must be met to ensure appropriate brain functioning. Glucose utilization cannot solely support neurons under intense energy demand, especially during hypoglycemia (14, 15). To protect the vital functions of neurons, the brain has adapted to utilize other mechanisms. When glucose is
plentiful, astrocytes store glucose as glycogen (5). When the nutrient demand from the neurons increases, stored glucose can be broken down to pyruvate (Appendix 7.1 for an overview of glycolysis). This pyruvate is metabolized to lactate using LDH and NAD. Lactate is subsequently diverted to the neurons using the ANLS (16). The donated lactate from the astrocytes and blood-derived lactate can then be used by the neurons to make pyruvate with the neuronal LDH, while yielding a molecule of NADH. The pyruvate and NADH can then be used to support ATP production by the neuron (5). This is the benefit provided by shuttling lactate from the astrocytes to the neurons as opposed to shuttling pyruvate. Shuttling lactate provides extra NADH to the neurons. In addition, pooling lactate in the astrocytes as opposed to pyruvate promotes the continued production of pyruvate and keeps NAD/NADH ratios in favour of pathways encouraging NADH production (ie. Pyruvate + NADH → lactate + NAD). The differential expression of LDH isoenzymes supports this idea (17). LDH5, favours the conversion of pyruvate to lactate, is expressed in higher amounts in astrocytes, while LDH1 favours the conversion of lactate to pyruvate, and is expressed in greater amount in neurons (18).

Acetate and ketone bodies, are likewise taken up by the astrocytes and diverted to acetyl-CoA production (2). The acetyl-CoA generated enters the TCA cycle to contribute to energy production and eventually toward pyruvate production (Appendix 7.1 and 7.2 for an overview of the TCA and ETC, respectively). The ketone body/acetate derived-pyruvate is utilized to supply the ANLS as described earlier and contribute to neuronal energy production. If energy is abundant, it can be diverted to astrocyte glycogen production.
1.1.3 - Astrocytes and Neurotransmitter Clearance:

One of the most important roles of astrocytes is neurotransmitter clearance and replenishment. 90% of neurons in the brain use Glu as their neurotransmitter, and 80-90% of the synapses in the brain are glutamatergic [19]. Astrocytes, couple tightly with neurons and Glu release by removing excess Glu from the synaptic cleft following neurotransmission [20]. Astrocytes then recycle the Glu back to the neuron as Gln, a process termed the Glu/Gln cycle [21] (Figure 1.3).

Figure 1.3 - The glutamate-glutamine cycle. Glu is vesicularized into synaptic vesicles for neurotransmission. Following the signal from an action potential in the presynaptic neuron, glutamate is released into the synaptic cleft. The signal is then propagated to the postsynaptic neuron through metabotropic and ionotropic glutamate receptors. The signal requires relief so another round of neurotransmission can take place. Glu is removed from the synaptic cleft by the pre/postsynaptic neurons and the astrocytes. Glu pools are replenished by both the presynaptic neuron and the astrocytes. Glu in the astrocytes is recycled into Gln by GlnS (Glu + ATP + NH₃ → Gln + ADP + P_i) and GluS (2Glu + NADP → Gln + KG + NADPH). GDH (KG + NAD(P)H → Glu + H₂O) helps by contributing fresh Glu for the
aforementioned reactions, requiring an input of KG from the TCA. Gln is subsequently shuttled to the presynaptic neuron to replenish Glu pools. Upon the arrival of the Gln, GluS (Gln + KG + NADPH \rightarrow 2Glu + NADP) metabolizes it to Glu. Glu is also pooled with the help of GDH by taking KG from the TCA. This Glu is vesicularized for another round of neurotransmission.

Glutamatergic activity is thought to account for 80% of the energy expenditure of the brain (22). This is a rational conclusion as there is a tremendous amount of nutrient diversion toward Glu production for neurotransmission and ATP production to support Na+/K+ ATPase for polarization of the neuron. Nutrients from both the astrocyte and the neuron must be diverted from the TCA cycle to supplement Glu pools through KG. Thus, as aforementioned, other carbon sources must be considered to fuel highly active metabolic pathways. Since Glu concentrations in synaptic vesicles can reach anywhere from 60-250mM (5), the "normally" active TCA cycle one might find in some other cells types is likely accelerated in order to support both neurotransmission functions as well as functions of cell maintenance. Indeed, a disruption in oxidative energy production within a neural cell could disrupt neurotransmission and therefore habitual brain functions. It is also logical to speculate that the production of ROS from this heightened oxidative metabolism would be augmented. It has been observed that neurons require augmented glutathione production to survive heightened ROS production through oxidative metabolism (23). Increased requirements for glutathione intrinsically implies a higher need for NADPH for its reduction, and Glu for its synthesis. Indeed, aging and neurological disorders have been associated with lowered levels of glutathione and/or the enzymes that produce it (24, 25).
Astrocytes also participate in the recycling of other neurotransmitters including adenosine, GABA (which also requires Gln for its synthesis), serine, glycine, acetylcholine, and ATP (5, 6, 26-28). All the preceding neurotransmitters have various functions, however they all require and input of energy and nutrients to release and recycle from both the astrocytes and the neurons. Therefore, it is not surprising that the energy requirements of the brain are extensive.

1.1.4 – Astrocytes, Lipids, and Cholesterol:

Lipid metabolism and trafficking in the brain is not very well understood (29). This is surprising because the brain is composed of a great deal of lipids, with white matter consisting of 50% of lipids in dry weight (5). The lipids are required in large amounts to form neural cells with extensive processes (dendrites, filopodia, and axons). In addition to the large cellular projections that need to be made, extensive myelination of axons is required for insulated neurotransmission (5). The burden of myelin synthesis is shouldered mainly by the oligodendrocytes (30). Where astrocytes play a major role in lipid metabolism in the brain, is with the synthesis of cholesterol, apolipoprotein E, and specialized fatty acids. The astrocytes appear to be the in-house cholesterol-producing units of the brain, supplying cholesterol that is essential for the stabilization of neuronal membranes and the development of the synapse (31, 32). Apolipoprotein E is a cholesterol and phospholipid carrier in the brain (31, 33). Apolipoprotein E is also almost exclusively formed within astrocytes (33, 34). Unfortunately, improper regulation and accumulation of apolipoprotein E has been associated with Abeta peptide
aggregation and Alzheimer's disease (34, 35). Specialized fatty acids such as docosahexaenoic acid (DHA) are synthesized within the astrocytes and delivered to the neurons (36). This n-ω3 polyunsaturated fatty acid has been shown to be important in proper brain development and cognitive function (37, 38). Oxidation of DHA and disruption in DHA homeostasis has been associated with cognitive impairment, aging, and neurological disorders (38-40). Again, astrocytes demonstrate their importance as metabolic couplers to neurons through lipid metabolism. Since neurons cannot make their own cholesterol they are dependent on astrocytic derived cholesterol. Specialized fatty acids, which are necessary for the proper functioning of the neuron, are also supplied by the astrocytes.

When exploring the physiology and biochemistry of the brain it is important to be cognizant that the neurons are not the only functional units involved. Astrocytes are another neural cell that have a dynamic functional role in the brain including providing structure, metabolic support, neurotransmitter clearance, cholesterol production, and essential fatty acid synthesis. These previously mentioned roles the astrocytes partake in are necessary for neurons to perform the job of neurotransmission. Without the tight coupling of astrocytes with neurons, appropriate brain functioning would be impossible. It is this dynamic role that astrocytes play in the brain that makes them a prime candidate for the experimentation of biochemical phenomena. Many studies on neurological disorders and neurotoxicology have typically been delineated in neurons. However, the unequivocal role the astrocytes play in neuronal functioning makes them a
target of opportunity for novel discoveries in neurochemistry, neurotoxicology, and neurological disorders.

1.2- Aspects of Astrocyte Biochemistry

Due to the unique requirements of astrocytes and their supportive role in the brain, a specialized biochemistry is required. The role of astrocytes in providing structural support in the brain and being a dynamic player in the BBB requires a highly functional cytoskeleton (41). Meeting the nutrient demands of neurons requires that astrocytes evolve a pliable carbon metabolism. The clearance of neurotransmitters and their recycling also necessitates a great deal of nutrient shunting toward neuronal needs, an attribute dependent on flexible metabolic networks. The demand for cholesterol, and specialized lipids in the brain, makes lipid metabolism an important function of astrocytes.

1.2.1- The Cytoskeleton of Astrocytes:

Astrocytes have a distinctive morphology and like neurons have a notable diversity in cell shapes (42). Just as other cells in the body, astrocyte morphology is closely connected to its function. The cytoskeleton plays a pivotal role in dictating the stellate morphology of the astrocytes, as well as being critical components of intracellular trafficking, and establishing metabolic compartments (43). The importance of the cytoskeleton in the brain can be observed by the distribution of cytoskeletal proteins. Cytoskeletal proteins comprise 1/3 of the total protein of the brain (5). Some of the main components involved in the astrocyte cytoskeleton are much the same as other cell types. The components are intermediary filaments and
actin filaments. However, astrocytes have a cell specific glial intermediary filament (GFAP), which differs from neuronal filaments but shares some sequence homology (5). The dynamics of the actin-based cytoskeleton are demonstrated in Figure 1.4.

**Figure 1.4 - Actin cytoskeleton dynamics.** Globular-actin (G-actin) bound to ADP is picked up by profilin, which increases the affinity of G-actin for ATP. When ADP is exchanged for ATP, profilin dissociates and the G-actin-ATP complex polymerizes to form a filamentous (F-actin). Upon the hydrolysis of ATP to ADP, the subunits of F-actin dissociate into their globular form once again.
The actin cytoskeleton is dynamic in that it is constantly being assembled and disassembled by polymerization and depolymerisation respectively of actin subunits. The key factor to this cellular process, like many other cellular processes, is the energy provided by ATP. Without ATP, the actin cytoskeleton cannot form a filamentous structure (44-46), a problem encountered in aging red blood cells (44). Profilin, or profilin-2 in the brain, aids in reloading ADP-actin monomers with ATP for their addition to the barbed ends of the actin filaments (47). Profilin is not required for actin polymerization however; actin polymerization significantly slows down in the absence of profilin (47, 48). Once ADP --> ATP exchange occurs, profilin dissociates and the ATP-actin complex is able to polymerize. ATP hydrolysis on the filamentous actin subunits leads to the subsequent depolymerisation of the actin filament. This cycle comprises the turnover of the cytoskeleton and it is intricately linked to cellular energy availability.

1.2.2 – Astrocytes and Energy Production:

Ensuring that neurons have a constant supply of energy is necessary to maintain proper brain functioning and is a burden shared with the astrocytes. Fortunately, astrocytes can elaborate many metabolic pathways and exploit an array of metabolites to support brain energy demand. To ensure that the neurons never go lacking energetically, the brain has been shown to store glycogen, utilize monocarboxylates (lactate and acetate), and ketone bodies (acetoacetate and β-hydroxybutyrate) as nutrient sources (5). The astrocytes have elaborated biochemical pathways to facilitate this requirement.
Glycogen metabolism

Astrocytes, like hepatocytes and myocytes have the ability to store glucose in the form of glycogen. When glucose is plentiful, glycogen stores are bolstered (49). However, during periods of intense neurotransmission or hypoglycemia, astrocyte glycogen stores are mobilized (50, 51). Glycogen dynamics in astrocytes are depicted in Figure 1.5.

Figure 1.5 - Glycogen metabolism in astrocytes. Glucose is imported into the astrocytes and is used to make pyruvate through glycolysis, or if nutrients are plentiful, the astrocytes fix "extra" glucose into glycogen. Glycogen synthesis is promoted by protein targeting to glycogen (PTG)/protein phosphatase - 1 (PP-1) complex, which dephosphorylates glycogen synthase (GS). Under a hypoglycemic state or when energy is required following neurotransmission, glycogen is mobilized to glucose. Glycogen breakdown is promoted in 2 ways: 1) GSK – 3 phosphorylates...
GS effectively inhibiting it. 2) Glutamate which is taken-up by the astrocytes following the neurotransmission of the neuron, activates adenylyl cyclase (AC) forming cAMP. The cAMP activates protein kinase A (PKA), which in turn phosphorylates glycogen phosphorylase (GP). Glycogen stores are then broken down into glucose (5, 49).

The fixing and mobilization of glycogen stores in astrocytes is a dynamic way in which the brain can ensure that it is not short on energy for neurotransmission. When glucose is not required, it can be stored as glycogen by promoting GS activity. This is accomplished by the promotion of PTG/PP-1 complex to dephosphorylate GS as well as the inhibition of GSK - 3 (which inhibits GS). When energy is not required, GSK - 3 is inhibited by phosphorylation, while PTG/PP-1 complex is active (49). Under hypoglycemic states, or following neurotransmission, nutrients are required (51). Thus, glycogen is mobilized to glucose to support the energy needs of the neurons. Glycogen breakdown is promoted in two ways 1) GSK - 3 is phosphorylated by PKA, which promotes its activity. GSK-3 has the duty of phosphorylating GS. Once GS is phosphorylated it no longer builds glycogen (49). 2) Glutamate uptake by the astrocyte following neuronal depolarization, promotes AC activity in a G-protein coupled receptor-mediated fashion. AC activity cyclizes ATP to cAMP which in turn activates PKA. PKA then phosphorylates GSK-3 (as described above) as well as phosphorylating GP, effectively activating it and promoting the breakdown of glycogen to glucose-1-phosphate (49).

Once the glycogen stores are broken down into glucose, the glucose is either supplemented as lactate to the needing-neurons, or directly as glucose. Whether it is the former, the latter, or both events that occur is still a matter of much debate in brain energetics (9, 16, 52, 53). Glucose is converted to pyruvate via glycolysis and
can be subsequently converted to lactate with the help of NADH and LDH (Figure 1.6).

**Figure 1.6 – Conversion of glucose into lactate.** Glucose is converted to 2 pyruvate molecules through glycolysis. This also yields 2ATP (2ATP invested, 4 ATP profited) and 2 NADH for the cell. The pyruvate is preferentially converted to lactate by LDH – 5 with an input of NADH. LDH-1 favours the conversion of lactate to pyruvate yielding an NADH.

The conversion of glucose to pyruvate by glycolysis provides energy and yields two pyruvate molecules that can be utilized to produce energy in the mitochondria. Pyruvate can also be used to make lactate with the investment of NADH by LDH – 5.
The lactate is then shuttled to the neuron to yield pyruvate and NADH by LDH-1 (54). The lactate-derived pyruvate can then be used to feed the neuronal TCA cycle and subsequently ATP production. Lactate-derived pyruvate can also be used to supply the neuron with a source of Glu for neurotransmission. As stated earlier, the preferential localization of LDH -5 in the astrocytes and LDH-1 in the neurons supports the idea that lactate is shuttled to the neurons from the astrocytes via the ANLS in an effort to support neuronal energy demand (18).

**Oxidative Lactate Metabolism**

The localization of LDH isoforms in neurons and astrocytes supports the idea that neurons use lactate as a food source and astrocytes shunt lactate toward neurons to support neuronal energy requirements. However, astrocytes have also been demonstrated to utilize lactate as a source of fuel for oxidative energy production (55-57). This once thought of end product of anaerobic metabolism is now being promoted as a supporter of oxidative energy production (58-60). Plasma lactate levels are normally 1mM, and can reach much higher levels during exercise (61-63). A significant level of lactate in the blood provides tissues, such as neural tissues, with an opportunity to capitalize on a free source of carbon. Though a mitochondrial LDH involved in oxidative energy metabolism hadn’t been described in astrocytes (until Lemire et al., 2008 and this thesis) mitochondrial LDH has been described in other highly oxidative tissues including the heart and muscle (54, 64, 65). The presence of an mLDH adds a novie twist on the brain lactate story (Figure 1.7).
Figure 1. 7 – Lactate oxidation by the neural mitochondria. Pyruvate derived from glycogenolysis and glycolysis in astrocytes is converted by 1) LDH – 5 in the astrocytes. Following lactate production in the astrocyte, the lactate is shuttled to the neurons via the ANLS. The lactate is then converted by 2) LDH – 1 in the neurons to yield pyruvate which enters the mitochondria and is combusted to produce ATP. Alternatively, lactate produced in the astrocytes and the lactate shuttled to the neuron can be brought into the mitochondria where it will be converted by 3) mitochondrial LDH – 1, to give pyruvate directly to the TCA cycle.

The presence of a mitochondrial LDH gives the neural cells the advantage of utilizing lactate as a source of carbon for oxidative energy production. The pyruvate, which is formed by breaking down glycogen and subsequently glucose, is broken down by a cytosolic LDH – 5, with an input of NADH. The lactate then enters the mitochondria where LDH-1 metabolizes it back to pyruvate yielding the invested NADH. In this manner, LDH – 5 coupling with LDH-1, turns lactate into a novel NADH shuttle (66). In addition, this gives the neural cells the ability to use lactate
oxidatively directly from the blood supply. Converting it into pyruvate in the
cytoplasm would require the shuttling of the NADH by means of other NADH
shuttles. Indeed, mitochondrial LDH provides new possibilities to understanding
brain lactate metabolism.

*Ketone bodies*

Another carbon source that the brain can utilize to meet its energy demands
are ketone bodies. Ketone bodies are released from the liver during periods of low
carbohydrate intake, and have been shown to be capable of supplementing the
brains energy demand (67, 68). Ketone bodies have also been demonstrated to play
a role in protecting the brain from oxidative stress and ischemia (68, 69). Both
acetoacetate and β-hydroxybutyrate can be used by astrocytes to make acetyl-CoA
(Figure 1. 8).
Figure 1.8 - Ketone body metabolism in astrocytes. Acetoacetate and β-hydroxybutyrate are obtained from the cerebral blood supply by the astrocytes. The β-hydroxybutyrate is metabolized to acetoacetate by β-hydroxybutyrate dehydrogenase. Acetoacetate is activated to acetoacetyl-CoA by acetoacetate - CoA ligase and ATP. Acetoacetyl-CoA splits to yield 2 acetyl-CoA by using a thiolase. The acetyl-CoA that is formed has two fates 1) acetyl-CoA can enter the mitochondria and participate in the TCA cycle and produce energy. The TCA cycle also gives rise to malate, which can be converted to pyruvate by NADP and ME. 2) Acetyl-CoA is also diverted to lipid production via acetyl-CoA carboxylase (ACC).
Ketone bodies within astrocytes provides a good alternative for a brain in need of energy. Acetoacetate and \( \beta \)-hydroxybutyrate are removed from the cerebral blood supply by the astrocytes. \( \beta \)-hydroxybutyrate is converted to acetoacetate by \( \beta \)-hydroxybutyrate dehydrogenase and NAD. The acetoacetate is subsequently converted to acetoacetyl-CoA by acetoacetate–CoA ligase with an input of ATP. The acetoacetyl-CoA then splits into two acetyl-CoA moieties by a thiolase. The acetyl-CoA that is generated enters the mitochondria to fuel the TCA cycle, which supports energy and Gln production. The Gln follows the Gln/Glu cycle and is shuttled over to the neuron as described earlier to support neurotransmitter production. The TCA cycle also provides malate that can be converted into pyruvate. Following its conversion, pyruvate is metabolized by LDH into lactate and again shipped to the neuron to support processes described previously. Acetyl-CoA is used to make lipids and cholesterol, another function of astrocytes. This is accomplished by ACC converting acetyl-CoA to malonyl-CoA. Lipid accumulation in the brain is abnormal, and thus acetyl-CoA would most likely be diverted toward cholesterol or the production of specialized lipids such as DHA (5).

*Acetate Utilization*

Another monocarboxylate that the brain can use to support its nutrient demand is acetate. In fact, acetate is used exclusively by astrocytes (70). Astrocytes remove acetate from the cerebral circulation in an effort to curtail the extensive nutrient demands of the brain (71). Acetate is used to fuel energy requirements in the neurons and astrocyte, gln production in the astrocytes, and lipid production (Figure 1.9).
Figure 1.9 - Acetate metabolism in astrocytes. Acetate is taken in by the astrocytes through the cerebral blood supply. The acetate is fixed into acetyl-CoA by ACS. The acetyl-CoA has multiple fates. 1) Acetyl-CoA enters the mitochondria where it participates in the TCA cycle, and subsequently adds to pyruvate and glutamate production (12). This requires an input of multiple carbon sources, as 2 carbons are lost for every turn of the TCA cycle. 2) Acetyl-CoA is utilized to manufacture lipids with the aid of ACC.
Acetate metabolism in astrocytes is much the same as ketone body metabolism. Acetate is taken up by the astrocytes through the cerebral blood supply. Acetate is then converted to acetyl-CoA with the aid of ACS. Again, acetyl-CoA can have multiple fates. It can enter the mitochondria where it can contribute to Gln and energy production (12). The Gln can be donated to support neuronal-Glu requirements and the energy can be used directly by the astrocytes. In addition, malate can be harvested from the acetyl-CoA-driven TCA cycle. This malate can be used to generate pyruvate, which can subsequently be used to make lactate and be shuttled via the ANLS to the neuron to support its energy requirements. The acetyl-CoA can also be diverted toward lipid production through ACC. These lipids will be used toward the synthesis of cholesterol, or to manufacture lipids such as DHA for utilization by the brain. However, as stated before, the brain does not normally store lipids as triglycerides (5). Most likely, the reasoning for neurons keeping clear of acetate uptake is that two carbon nutrient source would not be feasible for the energy demanding cell type. The acetate donated as lactate gives the neurons a useable carbon source at 3 carbons. However, acetate is only a source of 2 carbons. The carbon from acetate would be lost by the decarboxylation steps in the TCA cycle of the neurons. Astrocytes however, are the supportive cell to the neurons and thus the investment of making acetate into lactate or Gln to support energy and neurotransmitter production respectively is part of their altruistic nature (12). It is important to state that acetate utilization cannot be the sole source of nutrients. Other sources of carbon must be used to make the oxaloacetate that will fuse with
the acetyl-CoA produced from acetate to form the citrate required to prime the TCA cycle.

Evidently, astrocytes have many elaborate biochemical pathways in an effort to exploit as many carbon sources as possible to support the energy needs of the brain. Neurotransmission is a taxing process for neurons as nutrients are expended as both ATP and in neurotransmitter production. Replenishing both of the pools is necessary to fuel brain functions. Indeed, having a large brain is a luxury for a species that has an extravagance of nutrition available for its disposal. Glucose metabolism alone cannot keep pace with the high-energy demands of neurotransmission. Astrocytes have the ability however, to support neurotransmission by storing glucose as glycogen in times of need. They also exploit other sources of carbon such as lactate, ketone bodies, and acetate in an effort to meet energy demand. In addition, the presence of a mitochondrial LDH affords the astrocytes with the ability to use lactate in an oxidative fashion to support their own energy needs. An interesting facet of astrocytes is that in addition to supporting their own typical cellular biochemistry, they must also accommodate the metabolically demanding neurons. This cellular support system is also demonstrated by astrocytes having an active lipid metabolism.

1.2.3 – Lipid Metabolism and Astrocytes:

The pathways of lipid metabolism in astrocytes are much the same as those in other cell types. However, a caveat of the brain which differs from other cell types such as hepatocytes or adipocytes, is that lipid accumulation is not a normal
phenomenon (5). Astrocytes are the main consumers of fatty acids in the brain and have been demonstrated to use them as an alternative energy source to supplement neurons and oligodendrocytes (72). An increased level of lipids, or lipotoxicity, has been observed to induce a state of oxidative stress in neurons (73). When examining lipid metabolism, both production and degradation must be considered.

Lipogenesis in Astrocytes

There are two major lipogenic pathways in astrocytes. One is for the synthesis of cholestersols and isoprenoids. The second is for the synthesis of fatty acids. However, in both cases the precursor subunit is acetyl-CoA (5). One important lipogenic pathway is cholesterol synthesis (Figure 1.10).
Figure 1.10 - Cholesterol synthesis in astrocytes. 2 acetyl-CoA molecules combine to form acetoacetyl-CoA. Following the addition of another acetyl-CoA, HMG-CoA is formed by HMG-CoA reductase. Following a series of reactions, farnesyl-pyrophosphate is formed, which is the precursor to dolichols and ubiquinone. Finally cholesterol is synthesized (5).

Another important lipogenic pathway is the formation of fatty acids via elongation by acetyl-CoA moieties (Figure 1.11).
Figure 1.11 – Fatty acid synthesis in astrocytes. Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. Fatty acid synthase elongates the fatty acid by subsequent rounds of condensation, reduction, dehydration, and hydrogenation reactions with the help of NADPH. This process can occur up to a chain length of 12 carbons long (5).

Whether it is the synthesis of cholesterol or the synthesis of fatty acids, the precursor molecule is acetyl-CoA. Both have rate-limiting steps involved, HMG-CoA reductase is the rate-limiting step for cholesterol biosynthesis and creating farnesyl groups (involved in protein modification), and is the point of commitment (74). The rate-limiting step of fatty acids synthesis is the carboxylation of acetyl-CoA by ACC resulting in malonyl-CoA. Excess in acetyl-CoA promotes the activity of ACC, an
event that may lead to lipotoxicity. Polyunsaturated fatty acids are also synthesized in astrocytes. Polyunsaturated fatty acids like arachidonic acid (20:4 ω6) and DHA (22:6ω3) are essential for a healthy functioning brain (omega 3's and 6's), and are made in astrocytes by elongation and desaturation reactions within the endoplasmic reticulum (5, 75).

**β-oxidation in Astrocytes and the Involvement of L-carnitine**

In astrocytes, for lipids to contribute to energy production, the lipids are taken to the mitochondria where they can undergo β-oxidation reactions. The vessel that carries fatty acyl groups to the mitochondria for combustion is L-carnitine (76). The path of the synthesis of this non-essential amino acid is described in Figure 1.12.
Figure 1.12 – Carnitine biosynthesis. From protein degradation comes the primer for L-carnitine synthesis, trimethyllysine. Trimethyllysine undergoes a series of enzymatic steps to give rise to L-carnitine (77).

L-carnitine synthesis is a multi-enzyme process that employs KG, VitC, NADH, and divalent Fe in the path to its conception from trimethyllysine (77). L-carnitine is
synthesized within the cytoplasm for the purpose of transporting lipids into the mitochondria for β-oxidation (Figure 1.13).

**Figure 1.13 – Fatty acid transport for β-oxidation.** Long fatty acid chains are activated by acyl-CoA synthetase. Carnitine-palmitoyl transferase (CPT1) transfers L-carnitine onto the long fatty acyl-CoA. The acyl-carnitine then enters the mitochondria where CPT II removes the L-carnitine and reactivates the long acyl chain to an acyl-CoA for oxidation by the β-oxidation pathway in the mitochondria. β-oxidation yields acetyl-CoA and L-carnitine returns to the cytoplasm to retrieve more lipids (77).

In order for fatty acids to be burned and utilized, they must be transported to the mitochondria for β-oxidation. This process begins by the activation of a long fatty acid chain via acyl-CoA synthetase. Acyl-CoA synthetase activates the long chain fatty acid by tagging on CoA with the help of energy from ATP. CPT I which is located on the outer membrane of the mitochondria, transfers L-carnitine onto the
activated long fatty acyl-CoA chain (77). The acylcarnitine is then permitted to enter the mitochondria where CPT II exchanges the L-carnitine group on the acylcarnitine for CoA once again. The long fatty acyl-CoA can then undergo subsequent rounds of β-oxidation where one acetyl-CoA (2 carbons) is removed from the fatty acyl-CoA. If it is an even numbered fatty acid, the end product will be acetyl-CoA, which can then be utilized by the mitochondria to produce energy in an oxidative fashion. If it is an odd numbered fatty acid, the end product will be succinyl-CoA, which is also employed by the mitochondria to produce energy. The L-carnitine then returns to the cytoplasm to retrieve more lipids (77). The importance of L-carnitine cannot be understated. In its absence, lipids will not be broken down and lipids may accumulate, leading to lipotoxicity. L-carnitine has also been demonstrated to play other roles within the brain including acting like a fuel gauge for the brain to indicate body lipid levels (78). Knockout experiments of CPT in rats led to a state of obesity (78). Research also suggests that acetyl-L-carnitine may prevent Alzheimer’s by stabilizing Tau protein and by curtailing oxidative stress (79-81). L-carnitine has also demonstrated it’s efficacy as a chelator of metals, relieving metal stress (82).

Lipid metabolism in the brain is key to maintaining a healthy brain. Whether it is the production of specialized fatty acids [arachidonic (ω6) and DHA (ω3)] or cholesterol, astrocytes must perform these functions for the brain. In addition, astrocytes are the main site of β-oxidation of fatty acids, a process that yields acetyl-CoA, a moiety that can contribute to energy production.
1.2.4 - Metabolism in the Nucleus

Metabolism in the nucleus is a blackbox, just as understanding biochemistry in the mitochondria was some years ago. Today, we know quite a lot about the metabolic processes that occur within the mitochondria, but the same cannot be said about the nucleus. Recently, it was observed that there is a nuclear isoenzyme of CL (83). This nuclear CL provided acetyl-CoA (citrate + ATP + CoASH → oxaloacetate + acetyl-CoA) in-situ for the acetylation of histones (83). It appears as though the modification present on the histone affects the degree of condensation of the chromosome (84). Where ubiquitin "crowbars" the chromosome open and very little condensation can occur, acetylation does not repel the histones to the same extent, thus more DNA can be accessed by the transcriptional machinery when ubiquitin is attached to the histone in comparison to acetylation (84). Another study recently observed that propionyl and butyryl modifications could occur on histones (85). Thus, it appears as though the nucleus is ripe with metabolites available to communicate with the DNA and offer dynamic epigenetic feedback. If there are metabolites present, metabolic networks may be in place for the in-situ production of the modifying metabolites, such as the nuclear CL (83). Unravelling how these metabolic networks interact in the presence of cellular stressors would provide novel information on the metabolic link between physiological responses and genetic information. However, research on nuclear metabolism is in its infancy.

1.3 - Brain Metabolism and Disease

Clearly metabolism in the brain is quite intricate. Brain diseases which have been discovered to have an underlying metabolic dysfunction include epilepsy,
disorders of myelin sheath formation, aging, Alzheimer's disease, malfunction in neurotransmitter production, and toxic encephalopathies to name but a few (5, 86-90). Astrocytes are key metabolic determinants of the brain as their metabolic pathways support neuronal functioning in a variety of ways. Thus, any insult on astrocyte metabolism could have a deleterious effect on the brain.

1.4 - Environmental Pollutants and Neurological Disorders

Diseases of the brain such as Alzheimer's, Parkinson's, tauopathies, encephalopathies, and other neurodegenerative disorders have many linked underlying causes. The definitive primary source however, remains elusive and it is possible there are numerous effectors that act in concert to initiate or propagate neurological disorders (91, 92). Neurological diseases have been associated with numerous factors ranging from genetics, immune related inflammation, Abeta peptide accumulation, neurotoxicity to environmental pollutants, and ROS (93-97). One factor however, stands above all others, which is neurological diseases are linked to ageing (98, 99). The correlation between age and neurological diseases/disorders implies that there is an accumulation or compounding effect of the underlying cause over time. Environmental pollutants accumulate over time and they are indeed critical factors that may mediate neurological diseases. Interactions of pollutants and components of the brain are an ongoing crucial scientific endeavour if we are to understand these pathologies.

1.5 - The Blood-Brain Barrier and Protection from Xenobiotics

The BBB is the brain's primary defense against any xenobiotic insult. The majority of large molecules (peptides, proteins, and antibodies) do not have
unrestricted passage through the BBB (100). Even many small molecules have difficulty passing through the BBB (100). For a toxicant to pass through the BBB, it must either mimic an endogenous molecule and exploit its transporter, or the BBB must become "leaky". A leaky or dysfunctional blood brain barrier has been associated with age, stress, and neurological disorders (101-103). The ability of toxicants to pass more easily through the BBB as the brain ages may explain the correlation between neurological disorders and age. The passage of metals across the BBB is strictly controlled. This is not surprising as metal homeostasis throughout the body is also tightly regulated. The passage of Cu across the BBB is poorly delineated (104, 105). However, ATP7a has been suggested as a Cu carrier as it is expressed on vascular endothelial cells in the brain (106). The transport of Fe across the BBB has been characterized. Fe is bound by transferrin in the circulation. Transferrin interacts with the transferrin receptor and is internalized into the vascular endothelial cells by receptor-mediated endocytosis (107). The Fe can then be shuttled to the astrocytes, and is then delivered to the neurons in a process called the brain iron cycle (108). However, transferrin is also the vehicle via which the suspected neurotoxin, Al gains entrance across the BBB (109, 110).

1.6 - Metal Homeostasis and Toxicity in the Brain

Metal ions are involved in many biological processes, including those in the brain. Although metals are essential for all biological life forms, their homeostasis is stringently regulated due to their potential toxicity. Cu and Fe are arguably some of the most relevant transition metals required for life, but are also some of the most
toxic because of their potential to produce ROS (106). Both an excess and a lack of either of the metals has been observed to be detrimental to the cell (106). It is of no surprise then that cells have elaborated mechanisms to absorb, store, and dispose of these metals.

Copper

Cu is essential for cellular respiration (Cyt C Ox.), neurotransmitter biosynthesis (dopamine monooxygenase), anti-oxidative defense (SOD), and peptide amidation (111). It is clear from this short list of enzymes that Cu is essential to proper brain functioning. In fact, the absence of Cu in the brain leads to growth retardation in developing brains and neurodegeneration (112, 113). However, when concentrations of this metal reach elevated levels, the redox active Cu can produce ROS, bind to histidine, methionine, and cysteine in the active sites of enzymes (114). There are two heritable diseases that vocalize a great deal about Cu overload. The two most prominent diseases in which Cu accumulates is Wilson’s disease and Menke’s disease (106, 115). Wilson’s disease is an autosomal recessive disorder with a mutation in the Cu efflux pump ATP7b gene (116). In the liver this leads to copper accumulation. In the brain however, ATP7b is expressed in some regions, but it is the lack of synthesis of holo-ceruloplasmin, a Cu-carrying protein with ferrooxidase activity that may have a greater effect (106). Patients with Wilson’s disease experience neurodegeneration, psychiatric schizophrenia, and Parkinson’s like symptoms (106, 116). The biochemistry behind these neurological symptoms are correlational and have not been demonstrated, however they do reverse upon chelation therapy (106). It has been proposed that Cu may play a role in modulating
synaptic firing (117). Excess Cu is found to be associated with neurological diseases as well, and associated with senile plaques (118, 119).

Iron

Fe overload in the brain is linked to Parkinson's disease and to neurodegenerative disorders (120). Fe is involved in numerous processes in the brain including, oxidative energy production, neurotransmitter synthesis, and O$_2$ transport (121). Fe, like Cu, is redox active and can donate/accept electron readily and can thus participate in ROS production. Elevated levels of free Fe can damage macromolecules via the production of ROS (122). Therefore, the LIP is strictly policed in biological systems to ensure as little Fe as possible is free to damage the cell. Fe metabolism in the brain is unique compared to other tissue types. This observation gained momentum when individuals with genetic diseases related to systemic Fe homeostasis were studied, their brains did not experience the same iron overload or iron shortage that other tissues had (106, 123). So, although the proteins of systemic Fe metabolism are expressed in the brain, there are other factors involved in neural Fe homeostasis.

Fe (III) is shuttled into the brain from the cerebral blood supply, utilizing transferrin and the transferrin receptor (106). Nearly all Fe in the plasma is bound by transferrin. Transferrin is endocytosed in a clathrin-dependent fashion into the vascular endothelial cells, the diferric Fe is liberated from the transferrin by acidification of the endocytototic vesicle and subsequently pumped into the cell by a divalent metal transporter (following its reduction) to Fe (II) by an Fe-reductase. Fe is immediately picked up by a protein rich in acidic amino acid residues, called
ferritin and other ligands such as organic acids (citrate and lactate), polypeptides, and other low molecular weight ligands (124). This ensures that the Fe in the LIP is tightly bound to avoid undesired redox reactions in the cell. If Fe accumulates, ferroportin can be utilized to efflux excess Fe. If Fe is lacking, an iron response protein (apo-ACN), leaves its home in the mitochondria to bind to the iron response element in the nucleus to produce transcripts encoding the uptake of Fe and destabilizes transcripts involved in producing Fe-dependent enzymes such as SDH, and ferritin (125). One protein that plays more of a critical role in brain Fe metabolism is ceruloplasmin. Its absence in the brain has been found to be one of the only factors that can lead to lack of Fe in the brain (106). Ceruloplasmin is synthesized with astrocytes and is subsequently localized within the membrane of neural cells, regulating Fe release from Fe storage sites (126, 127).

Some of the most toxic metals are those that can donate electrons to generate ROS. Metals such as Fe and Cu donate electrons readily to O$_2$ to generate ROS and are thus tightly regulated to ensure that they do not participate in this event. One of the characteristic toxic effects of some non-essential metals such as Mn, Cd, Pb, and Al in the brain is that they interfere with Cu or Fe homeostasis.

1.7 - The Generation and Effects of ROS

The use of molecular oxygen (O$_2$), results in a conundrum for aerobic organisms. Using O$_2$ as the final electron acceptor for oxidative phosphorylation provides cells with an opportunity to capitalize on energy production. On the other hand, O$_2$ is consistently damaging our cells through its oxidative properties. The
cellular damage comes from the ability of O₂ to generate ROS. Triplet O₂, in its ground state, is a biradical with its outer valence electrons occupying two separate orbitals in parallel spin states (128). The ground state nature of O₂ restricts it from accepting pairs of electron, therefore making it difficult to react with organic molecules (129, 130). Thus, O₂ generally undergoes single electron reduction (Figure 1.14).

![Diagram showing the generation of free radicals from molecular oxygen]

**Figure 1.14 – The generation of free radicals from molecular oxygen.** Dioxgen (O₂), undergoes a series of univalent reductions. Reduction of these products leads to the formation of other common radicals (128).

The sequence of events that produces various radicals from O₂, are common reactions performed within biological systems. One of the most important uses of ROS generated in biological systems is that mediated by NADPH oxidase. NADPH oxidase is used by phagocytic cells and neutrophils to generate superoxide (O₂⁻) by reducing O₂ using NADPH as the electron donor (131). This superoxide is used to kill invading pathogens. Unfortunately, some abiotic stressors can elicit a similar response and generate undesired ROS (128). The reduction of O₂ is also commonly used by the machinery of the ETC to fuel the chemiosmotic production of a proton.
gradient for energy production. Cyt C Ox. (complex IV), the key respiratory complex in the mitochondria, catalyzes the reduction of O$_2$ to generate H$_2$O. The reaction consists of transferring 4 e$^-$ to O$_2$ (O$_2$ + 4H$^+$ 4e$^- \rightarrow 2$H$_2$O) (132, 133). This reaction is essential for the generation of ATP via the ETC. However, this utilization of O$_2$ as the terminal electron acceptor poses a threat to aerobic organisms. It is estimated that 2-5% of all the O$_2$ that is utilized by the ETC, is subject to incomplete reduction by e$^-$ leakage, resulting in the production of (O$_2$$^•$-) (132). Indeed, the mitochondria are the major site of ROS production in aerobic systems (134). The generation of ROS in the mitochondria takes place through electron leakage from complex I and III (Figure 1.15).

![Figure 1.15 - ROS production from the ETC](image)

**Figure 1.15 - ROS production from the ETC.** NADH passes its electrons to complex I. Complex I reduces coenzyme Q (CoQ) one electron at a time. CoQ delivers the electrons to complex III. Problems arise when the e$^-$ flow gets hindered. e$^-$'s leak from complex I and III and reduce O$_2$ to O$_2$$^•$-, which is abundant in the mitochondria (133).

The generation of ROS in the mitochondria occurs at the level of complex I and III (132). Under periods of intense activity (when ATP is being generated for neurotransmission) e$^-$ flow gets backed up at complex I because CoQ is saturated.
At complex III, a similar situation occurs, CoQ$^+$ cannot pass the electrons to complex III. This leads to electrons being passed to O$_2$ and the formation of O$_2^-$ (133). The event is further illustrated by the inhibition of complex I and III by compounds such as rotenone and myxothiazol. Genetic alteration of the Rieske Fe-S protein of complex III demonstrates this as well (132, 135). Other enzymes in the eukaryotic systems produce O$_2^-$ including the cytochrome P450 family, xanthine oxidase, and nitric oxide synthase (136).

Though O$_2^-$ is generated as a by-product of cellular metabolism, it has a long half-life, but does not inflict as much macromolecular damage as the hydroxyl radical (OH$^-$). ROS molecules interact with one another, and the reduction of O$_2^-$ by H$_2$O$_2$ can yield the highly reactive OH$^-$. Free copper and iron (redox active metals) can react with peroxide to produce the hydroxide radical via the Fenton reaction and the Haber-Weiss reaction (137, 138) (Figure 1.16).

\[
\begin{align*}
1) \text{Fe(II)} + O_2 & \rightarrow \text{Fe(III)} + O_2^- \\
Cu(I) + O_2 & \rightarrow Cu(II) + O_2^- \\
2) \text{Fe (II)} + H_2O_2 & \rightarrow \text{Fe (III)} + OH^- + OH^- \\
Cu (I) + H_2O_2 & \rightarrow Cu (II) + OH^- + OH^- \\
3) O_2^- + H_2O_2 & \rightarrow O_2 + OH^- + OH^- \\
4) \text{Fe (III)} + O_2^- & \rightarrow \text{Fe (II)} + O_2 \\
Cu (II) + O_2^- & \rightarrow Cu(I) + O_2
\end{align*}
\]

Figure 1.16 - Redox active metals catalyze ROS production. Fe$^{2+}$ and Cu$^{1+}$ 1) react with oxygen to make the superoxide. 2) Fe$^{2+}$ and Cu$^{1+}$ react with hydrogen peroxide to form the hydroxyl radical. 3) The Haber-Weiss reaction demonstrates
how the superoxide can react with peroxide to make the hydroxyl radical. 4) Fe$^{3+}$ and Cu$^{2+}$ can be regenerated back to Fe$^{2+}$ and Cu$^{1+}$.

Single electron transfer between ferric/ferrous and cupric/cuprous ions reacting with O$_2^-$ and other ROS molecules eventually leads to the production of OH$. Fe and Cu are recycled back to their II and I oxidation state, respectively. This information is troubling from a neurotoxicological standpoint as heightened levels of both Fe and Cu have been found in Alzheimer's brains and are associated with senile plaques (118, 138-140).

The toxicity of ROS molecules is due to their ability to react with cellular macromolecular structures. ROS are capable of extract the protons and electrons from the double bonds of polyunsaturated fatty acids, which subsequently produces fatty acyl radicals (141) [Figure 1.17].

**Figure 1.17 – Lipid Peroxidation by ROS.** The hydroxyl radical reacts with unsaturated fatty acids to form a lipid radical. The lipid radical can then react with oxygen and form the lipid peroxyl radical. The lipid peroxyl radical then interacts with another unsaturated fatty acid, which subsequently yields lipid peroxide and
another lipid radical. The end result is a non-functional lipid and a self-propagation of lipid peroxidation (141).

The mechanism by which unsaturated fatty acids get oxidized is a self-perpetuation mechanism, where the lipid radical can form another lipid radical through a lipid peroxyl radical intermediate. The unsaturated fatty acid peroxide formed by the termination of the reaction is not functional like the original unsaturated fatty acid. This phenomenon could lead to dysfunction of the cell membrane and interfere with membrane fluidity, and thus the importance of vitamin E (a membrane bound antioxidant). The break down of this lipid by-product can form reactive aldehydes that can interact with DNA and proteins (141). The hydroxyl radical has also been demonstrated to interact with proteins, by abstracting the \( \alpha \)-hydrogen of an amino acid, breaking open proline rings to form oxidized proline, and oxidize thiols (142). These modifications by ROS can render the protein functionless and can even promote protein aggregation, a phenomenon associated with Alzheimer's disease (143, 144). DNA is altered by ROS by the oxidation of guanine to 8-oxoguanine, a modification that creates lesions within the DNA (145).

Fe and ROS are intricately linked. Fe can form ROS by donating its electrons to \( O_2 \), thus the tight regulation of free Fe. ROS can also interfere with Fe homeostasis. The Fe-S clusters in metalloproteins are sensitive to increased levels of ROS within the cell (146). ROS can disrupt Fe-S clusters, which leads to an increase of free Fe in the cell. ACN in particular contains a labile Fe that can be removed under ROS, metal stress, and low Fe availability. The disruption of Fe in metalloproteins leads to an increase in free Fe and thus more formation of oxidative
stress (147). This process appears to self-perpetuate in a cyclical fashion and thus the cell must deal with ROS stress before it destroys the cell. The inability to do so has been associated with the aging process (148).

1.8 - Antioxidative Defense Systems

ROS are essential in biological organisms as they help guard against foreign intrusion. ROS is also produced unfortunately as a by-product of oxidative phosphorylation via the complexes in the electron transport chain (149, 150). The inherent production of ROS through oxidative metabolism does not deter the cell from utilizing its ATP producing ability. Instead, the cell implements a number of biological antioxidants to combat the deleterious ROS triggered by the use of oxygen as a terminal electron acceptor. Such biological molecules as vitamins C (ascorbic acid), vitamin E (α-tocopherol), taurine, arginine, citrulline, lipoic acid, and glutathione comprise a portion of the non-enzymatic antioxidant defense system (141). Proteins such as SOD, glutathione reductase, and glutathione peroxidases are examples of some of the antioxidant enzymes involved in the enzymatic antioxidant defense system (151).

Enzymatic Antioxidant Defense Systems

The detoxification of $O_2^{-}$ is accomplished by SOD. SOD catalyzes the disproportionation of $O_2^{-}$ to $H_2O_2$ and $O_2$. Eukaryotic systems express two separate isoenzymes that have different cellular location, and metal insertion pathways (152). The manganese (Mn)-dependent SOD is localized to the mitochondria, while the CuSOD is found in the cytosol. MnSOD is so important for quelling production of $O_2^{-}$.
in the mitochondria, that a mutation in the gene encoding MnSOD leads to a lethal phenotype (153). Since H$_2$O$_2$ is a product of SOD and is produced through the propagation of ROS, GPx and catalase work in concert to sequester H$_2$O$_2$. Catalase catalyzes the conversion of H$_2$O$_2$ into H$_2$O and O$_2$ (151, 154). GSH, GPx, and GR couple the enzymatic antioxidant defense system to the non-enzymatic antioxidant defense system. GSH is a tripeptide that is composed of a glutamate, cysteine, and glycine (141). Tissues that are very oxidative like the brain, liver, heart, and muscle tissues contain a large amount of GSH. Disorders such as Alzheimer’s disease, Parkinson’s disease, and other neurodegenerative disorders have been associated with reduced levels of GSH (155). GSH (reduced) and GPx, react with H$_2$O$_2$ yield H$_2$O and oxidized disulfide GSSG (151, 156). The regeneration of GSH from GSSG requires GR, an enzymes which requires NADPH to catalyze the reduction of GSSG (141). In fact, the anabolic reducing agent NADPH is also required for the reactivation of catalase and the reduction of the thioredoxin system (157, 158). Thus, NADPH-producing enzymes are just as necessary to the enzymatic antioxidant defense system as the enzymes directly involved. Indeed, NADPH-generating enzymes (NADP-ICDH, ME, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) are augmented under ROS stress (159, 160).

*Non-enzymatic Antioxidant Defense Systems*

Non-enzymatic antioxidant defense mechanisms rely on low-molecular weight moieties to detoxify ROS. Previously, the detoxification of H$_2$O$_2$ by GSH was mentioned. Other antioxidants such as the vitamins C and E (α-tocopherol), are also
potent antioxidants. Vit C, interacts with ROS to produce an ascorbyl free radical intermediate that becomes dehydroascorbate (161). The regeneration of dehydroascorbate back to ascorbate is performed by a GSH transhydrogenase. Thus, Vit C based antioxidant defense is also reliant on NADPH. Vitamin E, is a membrane bound antioxidant that protects the membrane from ROS-induced peroxidation of unsaturated fatty acids (162). Vitamin E is regenerated by partner antioxidants such as Vit C and GSH, again illustrating that its reductive power is hinged on NADPH availability.

Recent research has unravelled that α-keto acids can also participate in the defense of the cell against oxidative stress (163, 164). α-Keto acids such as KG and pyruvate have been demonstrated to sequester ROS via a non-enzymatic decarboxylation reaction (165, 166). The metabolic networks involved in the production of these α-keto acids are re wired for their accumulation. The subsequent non-enzymatic decarboxylation breakdown product for KG and pyruvate reacting with ROS is succinate and acetate, respectively. The benefit of utilizing α-ketoacids as antioxidants is that they can be made in-situ by the cell in a rapid manner and their by-products may help trigger signalling responses (167). In addition, the non-enzymatic decarboxylation products of the reaction of α-keto acids with ROS are intermediates of other metabolic pathways. Indeed, ROS is a reality of all aerobic biological systems. It can be beneficial to a system if kept in check. The cell has elaborated many methods to live in an oxidative environment (Figure 1.18).
**ENZYMATIC ROS DETOXIFICATION SYSTEMS:**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase</td>
<td>( \text{O}_2^- \xrightarrow{\text{H}_2\text{O}_2} \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>Catalase</td>
<td>( 2\text{H}_2\text{O}_2 \xrightarrow{\text{H}_2\text{O} + \text{O}_2} )</td>
</tr>
<tr>
<td>Glutathione Peroxidase</td>
<td>( 2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{} \text{H}_2\text{O} + \text{GSSG} )</td>
</tr>
</tbody>
</table>

**NON-ENZYMATIC ROS DETOXIFICATION SYSTEMS:**

- Glutathione (GSH)
- Ascorbic Acid (Vitamin C)
- \( \alpha \) - tocopherol (Vitamin E)
- \( \alpha \) - ketoacids

![Chemical Reaction](image)

**Figure 1.18 – The ROS defense mechanisms elaborated by biological systems.** Enzymatic and non-enzymatic antioxidant systems implemented by biological systems to combat oxidative stress.

Many systems exist to quell ROS. Having both enzymatic systems and non-enzymatic systems to battle oxidative stress is necessary as ROS molecules can interfere with biological macromolecules such as lipids, proteins, and DNA rendering them ineffective.

**1.9 - Aluminum, a Suspected Neurotoxin**

Al is the most abundant metal in the Earth’s crust, but has remained mostly isolated from biological systems due to its lack of bio-availability (168). Al is a non-redox active metal. Al is a hard acid so it readily interacts with hard bases such as silica, phosphates, oxides, and carboxylates. The lack of bioavailability is dictated by its chemistry. pH of the solution determines the species of Al, where Al is soluble at
an acidic pH, at a neutral pH (in biological systems for example) Al complexes undergo extensive hydrolysis and generate hydroxides [Al(OH)₃] that precipitate out of solution (169).

Three features have recently come into play that have made Al more bioavailable 1) An increase in anthropogenic acidification of soils, 2) the increased utilization of the metal for industrial and commercial purposes, and 3) its utilization as a flocculent in water treatment [Al₂(SO₄)₃] (168, 170, 171). This enhanced bioavailability has resulted in the accumulation of the metal in living organisms including humans. The main route of Al absorption in humans is uptake via the gastrointestinal tract most likely by binding to organic acids; it is then distributed throughout the body by binding to transferrin and citrate in the blood, and can ultimately cross the BBB (110, 169). The skeletal system, the liver, and the brain are some of the primary sites where Al deposits have been observed (172-174). Diseases such as encephalopathy, Alzheimer’s, Parkinson’s, and osteomalacia have been associated with the presence of this metal (93, 175-177). Al gains entrance into the cell via transferrin-mediated endocytosis and as complexes with organic acids (110, 178).

The molecular targets of Al toxicity are multifaceted and appear to involve the disruption of essential metal homeostasis such as calcium (Ca), magnesium (Mg), and Fe (170, 173, 179). Al has been demonstrated to replace Ca within the bone and interferes with Ca-based signalling events (170, 173, 180). Mg has been observed to be replaced by Al for binding to phosphate groups on the cell membrane, on ATP, and on DNA (181-183). Perhaps the main target of Al toxicity are Fe dependent
biological processes. Al's interference with Fe homeostasis also leads to the production of ROS (137, 184). The perturbation of Fe homeostasis and the subsequent generation of ROS both contribute to its toxicological effects (163, 167, 179, 184, 185) (Figure 1.19).

Figure 1.19 – The sources and effects of Al in biological systems.

As stated previously, perhaps the greatest toxicological effect of Al-toxicity is its ability to perturb Fe homeostasis as this leads to the creation of ROS. Oxidative metabolism is heavily reliant on Fe to execute the combustion of citric acid (186, 187). Enzymes of the TCA cycle such as ACN, SDH, and FUM contain Fe-S clusters (187). SDH is also a bridge to the ETC, where it functions as Complex II. The ETC also contains other Fe-dependent enzymes such as, Complex I, Complex III, and Complex IV (187). It then stands to reason that if Fe homeostasis was to be affected under Al-insult, oxidative ATP production would be severely compromised.
Previous studies from our research group have demonstrated that the activity and expression of Fe-dependent ACN was decreased under Al-toxicity (185). Similar observations were made with SDH, Complex I, and Complex IV (188). This results in a loss of NADH and subsequently a diminution of ATP production in an aerobic fashion (189). Similarly, when hepatocytes were treated with Al, Fe dependent enzymes such as ACN, SDH, Complex IV, and FUM were also inhibited (163, 167). This effect led to a decrease in the production of NADH and ATP (167). Figure 1.20.

**Figure 1.20 - Al inhibits oxidative energy production.** Al perturbs Fe dependent enzymes of the TCA cycle ACN, FUM, and SDH. NADH producing enzymes such as ICDH and KGDH are also inhibited. This leads to a loss in NADH production. Al also impedes ETC enzymes such as Complex I, II, and IV. The loss of NADH production and inhibition of the ETC results in a loss of mitochondrial ATP production. Red = decrease in levels.
Due to the inability of the TCA cycle to consume citrate, the HepG2 cells were transformed into lipid factories that produced and stored fatty acids (160). Another consequence of the Al toxicity was the switch to an anaerobic life style (190). Al has been demonstrated to induce ROS (191). ROS has also been shown to promote an anaerobic state within the cell (190, 192). HepG2 cells exposed to Al accumulate α-ketoglutarate (KG), a known antioxidant, by the downregulation of α-ketoglutarate dehydrogenase (KGDH) (163). This KG reacts with ROS to produce succinate (166). Succinate accumulates due to the diminished expression of SDH. This accumulation of succinate under Al-toxicity, promotes anaerobiosis by a prolyl-hydroxylase (PHD) and hypoxia inducible factor-1α (HIF-1α) dependent manner (190) (Figure 1.21).
Figure 1.21 – Summary of results from Mailloux et al in Al-treated HepG2 cells. Al interferes with Fe homeostasis and subsequently generates ROS. The free Fe and ROS disrupt the TCA cycle and the ETC enzymes that are Fe dependent. Interference with ACN (4Fe-4S) leads to the promotion of anaerobiosis and hinders the entrance of citrate into the TCA cycle. Citrate is instead shipped to the cytoplasm where it is stored as lipids by CL and ACC. The citrate that is funnelled into the TCA cycle is used to pool KG. KG gets pooled by an increase in NADP-ICDH and a decrease in activity of KGDH. NAD-ICDH is not used to make KG as NADH production would promote oxidative tension through the ETC. NADPH production however, lends to combating ROS. KG is also pooled to combat ROS. KG reacts with ROS by non-enzymatically decarboxylating to form succinate. The inhibition of SDH activity leads to the pooling of succinate, which subsequently results in the inhibition of prolyl hydroxylase (PHD). The inhibition of PHD leaves hypoxia-inducible factor 1-α (HIF-1α) active. Active HIF-1α promotes anaerobiosis. The lack of NADH and the interference with the Fe-dependent complexes of the ETC, results in a lack of ATP production. Red = decrease in levels, green = an increase in levels (160, 163, 167, 190).

The connection between Al and neurological disorders has eluded researchers for many years. Although progress has been made in this regard, the link between Al and neurological disorders such as Alzheimer’s and Parkinson’s diseases has yet to be precisely delineated. The discovery of Al in the brain of Alzheimer’s patients and the association of Al with amyloid plaques have provided some evidence to the connection between the metal toxin and Alzheimer’s disease (193-195). The involvement of Al in the induction and phosphorylation of Tau protein, as well as the promotion of neuroinflammatory transcripts has been demonstrated (93, 94, 196-199). Tau is a key protein in the formation of microtubules in neurons. When it becomes hyperphosphorylated, it attracts and accumulates Al, possibly inducing neurofibrillary tangles. Indeed, Al is a possible neurotoxin that requires attention.
Thesis Objectives:

The goals of these studies were to delineate the impact of a suspected neurotoxin, Al on various biochemical events in astrocytes. Since Al interferes with Fe metabolism leading to the production of ROS, the effects of ROS on various biochemical pathways in astrocytes were also studied. Little attention has been paid to astrocytes as far as the effects of Al are concerned. This is surprising as astrocytes are a key player in the functioning of the brain. Astrocytes participate in a variety of crucial roles in the brain including brain scaffolding (structural support), metabolic support for neurons, neurotransmitter homeostasis, and lipid metabolism in the brain. Thus, any insult imposed on astrocytes may effect the functioning of the brain. With a correlative connection between anthropogenic mobilization of Al and neurological disorders, the toxicological impact of Al on astrocyte physiology and biochemistry will provide critical molecular insights in understanding the link between this toxic element and neurological impairments.

Hypothesis

Previous work in our laboratory has demonstrated that Al perturbs numerous biochemical pathways in both prokaryotic and eukaryotic systems. The interference with Fe metabolism and the production of ROS by Al, will distress oxidative metabolism and lead to a lack of ATP production. The lack of ATP under Al and ROS stress will manifest in the disruption of cellular processes such as the turnover of the actin cytoskeleton. Using lactate as the delivery vehicle for the Al, will unravel findings in lactate metabolism. Since oxidative stress increases under Al toxicity, it is hypothesized that antioxidant defense systems will be augmented.
Specifically, the production of α-keto acids by the re-wiring of metabolic networks will be observed under Al and ROS treatment. Since mitochondrial metabolism is affected by Al and ROS stress, fatty acid accumulation should be observed as a consequence of a combination of increased lipid production and decreased lipid β-oxidation. Taken together, Al and ROS stress on astrocytes will manifest in cellular dysfunction that will provide molecular clues to the ill-defined link between Al and neurological disorders.
Section 2: Materials and Methodology

2.1 - Reagent List (alphabetical order)

- α-Ketoglutarate: Sigma-Aldrich (St.Louis, Missouri)
- α-Ketoglutarate Dehydrogenase: Sigma-Aldrich (St.Louis, Missouri)
- α – MEM (+1% streptomycin and penicillin): Princess Margaret Hospital (Toronto, Ontario)
- β-Mercaptoethanol: Sigma-Aldrich (St.Louis, Missouri)
- Acetonitrile HPLC grade: Fisher Scientific (Unionville, Ontario)
- Acetic Acid: Sigma-Aldrich (St.Louis, Missouri)
- Acetyl-CoA: Sigma-Aldrich (St.Louis, Missouri)
- Aconitase: Sigma-Aldrich (St.Louis, Missouri)
- Acrylamide: Sigma-Aldrich (St.Louis, Missouri)
- ADP: Sigma-Aldrich (St.Louis, Missouri)
- Agarose: Sigma-Aldrich (St.Louis, Missouri)
- Aluminum Chloride Hexahydrate (AlCl₃ • 6H₂O): Sigma-Aldrich (St.Louis, Missouri)
- Ammonium persulfate: Sigma-Aldrich (St.Louis, Missouri)
- 6 – aminohexanoic acid: Sigma-Aldrich (St.Louis, Missouri)
- ATP: Sigma-Aldrich (St.Louis, Missouri)
- Bis-acrylamide: Bio-Rad Laboratories (Mississauga, Ontario)
- Bistris: Fisher Scientific (Unionville, Ontario)
- BSA: Sigma-Aldrich (St.Louis, Missouri)
- L-Carnitine: Sigma-Aldrich (St.Louis, Missouri)
- Chemiglow Chemiluminescent Substrate: Alpha Innotech (San Leandro, California)
- Citric Acid: Sigma-Aldrich (St.Louis, Missouri)
- Coenzyme A: Sigma-Aldrich (St.Louis, Missouri)
- Coomassie G-250 and R-250: Thermo Fisher Scientific (Waltham, Massachusetts)
- Cy3 – GFAP: Sigma-Aldrich (St.Louis, Missouri)
- Cytochrome C: Sigma-Aldrich (St.Louis, Missouri)
- Deuterium Oxide 99.9%: Sigma-Aldrich (St.Louis, Missouri)
- Diaminobenzidine: Sigma-Aldrich (St.Louis, Missouri)
- 2,4 – Dinitrophenyl Hydrazine: ICN Biochemicals (Cleveland, Ohio)
- Dichlorofluorescein Diacetate: Sigma-Aldrich (St.Louis, Missouri)
- 2,6 – Dichlorindophenol: Sigma-Aldrich (St.Louis, Missouri)
- Digitonin: Sigma-Aldrich (St.Louis, Missouri)
- Dithiothreitol: Sigma-Aldrich (St.Louis, Missouri)
- DMSO cell storage media: Gibco (Carlsbad, California)
- EDTA: Sigma-Aldrich (St.Louis, Missouri)
- FBS: Invitrogen (Burlington, Ontario)
- Ferritin (Apo): Sigma-Aldrich (St.Louis, Missouri)
- FITC – conjugated anti-rabbit: Abcam (Cambridge, Massachusetts)
- Fumarase: Sigma-Aldrich (St.Louis, Missouri)
- Fumaric acid: Sigma-Aldrich (St.Louis, Missouri)
- Glacial Acetic Acid: CanLab (Toronto, Ontario)
- D-Glucose: Sigma-Aldrich (St.Louis, Missouri)
- L-Glutamate: Sigma-Aldrich (St.Louis, Missouri)
- Glycerol: Sigma-Aldrich (St.Louis, Missouri)
- Hexane CHROMASOLV Plus 95%: Sigma-Aldrich (St.Louis, Missouri)
- Hoechst 33528: Sigma-Aldrich (St.Louis, Missouri)
- 30% (w/v) hydrogen peroxide solution: Sigma-Aldrich (St.Louis, Missouri)
- Iodonitrotetrazolium Chloride: Sigma-Aldrich (St.Louis, Missouri)
- Isocitric Acid: Sigma-Aldrich (St.Louis, Missouri)
- Isopropanol 99%: Sigma-Aldrich (St.Louis, Missouri)
- Kaleidoscope Ladder and Precision Plus Standards: Bio-Rad (Mississauga, Ontario)
- Lactic Acid: Sigma-Aldrich (St.Louis, Missouri)
- 3-13C lactate: Cambridge Isotope Laboratories (Andover, Massachusetts)
- Lactate Dehydrogenase (porcine heart and muscle): Sigma-Aldrich (St.Louis, Missouri)
- L-Carnitine: Sigma-Aldrich (St.Louis, Missouri)
- Leupeptin: Sigma-Aldrich (St.Louis, Missouri)
- Magnesium Chloride: Sigma-Aldrich (St.Louis, Missouri)
- Malate Dehydrogenase: Sigma-Aldrich (St.Louis, Missouri)
- Malic Acid: Sigma-Aldrich (St.Louis, Missouri)
- Monobasic Potassium Phosphate HPLC Grade: Fisher Scientific (Unionville, Ontario)
- Morin Reagent: Sigma-Aldrich (St.Louis, Missouri)
- Mounting Solution: Fisher Scientific (Unionville, Ontario)
- N-Acetyl Cysteine: Sigma-Aldrich (St.Louis, Missouri)
- NAD: Sigma-Aldrich (St.Louis, Missouri)
- NADH: Sigma-Aldrich (St.Louis, Missouri)
- NADP: Sigma-Aldrich (St.Louis, Missouri)
- NADPH: Sigma-Aldrich (St.Louis, Missouri)
- Odyssey® blocking buffer: Loric (Lincoln, Nebraska)
- Oil Red O: Sigma-Aldrich (St.Louis, Missouri)
- Palmitic Acid: Sigma-Aldrich (St.Louis, Missouri)
- Periodic Acid: Sigma-Aldrich (St.Louis, Missouri)
- Pepstatin: Sigma-Aldrich (St.Louis, Missouri)
- Phalloidin (FITC and Rhodamine B conjugated): Sigma-Aldrich (St.Louis, Missouri)
- Phenazine Methosulphate: Sigma-Aldrich (St.Louis, Missouri)
- Phenylmethylsulfonyl fluoride: Sigma-Aldrich (St.Louis, Missouri)
- Potassium Cyanide: Sigma-Aldrich (St.Louis, Missouri)
- Potassium Phosphate (HPLC grade): Sigma-Aldrich (St.Louis, Missouri)
- Pyruvic Acid: Sigma-Aldrich (St.Louis, Missouri)
- Rhodamine B – conjugated anti-rabbit: Abcam (Cambridge, Massachusetts)
- **RNeasy Buffer**: Sigma-Aldrich (St.Louis, Missouri)
- **Sodium Chloride**: Sigma-Aldrich (St.Louis, Missouri)
- **Sodium Hydrosxide**: Fisher Scientific (Unionville, Ontario)
- **Silver Nitrate**: Sigma-Aldrich (St.Louis, Missouri)
- **Silver staining kit**: Bio-Rad Laboratories (Mississauga Ontario)
- **Succinic Acid**: Sigma-Aldrich (St.Louis, Missouri)
- **Sucrose**: Sigma-Aldrich (St.Louis, Missouri)
- **Sulfuric Acid, concentrated (HPLC grade)**: Sigma-Aldrich (St.Louis, Missouri)
- **TEMED**: Sigma-Aldrich (St.Louis, Missouri)
- **Tricine**: Sigma-Aldrich (St.Louis, Missouri)
- **Trizma Base**: Sigma-Aldrich (St.Louis, Missouri)
- **Trypsin**: Gibco (Carlsbad, California)
- **Trypan Blue**: Sigma-Aldrich (St.Louis, Missouri)
- **Tween 20**: Fisher Scientific (Unionville, Ontario)

2.2 – Equipment List (alphabetical order)

- **75 cm² collagen coated culture flasks**
- **96-well collagen coated plates**: Sarstedt (Montreal, Quebec)
- **175 cm² collagen coated culture flasks**: Sarstedt (Montreal, Quebec)
- **Accumet pH meter**: Fisher Scientific (Unionville, Ontario)
- **Alliance 2487 Dual Wavelength Absorbance Detector**: Waters Ltd. (Mississauga, Ontario)
- **Alliance 3695 separations module, High Performance Liquid**: Waters Ltd. (Mississauga, Ontario)
- **Axiocam HRm Digital Camera**: Zeiss Canada (Toronto, Ontario)
- **Axiovert 200M deconvoluting microscope**: Zeiss Canada (Toronto, Ontario)
- **Bio-Rad Mini-Protein II Dual Slab Cell**: Bio-Rad Laboratories (Mississauga Ontario)
- **Bright-line hemacytometer**: Hauser Scientific (Horsham, Pennsylvania)
- **Chemidoc XRS gel documentation system**: Bio-Rad Laboratories (Mississauga Ontario)
- **Compound Light Microscope CX2**: Olympus (America)
- **Confocal Microscope**: Nikon (Melville, New York)
- **2mL cryogenic vials**: Fisher Scientific (Unionville, Ontario)
- **Fisher model 500 sonic dismembrator**: Fisher Scientific (Unionville, Ontario)
- **Glass coverslips**: Fisher Scientific (Unionville, Ontario)
- **Gradient Former**: Fisher Scientific (Unionville, Ontario)
- **Haemocytometer**: Fisher Scientific (Unionville, Ontario)
- **HEPA class water jacketed CO₂ incubator**: Thermo Fisher Scientific (Waltham Massachusetts)
- **IEC Multi RF Refrigerated Centrifuge**: Fisher Scientific (Unionville, Ontario)
- **Odyssey® Infrared Imager**: Licor (Lincoln, Nebraska)
- **Olympus CX2 compound microscope**: Olympus America (Centre Valley, Pennsylvania)
- **Orion™ O₂ sensor**: Thermo Fisher Scientific (Waltham, Massachusetts)
- **35mm X 10mm Petri plates**: Sarstedt (Montreal, Quebec)
- **Sorvall Legend RT centrifuge**: Thermo Fisher Scientific (Waltham, Massachusetts)
- **Ultraspec 3100 Pro spectrophotometer**: Fisher Scientific (Unionville, Ontario)
- **Varian Gemini 2000 200MHz NMR spectrometer**: Varian (Palo Alto, California)
2.3 - Culturing of CCF-STTG1 (Astrocytes) and HepG2 (Hepatocytes) cells

2.3.1 - Astrocytes (CCF-STTG1):

The human astrocytoma cell line (CCF-STTG1) was acquired from the ATCC, Manassas, Virginia, USA. CFF-STTG1 cells are an adherent cell line established from a grade IV astrocytoma. 70–80% of cells in culture are found to be positive for glial fibrillary acidic protein (GFAP). The usefulness of this cell line is derived from its maintenance of normal astrocytic properties (200). The astrocytic cell line was sustained in α-Minimum Eagle Media (MEM) supplemented with 5% Fetal Bovine Serum (FBS) and 1% antibiotics (streptomycin, and penicillin). CCF-STTG1 cells were seeded at $1.0 \times 10^5$ cells/mL in 175 cm$^2$ culture flasks ($2 \times 10^6$ cells/flask), maintained in an incubator with 5% CO$_2$ in a humidified atmosphere operating at 37°C (66, 191, 201).

2.3.2 - Hepatocytes (HepG2):

HepG2 cells were a gift from Dr. Templeton (University of Toronto). It is a commonly utilized model system to study hepatic metabolism (202). The hepatic cell line was sustained in α-MEM supplemented with 5% FBS and 1% antibiotics (streptomycin, and penicillin). HepG2 cells were seeded at $1.0 \times 10^5$ cells/mL in 175 cm$^2$ culture flasks ($2 \times 10^6$ cells/flask), maintained in an incubator with 5% CO$_2$ in a humidified atmosphere operating at 37°C (160, 163).

2.3.3 - Culture Initiation and Passaging:

The cell cultures (both CCF-STTG1 and HepG2) were initiated by thawing the cells from cryogenic storage. The cryogenic vial was centrifuged at 150xg to remove
the cryogenic freezing medium and pellet the cells. The cellular pellet was then resuspended in α-MEM + 5% FBS and seeded in a 75cm² collagen-coated tissue culture flask and grown until confluence (7 days = CCF-STTG1, 6 days = HepG2). Cultures could then be routinely passaged from this initial culture by removing the adherent cells via trypsinization (9mL of PBS + 1mL of 10X trypsin to a final concentration of 1X trypsin) and diluting the cells to 1.0 X 10⁵ cells/mL in α-MEM + 5% FBS. The cell dilution was then introduced into a new 175cm² collagen-coated tissue flask containing 20mL total volume of culture media (α-MEM + 5% FBS). This dilution of cells maintained optimal growth conditions. The cell concentration was determined by the TBEA (203). The cells were passaged every 6 and 7 days to avoid the accumulation of waste products and to provide fresh nutrients to the HepG2 cells and CCF-STTG1 cells respectively. Each culture was maintained until passage number was 50 for the HepG2 and 35 for the CCF-STTG1 cells. Figure 2.1 provides an outline of the cell culturing and passage procedures.
Cryovial
= 2 X 10^6 cells

i) Thaw cells
ii) Remove storage media

75cm² flask
- Resuspend cells in
  10 mL of media

~ 6 days (HepG2)
~ 7 days (CCF-STTG1)

175cm² flask
- Resuspend cells in
  20 mL of media

Figure 2.1 – Synopsis of cell culturing. 2.0 X 10^6 cells are removed from a cryovial and resuspended in 10mL of fresh media in a 75cm² flask. Upon reaching confluence (6 days = HepG2; 7 days = CCF-STTG1), the cells are counted and seeded at 1.0 X 10^5 cells/mL in a 175cm² flask containing 20mL of media.

2.3.4 - Cell Storage Procedure:

Beginning from passage number one, the cells were cultured until confluence and isolated for cryogenic storage. The cells were detached from the flask by trypsinization. Following detachment, the cells were centrifuged at 150xg for 10min at 4°C. The cells were centrifuged at low speed to avoid damage to the plasma membrane. The supernatant was removed and the cellular pellet was rinsed twice with ice cold PBS [(136mM sodium chloride, 2.5mM potassium chloride, 1.83mM dibasic sodium phosphate, and 0.43mM monobasic potassium phosphate) pH 7.4]. The rinsed pellet was then resuspended in 2mL of DMSO containing cell storage
media (Gibco) and aliquoted into 2mL cryovials (Fisher). Each aliquot contained 2.0 X 10⁶ cells. Cell aliquots were frozen gradually, first at -20°C for 4h followed by 2 days at -80°C prior to cryogenic storage to avoid rapid freeze fracturing of the cells.

2.4 – Cell treatment and Viability

When a confluency of 75% was reached (culture day 5 for HepG2 cells; day 6 for CCF-STTG1 cells), the cell monolayer was washed with PBS. The astrocytic cells were re-supplemented with serum-free media containing 2.5 mM lactate chelated to varying amounts of Al (0.01mM – 0.1mM)¹ or 2.5 mM lactate with H₂O₂ (20μM, 40 μM, 80μM, and 100μM). Cells exposed to lactate (2.5mM) alone served as the control (191, 201). Some experiments required different concentrations of lactate utilization; 1mM and 5mM. HepG2 cells were resupplemented with serum-free media containing 2.5 mM citrate complexed to varying amounts of Al (0.01mM – 0.25 mM) or 2.5 mM citrate with 40μM H₂O₂. Cells exposed to citrate (2.5mM) alone served as the control (160, 163, 191). Serum-free media was used when introducing the stress, as it is common practice in toxicological studies. The absence of FBS from cell cultures is performed to maximize the observed effect of the toxicological agent (204).

Cells were tested for viability using the TBEA. Viability was assessed over a 10 day period by counting live cells and dead cells with a haemocytometer and a compound microscope (Olympus) operating at 100X magnification. Trypan blue

<table>
<thead>
<tr>
<th>1- Al concentration in tissues:</th>
<th>Intake:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum = 0.75mg/mL</td>
<td>Dietary: Canada = 8mg/day</td>
</tr>
<tr>
<td>Brain = 0.60 mg/mL</td>
<td>World Avg= 3-100mg/day</td>
</tr>
<tr>
<td>Aluminum Bone Disease = 0.40mg/mL</td>
<td>Inhalation: Polluted regions = 0.1mg/day</td>
</tr>
<tr>
<td></td>
<td>Occupational = 10mg/day</td>
</tr>
</tbody>
</table>

Srivastava R.A. et al; *Journal of Neurological Science*. 196 (2002); p. 45-52
Nayak Prasunpriya et al; *Environmental Research* 89 (2002) 101-115
Aluminum; Operational Guidance Value, 1998; *Health and Welfare Canada*
solution works by staining cells without an intact membrane (dead cells) with a blue colour. This allows for the discrimination between live cells and dead cells (total cells = # live cells + # of dead cells). The TBEA was therefore an effective method for determining the effects of the Al and H₂O₂ stress on cell viability.

2.5 – Metabolite Supplementation and Recovery Experiments:

To perform recovery experiments, after the 24h treatment period, the cells were incubated with serum-free media resupplemented containing the various recovery metabolites (Figure 2.2).

![Diagram showing the process of metabolite supplementation and recovery experiments]

**Figure 2.2 – Overview of cell treatments and recovery experiments.** Once cells reached 75% confluence the media was discarded. New serum-free media containing various treatments were introduced to the flask. Following a 24h treatment time, the media was collected for various analyses. The cells were then resupplemented with serum-free media containing various metabolites for recovery experiments. The cells were then exposed to the recovery media for various times. Following the recovery-media exposure the media was collected along with the treated cells for different analyses.
For recovery with α-ketoacids, the serum-free media was supplemented with 5mM KG or 5mM pyruvate for 8h. Other anti-oxidant recoveries were done with 1mM NAC for 24h. To assess the potential of L-carnitine to exert an anti-oxidant effect, cells were recovered with 5mM L-carnitine for 8h. Experiments carried out to determine the loss of the β-oxidative capacity of HepG2 cells, were performed by incubating the cells with 2mM palmitic acid for 24h following the stress treatment. The palmitic acid (100mM in 95% ethanol) was diluted 1:25 in PBS containing 20% fatty acid-free BSA (w/v) (205). Controls were performed using palmitate-free BSA. The cells and spent fluid were isolated at various time intervals for different biochemical analyses.

Glutamate uptake experiments were performed by incubating astrocytes (following their treatment with control, Al, or H₂O₂ containing media) in a serum-free media containing 5mM glutamate for 2h. The spent medium was then collected and the astrocytes were harvested.

2.6 - Fluorescence Microscopic Techniques:

For fluorescence microscopy, cells were grown on coverslips in a 35mm X 10mm petri plate. The cells were seeded at 1 X 10⁵ cells/mL in 2mL of α-MEM + 5% FBS. Once the cells grew to a minimal density (2-3 days), cells were exposed to treatment media as described previously. The cells were the treated for microscopic analysis.

2.6.1 - Intracellular Aluminum and the Morin Stain:
CCF-STTG1 cells (astrocytes) were grown to a minimal density on coverslips and then subsequently treated with serum-free media containing control and 0.1mM Al. Following a 24h treatment, the coverslips were washed twice with PBS and once with 0.5mM EDTA to remove any Al bound to the outermembrane of the cell. The astrocytes were fixed for 5min with a methanol:glacial acetic acid solution (3:1 v/v), followed by 5min of air drying. The coverslip was then washed 1X with PBS. The counterstain, Hoechst 33528 (nuclear stain) was then introduced for 10min (2.5μg/mL in PBS). The Hoechst 33528 was then removed and the coverslip was washed 3X with PBS. The coverslips were then treated with 0.1 mg/mL of Morin reagent [prepared in methanol:acetic acid 3:1 (v/v)] for 10min (206). The Morin stain was rinsed from the coverslips with PBS 3 times. Following the probing of the coverslips with the fluorophores, the coverslips were mounted onto microscopy slides using 1μL of mounting solution (Fisher) and then sealed with nail polish. When Morin reagent complexes with a trivalent metal it generates a green fluorescence that can be detected at λ_{excitation} = 425nm and λ_{emission} = 520nm. Hoechst 33528 was visualized at λ_{excitation} = 355nm and λ_{emission} = 465nm. Fluorescence was observed using a deconvolution microscope at 60X ocular magnification and in the dark to prevent photobleaching.

2.6.2 – Detection of Intracellular ROS:

Astroglia were grown to a minimal density on coverslips and treated with control, Al, and H2O2 conditions as described before. The coverslips were washed with 0.5mM EDTA and PBS and prepared for microscopic examination as described previously. For the detection of ROS levels within the astrocytes, the cells were
incubated with 20 μM of DCFDA in α-MEM and 10% FBS for 1h at 37°C. The coverslip was then washed 2X with PBS. The counterstain for the nucleus, Hoechst 33528 was then introduced for 10min (2.5μg/mL in PBS). The Hoechst 33528 was then removed and the coverslip was washed 3X with PBS. The coverslips were then mounted onto slides and deconvolution microscopy was performed at 40X and 60X ocular magnification. Fluorescence from DCFDA staining was visualized at $\lambda_{\text{excitation}} = 484\text{nm}$ and $\lambda_{\text{emission}} = 530\text{nm}$. Hoechst 33528 was visualized at $\lambda_{\text{excitation}} = 355\text{nm}$ and $\lambda_{\text{emission}} = 465\text{nm}$.

2.6.3 - Visualization of the Cytoskeleton of CCF-STT1 cells:

Primary analysis of astrocytic morphology was analyzed by phase contrast microscopy, using a Nikon confocal microscope at 20X ocular objective. For visualization of the actin cytoskeletal framework of the cell, following the fixing of the cells (as discussed previously), the slides were stained with phalloidin (1.5mg/mL) conjugated to FITC or Rhodamine B for a period of 20min. The status of intermediate filaments was made apparent by incubating cells in a Cy3 conjugated GFAP primary antibody. The cover slip was submerged in TTBS [Tween 20 0.1% (v/v) (20mM Tris-HCl, 0.8% (w/v) NaCl) at pH 7.6] with 5% FBS for 1h to block non-specific binding sites. The coverslip was then rinsed thrice with TBS. The coverslips were subsequently incubated in Cy3-GFAP (1/800) diluted TBS/5% FBS solution for a period of 45min. The cells were again treated with Hoechst 33528 as the counterstain. The coverslips were then mounted on to microscope slides and deconvolution microscopy was performed at 60X ocular magnification to discern the intricacies of the cytoskeleton under control and stress conditions. Fluorescence of
Cy3 was detected at at $\lambda_{\text{excitation}} = 550\text{nm}$ and $\lambda_{\text{emission}} = 570\text{nm}$, FITC at $\lambda_{\text{excitation}} = 495\text{nm}$ and $\lambda_{\text{emission}} = 520\text{nm}$, and Rhodamine B at $\lambda_{\text{excitation}} = 546\text{nm}$ and $\lambda_{\text{emission}} = 620\text{nm}$ respectively.

2.6.4 - Immunofluorescence Studies to Localize and Quantify Intracellular Proteins:

The cellular location and expression levels of CK, Profilin-2, and LDH were visualized by probing the cells with fluorescently labeled antibodies. Astrocytes were grown to a minimal density and exposed to control and stress media as described previously. The cells were washed, fixed, and counterstained with Hoechst 33528 as previously described. The coverslips were then washed with TBS twice prior to gently agitating them in TTBS containing 5% FBS for 1h. Treatment with tween-20 (a detergent) aids in permeabilizing the cell membrane to facilitate the uptake of the antibodies, while FBS acts to block non-specific binding sites. The blocking solution was then removed and the coverslips were washed three times in TBS for 5min. The coverslips were then incubated in the primary antibodies diluted in 2mL of TTBS containing 5% FBS for 2h. Excess antibodies were washed off the coverslip 3 times with TBS at 5min intervals. The secondary antibodies were then introduced with respective secondary antibodies conjugated to FITC. The secondary antibody binds to epitope on the primary antibodies (Table 2.1).

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-LDH</td>
<td>1/750</td>
<td>Anti-goat (FITC)</td>
<td>1/1000</td>
</tr>
</tbody>
</table>

Table 2.1 – Antibody incubation for immunofluorescence microscopy. A list of primary and secondary antibodies used for immunofluorescent microscopic analysis and their respective dilutions.
| Anti-CK (BB)  | 1/800 | Anti-mouse (FITC) | 1/1000 |
| Brain Isoform |       |                  |        |
| Anti-Profilin-2 | 1/800 | Anti-rabbit (FITC) | 1/1000 |

The secondary antibodies were washed away 3X with TBS to ensure that there was no background fluorescence. Green fluorescence from FITC was detected at $\lambda_{\text{excitation}} = 495\text{nm}$ and $\lambda_{\text{emission}} = 520\text{nm}$.

Co-localization of LDH with the mitochondria was performed by incubating the coverslips with rhodamine B (10µg/mL in 2mL of α-MEM containing 5%FBS) for 30min at 37°C following the PBS/EDTA washing step (described previously). Rhodamine B fluoresces when it is protonated by the acidic gradient created by an active mitochondria (207). The presence of a yellow fluorescence becomes evident as the green (FITC) overlaps with the red (rhodamine B). Similarly, co-localization of CK and profilin-2 (FITC) with the cytoskeleton (rhodamine B) is detectable (Figure 2.3).
2.6.5 Detection of Intracellular Lipid Droplets:

To detect lipids inside the HepG2 cells and astrocytes, Oil Red O (0.25% w/v in isopropyl alcohol) staining was utilized. Cells were treated as described previously. Following treatment, the cells were washed and fixed in the same manner as described previously. Oil Red O solution was diluted to 5% in PBS and introduced to the coverslips for 10min at 37°C. Upon the interaction of Oil Red O with triglycerides a red fluorescence could be detected at $\lambda_{\text{excitation}} = 563\text{nm}$ and $\lambda_{\text{emission}} = 650\text{nm}$ (208) using a deconvolution microscope at an ocular magnification of 40X. Hoechst 33528 was utilized as a counterstain.
2.6.6 – Visualization of Glycogen Granules:

Glycogen accumulation in astrocytes was detected using the periodic acid schiff base staining procedure (209). After the 24h exposure to control, Al, and H₂O₂ containing media, the cells were resupplemented with serum-free media containing 10mM D-glucose for 24h. The media was then discarded and the coverslips were washed 3X with PBS and once with 0.5mM EDTA to remove the excess D-glucose. The cells were then fixed to the coverslip as aforementioned, and then treated with 5% (v/v) of periodic acid solution for 5min. The periodic acid produces 2,3 aldehydic groups on D-glucose molecules. The coverslip was washed 5X with ddH₂O and the non-specific binding sites, such as aldehydes on exopolysaccharides and glycoproteins, were blocked with 5% (w/v) dimedone solution (note: dimedone does not react with glycogen granules). The dimedone is then washed with 4X ddH₂O rinses. A 10min incubation with the Schiff reagent was then used to stain the coverslips. The slides were then mounted onto coverslips as described previously. Bright field deconvolution microscopy was performed using the Red-Green-Blue option at 20X ocular magnification. Hoechst was used as a counterstain.

2.7 – Cellular Isolation and Subcellular Fractionation

CCF-STTG1 and HepG2 cells were treated with control, Al, and H₂O₂ containing media. CCF-STTG1 cells (astrocytes) required detachment from the flask using trypsinization as they are an adherent cell line. Briefly, 10X trypsin was diluted to 1X in PBS (2mL of 10X trypsin in 18mL of PBS), and introduced to the flask. Detachment of the astrocytes required 5min of incubation with the 1X trypsin.
Following trypsinization, the solution was discarded and the astrocytes were washed off the flask surface with 20mL of α-MEM + 5% FBS. The astrocytes were incubated at 37°C for 5min to allow for the cells to detach. HepG2 cells are adherent but were removed from the surface of the flask with a gentle tapping of the flask while constantly swirling PBS on the cell monolayer.

Following the removal of the cells from the flask surface, the cells were collected and placed in centrifuge tubes. The cells were then pelleted by centrifugation at 150Xg for 10min at 4°C. The pellet was washed twice in ice-cold PBS and then resuspended in CSB (50mM Tris-HCl, 1mM phenylmethylsulfonylfluoride, 1mM dithiothreitol, 250mM sucrose, 1mg/mL of pepstatin, 0.1mg/mL leupeptin, and 2mM citrate) and stored at -86°C.

When cells were needed, they were thawed from the -86°C storage and centrifuged at 150Xg for 10min at 4°C to pellet the cells. The cells were then resuspended in ice cold CSB (20μL/4.0 X 10^6 cells). The cell suspension was then homogenized on ice with a sonic dismembrator operating at an amplitude of 30% for the HepG2 cells, and 33% for the astrocytes. The suspension was sonicated 4 times for 10sec in 1sec bursts to disrupt the cell membrane. The cell suspension was kept on ice for 5min between each round of sonication to avoid damaging intracellular organelles and proteins. Following the sonication, the cell suspension was divided into subcellular fractions by using differential centrifugation (210-212).

Whole cells were first removed by centrifugation at 150Xg for 10min at 4°C to ensure a CFE. The pellet (whole cells) was discarded and the supernatant (CFE) was kept and subsequently centrifuged at 850Xg for 20min at 4°C to produce a pellet.
containing the nucleus. The supernatant was further centrifuged at 12,000Xg for 2h at 4°C to precipitate the mitochondria. The supernatant was finally centrifuged at 180,000Xg for 3h at 4°C to remove microsomes and ribosomes to obtain a “pure” cytosolic fraction. The nuclear and mitochondrial fraction was suspended in a minimal amount of CSB (20μL). These fractions could then be utilized for proteomic and metabolomics analyses. The purity of the fractions was confirmed by employing immunoblotting techniques for F-actin (cytosol), VDAC (mitochondria), and Lamin B or histone 2A (nucleus) (Figure 2.4).

Figure 2.4 – Cellular and Sub-cellular isolation procedure. An overview of the utilization of differential centrifugation to separate the compartments of the cell.
Subfractionation of the mitochondria involved treatment of the mitochondrial fraction with 1% digitonin for 30 min on ice, proceeded by high speed centrifugation at 10,000 g for 30 min at 4°C (213). The pellet consisted of the inner mitochondrial membrane and the matrix while the supernatant contained the outer membrane and the intermembrane space. The Bradford assay was utilized to quantitate the protein content and BSA was utilized as the standard (214). The homogeneity of the sub-fractions of the mitochondria were confirmed utilizing cytochrome C antibodies (Abcam) for the outer membrane/inner membrane space portion, while succinate dehydrogenase antibodies (a gift from Dr. Lemire, University of Alberta) were used for the inner membrane/matrix portion of the mitochondria.

2.8 – Measuring Oxidized Products in Al and ROS Treated Cells

2.8.1 – Oxidized Proteins:

Carbonyl content on proteins was assessed using the DNPH assay (215). Cells were treated, isolated, and the CFE was obtained as described in the previous sections. The CFE was then centrifuged at 180,000Xg for 3h to separate the soluble fraction from the membrane fraction. The soluble fraction was collected and mixed with 1mL of 2% (w/v) DNPH, followed by a 1h incubation. Two hundred microlitres of 50% (w/v) trichloroacetic acid was then added to the samples to precipitate the proteins. The precipitated proteins were then pelleted at 12,000Xg for 10min. The supernatant was decanted and the protein pellet was washed 3X with 10% (w/v) trichloroacetic acid solution. Following this washing step, the protein pellet was
further washed 3X in an ethylacetate:ethanol solution (1:1 v/v). The pellet was subsequently dissolved in 1mL of 6M guanidine-HCl and the absorbance was measured at 370nm using a spectrophotometer. The blanks for the spectrophotometry were prepared by making the solution as described previously without the addition of soluble protein. Negative controls were performed by making the solutions as described above without the addition of DNPH. The amount of oxidized protein was determined by using the extinction coefficient for hydrazones (ε = 21.5 nM⁻¹ • cm⁻¹).

2.8.2 – Oxidized Lipids:

Quantifying the level of oxidized lipids was accomplished by performing the TBARS assay (216). Approximately 4.0 X 10⁶ cells were isolated from each treatment and CFE was obtained as described previously. The membrane fraction was generated by centrifugation (as described above), and subsequently solubilized in a solution of 15% (w/v) trichloroacetic acid, 0.375% (w/v) trichloroacetic acid, and 0.25N HCl with a final volume of 1mL. The solubilized membrane mixture was heated at 95°C for 25min. When a pink-like colour developed, the membrane solutions were centrifuged at 21,000Xg for 10min. The supernatant was collected and absorbance was measured at 532nm using a spectrophotometer. Spectrophotometry blanks consisted of reaction solutions without membrane. Negative controls were performed devoid of trichloroacetic acid from the solution. The amount of oxidized lipids was calculated using the extinction coefficient (ε = 1.56 X 10⁵ M⁻¹ • cm⁻¹).
2.9 - HPLC Studies

Samples were filtered and diluted into a screw top HPLC vial. The dilution was 1 to 5, sample:mobile phase. The sample vials were then placed into the automated sampler of the HPLC. The EMPOWER software interface allowed for programming of the automatic injection protocol for the HPLC. The injection protocol consisted of 1) clearing the previous calibration 2) a 90min column equilibration, and 3) the sample set injection. Each sample preparation was injected at a volume of 10µL. The HPLC was calibrated by injecting varying concentrations of standard compounds (a 5-point calibration). The column was selected depending on the experiment, as was the mobile phase, and flow rate. The metabolites were detected using a UV-Vis dual wavelength detector operating at 210nm to detect C to O conjugation, and 254 to discriminate C to N conjugation. The metabolite detection produced peaks which were quantified using the EMPOWER software suite which accompanied the HPLC unit. The peaks were compared to known standard solutions (Appendix 7.4). Additionally, samples could be spiked with the suspected metabolite to confirm its identity or the sample could be collected at the given retention time and confirmed using enzyme specific reactions. The HPLC was cleaned weekly by running 100% HPLC grade isopropanol for 2h. This kept contamination low and inhibited bacterial growth.

2.9.1 - Spent Fluid Analysis:

To measure the metabolite profile of the spent media, the media were collected at various time points before, during, and after the treatment periods. These were treated with a 0.5% (v/v) solution of perchloric acid and mobile phase
(dependent on running conditions). The perchloric acid facilitated the precipitation of the protein in the spent fluid. Samples were placed on ice for 10min to allow for the precipitation to occur. The media was then centrifuged at 12,000Xg for 10min to remove the precipitated protein. The protein content of the spent fluid was then analyzed by SDS-PAGE and silver staining procedure (described later). The supernatant was filtered and diluted (1:5 in mobile phase) into screw top HPLC vials and loaded into the automated sampler in the HPLC. The mobile phase used for the spent fluid was 2.5mM H₂SO₄. The samples were run through a Rezex™ organic acid column operating at an elution rate of 0.6 mL/min at ambient temperature. The organic acids in the spent fluid were quantified at 210nm.

2.9.2 – HPLC Analysis of the CFE, Mitochondria, Nucleus, and Cytoplasm:

CFE was prepared by breaking open whole cells using sonication as described previously. Mitochondria, nucleus, and cytoplasm were obtained by differential centrifugation, again, as described previously. The protein content of each subcellular fraction and the CFE was ascertained by the Bradford assay (214). The CFE, mitochondria, nucleus, and cytoplasm obtained from control, Al and H₂O₂ culture conditions were normalized to 2mg/mL in mobile phase. When organic acids were the desired metabolites to analyze the mobile phase consisted of 20mM HPLC grade KH₂PO₄, pH 2.9, prepared in MilliQwater. When nucleotide analysis was required the mobile phase consisted of 20mM HPLC grade KH₂PO₄, 5% HPLC grade acetonitrile, pH 7.0, prepared in MilliQwater. The prepared samples were run through a C₁₈ reverse phase column with a polar cap from Phenomenex ® operating at a flow rate of 0.7mL/min for organic acids and 0.2mL/min at 26°C. The organic
acids were discriminated at 210nm and nucleotides at 254nm in the UV-Vis detector.

2.9.3 – Mitochondrial Reactions:

To measure mitochondrial lactate consumption, mitochondria isolated from control, Al and H₂O₂ treated CCF-STTG1 and HepG2 cells (2mg/mL protein equivalent), were incubated in a phosphate buffer [10 mM phosphate, 5mM MgCl₂ (pH 7.4)] containing 5mM lactate or 5mM citrate, 0.1mM NAD for varying time intervals at 37°C. To inhibit LDH 10mM oxamate were also added in select experiments (54, 66, 217). The reaction was stopped via boiling of the samples for 10min. The organic acids and nucleotides were subsequently extracted for HPLC analysis. The resultant suspension from the reaction was analyzed using a C₁₈-reverse phase column with the aid of an Alliance HPLC. The mobile phase utilized consisted of 20mM KH₂PO₄ (pH 2.9 with 6N HCl), operating at an elution rate of 0.7 mL/min at ambient temperature. To analyze nicotinamide nucleotide levels, the mobile phase was altered to a 20mM KH₂PO₄ (pH 7.0 with 6N HCl) containing 5% acetonitrile to accurately measure NAD(H) levels (218). The identities of the metabolites were compared with known standards, and the reaction mixtures were spiked with the appropriate standard metabolites. The initial levels of metabolites were obtained by running reaction mixtures at time zero. For confirmation of lactate consumption by the mitochondria, mitochondria isolated from the CCF-STTG1 cells were incubated in phosphate buffer including 10mM of 3-¹³C lactate, and 0.1mM NAD. To monitor TCA cycle intermediate accumulation, 1µM of NaN₃ was added to this reaction mixture. Aliquots were collected at varying time intervals, boiled and
analyzed. To make certain the metabolites being observed were indeed native to the TCA cycle, aliquots were collected during HPLC at the given retention times and lyophilized. Subsequently, enzyme specific assays were performed on the samples to confirm metabolite identity. To confirm citrate, 200μL of sample and 10 units of aconitase were placed in equilibration buffer [25 mM Tris-HCl, 5 mM MgCl₂ (pH 7.4)] and the formation of cis-aconitate was measured at 220nm. For the confirmation of fumarate, 200μL of sample and 10 units of fumarase were added to equilibration buffer and the disappearance of fumarate was measured at 220 nm. Succinate confirmation was ascertained utilizing 200μL of sample, 60μg/mL of membrane fraction collected from *P. fluorescens*, and 2.5mg/mL of DCIP in equilibration buffer. The oxidation of DCIP was measured at 500nm (66, 167).

The fate of pyruvate as an anti-oxidant in the mitochondria involved isolating control, Al and H₂O₂ mitochondria (2mg/mL protein equivalent) from the astrocytes and incubating them in a phosphate reaction buffer containing 5mM pyruvate, 5mM citrate, 0.1mM NAD for varying time intervals at 37°C. Boiling for 10min halted the reaction and the reaction mixture was then collected to assess pyruvate consumption and acetate production by the methodology previously described for HPLC of organic acids.

2.9.4 – Special HPLC Procedures for L-Carnitine and Palmitate:

Total carnitine was measured by a modified method described in (219). Briefly, the soluble cell free extracts were subjected to a 1:4 digestion in 1M KOH diluted in methanol for 60 min at 50°C to remove the acyl groups from the carnitine (219). The hydrolyzed carnitine was then injected a C₁₈-reverse phase column
working at a flow rate of 0.2mL/min. The mobile phase consisted of 20mM KH$_2$PO$_4$ (pH 7.0 with 6N HCl) and 20% acetonitrile \( (191) \). Free L-carnitine levels were measured using tandem HPLC analysis. L-carnitine and its derivatives were separated at a flow rate of 0.7mL/min, at a retention time of 4.8 – 5.2 min. The samples were collected and then subsequently reinjected in a C$_{18}$-reverse phase column working at a flow rate of 0.2mL/min. The mobile phase consisted of 20mM KH$_2$PO$_4$ (pH 2.9 with 6N HCl) and 20% isopropanol.

Palmitate content was measured by isolating the cytoplasm by centrifugation at 180,000g for 2h to remove any membranes. Following an extraction with hexane, the organic layer was analyzed for palmitate \( (220) \). The mobile phase utilized was 95% hexane: 5% isopropanol. Fatty acids were detected at 210nm. The separated metabolites were compared with known standards, and the metabolite mixtures were spiked with the given metabolites to confirm peak identities \( (191) \).

**2.10 - $^{13}$C-NMR Analysis of Lactate Consumption**

To evaluate the metabolism of lactate by the mitochondria, 2mg/mL protein equivalent of mitochondria was incubated in phosphate buffer including 10mM of 3-$^{13}$C lactate, and 0.1mM NAD. 1µM of NaN$_3$ was added to the reaction mixture to monitor the accumulation of TCA cycle intermediates \( (66) \). Reactions were performed in 1.5mL microcentrifuge tubes to a final volume of 250µL. The reaction mixture was mixed gently to ensure distribution of the cells. Samples were collected at various time points and the reactions were halted by heating the reactions to 100°C for 10min. To validate that the pyruvate interacts with ROS to produce
acetate, 10mM 3-\(^{13}\)C-pyruvate was incubated with 1mM H\(_2\)O\(_2\) for 1h in D\(_2\)O. NMR analyses were performed using a Varian Gemini 2000 spectrometer operating at 50.38 MHz for \(^{13}\)C (66, 185). Samples were analyzed with a 5mm dual probe (35° pulse, 1-s relaxation delay, 8 kilobytes of data, and 2000 scans). Chemical shifts were referenced to standard compounds under analogous conditions.

### 2.11 - Native Polyacrylamide Gel Electrophoresis (PAGE) Studies

#### 2.11.1 – Preparing BN-PAGE Gels:

Native PAGE gels were performed by a modified method described in (221). Mini-gels (8 X 7 cm\(^2\)) were cast using 0.75 or 1mm spacers using the Bio-Rad MiniProtean™ 2 system. To separate cytosolic, mitochondria, and nuclear proteins under native conditions, 4-16% linear gradient separating gels were utilized (Table 2.2).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>16%</th>
<th>4%</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>1874μL</td>
<td>234μL</td>
<td>273μL</td>
</tr>
<tr>
<td>(48% Acrylamide + 1.5% Bisacrylamide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3X BN Buffer</td>
<td>967μL</td>
<td>967μL</td>
<td>1136μL</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>664μL</td>
<td>169μL</td>
<td>2000μL</td>
</tr>
<tr>
<td>75% (v/v) Glycerol</td>
<td>773μL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>7.4μL</td>
<td>9.4μL</td>
<td>50μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>3μL</td>
<td>3μL</td>
<td>10μL</td>
</tr>
</tbody>
</table>

The 2.9mL of the 4% acrylamide gel was made in a solution separately from the 2.9mL of the 16% gel. The two gels were then mixed to yield a functional 4-16% linear gradient gel (resolving gel). The gradient was formed using a gradient former.
and peristaltic pump. When the gel had solidified, the stacking gel was poured over it to provide the wells where the proteins would be loaded. The stacking gel function to stack the proteins while the resolving gel acted to separate out the protein based on molecular masses.

The samples were then introduced into their respective wells along with molecular mass standards (Apo-ferritin = 480KDa, BSA = 120KDa, and 66KDa). The addition of the molecular weight standards ensured the gel was resolving appropriately. The gel cassettes were then assembled in the Bio-Rad MiniProtean™ 2 system. Following the loading of the wells, the gel was overlaid with blue cathode buffer or a ponceau S cathode buffer (both termed coloured cathode). Different cathode buffers are used to optimize the in-gel activity of certain enzymes (222). Enzymes that are involved in hydrolytic reaction yield activity bands when ponceau S is used as the cathode buffer (223). Enzymes such as glutamine synthase and citrate lyase are hydrolytic and require the use of ponceau S cathode. The inner chamber was then filled with the coloured cathode buffer and the outer chamber of the electrophoresis unit was filled with anode buffer.

The electrophoresis was performed at 4°C to ensure the protein maintained their native state. Eighty volts were applied to the gel until the protein penetrated from the stacking gel into the resolving gel. Upon reaching the resolving gel the voltage of the electrophoresis unit was increased to 150 volts, ensuring that the amperage did not exceed 15 milliamps. When the proteins had run halfway through the gel, the coloured cathode was replaced with a colourless cathode. The removal of the coloured cathode and its replacement with colourless cathode allowed for
partial removal of the stain, so activity bands could be visualized more readily.

When the proteins began to run out of the bottom of the gel the electrophoresis was halted.

2.11.2 - Native PAGE buffers:

**Blue Cathode Buffer (1L)**
- 8.96g of Tricine (50mM)
- 3.14g of BisTris (15mM)
- 0.2g of Coomassie G-250
- pH 7 at 4°C

**Ponceau S Cathode Buffer (1L)**
- 3.14g of BisTris (15mM)
- 0.00135g of Ponceau S
- 0.01g of Taurodexoycholic acid
- pH 7 at 4°C

**Colourless Cathode Buffer (1L)**
- 8.96g of Tricine (50mM)
- 3.14g of BisTris (15mM)
- pH 7 at 4°C

**3X BN Buffer (50mL)**
- 9.84g of 6-aminohexanoic acid (1.5M)
- 1.57g of BisTris (150mM)
- pH 7 at 4°C

**Anode Buffer (1L)**
- 10.45g of BisTris
- pH 7 at 4°C

**49.5% Acrylamide (100mL)**
- 48g of acrylamide
- 1.5g of Bisacrylamide

2.11.3 - In-gel Activity Staining:

Cytosolic proteins were prepared in BN buffer [500mM 6-aminohexanoic acid, 50mM BisTris (pH 7.0 at 4°C)]. Membrane proteins from the mitochondria were prepared in BN buffer with 1% β-dodecyl-D-maltoside for 30min on ice prior to storage. The maltoside is a mild detergent that helps solubilize the membrane-bound enzymes while maintaining their activity. Nuclear proteins were prepared much like mitochondrial proteins, however 1% DNase and RNase were added to the sample preparation to digest nucleic acids. The samples were prepared at 2mg/mL in a final volume of 200μL. Thirty micrograms of protein was loaded into the wells for electrophoresis. The electrophoresis unit was placed in a refrigerator at 4°C.
The electrophoresis took place as described previously. Following the completion of the electrophoresis, the gel slab was removed from the electrophoresis unit and equilibrated in a reaction buffer [25mM Tris, 5mM MgCl₂ (pH 7.0)] to wash off the buffer salts, for a period of 15-30min. The gels were then placed into labeled reaction vessels at 37°C (in the absence of light) along with a reaction mixture that consisted of the reaction substrates, cofactors, and coupling enzymes to a final volume of 1.5mL (per lane) in reaction buffer for the desired reaction. In-gel activity was visualized by various methods depending on the enzyme. Upon the appearance of an in-gel activity band, the gel(s) were placed in a destaining solution (50% methanol, 10% glacial acetic acid, 40% ddH₂O). The destaining solution facilitated the halting of the enzyme reaction as well as the removal of the colour leftover from the coloured cathode buffer. This helped yield activity bands on a clear gel. The gels were then be documented and the activity bands were quantified using Scion imaging for Windows (SCION Corporation, Frederick, MD). An additional gel was run with the control, Al, and H₂O₂ protein samples from each fraction, which was stained with Coomassie R-250 to ensure equal loading of protein. A molecular mass standard (Apo-ferritin = 480KDa, BSA = 120KDa, and 66KDa) was also ran and stained with Coomassie R-250 to ensure the BN gel ran properly. All reactions were performed in triplicate. A brief overview of the enzymes measured by BN-PAGE is given in Table 2.3.
Table 2.3 – Brief overview of enzymes measured using BN-PAGE technique. The in-gel activity was measured for these following enzymes. The detection method is present but is elaborated on in previous sections.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Analysis Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP-ICDH</td>
<td>NADP reduction</td>
</tr>
<tr>
<td>KGDH</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>ME</td>
<td>NADP reduction</td>
</tr>
<tr>
<td>LDH</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>SDH</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>Complex 4</td>
<td>Diaminobenzidine reduction</td>
</tr>
<tr>
<td>CK</td>
<td>G6PDH linked</td>
</tr>
<tr>
<td>NDPK</td>
<td>G6PDH linked</td>
</tr>
<tr>
<td>PDE</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>CL</td>
<td>MDH linked</td>
</tr>
<tr>
<td>ACC</td>
<td>Phosphate precipitation</td>
</tr>
<tr>
<td>ACS</td>
<td>Phosphate precipitation</td>
</tr>
<tr>
<td>ERBADDH</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>BBDOX</td>
<td>Vitamin C reduction</td>
</tr>
<tr>
<td>G6PD</td>
<td>NAD reduction</td>
</tr>
<tr>
<td>GluS</td>
<td>GDH linked</td>
</tr>
<tr>
<td>G6S</td>
<td>GDH linked</td>
</tr>
</tbody>
</table>

2.11.4 -NAD(P)H-generating Enzymes (LDH, NAD(P)-ICDH, KGDH, ME, and MDH):

The activity of these enzymes required the use of INT and PMS to monitor their in-gel activity. The electron transfer mechanism, which shuttles the electrons from NAD(P)H to INT using PMS as an electron mediator, was utilized to visualize in-gel activity of these enzymes. When INT accepts electrons from PMS, the INT becomes reduced and forms an insoluble pink-purple formazan precipitate at the site of the immobilized enzyme (Figure 2.5).
Figure 2.5 – The in-gel detection of NAD(P) dependent enzymes. NAD(P) is reduced to NAD(P)H. Electrons are used to reduce PMS, which is subsequently used to reduce INT, forming a pink-purple precipitate at the site of enzyme immobilization.
**NAD(P)-ICDH:**

For the activity detection of NAD(P)-ICDH, 5mM isocitrate, and 0.5mM NAD(P) was utilized. The activity of these enzymes was probed using 0.5mg/mL of INT, and 0.3mg/mL of PMS. As described previously the tetrazolium salt is reduced by NAD(P)H-generating enzymes (223). The activity of NAD-ICDH was probed in the mitochondrial fraction, while that of NADP-ICDH was probed in the cytosol.

**KGDH:**

The activity of KGDH in the mitochondria was discerned by the addition of 5mM KG, 0.5mM NAD, 0.25mM CoA, 0.5mg/mL of INT, and 0.3mg/mL of PMS (163).

**MDH:**

The activity of MDH in the mitochondria was visualized by the addition of 5mM malate, 0.5mM NAD, 0.5mg/mL of INT, and 0.3mg/mL of PMS (223).

**ME:**

The activity of ME in the cytosol was probed by the addition of 5mM malate, 0.5mM NADP, 0.5mg/mL of INT, and 0.3mg/mL of PMS.

**LDH Detection:**

LDH was probed in the cytoplasmic, mitochondrial, and nuclear fractions. The reaction mixture consisted of 5mM lactate, 0, 0.1, and 0.5mM NAD, 0.5mg/mL INT, and 0.3mg/mL PMS (66). 4 mM Silver Nitrate (AgNO₃) was included in the reaction mixture in order to identify LDH1 (224). Standards from porcine heart and muscle (5μg) were used as molecular weight standards and as markers for the specificity of the activity bands (66).
2.11.5 - FAD-reducing Enzymes (SDH):

FADH₂ producing enzymes were probed in a similar fashion to NADH producing enzymes (Figure 2.6).

![Diagram of SDH reaction](image)

**Figure 2.6 - The in-gel detection of FAD-dependent enzymes.** FAD is reduced to FADH₂. Electrons are used to reduce PMS, which is subsequently used to reduce INT, forming a pink-purple precipitate at the site of enzyme immobilization.

SDH activity was made apparent by incubating the gel lanes in a reaction solution consisting of reaction buffer, 5mM KCN (to interfere with downward
electron flow through the ETC), 5mM succinate, 0.5mg/mL of INT, and 0.3mg/mL of PMS. Exogenous FAD did not need to be added as it is covalently bound to SDH. However, the reaction would proceed much more rapidly if 0.1mM FAD were added to the reaction mixture. SDH activity was probed for in the mitochondrial fraction.

2.11.6 – Cytochrome C Oxidase Activity:

The in-gel activity of Cyt C Ox. was accomplished by using diaminobenzidene as a chromophore (225). Diaminobenzidene acts as an electron donor for cytochrome C. Once Diaminobenzidene accepts the electrons from cytochrome C, it undergoes oxidative polymerization at the site of enzyme activity. The polymerized diaminobenzidene becomes insoluble and precipitates out of solution with a brown colour. Cyt C Ox. activity was assessed in the mitochondrial fraction by adding the gel to a reaction buffer containing 10mg/mL diaminobenzidene, 10mg/mL of cytochrome C, and 562mg/mL of sucrose (to stabilize the cytochrome C (201). Diaminobenzidene was prepared fresh before each reaction by gentle heating of the solution. The precipitate is topical on the gel, unlike the formazan precipitate and thus the gel was documented as soon as the bands appeared.
2.11.7 – ATP-generating Reactions (Enzyme-linked Assays):

Detecting the activity of ATP-generating enzymes such as CK and NDPK, employed the help of exogenous hexokinase, and glucose-6-phosphate dehydrogenase to couple the production of ATP with INT precipitation (Figure 2.7).

Figure 2.7 – Enzyme linked in-gel detection of ATP producing enzymes. ADP is phosphorylated to ATP by the enzyme of interest. The ATP that is produced is used to fuel the phosphorylation of glucose by exogenously added hexokinase. The Glucose-6-phosphate that is produced is harvested by exogenous glucose-6-phosphate dehydrogenase. Glucose-6-phosphate reduces NADP to NADPH and INT is subsequently reduced to produce a pink-purple precipitate at the site of enzyme activity.
The in-gel activity of NDPK in the cytosol was established by the addition of 1mM ADP, 1mM GTP, 15mM glucose, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 1mM NADP, 0.5mg/mL of INT, and 0.3mg/mL of PMS to the reaction buffer. CK in the mitochondria and cytosol was monitored in a similar fashion, 1mM ADP, 5mM PCr, 15mM glucose, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 1mM NADP, 0.5mg/mL of INT, and 0.3mg/mL of PMS were all added to a reaction buffer.

2.11.8 – PDH and BN-Agarose Gel Electrophoresis:

Due to the large size of the mammalian PDH complex (10MDa), an agarose gel was required to resolve it. A BN-agarose gel was prepared as described in (226). Briefly, 0.05M 6-aminocaproic acid and 25mM Bis-Tris were added to 2.5% agarose solution. The solution was brought to a pH of 7.0 and allowed to solidify to a thickness of 3mm in a horizontal gel apparatus. The buffers used were the same as that of BN-PAGE. The gel was run at 5 milliamps for 3.5h with the buffers being replaced every 45min. Upon completion of the electrophoresis, the gel slab was placed in a reaction buffer containing 5mM pyruvate, 0.1mM CoA, 0.1mM NAD, 0.5mg/mL of INT, and 0.3mg/mL of PMS. The reaction was visualized as described previously for NADH producing enzymes.

2.11.9 – Lipogenic Enzymes (CL, ACS, and ACC):

The activity of the lipogenic enzyme, ATP-CL was probed using in-gel precipitation of INT. The in-gel activity of CL was detected using a reaction mixture
consisting of 10mM citrate, 0.75mM CoA, 0.37mM ATP, 1.5mM NADH, 5units/mL of MDH, 0.5mg/mL of INT, and 0.3mg/mL of PMS (223) (Figure 2.8).

**Figure 2.8 - Enzyme linked in-gel detection of ATP-CL.** Citrate is metabolized to oxaloacetate by CL. The resulting oxaloacetate is harnessed by exogenously added MDH, which subsequently oxidizes NADH to NAD. The electrons are harvested by dichloroindophenol, which reduces INT, producing a formazan precipitate at the site of immobilized enzyme activity that can be visualized.

The in-gel detection of ACC (cytosol) and ACS (in the cytosol and mitochondria) are performed with a modified method described in (227). The $P_i$
liberated as a consequence of the carboxylation of acetyl-CoA (ACC) or the PPi formed as consequence of the ligation of CoA with acetate (ACS) is detected. The reaction entails the complexation of P1 or PPi with ammonium molybdate [(NH4)6 (Mo7O24)] which forms an insoluble complex at acidic pH at the site of the in-gel activity of the immobilized enzyme. The precipitate is stabilized by the tertiary amine, triethylamine as it is a strong base. The reaction proceeded in three steps. 1) To detect ACC; the gel was incubated in 10mM ATP, 10mM HCO3-, and 1mM acetyl-CoA for 2.5h (191). To detect ACS; the gel was incubated in 10mM ATP, 5mM acetate, and 0.5mM CoA for 3h. 2) The gels were rinsed gently with ddH2O X5 to remove any residual P1 or PPi. 3) The gels were promptly exposed to the phosphate precipitation reagent (1.06g of ammonium molybdate in 1.37mL of triethylamine, and 9.2mL of concentrated HNO3). The reactions were allowed to proceed until the precipitate (grey-black) was present at the site of enzyme activity. The precipitate was topical, thus the gel was documented immediately following the presentation of the precipitate. Due to the high molecular mass of ACC, 7% isocratic BN-PAGE gels were preferred for proper protein separation. Negative reactions were performed without the addition of ATP for both ACC and ACS.

2.11.10 - Enzymes Involved in L-Carnitine Biosynthesis (BBADH and BBDOX):

BBADH activity was monitored by adding 5mM γ-butyrobetaine, 0.5mM NADH, 16.7μg/mL DCIP, and 0.4mg/ml INT in equilibration buffer. BBDOX activity was ascertained by the addition of 5mM γ-butyrobetaine, 5mM α-ketoglutarate, 2.5mM sodium ascorbate, 0.15mM ferrous sulphate (cofactor), and 0.4mg/ml INT in
equilibration buffer \((191)\). Activity bands were achieved by coupling of INT by ascorbate, a method modified from \((228)\). The reduction of the ascorbate by KG provided the reducing power to precipitate the INT directly. Reaction mixtures devoid of INT or NADH were used as negative controls for BBADH. While, reaction mixtures without ascorbate or KG were the implemented as negative controls for BBDOX.

2.11.11 – **Enzymes Involved in Glutamate Homeostasis (GDH, GluS, and GlnS):**

GDH activity was ascertained using the previously mentioned tetrazolium (INT) precipitation method. The gel was incubated in 5mM Glu, 0.5mM NAD, 0.5mg/mL of INT, and 0.3mg/mL of PMS.

The in-gel activity of GluS \([\text{Gln} + \text{KG} + \text{NADPH} \leftrightarrow 2 \text{Glu} + \text{NADPH}]\) in the cytosol and GlnS \([\text{Glu} + \text{ATP} + \text{NH}_3 \leftrightarrow \text{Gln} + \text{ADP} + \text{P}_i]\) in the cytosol were slightly more involved. The enzyme activities were linked to glutamate production. The glutamate produced was metabolized by exogenous GDH with the concomitant reduction of NAD to NADH. The reduction of NAD could then be captured by PMS and INT as mentioned previously. The reaction mixture for GluS relied on the addition of 5mM Gln, 5mM KG and 0.1mM NADPH in equilibration buffer to the gel for 2h. The reaction mixture was gently decanted; the gel was rinsed in reaction buffer. Following the first reaction step, the gel was then incubated with new reaction buffer containing 5units of GDH, 0.5mM NAD, 0.5mg/mL of INT, and 0.3mg/mL of PMS.
GlnS activity was deduced by incubating the gel in reaction buffer containing 5mM Gln, 0.1mM ADP, 0.1mM P_i, 5units of GDH, 0.5mM NAD, 0.5mg/mL of INT, and 0.3mg/mL of PMS.

2.12 – Oxygen Consumption Assays

To further determine whether lactate was contributing to mitochondrial respiration and the effects of Al and H_2O_2 on mitochondrial respiration, oxygen consumption was measured utilizing an Orion ® O_2 electrode. Isolated mitochondria were incubated with 5mM substrate (lactate, succinate, or pyruvate), 0.5mM NAD, and 0.5mM ADP in the equilibration buffer. Oxygen consumption was monitored over a 5min time interval. To inhibit the mitochondrial utilization of lactate, 5mM KCN was included in the reaction mixture.

2.13 – SDS-PAGE and Immunoblotting

2.13.1 – SDS-PAGE:

SDS-PAGE was performed using a discontinuous buffer system following a modified method described in (229). CFE, mitochondrial and cytosolic proteins from control, Al, and H_2O_2 treated cells were prepared in 6X sample buffer [62.5mM Tris-HCl (pH 6.80), 2% (w/v) SDS, and 2% (v/v) β-mercaptoethanol] at a final protein concentration of 1mg/mL to a final volume of 200μL. In preparation for electrophoresis the 30μL was boiled for 10min to denature the proteins. 10% isocratic gels were selected for SDS-PAGE analysis to separate the proteins efficiently. The 10% SDS-separating gel consisted of 9.2% acrylamide (w/v), 0.8%
bis-acrylamide (w/v), 0.375 Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.17% (v/v) TEMED, and 0.86% APS. The 30μg of protein samples were then loaded into the wells of the stacking gel 9.2% acrylamide (w/v), 0.8% bis-acrylamide (w/v), 0.375 Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.17% (v/v) TEMED [Note: the difference in pH between the stacking and resolving gel facilitated the stacking of the proteins]. Once the protein was loaded into the wells of the stacking gel, the electrophoresis buffer [0.025mM Tris, 0.192mM glycine, and 0.1% (w/v) SDS (pH 8.3)] was introduced in the electrophoresis unit and electrophoresis was performed at 80V until the proteins reached into the resolving gel. Once the protein running front entered the resolving gel, the voltage was increased to 150V until the protein completed running through the resolving gel. Gels were then treated for immunoblot blot procedure, treatment with Coomassie R-250 or the silver staining technique with a kit from Biorad (230) to determine protein expression levels. Coomassie staining involved fixing the gels in 10% acetic acid, 50% methanol and 0.2% Coomassie Brillant Blue R-250 for 45min at 65°C. Gels were then destained overnight. Kaleidoscope standards and the Precision Plus Dual Colour molecular mass markers were used to demarcate relative molecular masses.

**2.13.2 – SDS PAGE Buffers:**

**4X Tris/SDS pH 6.8**
- 1.5M Tris
- 0.4% (w/v) SDS
- Stored at 4°C

**6X Sample Buffer**
- 7mL of 4X Tris/SDS pH 6.8
- 3.8g of glycerol
- 10% (w/v) SDS
- 6% (v/v) β-mercaptoethanol
- 2mg of bromophenol blue

**4X Tris/SDS pH 8.8**
- 21.5M Tris
- 0.4% (w/v) SDS
- Stored at 4°C

**5X Electrophoresis Buffer (1L)**
- 15.1g of Tris
- 72g of glycine
- 5% (w/v) SDS
- Stored at 4°C
2.13.3 – Two-dimensional (2D) SDS-PAGE:

Following the in-gel activity from the BN-PAGE gels, the bands were precision excised and 2D SDS PAGE gels were performed in accordance with the modified method described by (223, 231). The 2D SDS-PAGE gel was effective for identifying if the protein responsible for the activity band in the native gel was indeed the protein of interest. For 2D immunoblot analysis, activity bands from native gels were precision excised from the gel and incubated in denaturing buffer (1% β-mercaptoethanol, 5% SDS) for 30 min, and then loaded vertically into a well of an SDS gel (66). The gels were ran as described previously for an SDS-PAGE analysis. Upon completion of the electrophoresis, the gels could be fixed using the silver staining protocol and kit from Biorad (230) or transferred onto a nitrocellulose membrane for 2D immunoblotting procedure. The molecular mass of the subunits could be deduced using the molecular mass marker and cross-referenced with the literature or an enzyme database such as the Brenda Enzyme Database.

2.13.4 – Immunoblotting Techniques:

Following SDS-PAGE or 2D SDS-PAGE, immunoblotting was then performed to monitor the expression levels of the specific proteins. It also served as an excellent method to confirm the presence of the proteins of interest being monitored during the in-gel activity analysis. The detection of the proteins that were resolved during the SDS-PAGE was achieved using primary antibodies and species-specific secondary antibodies conjugated to a reporter molecule. The reporter molecules
were either HRP or infrared (IR 680 or IR 700) conjugated secondary antibodies (Figure 2.9).

![Diagram of immunoblotting procedure]

**Figure 2.9 – Overview of the immunoblotting procedure.**

The primary antibodies and their corresponding secondary antibodies and detection methods are listed in Table 2.4.

**Table 2.4 – A List of the Antibodies Employed for Immunoblot Analysis.** The target proteins and their respective primary antibody, corresponding secondary antibody, and the method used to detect them. *Obtained from Dr. Lemire (University of Alberta). ** Obtained from Dr.R.Eisenstein (University of Wisconsin-Madison).

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Localization</th>
<th>Primary Antibody</th>
<th>Obtained From</th>
<th>Secondary Antibody</th>
<th>Obtained From</th>
<th>Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>CPE</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz</td>
<td>Anti-mouse (1/1000)</td>
<td>Santa Cruz</td>
<td>HRP</td>
</tr>
<tr>
<td>CK-BB</td>
<td>Cytosol</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>Anti-Rabbit (1/1000)</td>
<td>Biorad</td>
<td>HRP</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-actin</td>
<td>Cytosol</td>
<td>Mouse- polyclonal (1/750)</td>
<td>Abcam</td>
<td>Anti-Mouse (1/20,000)</td>
<td>Biorad</td>
</tr>
<tr>
<td>LDH</td>
<td>Cytosol</td>
<td>Rabbit- Polyclonal (1/800)</td>
<td>Santa Cruz</td>
<td>Anti-rabbit (1/1000)</td>
<td>Biorad</td>
</tr>
<tr>
<td>LDH1</td>
<td>Mitochondria</td>
<td>Goat- Monoclonal (1/800)</td>
<td>Abcam</td>
<td>Anti-goat (1/1000)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Mitochondria</td>
<td>Mouse- Monoclonal (1/1000)</td>
<td>Abcam</td>
<td>Anti-mouse (1/2000)</td>
<td>Biorad</td>
</tr>
<tr>
<td>VDAC</td>
<td>Mitochondria</td>
<td>Rabbit- Polyclonal (1/1000)</td>
<td>Abcam</td>
<td>Anti-rabbit (1/2500)</td>
<td>Biorad</td>
</tr>
<tr>
<td>PDE</td>
<td>Mitochondria</td>
<td>Rabbit- Monoclonal (1/5000)</td>
<td>Abcam</td>
<td>Anti-rabbit (1/2000)</td>
<td>Biorad</td>
</tr>
<tr>
<td>BBDOX</td>
<td>CFE</td>
<td>Mouse- Monoclonal (1/1000)</td>
<td>Abcam</td>
<td>Anti-mouse (1/20,000)</td>
<td>Licor</td>
</tr>
</tbody>
</table>

After electrophoresis, the stacking gel was removed and the separating gel was equilibrated for 15min in PTB. Nitrocellulose membrane was prepared by washing in PTB for 5min. The electroblotting cassette was prepared according to the instruction manual provided by Biorad. Two sponges and 8 X 9cm pieces of 3mm Whatman™ paper were pre-wet with PTB prior to assembling the electroblotting apparatus. The nitrocellulose membrane was sandwiched between two pieces of Whatman™ paper with two sponges on the outside. The
electroblotting apparatus was subjected to an electric current. The electric current applied helped the migration of the proteins from the gel onto the porous nitrocellulose membrane. The transfer was carried out for 16h at 4°C with a constant voltage of 25 volts and 50 milliams. Following the transfer, the membrane was removed and placed in 5% blotto [5% nonfat skim milk in TTBS (20mM Tris-HCl, 0.8% (w/v) NaCl, 1% (v/v) Tween-20, at pH 7.6)] to block the non-specific binding sites. The membrane was blocked for 1h – 24h. Following the membrane blocking, the membrane was washed 2X for 10min with TTBS. The membrane was then probed with the primary antibody diluted in 5% blotto for a period of 1h – 24h. Following the probing with the primary antibody, the membrane was washed 3X with TTBS to remove any residual primary antibody. The membrane was then probed with the secondary antibody diluted in 5% blotto for a period of 1-2h. Following the probing with the secondary antibody, the membrane was then washed 3X with TTBS to remove residual secondary antibody. The membranes were then ready for detection.

2.13.5 – Immunoblot Detection Methods:

The visualization of the proteins following the electroblotting was achieved by the use of Chemiglow™ chemilluminescent substrate system for the HRP conjugated secondaries. The membrane was exposed to the detection reagents consisting of Solution A (luminol enhancer solution) and Solution B (stable peroxide buffer), for 5min at room temperature. The detection solution were mixed 1:1 and distributed over the membrane evenly on the protein containing face. The
membrane was then visualized using Chemidoc XRS system set to high sensitivity chemiluminescence.

The infrared-tagged secondary antibodies were detected by placing the membrane on an Odyssey™ infrared imager from Licor. The gel was scanned using the accompanying Odyssey™ software, which detected the infrared signal (680nm or 700nm) from the infrared conjugated secondary antibodies.

### 2.13.6 – Immunoblotting Buffers:

<table>
<thead>
<tr>
<th>Protein Transfer Buffer (1L)</th>
<th>TTBS (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.03g of Tris</td>
<td>2.42g of Tris</td>
</tr>
<tr>
<td>14.4g of glycine</td>
<td>8g of NaCl</td>
</tr>
<tr>
<td>stored at 4°C</td>
<td>1% (v/v) Tween-20</td>
</tr>
<tr>
<td></td>
<td>pH 7.6</td>
</tr>
</tbody>
</table>

### 2.14 – In-cell Immunoblotting

In-cell western assays were modified from the Odyssey® Infrared Imaging System protocol document (Li-COR doc# 988-08599). Briefly, CCF-STTG1 cells were seeded in 96-well plates at 1.0 X 10⁶ cells/mL. Following seeding (48h), the cells were grown to 75% confluence and then stressed as described above. After treatment, the media was removed and the cells were washed thrice with PBS. The cells were then fixed with 37% formaldehyde for 20min at room temperature. The fixing solution was then removed and the cells were rinsed with 0.1% tween-20 in PBS. Blocking ensued using Odyssey® blocking buffer for 2h. Primary antibody incubations occurred over a 1h period with gentle shaking. Primary antibodies for F-actin, LDH, VDAC, BBDOX, and LDH (refer to Table 2.3) were diluted to 1:200 in
blocking buffer. Secondary antibody incubation took place over 2h with gentle shaking and in the dark. Secondary antibodies for the corresponding primaries were diluted 1/200 in blocking buffer. The infrared signal was detected using an Odyssey® Infrared Imager.

2.15 - Quantitative PCR for LDHA-like 6B:

Control and H₂O₂ stressed astrocytes were harvested as described previously. The cells were suspended in RNeasy buffer, and packed on dry ice. The cells were then sent to MCLAB (molecular cloning laboratories) in San Francisco, CA. mRNA isolation, cDNA synthesis, probe/primer design and validation (Taqman probe), and qPCR analysis for LDHA-like 6B was performed. The levels of LDHA-like 6B in the control and H₂O₂ stressed astrocytes were compared to the internal standard GAPDH. The statistical analysis was performed by MCLAB and the data was sent in a Microsoft excel file. The cDNA sequence for LDHA-like 6B is shown in Appendix 7.5

2.16 - Statistical Analysis

The student T-test was calculated to determine the significance in the different between the control and each stress (0.01mM Al, 0.1mM Al, and 40μM H₂O₂). All experiments were performed at least two times (biological replicates) with 3 experimental replicates of each. The confidence interval of 95% was chosen (p ≤ 0.05), with the exception of the data from Glu/Gln cycling and nuclear metabolism, which are preliliminary data.
Section 3: Results

3.1 - Preliminary Observations

To identify an appropriate point to introduce the Al and H$_2$O$_2$ stress, it was important to ascertain the growth profile of the astrocytes. Utilizing the TBEA, it was determined that the astrocytes reached 75-80% confluency at approximately 6 days and 100% at approximately 7 days (Appendix 7.6). This provided a target period in which to introduce the stressors. Previous work from our lab found that hepatocytes (HepG2) were sensitive to 0.25mM Al (163, 190, 192). A lower concentration of Al (0.1 and 0.01mM) was chosen for these studies as astrocytes are known to be more susceptible to toxicants then hepatocytes and these concentration are within the range of Al found in the brain of individuals with neurological disorders (93, 232, 233). The effect of Al on the growth of the astrocytes was assessed over a 24h period (Figure 3.1).
Figure 3.1 – Growth profile of astrocytes treated with 0.1mM Al over a 24h period. Cell viability was ascertained via the TBEA. A) Control B) 0.1mM Al. n=4 ± SD. P ≤ 0.05

Following a 24h treatment with 0.1mM Al, the astrocytes experienced a lack of growth (25% less than the control). The difference in viable cells was indeed due to an inhibition of growth as opposed to cell death, as demonstrated by an equal number of dead cells within the control and 0.1mM treated samples (Appendix 7.7). Additionally, when the CFE was probed via immunoblot for caspase 3, the presence of cleavage products was not apparent (Appendix 7.8).

In addition to stressing the astrocytes with Al, a H2O2 stress was also performed to compare the treatment response as Al has been shown to generate an oxidative environment. A growth response with varying concentrations of H2O2 was performed (Figure 3.2).
Figure 3.2 – Viable cell growth of astroglial cells treated with H₂O₂ for varying time periods. CCF-STTG1 cells were grown to confluency and then exposed to A) control B) 20 μM H₂O₂ C) 40 μM H₂O₂ and D) 100 μM H₂O₂ for up to 24h. Viable cells were determined utilizing the TBEA. n=3 ± SD. P ≤ 0.05.

Stressing the cells with 40μM H₂O₂ provided a reduction of about 50% in cell viability, whereas 20μM H₂O₂ yielded a negligible effect, and 100μM H₂O₂ produced too great a loss of cell viability. Again, counting the number of dead cells using the TBEA, disclosed a diminished cell growth rather than an increase in cell death. However, a great deal of cell death was observed when the astrocytes were treated with 100μM H₂O₂ (Data not included).

The choice of the delivery vehicle was lactate, and Al was provided as its lactate complex. Primary experiments focused on ascertaining whether or not Al
was being delivered into the astrocytes. Astrocytes were grown to confluency on microscope coverslips and treated with 0.1mM Al for 24h. Following the stressing period, Morin stain was utilized to measure Al levels within the cell (Figure 3.3).

![Image of Figure 3.3](image_url)

**Figure 3.3 – Morin stain for Al in astroglioma cells.** A) control and B) 0.1mM Al treated cells were accessed for Al levels using the Morin stain (green). The cells were counterstained with Hoechst to identify the nucleus (blue). Scale bar = 10μm. ** Note: background fluorescence from morin in the control is due to reaction with trivalent Fe.

Using deconvolution microscopic techniques, it was clear that Al was indeed being delivered into the astrocytes (Figure 3.3). Having established that Al entered inside the cell it was crucial to make certain that any changes observed were indeed due to the presence of the metal. One of the effects that Al has been demonstrated to have on cellular systems is the generation of ROS (137, 234, 235).
Dichlorofluorescein diacetate (DCFDA) staining is commonly used to measure the levels of ROS within the cell (236, 237). Following treatment with Al and H₂O₂, CCF-STTG1 cells were probed with DCFDA stain to ascertain ROS levels (Figure 3.4).

**Figure 3.4 – Al and H₂O₂ induced ROS.** Astrocytic cells were analyzed under A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ conditions. Fluorescence microscopy was performed at 20X ocular magnification. Blue = Hoechst (nuclei), Purple =DCFDA (ROS). Scale bar = 10 μm. Arrows indicate fluorescence generated by ROS.

Using the DCFDA stain, there was an observed increase in DCFDA fluorescence, indicative of an increase of ROS levels within the Al and H₂O₂ treated cells. To confirm that a state of oxidative stress was occurring, the levels of oxidized proteins and lipids were measured (Table 7.1).
Table 3.1 - Oxidized lipids and proteins profile in CCF-STTG1 cells exposed to Al and ROS stress. (* Denotes a significant increase in oxidized lipids; ** signifies a significant increase in oxidized protein levels)

<table>
<thead>
<tr>
<th></th>
<th>Oxidized Lipids (μmol MDA/ 4 X 10^6 cells)</th>
<th>Oxidized proteins (pmol carbonyl/ 4 X 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.036 ± 0.010</td>
<td>0.625 ± 0.005</td>
</tr>
<tr>
<td>0.01mM Al</td>
<td>0.139 ± 0.006*</td>
<td>0.983 ± 0.012</td>
</tr>
<tr>
<td>0.1mM Al</td>
<td>0.785 ± 0.017*</td>
<td>1.750 ± 0.097**</td>
</tr>
<tr>
<td>40μM H₂O₂</td>
<td>0.810 ± 0.079*</td>
<td>1.469 ± 0.047**</td>
</tr>
</tbody>
</table>

n=3 ± SD; p ≤ 0.05

Indeed, the levels of oxidized proteins and lipids increased under the Al and H₂O₂ stress. This observation along with increased quantities of H₂O₂ has been established in HepG2 cells as well (163).

Preliminary observations about the metabolic state of the cells can also be established from analysis of the spent fluid components. Following the 24h treatment period with Al and H₂O₂, the spent fluid was collected. A metabolite profile was obtained using HPLC (Figure 3.5).
Figure 3.5 – Metabolite profile of the spent fluid from astrocytes spent. Spent fluid was collected following treatment with A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂. HPLC analysis was performed to qualify differences in the metabolite profile.

The metabolite profile obtained by performing HPLC on the spent fluid revealed an alteration of metabolite being exuded from the cell under Al and H₂O₂.
stress. This altered metabolite profile of the spent fluid is a primary indicator that a greater shift of metabolism is occurring within the stressed astrocytes. In addition, the spent fluid metabolite profile also revealed an accumulation of α-keto acids in the Al and \( \text{H}_2\text{O}_2 \) stressed cells. This phenomenon has been observed previously in HepG2 cells (163).

Another indicator of cellular alteration that may be reflected in the spent fluid, are proteins. Proteins were analyzed in the spent fluid following Al and \( \text{H}_2\text{O}_2 \) treatment using SDS PAGE. The proteins were made evident by the silver staining procedure (Figure 3.6).

![Figure 3.6](image)

**Figure 3.6 – Protein profile of the spent fluid from astrocytes.** The spent fluid was collected at 0, 4, 8, and 24h intervals from cells treated with A) Control B) 0.1mM Al. The spent fluid was then run in a 10% SDS PAGE and silver stained. A molecular weight standard (STD) was added as a running control.

The silver stain analysis revealed that proteins were being released into the spent fluid within both the control and Al stressed conditions. The quantity being exuded seemed to increase over the incubation period and certain bands within the
Al-treated spent fluid appeared more prominent after the 24h treatment period. This altered protein profile within the spent fluid is a preliminary observation which suggests alterations within the Al-treated astrocytes.

Preliminary observations from the treatment of astrocytes with Al and H$_2$O$_2$ demonstrated that the presence of the stressors has a negative impact on viable cell numbers. In addition, the treatment with Al and H$_2$O$_2$ increased the levels of ROS within the astrocytes. Indeed, microscopy confirmed the delivery of Al into the cell body of the astrocytes. Analysis of metabolites and proteins in the spent fluid following Al and H$_2$O$_2$ stressing suggest an altered metabolism and biochemistry within the cell.

3.2 - Al and oxidant induced loss of cytoskeletal architecture

One of the first observations made on the astrocytes treated with Al, was the loss of typical cellular morphology (the presence of dendritic processes). The preliminary observation was made using a light microscope. The loss of morphological features is depicted in Figure 3.7.

![Figure 3.7](image-url)
Figure 3.7 – Phase contrast microscopic studies of human astrocytoma cells. A) Control cells B) cells exposed to 0.1mM Al. Microscopy was performed at 20X ocular objective. Note: solid arrows (→) indicates well defined structures. Broken arrows (- - →) are indicative of changes in the stressed cells. Scale bar = 10 μm (Lemire et al., 2009).

The loss of "normal" morphology of the astrocytes was a primary indication that there was a dysfunction of cellular biochemistry. It is important to note that the stressed cells appear to have a more rounded morphology whereas the control cells have nice defined processes.

To further elucidate these structural changes of what was happening with the Al-stressed astrocytes, deconvolution fluorescence microscopy was performed. Since actin is the main component of the cytoskeleton, and provides shape and structure to the cell, it was a prime candidate as a target of the Al-induced change in morphology (238). Using the fungal derived toxin phalloidin, tagged with FITC, the actin component of the cytoskeleton could be observed (Figure 3.8).
Figure 3.8 - Fluorescence microscopic studies of human astrocytoma cells. A) Control cells B) cells exposed to 0.1mM Al. Astrocytommas were strained with Hoechst (nucleus) and FITC-Phalloidin (actin). Microscopy was performed at 60X ocular objective. Scale bar = 10μm. Note: solid arrows (→) indicate well-defined structures. Broken arrows (-----→) are indicative of changes in the stressed cells (Lemire et al., 2009).

Fluorescence microscopic analysis revealed that the actin cytoskeleton under Al treatment formed a globular structure (the actin did not seem to be able to polymerize), while the control cells were able to form a nice filamentous actin cytoskeleton. This observation was further studied with a lower concentration of Al and H₂O₂ (Figure 3.9).
Figure 3.9 - Microscopic studies of human astrocytoma cells. A) Control cells B) cells exposed to 0.01mM Al C) cells exposed to 0.1mM Al and D) cells exposed to 40μM H$_2$O$_2$ for 24 h respectively. Following treatment cells were stained with Hoechst (nucleus), Cy3-GFAP, and FITC-Phalloidin (actin). Microscopy was performed at 40X ocular objective, scale bar = 10 μm. Note: solid arrows (→) indicates well defined structures. Broken arrows (---→) are indicative of changes in the stressed cells (Lemire et al., 2009).
Again, observations with a lower concentration of Al (0.01 mM) and H₂O₂ treatment confirm the inability of the actin cytoskeleton to polymerize under stress. A Cy3-labeled GFAP antibody was used as an astrocytes specific marker. GFAP is an intermediary filament. The expression of GFAP does not appear to be affected by Al or H₂O₂ stress (201). It is also important to note that the overall expression of actin does not appear to decrease as a consequence of Al and H₂O₂ stress respectively. The effects of Al and H₂O₂ on the cytoskeleton appear to be a perturbed polymerization of actin.

Actin polymerization, like most processes in the cell, are heavily dependent on ATP (238). Since we have previously observed that ATP production is perturbed under both Al and H₂O₂ treatment, elucidating the ATP status of the astrocytes under similar conditions was essential (159, 160, 163). Figure 3.10 displays some of the attributes of the ATP producing machinery within the astrocytes.
**Figure 3.10 - Energy status of stressed human astrocytoma cells.** Cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40µM H₂O₂ for 24 h. I) In-gel activity stain for Cyt C Ox. II) In-gel activity stain for NAD-ICDH. III) HPLC analysis of cellular ATP levels. n=3 ±SD; p ≤ 0.05. * Denotes a significant decrease of ATP compared to control treatment. IV) In-gel activity stain for NDPK. V) In-gel activity stain for MDH (Lemire et al., 2009).

Astrocytes exposed to Al and H₂O₂ were observed to have lowered activities of NAD-ICDH and Cyt C Ox, enzymes involved in NADH and ATP production respectively. In addition, SDH or Complex II is also down in activity in Al treated cells when compared to the control (Appendix 7.9). HPLC analysis of ATP levels indicated that there is a loss in the amount of ATP being produced. The phosphate buffering system NDPK also appeared to be downregulated in activity in the Al and H₂O₂ treated cells. It does not appear to be a general downregulation of all enzymes involved in the energy producing machinery. The activity of mMDH does not appear to be altered under Al and H₂O₂ treatment, thus the downregulation in activity seems to be targeted. Further evidence that Al and H₂O₂ affect oxidative energy production are shown in Figure 3.11 and 3.12. **Figure 3.11** revealed that the mitochondrial potential [indicated by rhodamine (red) fluorescence] was diminished under Al-treatment.
Figure 3.11 – Mitochondrial membrane potential in astrocytes exposed to A) control and B) 0.1mM Al. The cells were subsequently stained with Hoechst (nucleus), and Rhodamine (membrane potential). Note: the lack of red fluorescence in the Al-treated cells. Microscopy was performed at 60X ocular objective, scale bar = 10 μm.

Figure 3.12 demonstrates that the ability of the mitochondria from Al and H₂O₂ treated cells; to consume O₂ (the terminal electron acceptor of the ETC) has decreased.
Figure 3.12 – Oxygen consumption assay of astroglial mitochondria exposed to with A) control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂. Oxygen consumption was measured utilizing an Orion ® O₂ electrode. Isolated mitochondria were incubated with 5mM substrate lactate, 0.5mM NAD, and 0.5mM ADP in equilibration buffer. Oxygen consumption was monitored over a 5min time interval. To demonstrate inhibition of mitochondrial respiration, 5mM KCN was included in the reaction mixture. n=3 ± SD; p ≤ 0.05

The loss of mitochondrial potential as well as a decrease in oxygen consumption is indicative of the inability of the mitochondria to function properly under Al and H₂O₂ stress. The decreased capacity of oxidative phosphorylation has been demonstrated previously with our research group in *P. fluorescens* and HepG2 cells (163, 167, 188).

ATP production was found to be perturbed in the cells subjected to Al and H₂O₂ stress. CK coupled with PCR has been demonstrated to provide local energy for actin polymerization (239, 240). Determining the status of creatine (Cr) homeostasis under Al and H₂O₂ treatment was a crucial aspect of linking ATP to a
loss of cellular morphology. The brain isoform of CK (CK-BB) was probed for activity and expression. While HPLC was used to measure Cr/PCr ratios (Figure 3.13).

![Figure 3.13](image)

**Figure 3.13 – Activity and expression of CK in astrocytoma cells under Al insult.** CCF-STTG1 cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. I) In-gel activity stain for CK. II) Bands were excised from activity gel (I) and subsequently ran on a 10% SDS-PAGE gel and silvered-stained for protein expression. 10 μg of a CK standard was run in tandem with both experiments (sigma). III) Immunoblot assay for the brain isoform of CK. IV) Cr (closed bar ■) and PCr (open bar □) levels were measured utilizing a Waters HPLC with a C18-reverse phase column. n=3 ± SD; p ≤ 0.05. * Denotes significant decrease in Cr levels compared to control treatment, while ** denotes significant decrease in PCr levels compared to control treatment. (Note: Activity bands were observed in the stressed conditions only after prolonged incubation periods) (Lemire et al., 2009).

CK activity in Al and H₂O₂ stressed cells was markedly decreased compared to the control cells. When the activity bands were precision excised, run in a 2D denaturing gel, and probed for protein expression levels, the diminished activity of
CK was due to the decrease in enzyme levels in both Al and H$_2$O$_2$ stressed conditions. A CK-BB antibody was utilized to confirm the decrease in expression levels in the Al and H$_2$O$_2$ stressed conditions. Analysis of the levels of Cr and PCr revealed that the amount of these metabolites decreased under Al and H$_2$O$_2$ stress. Hence the syntheses of both these metabolites were affected.

To demonstrate that CK associates with the actin cytoskeleton to donate local energy for polymerization, microscopy was performed to localize and assess CK levels (Figure 3.14).
Figure 3.14 - CK and the actin cytoskeleton in astrocytes incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ media for 24 h. The cells were subsequently stained with Hoechst (nucleus), Rhodamine-Phalloidin (actin), and FITC-CK. Note: the yellow demarcates the association of CK with the actin filaments. Microscopy was performed at 60X ocular objective, scale bar = 10 μm. Solid arrows (→) indicate filamentous structure of actin (control conditions), while broken arrows (· · →) are indicative of loss of filamentous actin (stressed conditions) (Lemire et al., 2009).
These microscopic analyses helped confirm the biochemical data i.e the decreased activity of CK under these stressed conditions was due to diminished expression of the enzyme and the actin levels were not affected. However, a lack of localized ATP levels were responsible for the globular structure associated with the Al-stressed astrocytes.

Since the inability of the actin cytoskeleton to polymerize appeared to be related to a lack of ATP, it was thought that there might be a link to profilin-2. Profilin-2, aids in the regulation of actin polymerization and ATP exchange (241). Profilin-2 expression was assessed by immunoblot analysis (Figure 3.15).

![Figure 3.15 - Analysis of profilin-2 levels. Astrocytoma cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. I) Immunoblot for profilin-2. II) Immunoblot for F-actin as a loading control. (Note: Although the actin levels are similar, in the stressed conditions the structural features were markedly altered) (Lemire et al., 2009).](image)

Immunoblot analysis of profilin-2 revealed that its expression had decreased under Al and H₂O₂ stress. However, actin levels did not seem to change in the stressed conditions, an observation previously seen in the microscopic studies.
Microscopy was performed to confirm the immunoblotting observations for profiling-2 expression, as well as to provide information on the localization (Figure 3.16).

Figure 3.16 - Al-induced cytoskeletal dysregulation and profilin-2 expression. Astrocytoma were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40µM H₂O₂ for 24 h. The cells were stained with Hoechst (nucleus), Rhodamine-Phalloidin (actin), and FITC-profilin-2. Note: yellow demarcates the association of profilin-2 with the actin filaments. Microscopy was performed at 40X ocular objective, scale bar = 10 µm. Solid arrows (→) point to filamentous actin (control conditions), while broken arrows (- - →) are indicative of changes in the actin arrangement (stressed conditions) (Lemire et al., 2009).
The observations provided by the microscopy data demonstrated that profilin-2 expression has indeed decreased, in accordance with the immunoblot analysis, in the Al and H$_2$O$_2$ stressed conditions. The co-localization of profilin-2 with the cytoskeleton was visualized by the presence of yellow fluorescence. Indeed, it was also noted that actin again appeared to be forming globular structures as opposed to filamentous ones that were characteristic of the control cells.

As stated previously there was an observed increase in ROS levels with the Al and H$_2$O$_2$ treated cells when compared to the controls. To analyze whether or not this increase in ROS could be attenuated, the cells were recovered with 1mM NAC (a commonly utilized antioxidant). ROS levels were qualified using DCFDA staining (Figure 3.17)
Figure 3.17 - Al-evoked ROS in the astrocytoma cell line. Cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H$_2$O$_2$ for 24 h. The cells were stained with Hoechst (nucleus), and DCFDA (ROS). I) 24h incubation in control or stressed conditions. II) 24h incubation in 1mM NAC, following exposure to control or stress conditions (A significant decrease in ROS after NAC treatment was evident). Microscopy was performed at 60X ocular objective, scale bar = 10 μm. Note: blue colouration signifies the nucleus, whereas red indicates the presence of ROS (Only minute amounts of ROS were detected in the control cells (Lemire et al., 2009).

As observed previously, Al generates ROS as does H$_2$O$_2$ treatment. Microscopic studies revealed that the ROS that were generated under the Al and H$_2$O$_2$ stress could be reversed with the addition of NAC, an observation that was evident with the DCFDA fluorescence (red).

With the observation that NAC reverses the formation of ROS in the Al and H$_2$O$_2$ stressed astrocytes, it was imperative to see if NAC could reverse the loss of energy production and malformed cytoskeleton. Microscopy was used to monitor the recovery of the cytoskeleton, while HPLC was used to measure the recuperation of ATP levels (Figure 3.18).
Figure 3.18 - Anti-oxidant treatment and the recovery of cytoskeletal architecture and energy status in human astrocytes (CCF-STTG1). A) Control cells B) cells treated with 0.01mM Al C) cells exposed to 0.1mM Al and D) cells treated with 40μM H$_2$O$_2$ for 24h respectively. Cells were subsequently exposed to 1mM NAC for 24h. I) Immunofluorescence staining with Hoechst (nucleus), Cy3-
GFAP, and FITC-Phalloidin (actin). Microscopy was performed at 40X ocular objective, scale bar = 10 μm. Arrows point to the return of the filamentous structure of actin. II) HPLC analysis of cellular ATP levels. n=3 ±SD; p ≤ 0.05. (Lemire et al., 2009)

Incubation with the antioxidant NAC led to the partial recovery of the actin cytoskeleton that was disturbed under Al and H₂O₂ stress. This was observed by what appeared to be the beginnings of the reformation of a filamentous actin cytoskeleton, visualized with the fluorescence of phalloidin (actin). More intriguing was the recovery of ATP levels in the Al and H₂O₂ stress after a 24h treatment with the antioxidant NAC. Hence, the loss of cytoskeletal architecture observed within the astrocytes under Al and H₂O₂ stress was due to ineffective actin polymerization. This was linked to perturbed energy production in the stressed cells. Loss of ATP production was demonstrated to result from the downregulation in the ATP producing pathways (oxidative phosphorylation). Mediators in ATP homeostatsis and actin polymerization (CK and profilin-2) were also downregulated in activity and expression under Al and H₂O₂ treatment. The result of the dysregulation of these biochemical pathways is thought to be responsible for the observed loss of cellular morphology. Recovery with the antioxidant NAC appeared to reverse the loss of actin polymerization and ATP loss caused by Al and H₂O₂ derived ROS (Figure 3.19).
Figure 3.19 - A molecular link between Al toxicity and morphological perturbation in human astrocytoma cells (Lemire et al., 2009).
3.3 - *Astrocytes and the Lactate Conundrum*

The role of lactate in the brain remains elusive. Astrocytes can utilize lactate as a carbon source in order to support their growth. However, the exact mechanisms are not fully understood. In this study, Al was given to the astrocytes as a complex of lactate. Hence the fate of lactate was monitored by HPLC (Figure 3.20).

![Graph showing lactate utilization by an astrocytic cell line.](image)

**Figure 3.20 - Lactate utilization by an astrocytic cell line.** CCF-STTG1 cultures were supplemented with 2.5mM lactate in serum free media. Lactate measurements were performed using HPLC at various time intervals. Viable cell counts were performed using the TBEA. **A** - Corresponds to viable cell number of the astrocytic cells grown in serum free α-MEM+2.5 mM lactate. **B** - Corresponds to viable cell number of astrocytic cells grown in serum free α-MEM. **C** - Corresponds to relative amount of lactate levels in the spent fluid (lactate cultures). n = 3, mean ±SD, p ≤ 0.05. (Lemire et al., 2008)
When compared to serum free media, astrocytes supplemented with lactate for 24h, reach a higher cell number. HPLC analysis on the spent fluid also demonstrates that the astrocytes were capable of consuming 75% of the lactate within a 24h period. As the lactate was being used by the astrocytes to support growth under aerobic conditions, it was important to delineate how this metabolite was utilized to produce ATP. Metabolomic analysis was implemented to assess lactate consumption by the astrocytic mitochondria (Figure 3.21).

![Figure 3.21 - Lactate consumption by mitochondria derived from this astrocytic cell line. I] Mitochondria were incubated with 5mM lactate and 0.1mM NAD. The time dependent consumption of lactate by the mitochondria was measured using HPLC. II] The purity of the mitochondrial and cytoplasmic fractions were confirmed using VDAC and F-actin respectively. III] Mitochondrial lactate consumption was confirmed using NMR spectroscopy. Data were acquired after 15min and 60min respectively. n = 3, mean ± SD, p ≤ 0.05. (Lemire et al., 2008).
When the astrocytic mitochondria were isolated and incubated with lactate and NAD, the lactate was consumed in a time-dependent fashion. The longer the incubation time the greater amount of lactate was consumed. This observation was confirmed by incubating the mitochondria with $^{13}$C$_3$-lactate and NAD. The purity of the fractions was ascertained by immunoblot analysis for VDAC (mitochondria) and F-actin (cytoplasm). After determining that the mitochondria were consuming lactate, attempts were made to deduce whether or not it was being used to make energy. HPLC analysis was performed to measure the levels of NADH and ATP in the mitochondria (Figure 3.22).
Figure 3.22 - Lactate as a source of mitochondrial energy in an astrocytic cell line. Mitochondria were incubated with 5mM lactate or 5mM citrate, 0.1mM NAD, and 0.1mM ADP for varying time intervals. Nucleotide levels were measured by HPLC using a C18 reverse phase column. I) ATP/ADP ratio in mitochondria. Open bar □ = ADP, and closed bar ■ = ATP II) NAD/NADH ratio in mitochondria incubated with lactate. Open bar □ = NADH, and closed bar ■ = NAD. Peaks were confirmed by utilizing known standards and by spiking the samples. n = 3, mean ± SD, p ≤ 0.05 (Lemire et al., 2008).

HPLC analysis of mitochondria incubated with lactate, NAD, and ADP revealed the ability of the astrocytic mitochondria to use the lactate to produce NADH and ATP. The utilization of lactate increased with the incubation time. Albeit at slightly lower levels, lactate like citrate, a metabolite known to be used by the TCA cycle to produce NADH, also enabled the cells to generate NADH. However, there was a coomitant reduction in both the NAD and ADP the mitochondria incubated with lactate.

To confirm that lactate was being diverted toward the TCA cycle, mitochondria were incubated with 13C3-lactate, NAD, and NaN3 (to inhibit the ETC). HPLC and NMR analysis were performed to monitor the fate of the lactate (Figure 3.23).
Figure 3.23 - Oxidative metabolism of lactate in astrocytic mitochondria. Mitochondria isolated from CCF-STTG1 cells were incubated in 10mM 3-^{13}C-lactate, 0.1mM NAD and 1µM NaN₃ for varying time intervals. Accumulation of TCA cycle intermediates were measured via I) HPLC and II) NMR spectroscopy. HPLC fractions were also confirmed by enzymatic assays (citrate, succinate, and fumarate) (Lemire et al., 2008).

Following the ^{13}C-labeled lactate in the mitochondria, it was discerned that the lactate was being devoted to metabolites of the TCA cycle (fumarate, α-ketoglutarate, succinate, citrate, isocitrate, and malate). This was observed in the ^{13}C NMR analysis and confirmed with HPLC. In fact, more TCA cycle metabolites were evident in the HPLC analysis. Citrate, succinate, and fumarate were confirmed by isolating the metabolites following HPLC and performing enzyme specific reactions on them. After establishing lactate as a contributor to the TCA cycle, our goal was to identify if lactate was playing a part in oxidative metabolism. Isolated mitochondria were incubated with various substrates, and markers of oxidative
metabolism were measured, including Complex IV activity (Cyt. C. Ox.) and O₂ consumption (Figure 3.24).

![Graph showing oxygen consumption](image)

**Figure 3.24- Lactate promotes aerobic respiration in astrocytic mitochondria.**
I) Mitochondria from an astrocytic cell line were isolated and oxygen consumption was measured over a 5 min period, utilizing an oxygen electrode (Orion®). Mitochondria were incubated with 5mM substrate, 0.5mM NAD, and 0.5mM ADP. A reaction buffer blank was also analyzed to ensure proper instrument calibration. II) In gel activity of cytochrome C oxidase. A) CCF-STTG1 cells incubated with 2.5mM lactate. B) CCF-STTG1 cells incubated with 2.5mM glucose. C) CCF-STTG1 cells; incubated with 2.5mM citrate. n = 3, mean ± SD, p ≤ 0.05. (Lemire et al., 2008).

It was evident that lactate was contributing to mitochondrial consumption of O₂. Compared to succinate and citrate, the O₂ consumption was a slightly less. However, when lactate was incubated with mitochondria in the presence of CN⁻ there was very little O₂ consumption. Complex IV of the ETC was equally as active.
when astrocytes were incubated in lactate as when they were incubated in glucose and citrate.

As lactate was metabolized by the mitochondria, it was logical to speculate that there was an isoenzyme of LDH associated with the mitochondria. To determine if it was truly LDH that was consuming the lactate, the mitochondria were incubated with lactate and NAD in the presence or absence of oxamate (an LDH inhibitor). HPLC analysis was utilized to monitor lactate consumption (Figure 3.25).

![Diagram of lactate metabolism](image)

**Figure 3.25 - Mitochondrial lactate metabolism.** Mitochondria from CCF-STTG1 cells were isolated and incubated with 5mM lactate, and 0.1mM NAD for varying time intervals in the presence or in absence of 10mM oxamate. Relative lactate levels were measured by HPLC. • = mitochondria in the absence of 10mM oxamate. ▲ = mitochondria in the presence of 10mM oxamate. n = 3; mean±SD, p ≤ 0.05 (Lemire et al., 2008).
In the control (ie. Without oxamate) the mitochondria readily consumed the lactate. Most of the dicarboxylic acid was utilized over a 2h period. However, when oxamate, an LDH inhibitor, was introduced, very little of the lactate was consumed. This finding that mitochondria lost their ability to consume lactate when treated with oxamate, prompted us to probe for the presence of LDH activity. BN-PAGE was implemented to determine if there was an active LDH in the mitochondria (Figure 3.26).

![Figure 3.26](image)

**Figure 3.26 - BN PAGE analyses of LDH in an astrocytic cell line.** I) Mitochondrial fraction. II) Soluble fraction. A) Coomassie stain for LDH from porcine heart (Sigma). B) Coomassie stain for LDH from porcine muscle (Sigma). C) In gel detection of LDH activity with 0mM NAD. D) In gel detection of LDH activity with 0.1mM NAD. E) In gel detection of LDH activity with 0.5mM NAD. F) In gel detection of LDH activity with 0.5mM NAD and 2mM AgNO₃ (Lemire et al., 2008).

Probing for LDH activity in the mitochondria demonstrated that there were two isoenzymes present compared with the cytoplasm, which only contained one isoenzyme. The activity band produced by the LDH increased with amount of NAD that was added to the reaction mixture (note the lack of activity in the reaction mixtures supplemented with 0mM NAD). The cytoplasmic LDH matched the profile of the LDH standard from porcine heart, while the mitochondrial LDH matched the profile of the standard of porcine muscle. When the activity of LDH was probed in
the presence of AgNO₃ (an inhibitor of all LDH isoenzymes aside from LDH1), it was discovered that the upper band from the activity stain for LDH in the cytoplasmic and mitochondrial fraction was LDH1.

To confirm that the enzyme identified as LDH in the activity stain analysis was indeed LDH, the bands were precision excised and run on a 2D SDS-PAGE gel. The bands were then probed for LDH expression via an immunoblot (Figure 3.27).

![Figure 3.27 - 2D immunoblot analysis of LDH. Activity bands were excised from BN PAGE experiment and ran on a 10% SDS-PAGE. A) Upper band (mitochondrial fraction). B) Lower band (mitochondrial fraction). C) Band from soluble fraction. (Lemire et al., 2008).](image)

2D immunoblot analysis revealed that the upper and lower bands from the mitochondrial LDH probed in the activity stain were indeed LDH, as was the band excised from the activity probing of the cytoplasmic fraction. To further confirm that the LDH was localized in the mitochondria, fluorescence microscopic analysis was performed (Figure 3.28).
Figure 3.28 - Lactate dehydrogenase localization in an astrocytic cell line. A) Hoechst stain for the nucleus. B) Rhodamine B stain utilized for mitochondrial localization. C) FITC tagged secondary for anti-LDH. D) Merged image of Hoechst, Rhodamine B, and FITC. Note: Yellow spots are indicative of LDH associated with the mitochondria (Lemire et al., 2008).

The microscopy studies demonstrated that LDH was indeed localized to the mitochondria. This observation was made by the co-localization of the red fluorescence (rhodamine = mitochondria) with the green fluorescence (FITC = LDH), giving a yellow fluorescence. Since it was deduced that the LDH was indeed found in the mitochondrial fraction, our next endeavour was to determine which sub-fraction of the mitochondria the LDH was located within. Immunoblot analysis was performed on the mitoplast (inner membrane and matrix) and the
outermembrane/inner membrane space fractions of the mitochondria (Figure 3.29).

![Image of a gel with bands labeled as STD, A, B, A, B, Cyt C, SDH]

**Figure 3.29 - Localization of LDH in the mitochondria.** Mitochondria were isolated and separated into A) Mitoplast and B) Outer membrane and inner membrane space fractions. I) Immunoblot for LDH1 in the mitochondrial fractions. II) Immunoblot for Cyt C and SDH to determine purity of mitochondrial fractions. STD corresponds to LDH from porcine heart (Sigma). (Lemire et al., 2008).

Probing the sub-fractions of the mitochondria divulged that mLDH was located within the mitoplast portion. The signal for mLDH was absent from the outer membrane/inner membrane space fraction. The fraction where the mLDH was discovered was confirmed by probing for SDH (mitoplast) and Cyt C (inner membrane).

Discovering a mLDH in these astrocytic cell lines was an important step to deciphering brain lactate metabolism. We observed this astrocytic cell line metabolized lactate in an oxidative fashion. This lactate was destined for the TCA cycle and then oxidative production of ATP. When lactate entered the mitochondria, it was acted-upon by a mitochondrial isoenzyme of LDH, which resembled LDH1 (as it was sensitive to oxamate but not AgNO₃ treatment). This mitochondrial isoenzyme of LDH1 was localized in the mitoplastic fraction of the mitochondria (Figure 3.30).
Figure 3.30 – The discovery of a mitochondrial LDH.

3.4 - The Involvement of mLDH in Al and ROS Stress Attenuation

Another important observation that was made was that Al and ROS stressed astrocytes consumed a greater amount of lactate than control cells (Figure 3.31). It also appeared as though the Al and ROS stressed cells were accumulating other monocarboxylates in the spent fluid (Figure 3.31).
Figure 3.31 – Monocarboxylate profile of astrocytic spent fluid. HPLC analysis was performed on the spent fluid from A) control B) 0.01mM Al C) 0.1mM Al and D) H₂O₂ stressed astrocytes. Spent fluid was collected after a 24h treatment.

Profiling the spent fluid of the control and stressed astrocytes, it was evident that lactate was being consumed more in the Al and H₂O₂ treated cultures. As the concentration of Al increases, the amount of lactate consumed does as well. In addition, pyruvate and acetate accumulated in the spent fluid in the Al and H₂O₂ stressed cultures. The levels of these monocarboxylates in the CFE were monitored by HPLC (Figure 3.32).
Figure 3.32 - Monocarboxylate levels in the CFE. CCF-STTG1 cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. Following the treatment, levels of lactate, pyruvate, and acetate were measured utilizing a Waters HPLC with an organic acid column. n=3 ± SD; p ≤ 0.05. * Denotes significant change in metabolite levels.

HPLC analysis on the CFE helped establish that Al and H₂O₂ treated cells had lower levels of lactate compared to control cells. The levels of both pyruvate and acetate within the Al and H₂O₂ stressed cells on the other hand were found to be elevated when compared to control CFE. As the concentration of Al increased more lactate was consumed. The increase in pyruvate and acetate was concomitant with the increase of Al.

There appeared to be a difference in lactate metabolism within the Al and H₂O₂ stressed cells in comparison to control astrocytes. Thus, measuring the levels of LDH in the astrocytes seemed a logical step to follow. LDH levels were ascertained utilizing the in-cell western blot technique (Figure 3.33).

![Figure 3.33 - In-cell western blot for LDH expression.](image)

Astrocytes were seeded on a 96-well plate and then treated with A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. Following the stressing, the astrocytes were probed for the expression of I) LDH (green) and II) F-actin (red). Infrared fluorescence was quantified using an Odyssey Infrared Imager™. n=5 ± SD; p ≤ 0.05.
Quantifying the expression levels of LDH revealed that the levels of the protein were slightly elevated in the Al and H$_2$O$_2$ stressed astrocytes, when compared to the expression levels of F-actin. With the observations that the LDH expression pattern did not correspond well with the lactate consumption pattern monitored via HPLC in the Al and H$_2$O$_2$ treated astrocytes, further analysis of LDH was undertaken. LDH activity and expression levels were measured using in-gel activity and 2D silver staining/immunoblotting respectively within the cytoplasmic and mitochondrial fraction (Figure 3.34).

![Image](image.png)

**Figure 3.34 - Activity and expression levels of LDH.** CCF-STTG1 cells were incubated in **A** Control **B** 0.01mM Al **C** 0.1mM Al and **D** 40μM H$_2$O$_2$ for 24 h. **I** In-gel activity stain for LDH in the cytoplasm. **II** Bands were excised from activity gel (I) and subsequently ran on a 10% SDS-PAGE gel and silvered-stained for protein expression. 10 μg of a LDH standard was run in tandem (Sigma). **III** Immunoblot
assay LDH in the cytoplasm. **IV)** In-gel activity stain for LDH in the mitochondria. **V)** Bands were excised from activity gel (IV) and subsequently ran on a 10% SDS-PAGE gel and silvered-stained for protein expression. 10 μg of a LDH standard was run in tandem (sigma). **VI)** Immunoblot assay LDH in the mitochondria. **VII)** An immunoblot assay for purity of the cytoplasmic (F-actin) and mitochondrial (VDAC) fractions. STD corresponds to 10 μg of a LDH (Sigma).

The activity and expression analysis for LDH within the separate fractions (mitochondrial and cytoplasmic) revealed a differential profile. In the cytoplasmic fraction, the activity and expression of LDH diminished within the Al and H₂O₂ stress. However, in the mitochondrial fraction the activity and expression of LDH were markedly augmented in the Al and H₂O₂ stressed astrocytes. Immunoblot analysis on the cytoplasmic and mitochondrial fraction for F-actin and VDAC expression respectively, demonstrated that the fractions were relatively pure and protein loading was equal. It is important to note that both the 0.01mM and 0.1mM Al stress immunoblot analysis for F-actin showed the expression of two bands. The observation that the activity of LDH decreased in the cytoplasmic fraction while increasing in the mitochondrial fraction was duplicated in HepG2 cells (**Appendix 7.10**).

In-gel activity analysis of mLDH revealed an increase under Al and H₂O₂. The activity of the mLDH also increased with increasing concentrations of Al. It was then pertinent to determine if increasing the amount of H₂O₂ or lactate would have the same effect (**Figure 3.35**).
**Figure 3.35 - Activity of mLDH under the influence of H\(_2\)O\(_2\) and lactate.** CCF-STTG1 cells were incubated I) varying concentrations of H\(_2\)O\(_2\) (20\(\mu\)M, 40\(\mu\)M, and 80\(\mu\)M) and II) different concentrations of lactate (1\(\text{mM}, 2.5\text{mM}, \text{and } 5\text{mM}) for 24\text{h. In-gel activity analysis for mLDH was then performed.}

In-gel activity analysis of mLDH under varying concentrations of H\(_2\)O\(_2\), revealed that as H\(_2\)O\(_2\) concentrations increased, the activity of mLDH increased. However, when the concentrations of lactate were increased, the activity of mLDH did not increase.

As mLDH expression and activity were increased, it was interesting to assess if there was a concomitant elevation of the transcript level. A specific mitochondrial isozyme of LDH was discovered using a bioinformatics approach, namely the LDHA like 6B (242). RNA was isolated from control, Al and H\(_2\)O\(_2\) treated astrocytes and transcript levels for LDHA like 6B was measured by qPCR (Table 3.2).
Table 3.2 – relative transcript levels of LDHA like 6B (a mitochondrial isoenzyme of LDH). mRNA from control and H2O2 treated cells was isolated and qPCR was performed to measure relative transcript levels. GAPDH was used as an internal standard. Values are expressed as transcript levels compared to GAPDH transcript levels.

<table>
<thead>
<tr>
<th></th>
<th>Trial #1</th>
<th>Trial #2</th>
<th>Average</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.21</td>
<td>1.105</td>
</tr>
<tr>
<td>40 µM H2O2</td>
<td>1.61</td>
<td>1.82</td>
<td>1.715</td>
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The transcript levels of LDHA like 6B, increased about 50% in astrocytes which were treated with H2O2 compared to the control. These levels were expressed in terms of the housekeeping gene GAPDH, therefore LDHA like 6B was expressed at greater levels in both the control and H2O2 stressed astrocytes.

With the discovery that Al and H2O2 stressed astrocytic mitochondria were consuming more lactate, a reaction was prepared to monitor the products that were formed. The mitochondria were incubated with lactate and NAD, and the resulting reaction mixture was subjected to HPLC analysis (Table 3.3).

Table 3.3 – Lactate consumption and monocarboxylate production in astrocytic mitochondria. Control, Al (0.01mM and 0.1mM), and 40 µM H2O2 mitochondria (0.2mg/mL) from astrocytes were incubated with 5mM lactate and 0.1mM NAD for 1h. The reaction mixture was then subjected to HPLC analysis. Values are expressed as percentage compared to control. n=3 ± SD; p ≤ 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Acetate</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 17.6%</td>
<td>100 ± 18.9%</td>
<td>100± 17.5%</td>
</tr>
<tr>
<td>0.01 mM Al</td>
<td>68.5 ± 11.3%</td>
<td>162.8 ± 3.5%</td>
<td>712.2 ± 6.8%</td>
</tr>
<tr>
<td>0.1 mM Al</td>
<td>29.9 ± 7.4 %</td>
<td>323.6 ± 8.0%</td>
<td>1660.1 ± 4.6%</td>
</tr>
<tr>
<td>40 µM H2O2</td>
<td>24.4 ± 8.2 %</td>
<td>335.9 ± 6.3 %</td>
<td>1699.2 ± 5.5 %</td>
</tr>
</tbody>
</table>
HPLC analysis of the mitochondrial incubations with lactate and NAD demonstrated that Al and H$_2$O$_2$ stressed mitochondria metabolized lactate more readily than control mitochondria. Additionally, other monocarboxylates such as pyruvate and acetate increased within the Al and H$_2$O$_2$ stressed mitochondria compared to the control. These aforementioned trends were more pronounced as the concentration of Al increased from 0.01mM to 0.1mM. These observations were reflected in mitochondria from HepG2 cells that were treated in a similar manner (Appendix 7.11).

The accumulation of pyruvate in the Al and H$_2$O$_2$ treated cells prompted an investigation of PDH. The activity and expression of PDH in the astrocytic mitochondria was measured using BN-PAGE, 2D immunoblotting, and in-cell western blotting techniques (Figure 3.36).

![Figure 3.36 - Activity and expression of PDH in astrocytic mitochondria. CCF-STTG1 cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H$_2$O$_2$ for 24 h. I) In-gel activity stain for PDH. 1mM Br-pyruvate was used as an inhibitor to demonstrate band specificity. II) Bands were excised from activity gel (I) and subsequently ran on a 10% SDS-PAGE gel and an immunoblot was performed for the E3 subunit of PDH. 10 μg of a PDH standard was run in tandem (Sigma).](image)

In-gel activity staining and 2D-expression analysis demonstrated that PDH activity and expression were perturbed in the Al and H$_2$O$_2$ treated astrocytes. It is
important to note that the 2D expression of PDH in the H$_2$O$_2$ stress was not affected as greatly as the Al, however it was still lower than the control. The signal from the PDH expression was at the same level in the gel as that of the PDH standard which was run. The activity of PDH was effectively inhibited by 1mM Br-pyruvate (an inhibitor of PDH), indicative of band specificity. The lowered activity of PDH in Al and H$_2$O$_2$ stressed cells was evident in HepG2 cells (**Appendix 7.12**).

The activity of LDH had increased and that of PDH had decreased. In addition pyruvate was being accumulated. Previously we have discovered KG (an $\alpha$-keto acid) was accumulated in HepG2 cells and *P. fluorescens* to combat oxidative stress (163, 243). Our goal was to determine whether pyruvate, an $\alpha$-keto acid was contributing to the attenuation of oxidative stress (**Figure 3.37**).
Figure 3.37 – Pyruvate as an antioxidant. Astrocytes were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. I) The cells were stained with Hoechst (nucleus) = blue, and DCFDA (ROS) = green, following the treatment period (No recovery). Following the treatment the cells were recovered with 5mM pyruvate for 8h (5mM pyruvate recovery). Microscopy was performed at 60X ocular objective, scale bar = 10 μm. II) 10mM 3-¹³C-pyruvate was incubated with 1mM H₂O₂ for 1h. ¹³C-NMR was then performed. Note the shift from a CH₃ group from pyruvate (~24ppm) to the CH₃ group from acetate (~21ppm).

Using DCFDA staining to measure ROS levels, it was determined that ROS levels had increased in the Al and H₂O₂ stressed astrocytes compared to the control.
When recovered for 1h with pyruvate there was a significant reduction in DCFDA fluorescence, thus less ROS. Incubation of $^{13}$C-labeled pyruvate with ROS (H$_2$O$_2$) led to the production of acetate (the non-enzymatic decarboxylation product of pyruvate).

PDH activity was observed to be inhibited in the Al and H$_2$O$_2$ treated astrocytes. Thus, to ensure PDH was contributing to pyruvate consumption or acetate production, Br-pyruvate was used to inhibit PDH. Pyruvate consumption and acetate production by the mitochondria was then ascertained using HPLC analysis (Figure 3.38).

**Figure 3.38 – Pyruvate reacts with ROS to produce acetate.** Astrocytes were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H$_2$O$_2$ for 24h. Following treatment, the 0.2mg/mL of mitochondria were isolated and incubated in 5mM Br-pyruvate, 5mM pyruvate, and 0.1mM NAD for 4h. I) Pyruvate levels were analyzed by HPLC. II) Acetate levels were measured by HPLC. ■ (closed bar) = levels prior to reaction. □ (open bar) = levels following 4h with reactants. n=3 ± SD; p ≤ 0.05.

As observed in previous experiments, the Al and H$_2$O$_2$ stressed mitochondria contained more pyruvate and acetate in comparison to the control. When Br-
pyruvate (a PDH inhibitor) was introduced into the reaction mixture, the control, Al, and H₂O₂ treated mitochondria accumulated pyruvate. However, the Al and H₂O₂ stressed astrocytic mitochondria accumulated a greater amount of acetate than the control.

To determine the fate of the acetate that was being accumulated, acetate-metabolizing enzymes were probed in the astrocytes. BN-PAGE analysis was implemented to study the in-gel activity levels of cytoplasmic acetyl-CoA synthase (cACS), mitochondrial acetyl-CoA synthase (mACS), and acetyl-CoA carboxylase (ACC) (Figure 3.39).

![Image](https://via.placeholder.com/150)

**Figure 3.39 – The fate of acetate under Al and ROS stress.** Astrocytes were incubated in **A)** Control, **B)** 0.01mM Al, **C)** 0.1mM Al and **D)** 40μM H₂O₂ for 24 h. In-gel activity was performed on **I)** mACS, **II)** cACS, and **III)** ACC. **STD** corresponds to a BSA (10 μg) and Apo-ferritin (10 μg) standard that was coomassie stained for protein expression.

In-gel activity staining of enzymes involved in acetate metabolism demonstrated that the mitochondrial isoform of ACS was decreased in the Al and
H$_2$O$_2$ stressed astrocytes. Alternatively, the cytoplasmic isoform of ACS was augmented in Al and H$_2$O$_2$ stressed conditions. In-gel activity analysis of ACC showed that the levels of the enzyme increased under Al and H$_2$O$_2$ treatment when compared to the control cells.

Since the activity of enzymes that devote acetate to lipid production were increased, measuring the levels of lipids within the cell was the logical route to follow. Oil Red O staining was used to visualize lipid levels using fluorescence microscopy (Figure 3.40).

![Fluorescence microscopy of triglyceride levels in astrocytes.](image)

**Figure 3.40** - Fluorescence microscopy of triglyceride levels in astrocytes. Astrocytes were incubated in **A)** Control **B)** 0.01mM Al **C)** 0.1mM Al and **D)** 40μM H$_2$O$_2$ for 24 h. Post-treatment, the cells were treated with Hoechst (nucleus) = blue and Oil Red O (triglycerides) = red. Scale bar = 10 μm.
Fluorescence microscopy demonstrated that the levels of lipids increased in the astrocytes treated with Al and H₂O₂ in comparison to the control cells. This observation was replicated in HepG2 cells (Appendix 7.13). Al and H₂O₂ treated astrocytes were observed to consume lactate at a greater rate than control cells. In addition, Al and H₂O₂ stressed astrocytes were found to accumulate the monocarboxylates; pyruvate and acetate. The heightened consumption of lactate and accumulation of pyruvate was attributed to an increased activity of mM LDH and a concomitant reduction in the levels of PDH. The accumulated pyruvate appeared to be utilized for the detoxification of ROS present in the Al and H₂O₂ stressed cultures, to produce acetate by non-enzymatic decarboxylation. The acetate that was produced was directed toward lipid storage through cACS and ACC (Figure 3.41).

![Diagram](image)

Figure 3.41 - The mitochondrial pathway of lactate metabolism in Al and ROS stressed astrocytes.
3.5 - Disruption of L-carnitine Metabolism and Lipid Accumulation

An accumulation and redirection of α-keto acids was apparent in the Al and H\textsubscript{2}O\textsubscript{2} stressed cells when compared to the control cells. As pyruvate was being redirected toward the scavenging of ROS, the status of another α-keto acid, KG was ascertained. We have recently uncovered the anti-oxidative role of this keto acid (163). HPLC analysis was implemented to detect the levels of KG and its non-enzymatic decarboxylation product, succinate (Figure 3.42).

![Graph](image)

**Figure 3.42 - KG and Succinate levels in astrocytoma cells.** CFE was obtained from CCF-STTG1 cells and **A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H\textsubscript{2}O\textsubscript{2} containing media.** HPLC analysis was performed to ascertain □ = KG levels (open bar) and ■ = succinate levels (closed bar) in the CFE. n=3 ± SD. P ≤ 0.05. (*Indicates a significant change KG levels, whereas ** is indicative of a significant change in succinate levels). (Lemire et al. 2011)

HPLC analysis of the CFE from astrocytes demonstrated that both the levels of KG and its non-enzymatic decarboxylation product, succinate increased in the Al
and H$_2$O$_2$ treated cultures. This observation was mirrored in HepG2 cells (Appendix 7.14).

The prospect that KG is utilized to attenuate ROS was investigated by performing in-gel activity on enzymes involved in KG accumulation (NADP-ICDH and KGDH) (Figure 3.43)

![Activity of KG pooling enzymes](image)

**Figure 3.43 – Activity of KG pooling enzymes.** Astrocytic cells were analyzed under A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H$_2$O$_2$ conditions. I) In-gel activity analysis of mitochondrial CFE for KGDH. (Lemire et al. 2011)

Microscopic analysis on ROS levels in the presence and absence of KG (Figure 3.44).
Figure 3.44 – KG and ROS detoxification. Astrocytic cells were analyzed under A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H₂O₂ conditions. Fluorescence microscopy was performed at 20X ocular magnification. Blue = Hoechst (nuclei), Purple = DCFDA (ROS). Following a 24h treatment the cells were recovered with 5 mM KG 8h (note the sharp decrease in ROS). Scale bar = 10 μm. Arrows indicate fluorescence generated by ROS. (Lemire et al. 2011)
In-gel activity demonstrated that the NADP-ICDH increased in activity in Al and H$_2$O$_2$ stressed cells. Meanwhile, the activity of KGDH had decreased in activity in the Al and H$_2$O$_2$ stressed cells when compared to control cells. Microscopic analysis revealed that ROS levels are intensified in Al and H$_2$O$_2$ treated cells. The treatment with 5mM KG for 8h relieved the ROS.

Since we established that there was an accumulation of lipids (Figure 3.40), and that KG was being rerouted, it was relevant to evaluate if the lipid-carrying molecule L-carnitine (a KG dependent moiety) was also affected. HPLC analysis was used to establish the status of L-carnitine and acyl-carnitine in Al and H$_2$O$_2$ treated astrocytes (Figure 3.45).

![Figure 3.45](image)

**Figure 3.45 – L-carnitine homeostasis in astrocytoma cells.** HPLC analysis was done to measure total and free carnitine levels in the CFE from **A** Control **B** 0.01 mM Al **C** 0.1 mM Al and **D** 40 μM H$_2$O$_2$ treated. **I** free-carnitine levels in astrocytoma cells, **II** Total carnitine levels in astrocytoma cells. Where the open bar □ is non-recovered cells and the closed bar ■ are cells recovered with 5mM KG for 8h. n=3 ± SD. P ≤ 0.05. (*Indicates a significant change in metabolite levels) (Lemire et al. 2011)
Free L-carnitine, a moiety that relies on KG to be synthesized, was found to be lower in cells exposed to Al and H$_2$O$_2$ compared to the control cells. When the cells were recovered with KG for 8h, the levels of free L-carnitine in the Al and H$_2$O$_2$ treated cells returned to levels comparable to the control cells. This trend was replicated in HepG2 cells (Appendix 7.15). Total L-carnitine levels (carnitine bound to acyl groups in addition to free carnitine) was found be much lower in the astrocytes exposed to 0.1mM Al and H$_2$O$_2$ compared to the control astrocytes. However, cells exposed to 0.01mM Al did not experience a great reduction in total L-carnitine levels compared to control cells. The ability of L-carnitine to attenuate ROS stress was also studied (Figure 3.46).
Figure 3.46 – L-Carnitine and ROS detoxification. Astrocytic cells were analyzed under A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H₂O₂ conditions. Fluorescence microscopy was performed at 20X occular magnification. Blue = Hoechst (nuclei), Purple =DCFDA (ROS). Following a 24h treatment the cells were recovered with 5 mM carnitine for 8h. Scale bar = 10 μm. Arrows indicate fluorescence generated by ROS. (Lemire et al. 2011)
A recovery with 5mM L-carnitine failed to release ROS levels in the H₂O₂ treated cells, however it was able to attenuate ROS fluorescence in the Al treated astrocytes. Since L-carnitine levels were lower in Al and H₂O₂ stressed cells when compared to control cells, measuring the levels of the enzymes that synthesize L-carnitine was undertaken. In-gel activity analysis and subsequent 2D expression analysis of BBADH and BBDOX, two enzymes in the L-carnitine synthesis pathway was performed (Figure 3.47).

![Figure 3.47 - In-gel activity analysis and expression of enzymes involved in carnitine biosynthesis.](image)

Astrocytoma cells were treated in A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H₂O₂ containing media. I) In-gel activity analysis of cytoplasmic CFE for BADH (negative controls were performed in the absence of either NADH or INT from the reaction mixture) and II) In-gel activity analysis of cytoplasmic CFE for BBDOX (negative controls were performed in the absence of either ascorbic acid (VitC) or KG from the reaction mixture). Bands were subsequently precision excised and run on a 10% isocratic SDS-PAGE gel and silver stained for expression analysis of III) BBADH and IV) BBDOX. (Lemire et al. 2011)
In-gel activity staining of the enzymes involved in the synthesis of L-carnitine, BBADH and BBDOX, showed the activity to be perturbed under Al and H₂O₂ stressed conditions when compared to control conditions. A molecular mass standard of 10µg of BSA and Apo-ferritin were ran along side the protein samples to ensure the gel had run properly. In addition negative controls, reaction mixtures without NADH or INT (BBADH) and VitC or KG (BBDOX), were performed to ensure the specificity of the in-gel reaction. There was no activity present in the negative controls. When the in-gel activity bands were excised and run on a 2D isocratic SDS-PAGE gel, the expression levels were also found to have decreased in the Al and H₂O₂ stressed cells in comparison to the control cells. The molecular mass ladder that was run demonstrated that the protein showed up at the level on the gel which corresponded to their approximate predicted molecular masses of 54 kDa and 42 kDa for BADH and BBDOX respectively (244, 245).

To confirm that the expression levels of BBDOX were inhibited in Al and H₂O₂ treated cultures compared to control cultures, immunoblotting technique was used to measure protein levels (Figure 3.48).

Figure 3.48 - Expression levels of BBDOX in astrocytoma cells. Immunoblot analysis of CFE for BBDOX was performed in A) Control, B) 0.01mM Al, C) 0.1mM Al, and D) 40 µM H₂O₂ stressed I) astrocytoma. VDAC served as a loading control for these experiments. (Lemire et al. 2011)
Measuring expression levels of BBDOX by immunoblotting, revealed that the levels were reduced in Al and H$_2$O$_2$ treated astrocytes compared to the control astrocytes. These levels were compared to the mitochondrial marker, VDAC, which had expression patterns which were similar in all the conditions. This observation was found to be the same in HepG2 cells (Appendix 7.16).

To confirm the expression patterns delineated by immunoblotting of BBDOX in astrocytes, in-cell immunoblotting was implemented for confirmation (Figure 3.49).

![Figure 3.49 - In-cell immunoblot analysis of BBDOX in astrocytoma cells. Cells were seeded in a 96 well plate and treated with A) Control, B) 0.01mM Al, C) 0.1mM Al, and D) 40 μM H$_2$O$_2$. In-cell immunoblots were then performed for I) BBDOX and II) Actin. n=4 ± SD. P ≤ 0.05. (*Indicates a significant change BBDOX expression levels). (Lemire et al. 2011)](image)

In-cell immunoblot analysis for BBDOX, confirmed the observations revealed by the immunoblot analysis. The expression of BBDOX was decreased in the Al and
H$_2$O$_2$ stressed cells when compared to control conditions. This was compared to the
expression levels of F-actin which were unaltered between treatment conditions.
The loss of L-carnitine and the subsequent inability of the cell to oxidize lipids when
stressed with Al and H$_2$O$_2$ was evident by the accumulation of lipids visualized using
fluorescence microscopy and Oil Red O staining (Figure 3.40 and Appendix 7.13).
In addition when Al stressed HepG2 cells were given palmitic acid, they were unable
to consume them. This was assessed using HPLC, where there was a greater
amount of palmitate in the Al stress compared with the control. BSA, which was
used as the delivery vehicle for the palmitate, was also given as a control to Al and
control HepG2 cells. There was very little palmitate evident in the control and Al
treated HepG2 cells in the BSA control. When the control and HepG2 cells were
recovered with 5mM KG for 8h, the palmitate was subsequently metabolized
(Appendix 7.17).

To ascertain if the accumulation of lipids in Al and H$_2$O$_2$ stressed cells was
solely due to a lack of β-oxidation, or if there was also an increase in lipogenic
enzymes. The in-gel activity of CL and ACC were measured in control, Al, and
stressed astrocytes and hepatocytes (Table 3.4).
Table 3.4 - In-gel activity analysis for lipogenic enzymes. The activities of both ACC and CL were ascertained via in-gel activity assay. The bands were subsequently quantified utilizing Scion Imaging™ software. n=3 ± SD. P ≤ 0.05. (*Indicates a significant difference in enzyme activity). (Lemire et al. 2011)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Astrocytoma</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.01 mM Al *</td>
<td>0.1 mM Al *</td>
</tr>
<tr>
<td>Citrate Lyase</td>
<td>337 ± 58</td>
<td>1179 ± 112</td>
<td>6956 ± 79</td>
</tr>
<tr>
<td>Acetyl-CoA Carboxylase</td>
<td>3050 ± 92</td>
<td>15870 ± 877</td>
<td>16738 ± 1347</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HepG2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Lyase</td>
<td>Control</td>
<td>0.25 mM Al *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2998 ± 407</td>
<td>11306 ± 761</td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA Carboxylase</td>
<td>10784 ± 780</td>
<td>27014 ± 250</td>
<td></td>
</tr>
</tbody>
</table>

In-gel activity analysis and subsequent quantification using Scion Imaging™ software revealed that CL and ACC activities were elevated in the Al and H₂O₂ stressed cells in comparison to the control cells. The channeling of KG to attenuate ROS stress in Al and H₂O₂ treated cells led to the accumulation of succinate and a lack of L-carnitine synthesis. The enzymes which synthesize L-carnitine (BBADH and BBDOX) were also inhibited by Al and H₂O₂ stress. This lack of L-carnitine production resulted in the inability of the Al and H₂O₂ stressed cells to oxidize lipids. Lipogenic enzymes were also augmented in Al and H₂O₂ treated cells. Al and H₂O₂ stressed cells were found to accumulate a greater amount of lipids when compared to control cells (Figure 3.50).
Figure 3.50 – Al and ROS induced lipid accumulation. Al generates ROS. The ROS is attenuated by pooled KG with the subsequent production of succinate. There is less KG available for L-carnitine production, thus lipids cannot be carried to the mitochondria to be oxidized.

3.6 – The Impact of Al and ROS on Other Functions of Astrocytes

Since another key function of astrocytes is the recycling of neurotransmitters, it was important to elucidate if indeed Al and H$_2$O$_2$ affected this critical role. Glutamate is the main neurotransmitter in the brain, so determining the ability of the astrocytes to uptake glutamate under Al and H$_2$O$_2$ treatment was monitored. HPLC was implemented to measure glutamate clearance from the spent media of control, Al, and H$_2$O$_2$ stressed astrocytes (Figure 3.51).
Figure 3.51 - Glutamate clearance in Al and H$_2$O$_2$ treated astrocytes. Astrocytoma cells were treated in A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 $\mu$M H$_2$O$_2$ containing media. I) Following the treatment period the spent media was subjected to HPLC to measure glutamate levels. II) Following the treatment period the astrocytes were recovered with 5mM glutamate for 2h. The consumption was then measured via HPLC. n=1.

Measuring the levels of glutamate in the spent fluid using HPLC revealed that the Al and H$_2$O$_2$ stressed cells took up more glutamate from the media when compared to the control. When the astrocytes were incubated with 5mM glutamate for 2h, the Al and H$_2$O$_2$ stressed cells removed more of the neurotransmitter from the media compared to the control astrocytes. Upon entry into the cell, glutamate must be acted upon by enzymes to be recycled. Since there was an observed difference in glutamate clearance from the spent media, ascertaining the activity of enzyme involved in glutamate clearance under Al and H$_2$O$_2$ treatment was performed. BN-PAGE was utilized to determine the in-gel activity of enzymes involved in glutamate clearance (Figure 3.52).
Figure 3.52 - In-gel activity analysis of enzyme involved in glutamate metabolism. Astrocytoma cells were treated in A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 µM H₂O₂ containing media. Following the treatment period the astrocytes were recovered with 5mM glutamate for 2h. I) In-gel activity of GDH. II) In-gel activity analysis of GluS. III) In-gel activity analysis of GlnS.

The in-gel activity of glutamate metabolizing enzymes (GDH, GluS, and GlnS) were inhibited by Al and H₂O₂ stress when compared to controls. GlnS appeared to be particularly sensitive to low doses of Al (0.01mM), while a slight reduction of activity was observed in the the 0.1mM Al and 40 µM H₂O₂ treated cultures. When the astrocytes were given 5mM glutamate for 2h, the enzyme activities of GDH and GluS appeared to shift activity patterns. In the 0.1mM Al and 40 µM H₂O₂ treated astrocytes GDH and GluS experienced an increase in activity compared to 0.01mM Al treated and control cells. However, GlnS activity was lowered in the stressed cells compared to the control.

Preliminary observations on the effects of Al and H₂O₂ stress on neurotransmitter clearance by astrocytes demonstrated that glutamate is more readily taken up by Al and H₂O₂ stressed astrocytes. The enzymes which metabolize glutamate are down under Al and H₂O₂ stress when glutamate is not present. However, when the astrocytes are introduced to 5mM glutamate in their media, the Al and H₂O₂ stressed astrocytes activate GDH and GluS. GlnS remains impaired under no glutamate recovery as well as under the presence of glutamate. Thus,
enabling these Al and ROS-challenged cells to divert Glu to the production of the antioxidant KG.

Astrocytes serve the additional function of being glucose stores for the brain as glycogen granules. Since there was an observed dysfunction of astrocytic metabolism under Al and H$_2$O$_2$ stress, we explored the status of glycogen. A periodic acid-Schiff stain was performed on control, Al, and H$_2$O$_2$ treated astrocytes to quantify glycogen by microscopy (Figure 3.53).

![Figure 3.53 - Glycogen levels in astrocytes under Al and ROS treatment.](image)

Astrocytes were stressed with A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H$_2$O$_2$ containing media. The cells were subsequently treated with Hoechst (nucleus) = blue and periodic acid-Schiff stain (glycogen) = red. Scale bar = 10 μm.

Microscopy revealed that astrocytes that were treated with Al and H$_2$O$_2$ accumulated a greater amount of glycogen within their cytoplasm when compared
to control cells. Indeed, the function of neurotransmitter recycling and energy storage are affected by the presence of Al and H$_2$O$_2$.

3.7 – *Nuclear Metabolism under Al and ROS Stress*

As metabolic networks in the nucleus are involved in a variety of nucleic acid and histone modifications, it was important to evaluate if these toxins have an impact on nuclear metabolism. The levels of metabolites of interest were monitored by performing HPLC analysis on the control, Al, and treated astrocyte nucleus *(Table 3.5)*.

**Table 3.5 – Metabolite levels of Al and ROS treated astrocyte nucleus.** Control, Al (0.01mM and 0.1mM), and 40 μM H$_2$O$_2$ nucleus (0.2mg/mL) from astrocytes were subjected to HPLC analysis. Values are expressed as percentage compared to control. n=1.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>0.01mM Al</th>
<th>0.1mM Al</th>
<th>40μM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>120.2</td>
<td>201.2</td>
<td>126.3</td>
</tr>
<tr>
<td>Acetate</td>
<td>308.3</td>
<td>333.7</td>
<td>283.8</td>
</tr>
<tr>
<td>Citrate</td>
<td>103.1</td>
<td>183.9</td>
<td>109.7</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>103.9</td>
<td>175</td>
<td>105.8</td>
</tr>
<tr>
<td>Succinate</td>
<td>99.6</td>
<td>184.6</td>
<td>126.9</td>
</tr>
</tbody>
</table>

The levels of keto acids such as KG and pyruvate increase under Al and H$_2$O$_2$ treatment, as does their subsequent non-enzymatic decarboxylation producte succinate and acetate, respectively. It is also interesting to note that citrate levels
increase under 0.1 mM Al treatment. It is apparent that 0.1 mM Al has a greater effect on metabolite levels alteration than does 0.01 mM Al and H$_2$O$_2$ stress. To further assess the effects of Al and H$_2$O$_2$ on nuclear metabolism, the in-gel activity of some metabolic enzymes was measured by BN-PAGE (Figure 3.54).

![Figure 3.54 - In-gel activity analysis of nuclear metabolic enzymes.](image)

Astrocytoma cells were treated in A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 $\mu$M H$_2$O$_2$ containing media. Analysis was performed for nLDH, nMDH, nME, and nCL.

Pre-liminary in-gel activity analysis revealed the presence of a nuclear isoenzyme of LDH (possibly two isoforms), MDH, ME, and CL. All of these aforementioned nuclear enzymes experienced higher activity in the Al and H$_2$O$_2$ treatment conditions. These nuclear reactions do necessitate the need for numerous metabolites like KG and pyruvate for antioxidative defense as well as acetate for histone modification.
Section 4: Discussion

Al is a toxicant with many facets. The interference with Fe homeostasis and the production of ROS are two of the paramount biochemical implications of Al stress (179, 246). Indeed, the disruption of Fe metabolism and ROS production are hallmarks of other environmental toxicants, numerous disease states, and aging (247-249). The bioavailability of Al is on the rise due its industrial utilization and use in everyday activities. This is a cause for concern because of the connection between the metal toxin and neurological disorders. In the case of Alzheimer's disease, the induction and phosphorylation of Tau protein, and the promotion of neuroinflammatory transcripts has been linked to Al toxicity (93, 193-195, 198). However, other molecular aspects of the interaction of Al with the brain have yet to be fully delineated.

The effects of Al and H$_2$O$_2$ on astrocytes, in this study were found to alter mitochondrial metabolism, an event that leads to a loss of oxidative energy production. The lack of cellular ATP levels resulted in the inability of the actin-based cytoskeleton to polymerize and form a filamentous structure. The inability of the cytoskeleton to form under Al and ROS presence resulted in a loss of astrocytic morphology, a feature that could be deleterious to proper brain functioning. This research also revealed a mitochondrial isoform of LDH, which bestowed the ability of this astrocytic cell line, to metabolize lactate in an oxidative fashion to produce energy. The role of the mLDH seemed to switch under Al and ROS stress to a generator of the anti-oxidant, pyruvate. This pyruvate reacted with the ROS non-enzymatically to yield acetate. The acetate was utilized by the astrocytes to produce
lipids. In addition to an increase in lipid synthesis under Al and ROS stress, the astrocytes experienced a decrease in the β-oxidation of lipids due to the diminished synthesis of L-carnitine, the lipid-cargo importer to the mitochondria. The lack of L-carnitine is due to a shunting of another α-keto acid, KG toward ROS detoxification. The molecular details of these aforementioned events are seminal findings on a link between Al, ROS, and neurological disorders and are discussed in further detail (Figure 4.1).

**Figure 4.1 - A global view of the effects of Al on astrocyte physiology.** Al interferes with mitochondrial metabolism by the production of ROS and the interference with Fe homeostasis. This leads to a lack of energy production, a disparate metabolite profile, and disturbance in Glu/Gln cycling. All of the previously mentioned biochemical pathways are crucial to astrocytes functional role in brain physiology.


4.1 - Al Induces ROS and Altered Astrocytic Metabolism

Preliminary observations on the effects of Al and H$_2$O$_2$ (an ROS moiety) on an astrocytic cell line led to cellular dysfunction and altered metabolism. The fashion in which Al disrupts astrocyte biochemistry and cell physiology is likely due to the interference with Fe homeostasis (185, 250). An increase in bioavailable Fe leads to the production of ROS and the disturbance of macromolecules such as proteins, DNA, and lipids (137, 251). In addition, Al can interact with the phosphate head groups in the lipid bilayer, exposing the fatty acyl chains to oxidative damage (252). Thus, Al and Fe-derived ROS act in concert to damage the cell membrane.

In this study, an Al-lactate complex (2.5mM lactate:0.01mM Al, and 2.5mM lactate:0.1mM Al) was used to deliver the Al. Use of the morin stain, revealed that Al was gaining entrance to the astrocytes (Figure 3.3). The choice of an Al-lactate chelate was due to its physiological relevance. Organic acids such as citrate and lactate are found to bind Al in the body and can help with the passage of Al across the BBB (253, 254). Al-lactate also proved to be a useful Al delivery vehicle in other studies involving the brain (255-257).

Al and Cell viability

Al’s entrance into the astrocytes resulted in a loss of viable cells (Figure 3.1). 40µM H$_2$O$_2$, a direct ROS stressor, likewise lead to a decrease in viable cells (Figure 3.2). It is interesting to note that the astrocytes were not dying under Al and ROS treatment, but growth was perturbed. This was apparent by the absence of non-viable cells found during cell counts (Appendix 7.7). A lack of Caspase-3 cleavage products also confirmed that the astrocytes were not dying via apoptosis.
Treatment with these concentrations of Al and ROS were inhibiting cell growth rather than increasing cell death. However, other research has demonstrated that much higher concentrations of Al (1mM) can induce cell death in neural cells (259).

*Al and H₂O₂ promote ROS production*

Indeed, Al and H₂O₂ stress induces a state of ROS in the astrocytes (Figure 3.4). Al generates ROS through its interference with Fe homeostasis (179, 183). Labile Fe generates ROS, which in turn can oxidize lipids, proteins, and DNA (137). Indeed, the levels of oxidized protein and lipids were elevated under Al and ROS treatment when compared to control (Table 3.1). Al, as well as other metals (Fe and Cu), are found to associate with Abeta peptide in senile plaques (183). The disruption of Fe homeostasis by Al, and the association of Fe and Al with senile plaques, may lead to augmented oxidative stress in the brain and explain some pathological findings in Alzheimer’s disease.

*Altered metabolism*

The accumulation of metabolites (Figure 3.5) and proteins (Figure 3.6) in the culture media of Al and ROS treated cells is indicative of cellular dysfunction, since metabolites will accumulate when the enzymes that metabolize them are dysfunctional (260). Accumulation of metabolites (such as KG) in the extracellular environment has been described previously in other stressed conditions including oxidative, hypertensive, and ischemic (260-262). Previously, we have demonstrated that Al can induce the efflux of metabolites into the culture media of hepatocytes (167). As stated previously, Fe homeostasis is altered under Al and ROS treatment.
Since oxidative metabolism is inherently dependent on Fe (ACN, FUM, SDH, Cyt C. Ox.) the accumulation of metabolites under Al and ROS treatment is likely due to an impairment of Fe dependent metabolic pathways. A phenomenon that has been previously demonstrated in hepatocytes and *Pseudomonas fluorescens* (163). Figure 4.2 summarizes these preliminary observations.

**Figure 4.2 - Cellular dysfunction under Al and ROS stress.** Al-lactate and H$_2$O$_2$ enter the astrocyte where they interfere with Fe homeostasis. The free Fe then generates ROS. The ROS leads to the oxidation of cellular components (lipids, proteins, and nucleic acids). Interference with Fe homeostasis, oxidized cell components, and ROS all inhibit oxidative metabolism and lead to cellular dysfunction. The enzymes of oxidative metabolism are no longer available to consume the metabolites and thus the astrocytes efflux them. These factors contribute to the observed loss of viable cell growth and the accumulation of metabolites in the spent fluid.
Under Al and ROS stress, a loss in cell viability was observed. Al was found to gain entrance into the astrocytes where it induced an oxidative state, likely due to interference with Fe homeostasis. The increase in ROS lead to the creation of oxidized cellular components, namely lipids and proteins. The oxidized lipids and proteins, the interference with Fe homeostasis, and the generation of ROS, interfered with cellular metabolism and imposed a state of cellular dysfunction.

4.2 - Al and oxidant induced loss of cytoskeletal architecture

Al and ROS treated astrocytes were observed to have an altered cellular morphology. The filopodia of the astrocytes appear to be absent under Al stress. The cells look round and globular as opposed to having nice defined processes (Figure 3.7). The filopodia of neural cells are their defining structures and integral to their functioning. The end-feet of astrocytes are responsible for many key physiological functions including brain scaffolding, nutrient exchange, and neurotransmitter clearance (263). Thus, their absence would be detrimental to brain physiology. In fact, many neurological diseases are characterized by a loss of neuronal processes including Alzheimer’s disease, Parkinson’s disease, aging, and other neurotoxic events (264-266). Indeed, Al and other metal toxicants have also been demonstrated to cause a loss in neural processes (256, 267-269).

The actin-cytoskeleton

The filopodia of astrocytes are actin-rich cytoskeletons (270). The actin-based cytoskeleton is necessary to maintain the morphological features of the astrocyte, and is pivotal to the cells proper functioning (271). CCF-STTG1 cells
exposed to Al and ROS stress revealed that the actin was unable to polymerize to form a filaments and remained globular \((201)\) (Figure 3.8 and 3.9). Other studies have demonstrated the dysfunctional actin-cytoskeleton under Al treatment in neurons \((272)\) and plants \((273)\). It has also been observed that the cytoskeleton is sensitive to ROS \((274, 275)\) and cadmium \((276)\). These toxicants, like Al promote an oxidative environment within the cell and could be responsible for neurological disorders. Other filaments have also been found to be affected under Al stress, such as microtubules \((277)\). What other studies have failed to demonstrate is a definitive underlying cause for the inability of actin to polymerize. It is important to note that it is not the expression of actin that was affected by Al and ROS stress, it was the inability of the actin to polymerize and form a defined filamentous structure (Figure 3.8 and 3.9).

Al and ROS-induced ATP depletion

Actin polymerization is an ATP demanding cellular process \((238, 278)\). It stands to reason that a lack of ATP would affect many cellular processes, including the turnover of the actin cytoskeleton. Previously, research from our lab has found that oxidative energy production is diminished under Al treatment \((159, 160)\). As stated previously, Al interferes with Fe homeostasis, which consequently increases cellular ROS levels. Fe-dependent enzymes of the TCA cycle and ETC were found to be diminished under Al and ROS stress leading to a loss in ATP production \((163, 167)\). Treatment of astrocytes with Al and ROS also lead to a decrease in oxidative energy production (Figure 3.10). The decrease in activity of Cyt C Ox. could be occurring for two reasons; 1) Cyt C Ox. is an Fe dependent enzyme and Al and ROS
interfere with Fe dependent enzymes. 2) The ETC inherently produces ROS, and thus the cell might attenuate the activity of oxidative phosphorylation under Al and ROS stress to minimize its contribution to the oxidative state. The activity of NAD-ICDH may also be decreased under Al and ROS stress for this same reason. Production of NADH would drive oxidative phosphorylation, and thus decreasing the amount of NADH production would limit the contribution of the ETC to ROS production (279). The consequence of a lack of ATP can be demonstrated by the lack of activity of NDPK in Al and ROS treated astrocytes. NDPK transfers high-energy phosphates from ATP to other nucleotide diphosphates (280). A lack of ATP would result in a lack of fuel for NDPK to fix phosphates onto other nucleotide diphosphates.

The Al and ROS induced decrease in oxidative phosphorylation was also observed by a loss of mitochondrial membrane potential and a lack of mitochondrial O₂ consumption (Figure 3.11 and 3.12). The production of a proton gradient is key to driving oxidative energy production (281). The loss of the production of a proton gradient in Al-stressed astrocytes is a clear indication of mitochondrial dysfunction. O₂ is the terminal electron acceptor of the ETC. The lack of consumption in O₂ indicates that Al and ROS are affecting movement of reductive power down the ETC. Taken together mitochondrial metabolism is perturbed by Al and ROS causing a loss of ATP production. The inability to preserve cellular ATP levels as a consequence of Al and ROS can be disastrous to the cell as ATP is required to drive various cellular functions including maintaining the structure of the cytoskeleton and ultimately cell morphology (282).
CK and ATP buffering

CK in the brain is a crucial enzyme for the maintenance of astrocyte morphology. CK buffers ATP into phosphocreatine as a means to build energy reserves. This provides ATP storage for periods of intense energy demands, while at the same time promoting the continued production of ATP by maintaining ADP pools. CK has been found to associate with the actin filament to produce energy locally for maintenance of cytoskeletal structure and function (283). Similarly, glycolytic enzymes have been found to also interact with polymerized actin, forming multi-enzyme complexes for cytoskeletal rearrangements and organelle transport (284). Having enzymes that can generate ATP locally provides an advantage, as mitochondria are too large for the confines of the fine processes of the cell.

Cells such as neurons and astrocytes rely on local substrate level phosphorylation to generate ATP for actin turnover in their processes (dendrites, axons, and filopodia), while the mitochondria supplies ATP to the cell body. The lack of ATP under Al and ROS treatment and the combined lack of CK activity/expression, lack of Cr/PCr, and the inability of CK to associate with actin lead to a set of circumstances that appear to be preventing the cytoskeleton from forming and subsequently leading to a loss of cellular morphology (Figure 3.13 and 3.14)(201). The reduction in Cr levels suggests that Al and ROS could be affecting the synthesis of Cr. Lack of PCr could be partially due to the aforementioned lack of Cr synthesis or that there is not enough ATP available to store it in PCr. The loss in activity of CK has been demonstrated in the presence of ROS the brain and heart (138, 285).

Profilin-2; the actin polymerization catalyst
The low-molecular weight protein, Profilin, aids in the ADP to ATP exchange on globular actin monomers and its absence slows down the process of actin polymerization (47, 48, 286). Lack of ATP and the decreased expression of profilin-2 under Al and ROS treatment may both be contributing factors to the loss of actin polymerization (Figure 3.15 and 3.16).

*Antioxidant recovery of Al and ROS stress*

It was evident that Al and H₂O₂ were inducing an ROS stress on the astrocytes (Figure 3.17). Treatment with the anti-oxidant NAC recovered the energy status and the cytoskeleton of the astrocytes (Figure 3.18). The recovery of ATP levels signals that oxidative energy production was likely resumed following the relief of oxidative stress. The TCA cycle and the ETC would resume the production of NADH production and ATP production respectively, because the threat of contributing to ROS production would be relieved. The recovery of cellular ATP levels and the ability of the cytoskeleton to polymerize following antioxidant recovery supports the observations that astrocytes exposed to 0.01mM Al, 0.1mM Al, and 40μM H₂O₂ were not undergoing apoptosis. Although Al has been demonstrated to mediate cytoskeletal disassembly by apoptosis (287), the reversibility of the effects with an antioxidant obviates this possibility in these present studies.

The cytoskeleton performs a crucial role in astrocytes, allowing these cells to support brain functions (288). The ability of Al and ROS to decrease ATP levels and interfere with the formation of the cytoskeleton would perturb the functions of astrocytes in the brain. The loss of astrocyte morphology has been characterized in numerous neurological disorders (288). Here we discovered a mechanism by which
Al and ROS interfere with morphology by affecting the biochemical pathways involved in energy production (Figure 4.3).

![Diagram of actin polymerization and creatine kinase reaction](image)

**Figure 4.3 - Al and ROS induced interference with actin polymerization.** Al and ROS interfere with Fe homeostasis, which leads to a decrease in oxidative energy production. Cellular pools of ATP become depleted and CK cannot produce PCR. There is also no ATP available for NDPK to make other nucleotide triphosphates. Lack of ATP and a downregulation in Profilin-2 prevent the polymerization of the actin-cytoskeleton. Red = a downregulation under Al and ROS treatment.

This is the first demonstration of a molecular link between Al toxicity, perturbed cellular energy metabolism, and a loss of cytoskeletal structure in human astrocytoma cells (201). These data also demonstrate the importance of CK in buffering the energy that is necessary for fueling biochemical reactions such as maintenance of the cytoskeleton. Al and ROS disrupt cellular energy production and
results in the inability of CK and profilin-2 to contribute to the maintenance of cellular morphology (201). Taken together, these data provide a molecular link to Al, ROS, energy metabolism, and neurological dysfunction.

4.3 – Astrocytes and the Lactate Conundrum

Lactate is still regarded as a metabolic end product of anaerobic respiration that must be shipped to the liver to be converted back into something useable (glucose) by a process called the Cori cycle (289). Levels of lactate in the plasma can reach up to 1mM and exceed that during exercise (61-63). Under sustained periods of elevated stimulation, the brain has also been demonstrated to produce large amounts of lactate (290). The elevated levels of lactate are derived from astrocytes producing lactate from glucose to feed hungry neurons through the ANLS (291-293). This lactate provides carbon to support neuronal energy and neurotransmitter production. However, astrocytes also require an oxidizable source of energy as they actively expend ATP to support a variety of neuronal functions. Other highly oxidative tissues such as the heart and skeletal muscle have been found to utilize lactate in an oxidative fashion to produce energy (54, 59). This utilization of lactate in an oxidative fashion was found to be mediated by a mitochondrial isoenzyme of LDH (294). Since our research relied on lactate as our control treatment and our delivery vehicle for Al, and the astrocytes appeared to be growing very well when given lactate as a carbon source, we set out to discover if astrocytes could utilize lactate as a source of carbon for mitochondrial energy production.

Lactate consumption
When astrocytes were supplemented with lactate they readily consumed it to support their growth and cell division (Figure 3.20). If this metabolite was being used to support growth it was hypothesized that the mitochondria might be using it as a source of carbon to produce ATP. Adding NAD to support the reaction revealed that mitochondria from the astrocytes were capable of consuming lactate in a time-dependent manner (Figure 3.21). This observation was crucial, as the mitochondria of neural cells, until this point hadn’t been observed to consume lactate. The lactate dogma in the brain is that lactate is utilized by neurons and supplied by astrocytes as part of the ANLS to support energy demands of neurons.

*Lactate is a source of mitochondrial energy in astrocytes*

Since lactate was supporting the growth of astrocytes and the mitochondria was consuming the lactate, it was likely that lactate was contributing to oxidative metabolism by entering the TCA cycle and producing NADH. This NADH could then be used by the ETC to produce ATP and support growth and cell functions. The mitochondria were indeed using lactate to support oxidative energy production (Figure 3.22). Comparing the capacity of the mitochondria to produce NADH in the presence of lactate to that of citrate revealed that they are both capable of manufacturing the reducing factor. Equal molarities of lactate and citrate were utilized for this experiment. Therefore the increased NADH production from citrate is likely due to it being a six-carbon moiety compared to lactate, which is a 3-carbon moiety. Citrate would be expected to produce twice as much NADH under ideal conditions.
If lactate was truly being diverted toward the TCA cycle for oxidative energy production, there should be the accumulation of TCA cycle intermediates. Metabolomic analysis was performed on isolated mitochondria incubated in lactate, NAD, and NaN₃ (to halt the ETC). TCA cycle intermediates were found to accumulate in the reaction mixture following the incubation period (Figure 3.23). NaN₃ was needed to slow the complete combustion of all the lactate. By backing up the ETC, TCA cycle intermediates would eventually pile up. The TCA cycle is the gateway between carbohydrate combustion and the ETC where ATP is formed. Previous studies have demonstrated that neurons when given ¹³C-labelled lactate, produced glutamate (63). This supports the idea that neurons given exogenous lactate from the blood or from the astrocytes via the ANLS supplements neurotransmitter production.

Following the observation that lactate contributes to the TCA cycle and produces NADH, it was necessary to ascertain if the energy that was being produced in the presence of lactate was indeed derived from the ETC. O₂ is the terminal electron acceptor of the ETC, and its consumption is directly related to the activity of oxidative energy production. The mitochondria incubated in lactate were discovered to be consuming O₂ at a rate comparable to that of pyruvate and succinate (Figure 3.24). In addition, the activity of Cyt. C. Ox. was observed to be just as active in cultures incubated in lactate as those that were given glucose or citrate as their primary carbon source (Figure 3.24). Cyt. C. Ox. is the final complex in that ETC and donates its electron to the terminal electron acceptor O₂ to produce H₂O. These data taken together reveals that lactate was contributing to ATP
production through the ETC. Lactate being used to produce energy through the ETC, would be beneficial to the cell and could indeed support the cell as a source of carbon, due to the high-energy yield obtained through oxidative phosphorylation.

*LDH in the mitochondria*

There are few enzymes known to consume lactate. One of the enzymes known to consume lactate is LDH. LDH has been discovered in the mitochondria of myocytes and spermatozoa previously (295, 296). To determine if it was LDH committing lactate to oxidative energy production, lactate consumption by the mitochondria was assessed in the presence of an LDH inhibitor, oxamate (54). Mitochondria that were incubated with lactate in the presence of oxamate did not consume the lactate. However, mitochondria that were given lactate in the absence of oxamate were found to readily consume the lactate (*Figure 3.25*). This experiment hinted that the lactate-consuming enzyme was LDH. Further experiments were required to confirm the presence of a mitochondrial LDH. In-gel activity analysis for LDH on mitochondrial protein extract revealed the presence of two isoenzymes of LDH (*Figure 3.26*). However, the cytoplasmic extract contained only one isoenzyme. Using AgNO₃ helped identify the upper band as LDH1. AgNO₃ reacts with the sulphydryl groups on the α-subunit of LDH, thus inhibiting every isoenzyme except LDH1 (224). LDH1 preferentially metabolizes lactate to pyruvate (18). This supports the idea that this mitochondrial LDH facilitates the consumption of lactate for oxidative energy production. The lower band could be LDH2 – 5, as they all contain at least one α-subunit. Confirmation that the activity was derived from LDH was accomplished by doing a 2D immunoblot (*Figure 3.27*). Indeed the
upper and lower activity bands from mitochondrial fraction and the activity band from the cytoplasm was LDH. Colocalization studies confirmed that LDH was located in the mitochondria (Figure 3.28). Previous studies have found that cytosolic LDH can associate with the outer membrane of the mitochondria (297). Here we demonstrate by subfractionation of the mitochondria, that this mitochondrial isoenzyme of LDH is localized to the inner membrane/matrix portion of the mitochondria (Figure 3.29).

These observations demonstrate the presence of a mitochondrial LDH in this human astrocyte cell line and its involvement in oxidative metabolism, a seminal finding, that is important to our understanding of brain lactate metabolism. The neuronal consumption of lactate and the ANLS has been well characterized, however the astrocytes were not considered as possible sites of lactate consumption. This research characterizes the utilization of lactate by astrocytes (Figure 4.4).
Figure 4.4 - The versatile role of a mitochondrial LDH in human astrocytes. Lactate is either taken up through the cerebral vasculature or obtained through the glycolytic breakdown of glucose. Lactate can then be shuttled into the mitochondria where mitochondrial LDH can convert it into pyruvate (66).

A mitochondrial LDH is beneficial to astrocytes as it provides a source of carbon to fuel oxidative energy production through the TCA cycle and subsequently the ETC. It also affords the mitochondria with an additional method of shuttling NADH from the cytoplasm (a novel NADH shuttle). The pyruvate that is produced can be utilized in a variety of processes including antioxidant defense, as α-keto acids such as pyruvate have been shown attenuate oxidative stress (298, 299). The finding of LDH in the mitochondria of astrocytes could help explain the importance of lactate in brain biochemistry as this relationship between lactate in the mitochondria would impart multiple benefits to a high-energy demanding organ like the brain (66).

4.4 - The involvement of mLDH in Al and ROS stress attenuation

Previous research from our laboratory has demonstrated the rewiring of metabolic networks to generate the antioxidant, KG under Al and ROS stress (163, 164). This was characterized by an accumulation of KG and its subsequent breakdown product from reacting with ROS, succinate in the spent media and the CFE. Preliminary observation from astrocytes supplemented with lactate show an accumulation of the α-ketoacid, pyruvate and its subsequent breakdown product from interacting with ROS, acetate in the media. Since, pyruvate is also characterized as an antioxidant (298, 299), there was a potential that these human
astrocytic cells were using lactate to fuel antioxidant production under Al and ROS treatment.

*Lactate leads to pyruvate and acetate accumulation*

Analysis of the spent media from astrocytes revealed that the Al and ROS treated cells were consuming more lactate when compared to the control. In addition, the Al and ROS treated astrocytes were characterized by an accumulation of two other monocarboxylates in the spent media, pyruvate and acetate (Figure 3.31). When the CFE was probed for the levels of these aforementioned monocarboxylates, a similar trend was observed (Figure 3.32). These trends were more apparent in the 0.1mM Al as opposed to the 0.01mM Al treated cultures, indicative of a dose-dependent response. Pyruvate has also been observed to be exuded in the presence of other toxicants and xenobiotics (300). The more prominent consumption of lactate and its utilization in the CFE of Al and ROS treated astrocytes were indicative of a differential metabolic profile in the stressed astrocytes. The accumulation of pyruvate and acetate in the spent media and the CFE of Al and ROS stressed astrocytes, prompted the hypothesis that lactate was being diverted to pyruvate and that this pyruvate might be contributing to the antioxidant defense of the astrocytes. The pyruvate would react with the ROS to nonenzymatically decarboxylate to the observed acetate (301). Exuding the pyruvate into the spent media could provide a mechanism by which the astrocytes could protect themselves from the ROS extracellularly, in an effort to prevent ROS from exerting its toxic effects to the intracellular environment. This data would compliment previous studies from our group, where Al stressed hepatocytes were
found to consume a greater amount of citrate, to produce KG. The KG reacted with ROS to nonenzymatically produce succinate (163).

*The involvement of mLDH in antioxidative defense*

If lactate was indeed being directed to pyruvate accumulation to combat ROS in the Al and ROS treated astrocytes, LDH would be necessary to facilitate the transition. The expression pattern of LDH in the astrocytes (whole cells) did not correspond with the increase in pyruvate levels (*Figure 3.33*). Thus, the mitochondrial and cytoplasmic compartments were probed separately for the activity of LDH. Activity and expression analysis for LDH revealed decreased activity and expression in the Al and ROS stressed cytoplasm, while the activity and expression for the mitochondrial isoenzyme increased under Al and ROS treated conditions (*Figure 3.34 and 3.35*). This observation was also replicated in Al and ROS treated HepG2 cells (*Appendix 7.10*). The immunoblot for fraction purity revealed an interesting observation. It appears as though there are two bands present for F-actin in the Al treated samples that were not present in the control and ROS treated samples (*Figure 3.34*). Actin has been found to be the backbone of many scaffolding proteins (302). It is possible that actin is binding Al, but since the sensitivity of the infrared imager is nano-gram range compared to traditional immunobloting, which is typically in micro-grams, this may be the first observation of this nature.

More diligent analysis of LDH activity and expression in the mitochondrial and cytoplasmic fractions revealed that LDH showed a differential pattern between the two fractions. LDH activity and expression in the cytoplasm had decreased in
the Al and ROS stressed cultures indicating that lactate was not being consumed in the cytoplasm. However, LDH activity and expression in the Al and ROS stressed mitochondria increased. Thus, lactate was being consumed instead in the mitochondria under Al and ROS treatment. This differential activation of LDH in the mitochondria and deactivation of LDH in the cytoplasm under Al and ROS is an interesting biochemical observation. It appears as though an Al and ROS stress direct pyruvate production to the mitochondria. The puzzling aspect is that mitochondrial metabolism is perturbed under Al and ROS treatment. So any pyruvate production in the mitochondria would lead to the pooling of the metabolite. As discussed previously, pyruvate can act as an antioxidant. Perhaps LDH increase in the mitochondria is an adaptive mechanism to deter ROS production. The purpose of an LDH that is modulated by ROS in the mitochondria would contribute to the control of the oxidative environment of this organelle. When mitochondria produce ROS via the ETC, perhaps mitochondrial LDH is invoked to attenuate the ROS.

It is likely that cytoplasmic LDH decreases its activity under oxidative stress to save the lactate for the mitochondrial LDH to use, as it is the more oxidative environment. Thus, cytoplasmic and mitochondrial LDH work in tandem to attenuate ROS stress by diverting pyruvate pooling to the mitochondria. The ability of ROS to induce the activity of mitochondrial LDH was assessed and the ability of increasing doses of lactate to induce mitochondrial LDH activity was also probed (Figure 3.35). Lactate did not share H$_2$O$_2$’s ability to upregulate the activity of mitochondrial LDH. The inducibility of mitochondrial LDH by increasing the dose of
ROS supports the hypothesis that mitochondrial LDH can be used as a tool to pool pyruvate for antioxidant defense. Using bioinformatics tools, Holmes and Goldberg, 2009 identified an isoenzyme of LDH, LDHA like 6B, localized in the mitochondria (242). The name, LDHA like 6B stems from its homology to LDHA aka. The number 6 denotes a new subtype and B relates to different sequences of which there are 3 types namely, A, B, and C. Of these LDHA like 6 isoenzymes only B has a mitochondrial targeting sequence. Its presence would confirm our observation that the LDH we had discovered in the mitochondria was an LDH1 isoenzyme that preferentially consumes lactate to form pyruvate. Probing the transcript levels for LDHA like 6B revealed that this mRNA level increased about 50% when the astrocytes were treated with ROS (Table 3.2).

Accumulation of the antioxidant; pyruvate

Our hypothesis was that LDH activity had increased in the mitochondria in an effort to pool pyruvate for the attenuation of ROS. Thus, we would expect to see an accumulation of pyruvate and its nonenzymatic decarboxylation product, acetate. The mitochondria isolated from Al and ROS stressed astrocytes were consuming lactate more readily to pool pyruvate and acetate in a dose dependent fashion (Table 3.3). This observation was also replicated in a HepG2 cell lines' mitochondria treated under the same conditions (Appendix 7.11). This observation confirms the hypothesis that the Al and ROS treated mitochondria were consuming lactate to pool pyruvate. The concomitant production of acetate provides evidence toward the utilization of this pyruvate as an antioxidant to detoxify ROS.
To pool pyruvate, not only would LDH have to increase in activity, PDH would have to decrease so as not to consume the pyruvate. PDH typically consumes pyruvate to prime the TCA cycle with a source of acetyl-CoA. The TCA cycle was demonstrated to be less active in Al and ROS stressed cells. PDH, was found to down in activity in the Al and ROS treated cells. The expression of PDH was also downregulated in the Al treated cells compared to the control, but the expression of PDH in the ROS stressed cells was not as severely affected (Figure 3.36). The observations for PDH activity were replicated in HepG2 cells (Appendix 7.12). PDH is characterized by a lipoic acid residue on its E2 subunit (303). This lipoic acid (di-thiol) has the potential to attenuate ROS as a reducing factor (304). This might explain the greater loss of activity but the maintenance of expression in the direct ROS stressor, H$_2$O$_2$. Perhaps the lipoic acid residue containing PDH is expressed as a tool to fend off the ROS. This observation has been made with KGDH, another lipoic acid dependent enzyme (163). The activity of KGDH under Al, Zn, and ROS was abolished while the expression was maintained. In this astrocytic cell line, only the ROS stress maintained the expression of PDH, while Al lowered the activity and expression. Perhaps PDH transcription or translation is more sensitive to the presence of a toxic metal than its sister protein KGDH or perhaps a protein that regulates its expression is more sensitive to Al than ROS. One might speculate it may be an Fe problem since Al interferes with Fe homeostasis. However, ROS also interferes with Fe homeostasis. Indeed, the complex results with PDH under Al in contrast to ROS stress are intriguing. However, the results corroborate with the
data demonstrating the accumulation of the \(\alpha\)-keto acid and potential antioxidant, pyruvate.

The ROS scavenging potential of pyruvate was tested in-vitro by incubating 10mM \(^{13}\)C3-pyruvate with 1mM \(\text{H}_2\text{O}_2\) for 1h. \(^{13}\)C NMR analysis revealed that acetate was generated. Indeed, incubating the Al and ROS stressed astrocytes with pyruvate attenuated ROS levels (Figure 3.37). Thus, pyruvate was being pooled by the combined increased activity of LDH and decrease in activity of PDH, under Al and ROS stress, as a method to detoxify oxidative stress. To assure that pyruvate was taking the route of nonenzymatic decarboxylation to produce acetate, Br-pyruvate was used to inhibit PDH. While PDH was being inhibited, the mitochondria from the Al and ROS treated cells were still observed to produce acetate from pyruvate, where the control cells were simply accumulating the metabolite (Figure 3.38). This proves that, the acetate that was being produced in the mitochondria of Al and ROS treated astrocytes, was a result of the non-enzymatic decarboxylation of pyruvate with ROS and not through a metabolic pathway evoked by ROS treatment.

*The fate of acetate*

The accumulation of acetate as a product of ROS detoxification by pyruvate was observed in Al and ROS treated astrocytes, in the mitochondria, the CFE, and the spent media. However, astrocytes have been demonstrated to consume acetate as a source of carbon to fuel their metabolism (305, 306). To utilize acetate it must be fixed as acetyl-CoA via ACS (305). The activity of mitochondrial ACS was down in Al and ROS stress, while the activity of cytoplasmic ACS had increased in Al and ROS stress when compared to control cells (Figure 3.39). The differential activity in the
mitochondrial and cytoplasmic fraction is a hint to what the fate of the acetyl-CoA will be once the ACS fixes it. The acetate was being fixed into acetyl-CoA more readily within the cytoplasm than the mitochondria under Al and ROS treatment. Since, the TCA cycle is hindered under Al and ROS stress, the acetyl-CoA would be irrelevant in the mitochondria. However, acetyl-CoA production within the cytoplasm would lead to the production of lipids (307). For acetyl-CoA to become lipids it must pass through ACC to become malonyl-CoA first. ACC activity also increased in the Al and ROS stressed astrocytes (Figure 3.39). This implies that the acetyl-CoA generated by cytoplasmic ACS was being routed toward lipid production. Indeed, when the level of lipids within the astrocytes were measured, it was determined that there was a significant increase within the Al and ROS treated cells (Figure 3.40). An increase of lipogenesis has been observed previously in hepatocytes treated with Al (160). This observation was duplicated in hepatocytes treated with Al and ROS (Appendix 7.13). Thus, the acetate that was being produced as consequence of the interaction of pyruvate with ROS in the Al and ROS treated cells, was being metabolized by cytoplasmic ACS to produce acetyl-CoA, the acetyl-CoA was then being used to bolster lipid stores through ACC.

LDH plays a key role in the attenuation of oxidative stress to generate pyruvate, which is subsequently utilized to combat ROS during Al and H₂O₂ stresses. The pooled pyruvate undergoes non-enzymatic decarboxylation when it reacts with ROS to form acetate. The acetate produced through the detoxification of ROS is then metabolized by cytoplasmic ACS to produce acetyl-CoA in the cytoplasm, which is subsequently routed to lipogenesis by ACC. Acetyl-CoA direction toward lipogenesis
happens in lieu of its commitment to the TCA cycle as it is inhibited by Al and ROS stress (Figure 4.5).

![Diagram of mitochondrial metabolism](image)

**Figure 4.5 – Mitochondrial LDH is involved in attenuating ROS and lipid production.** Red = decreased levels. = increased levels

It appears that mitochondrial LDH in astrocytes has a more relevant role than simply a means to metabolize lactate to support energy expenditures within the cell. The mitochondria have this enzymatic tool that can be invoked to combat oxidative stress, within an organelle that is under consistent threat from ROS. Under Al and ROS challenges, lactate is diverted to the mitochondria where it becomes a supplier
to the anti-oxidant pool of pyruvate. Cytoplasmic LDH is relieved of its metabolic duty to allow lactate to be consumed by its mitochondrial partner. The pooled pyruvate is then harvested to attenuate ROS. Pyruvate has been demonstrated to be an effective anti-oxidant in against other toxicants (300) and has been demonstrated to be a potent antioxidant for the heart, even protecting it from periods of high oxidative stress following reperfusion (308, 309). Acetate is formed by the reaction of pyruvate with ROS and fixed into lipids. Again, a pair of sister enzymes one in the mitochondria and the other in the cytoplasm conjugate their efforts to ensure that the pathway of lactate to lipids is streamlined. Mitochondrial ACS is downregulated while cytoplasmic ACS is upregulated to make acetyl-CoA for lipogenesis and not for the TCA cycle. Both PDH and mitochondrial ACS are down in activity due to the inhibition of the TCA cycle by Al and ROS. Indeed, this phenomenon appeared to be a cellular event as opposed to just an astrocyte specific adaptation as some of the observations were also uncovered in hepatocytes.

4.5 – Disruption of L-carnitine metabolism and lipid accumulation

As there was an observed accumulation of lipids in the Al and ROS treated astrocytes, we hypothesized that there was a lack of β-oxidation of lipids due to mitochondrial dysfunction under Al and ROS treatment. It is also known that the amino acid L-carnitine is required for the transport of lipids into the mitochondria for β-oxidation (77). L-carnitine is a metabolite that relies on KG for its biosynthesis (77). Previously, our research group characterized the accumulation and redirection of KG to attenuate ROS stress (163, 164). If KG was being diverted from other pathways in an effort to quell ROS, perhaps L-carnitine production was not a priority
in Al and ROS challenged astrocytes. This lack of L-carnitine would be a contributing factor to the observed lipid accumulation under Al and ROS treatment.

*The accumulation of KG and succinate*

Clearly an effort was being mounted to detoxify the ROS in the Al and ROS treated cells, as was observed by the accumulation of pyruvate. Another α-keto acid, KG might also be being accumulated under Al and ROS treatment to combat ROS. The non-enzymatic decarboxylation product of the reaction being KG and ROS is succinate (163). The spent fluid from the astrocytes treated with Al and ROS revealed an accumulation of KG and succinate (*Figure 3.42*). These observations were replicated in HepG2 cells (*Appendix 7.14*). To understand how KG was being accumulated, one of the enzymes involved in its production (NADP-ICDH) and another that consumes it (KGDH) was probed (*Figure 3.43*). Indeed, NADP-ICDH and KGDH were working in concert to pool KG by the increased activity of the former and decreased activity of the latter. These two enzymes working in tandem with each other under Al and ROS toxicity could account for the accumulation of KG and its non-enzymatic decarboxylation product, succinate. Previously, we have found that the activity of NAD-ICDH also decreases in activity due to its NADH producing capacity (163). The cell prefers to produce its KG and make NADPH which is an anabolic reducing factor and a contributor to anti-oxidant defense systems, as opposed to making it with the generation of NADH, a contributor to oxidative metabolism, when it is subjected to Al and ROS stress (163). Other metabolites that have been demonstrated to contribute to this accumulation of KG are glutamate and histidine (164, 243). The antioxidant capacity of KG was
demonstrated by recovering Al and ROS stressed astrocytes in 5mM KG for 8h (Figure 3.44). The levels of ROS within the Al and ROS treated astrocytes did indeed decrease following the recovery with KG. The antioxidant capacity of KG was the logical explanation for the observed accumulation of KG in the spent fluid. The observed accumulation of the non-enzymatic breakdown product succinate in the spent fluid of the astrocytes treated with Al and ROS is evidence that KG was being used to attenuate ROS.

*The loss of L-carnitine production*

The rerouting KG is necessary to attenuate ROS, however the consequence of pooling a metabolite would be that the other biochemical pathways that require the metabolite would have to be downgraded. One pathway that requires KG for its synthesis is the production of L-carnitine (77). The implications of a lack of L-carnitine could be a resultant inability to consume lipids. L-carnitine escorts lipids to the mitochondria through an intermediate called acyl-carnitine. Acyl-carnitine is L-carnitine with an attached fatty acyl chain that gains entrance into the mitochondria (77). Both the levels of L-carnitine (free) and acyl-carnitine (+ free L-carnitine = total carnitine) were decreased in Al and ROS treated cells (Figure 3.45). Again, the trends observed for free carnitine were duplicated in HepG2 cells (Appendix 7.15). The levels of total carnitine only decreased slightly in the lower dose of Al (0.01mM) while the levels of free L-carnitine decreased dramatically. Most carnitine found in the cell is indeed acylated (77). Thus, the data would suggest that 0.01mM Al had little effect on total carnitine levels. L-carnitine has been demonstrated as a chelator of metals (82), which explains the attenuation of
ROS stress when the cells were recovered with L-carnitine (Figure 3.46). L-carnitine in the Al treated cells may be implemented in the chelation of the trivalent metal. The Al is no longer available to interfere with Fe homeostasis and less ROS is produced. Alternatively, Al is interfering with Fe homeostasis, and L-carnitine is chelating the Fe and preventing it from producing ROS. A recovery with KG not only attenuated ROS in the Al and ROS treated cells; it also recovered the levels of L-carnitine in astrocytes (Figure 3.45) and hepatocytes (Appendix 7.15).

The biosynthesis of L-carnitine is a multi-step enzymatic pathway that relies on KG as a cofactor for its synthesis (77). Two enzymes in the L-carnitine biosynthetic pathway were analyzed for activity and expression BBADH and BBDOX. The latter is directly dependent on KG as a cofactor (77). The activity and expression of both BBADH and BBDOX was inhibited by Al and ROS treatment (Figure 3.47, 3.48, and 3.49). In addition, hepatocytes that were treated with Al and ROS were probed for BBDOX activity (Appendix 7.16). The loss of BBDOX activity could be attribute to the absence of KG. However, the expression analysis along with the loss in an active BBADH supports the idea that the entire L-carnitine biosynthetic pathway is negated in the presence of Al and ROS in an effort to preserve KG for ROS detoxification.

*Lack of L-carnitine contributes to lipid accumulation*

The consequence of a lack of L-carnitine would be an inability to metabolize lipids in the mitochondria via β-oxidation. The TCA cycle is found to be perturbed under Al and ROS toxicity due to an interference with Fe homeostasis, an event that is characterized by the inability to consume citrate and an accumulation of lipids.
(167). However, lipids cannot not even pass into the mitochondria without the carrier, L-carnitine. The lack of β-oxidation by Al and ROS stressed cells is observed by an increase in lipid levels and the inability to consume palmitate (Appendix 7.17). KG is important in a variety of biochemical pathways. The β-oxidation of lipids is another pathway that suffers when KG is diverted to combat an oxidative stress. When lipids are found to accumulate there are two ways in which they can do so. 1) Is a decrease in the ability to consume lipids (a decrease in β-oxidation) and 2) an increase in lipogenesis. The former was characterized under Al and ROS treatment due to a KG-mediated lack of L-carnitine. The latter was ascertained by probing for the activities of the lipogenic enzymes CL and ACC (Table 3.4). Both lipogenic enzymes, CL and ACC, were elevated in Al and ROS stressed cells. Under Al and ROS stress the TCA cycle functions at a reduced rate (167). Citrate, or citrate derived from lactate, is shipped from the mitochondria to the cytoplasm where it activates CL. CL converts citrate to acetyl-CoA. The acetyl-CoA is devoted to lipogenesis by carboxylation with ACC. This network funneled potential oxidative energy producing fuels toward lipid synthesis is augmented under Al and ROS treatment. Thus, the lipid accumulation observed in Al and ROS stress is characterized both by a decrease in β-oxidation of fatty acids, and an increase of lipogenesis.

The redirecting of metabolites to attenuate an induced oxidative stress is again observed with the shunting of KG toward ROS detoxification. Like the pooling of pyruvate, KG is pooled to aid in the reduction of ROS. However, this process has
consequences. The pathways which depend on KG for their functioning can be altered (Figure 4.6).

![Diagram of metabolic pathways involving L-carnitine metabolism]

**Figure 4.6 – The effects of Al and ROS on L-carnitine metabolism.** KG is diverted to ROS detoxification, leading to a perturbation of carnitine synthesis. Fatty acids are directed to lipid storage, as β-oxidation can no longer take place. $\downarrow$ = a decrease in levels. $\uparrow$ = an increase in levels. PHD = Prolyl hydroxylase, HIF-1α = Hypoxia inducible factor 1 alpha (310).

The diversion of KG in Al and ROS treated cells alters the production of L-carnitine through the L-carnitine biosynthetic pathway. Two enzymes that contribute to this pathway, BBADH and BBDOX are downregulated under Al and ROS stress in an effort to preserve KG. This results in a loss of L-carnitine production. L-carnitine levels along with acyl-carnitine levels were observed to be decreased in Al and ROS stressed cells. The loss of L-carnitine lead to the inability of
lipids to enter the mitochondria for consumption by for β-oxidation, effectively contributing to lipid accumulation. Lipid accumulation was exacerbated by the heightened levels of the lipogenic enzymes CL and ACC, which siphoned the nutrients destined for the TCA cycle toward lipogenesis. The accumulation of lipids could be detrimental to the cell. Left unchecked lipid accumulation can lead to lipotoxicity of the cell (311). Lipotoxicity is observed to be an underlying cause to neurological disorders, as it leads to cellular dysfunction and initiates cell death in astrocytes (311-313). KG reacting with ROS leads to the production of succinate via non-enzymatic decarboxylation. The accumulation of succinate has been demonstrated to induce a state of anaerobiosis in a PHD-HIF-1α dependent manner (190). The induction of an anaerobic state by succinate accumulation is another factor that reduces flow of the TCA cycle and can contribute to lipid accumulation. It seems that Al and ROS stressed cells are faced with the dilemma of shutting off biochemical pathways that produce necessary cofactors for cellular functioning and the production of molecules to attenuate the stress, ie. α-keto acids. The logic of the cell is likely one to primarily eliminate the stress so that ‘business as usual’ can resume. This would mean redirecting necessary metabolite temporarily to attenuate the stress. This is exemplified here by the redirection of KG to combat oxidative stress much to the chagrin of L-carnitine production.

4.6 - The impact of Al and ROS on other functions of astrocytes

Astrocytes are pivotal in neurotransmitter clearance (263). The main neurotransmitter in the brain is Glu, which astrocytes are involved in removing from
the synaptic cleft following neurotransmission so another round of synaptic firing can ensue (5, 314). Glu is recycled back to neurons by a process called the Glu/Gln cycle (as described earlier). Any disruption of this process under Al and ROS could lead to a malfunction in neurotransmission.

Glutamate clearance

Spent media from the astrocytes treated with Al and ROS contained less Glu compared to the control cells (Figure 3.51). Glu, can be used by both GDH and GluS to contribute to the production of KG. It is quite probable that Al and ROS stressed astrocytes are using Glu to produce the antioxidant KG. Indeed when the astrocytes were supplemented with excess Glu, Al and ROS stressed cells consumed more (Figure 3.51). This experiment was a crude mock-up of neurotransmitter release from the neurons, and the subsequent removal of the Glu from the synaptic cleft. The activity of GDH, GluS, and GlnS were all perturbed in the Al and ROS treated astrocytes (Figure 3.52). The decrease in activity of these three enzymes under Al and ROS treatment may be in an effort to conserve KG. All of the aforementioned enzymes require KG to construct their respective metabolites and thus if KG is being pooled to attenuate ROS stress, the enzymes that require them would experience a loss in activity. Interestingly, when the activities of GDH, GluS, and GlnS were probed again in the Al and ROS treated astrocytes, following a recovery with Glu, the activities of GDH and GluS increase while that of GlnS decreased (Figure 3.52). Again, these observations can be explained by the desire of the astrocytes to accumulate KG under Al and ROS treatment. When Glu is plentiful (following the recovery), the amino acid is sacrificed to pool KG for the detoxification of oxidative
stress. This was observed by the increase in activity of GDH and GluS, both KG producing enzymes (when equilibrium shifts to Glu as the reactant), in Al and ROS treated cells following the introduction of Glu into the media. However, GlnS is down in activity in the Al and ROS treated astrocytes. The explanation for this observation is that Glu would be diverted toward KG production and thus would not be used for the production of Gln. These observations are outlined briefly in Figure 4.7.

![Diagram of Glutamate metabolism](image)

**Figure 4.7** – Glu is shifted toward KG production and not neurotransmitter recycling.

The production of KG to attenuate oxidative stress seems so pertinent, that Glu is redirected toward its production. Under resting states, Al and ROS stressed astrocytes maintain their KG pools by downregulating GDH and GluS. GlnS is downregulated as the Glu needed for it to react is redirected to KG. When Glu is present, GDH and GluS increase in activity to pool KG while GlnS decreases its activity. Ultimately, the recycling of Gln back to the neuron would be halted if this were to occur in-vivo, leading to a hindrance of neurotransmission. In fact, disturbances in the Glu/Gln cycle have been demonstrated in some neurological disorders (315).
Glycogen accumulation

Another key function of astrocytes is the storage of glycogen for periods of intense prolonged neural activity and hypoglycemia (13). When glycogen levels were probed in astrocytes treated with Al, ROS, and control conditions, glycogen was found to have accumulated within the cell (Figure 3.53). The enhanced accumulation of glycogen is an intriguing observation. This accumulation of glycogen may lead to astrocytic cell death as inhibition of GSK-3, a phenomenon that would also lead to the accumulation of glycogen, results in cell death of astrocytic cells (316). However, heightened expression of GSK-3, a phenomenon which would lead to lack of glycogen production has been associated with Alzheimer’s disease (317). Though this observation is preliminary, discovering the mechanisms by which this glycogen accumulates under Al and ROS treatment might divulge some interesting biochemical events. Indeed, what would be more detrimental to the astrocytes and brain metabolism in general would be the inability to breakdown the glycogen to glucose, a process initiated by glycogen phosphorylase.

In addition to some of the other observations of the affects of Al and ROS in this human astrocytic cell line, other functions such as neurotransmitter recycling and glycogen metabolism appear to be affected. These are functions of the astrocytes that are crucial to the operation of the brain. Their alteration under Al and ROS stress may provide additional clues to Al, oxidative stress, astrocytes, and neurological disorders.
4.7 – *Nuclear metabolism in astrocytes*

Recent discovery of a nuclear CL that contributes to acetyl-CoA production for acetylation (83) has prompted the search for a metabolic network in the nucleus to supply modification metabolites that may regulate transcription. Metabolic profiling of the nucleus using HPLC demonstrated elevated levels of some interesting metabolites (*Table 3.5*). In the Al and ROS stress there was an accumulation of pyruvate and acetate. Since LDH was found to be more active in the nucleus from Al and ROS treated cells (*Figure 3.54*), it is possible that lactate is being pooled as pyruvate for the attenuation of ROS. The non-enzymatic decarboxylation product would be acetate, which was found to have increased under Al and ROS stress in the nucleus. The accumulation of KG and its non-enzymatic decarboxylation product, succinate increase was also observed in the 0.1mM Al treated nuclei (*Table 3.5*). The accumulation of KG and succinate support a possible increase of nuclear ROS and an effort by the cell to curb it via antioxidative defense. Under Al and ROS treatment there was also an accumulation of citrate in the 0.1mM Al stress. The accumulation of citrate coupled with an increase in nuclear CL, provides evidence for a metabolic module in the nucleus to provide acetyl-CoA for histone acetylation under Al stress (83). The upregulation of CL, coupled with that of MDH and ME, provide a metabolic pathway that may continually supply acetyl-CoA for histone modifications (*Figure 4.8*).
Figure 4.8 – A hypothetical metabolic module in the nucleus of astrocytes. CL diverts citrate to acetyl-CoA. The oxaloacetate is redirected to pyruvate by the upregulation of MDH and ME. In addition, pyruvate is pooled by the increased activity of LDH in the nucleus. The pyruvate can then react with ROS to produce acetate; a moiety that may also be fixed in acetyl-CoA through ACS. The pool of acetyl-CoA may be devoted to histone modification.

These are preliminary observation on the nucleus from astrocytes treated with ROS and Al. There are certainly some deviations in the trends between the 0.1mM Al stress and the H$_2$O$_2$ stress. This is interesting because throughout most of the observations the data between the Al and ROS stress has been relatively similar. However, this provides evidence that perhaps an Al stress may signal a different response in the astrocytes, as Al stressed nuclei accumulated citrate and KG while the ROS stressed nuclei did not. Also, pyruvate, acetate, and succinate were found at higher levels in the nuclei from Al stressed cells compared to the nuclei from the ROS treated cells. This metabolic interplay may provide acetyl-CoA for histone
modification, an event critical in genetic regulation. The link between nuclear metabolism and modulation of genetic information is only beginning to enlarge.
Section 5: Conclusion

Due to its ability to interfere pre-dominantly with Fe-metabolism, Al is a neurotoxin. In this work, Al toxicity leads to dysfunctional astrocyte biochemistry. One cellular biochemical module which was observed to be affected by Al was oxidative energy production. The loss of ATP production led to the inability of the actin-cytoskeleton to polymerize. The end-result of a perturbed actin-cytoskeleton is a loss in cellular morphology. Since the unique morphology of astrocytes lends to its physiological role in the brain, this loss of morphology would be detrimental to the function of the astrocytes in the central nervous system. Astrocytes appeared to respond in a similar fashion to ROS (H₂O₂) stress.

Lactate was found to be an oxidative fuel for this astrocytic cell line, an observation that may have implications to the rethinking of the brain energy budget. In addition, this mitochondrial LDH was demonstrated to have a greater role in Al and ROS stressed astrocytes. Al and ROS evoked an increased activity of mitochondrial LDH, which with a concomitant decrease in PDH lead resulted in the pooling of the antioxidant pyruvate. The formation of acetate as a consequence of the interaction between pyruvate and ROS was subsequently devoted to fat production. The inability of the mitochondria to oxidize fatty acids and diminished synthesis of L-carnitine further promoted the lipogenic characteristic of these stressed astrocytes. L-carnitine production was lost due to the rerouting of KG, another antioxidant toward ROS sequestration. Thus, the perturbation of the
mitochondria under Al and ROS, coupled with the accumulation of lipids resulted in dysfunctional astrocytes; cells critical for the proper functioning of the brain.

Other functions of the astrocytes were also affected by Al and ROS treatment. The redirection of Glu toward KG accumulation was observed under Al and ROS stress, a phenomenon that would alter the workings of the Glu/Gln cycle. Ultimately the Glu/Gln cycle is crucial to the recycling of the neurotransmitter Glu back to neurons. Another astrocyte-specific task that was altered under Al and ROS stress was glycogen metabolism.

The loss of astrocyte morphology, the perturbation of mitochondrial metabolism, the accumulation of lipids, the disruption of the Glu/Gln cycle, and the altered glycogen metabolism were all mediated by the toxic influence of Al and ROS. Since these biochemical processes are critical to the role of astrocytes, it is clear that the functioning of the brain will be severely affected, hence the neurotoxicity of Al and ROS. This holistic molecular studey establishes an intricate metabolic link between Al toxicity and astrocyte dysfunction that may help explain Al-induced neurological disorders such as Alzheimer’s, Parkinson’s, cognitive impairment and Multiple Sclerosis (Figure 5.1).

Future Works:

These findings remain seminal and require further discovery. The links between Al and astrocytic dysfunction must be proven in primary cell models and/or co-culture systems. In addition, whole animal modelling of these findings would add credence to the discoveries found in this model system. A more in-depth look at neurotransmitter homeostasis (Glu, adenosine, D-serine) might provide
more clues to the metabolic link between Al, astrocytes, neurological disorders as neurotransmitter metabolism is intricately linked to astrocytic functioning. Indeed, there is a hearth of knowledge left to unravel to solidify a concrete link between Al and neurological disorders.

Figure 5.1 – A global perspective on Al-toxicity in Astrocytes: Implications for neurological disorders.
Section 6: Literature Cited

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** = Published work from this thesis
Section 7: Appendix

Appendix 7.1 - The glycolytic pathway. The process of converting glucose to pyruvate
Appendix 7.2 - The tricarboxylic acid cycle. The combustion of pyruvate to yield NADH.
Appendix 7.3 - The electron transport chain. The process of harnessing the reductive power of NADH to produce a proton gradient. The proton gradient is then used to produce ATP.
Appendix 7.4 – HPLC chromatograms for common metabolites. 1mM of each standard was run in the HPLC under the given conditions.
1 gtcgagagcg ggttagagtgc tgaagtcctt gccaaagcgtt cctgtgacgt tctcaacgtc
61 gatcaagcag ctttttttcca ttcttctctt ccacttccctt acgtgagcag ctaggtgtg
121 acttggtccctg tggagtgggc cagcccgaga atagcgtcgg tggagtgcag cttctctatgc
181 ctggtggttgg cgctgctgcct gctgatgcga cgcgctgatcc gctgctgtgctc
241 ttccaaccccg gtagcaagat tggagctgtg aagtgagcgc tttatgcctcc
301 gagaagcccg ttcctcagac taaggtcttc tctacgagaa cttgatcccg ggagatgcgc
361 tgcgtacctct tctccttatt aaaaagtttg agtttctgttc cttctcctcttt gcagcttattg
421 gagaacaacag tggagctgtg gtagcaggg ctcccttcct ttccagataat ccagaaatgtg
481 ccaaaaccttt ttgtagtcag aagacctttt tctcaagcag aagctcaacctt actgtttatgc
541 acacgaggttg cagcccaagaa aaaaagaaag acgcgcctttta atttatgtcag gcagaagatg
601 gcctaacctca aagtataaat tttcaggttt gttcagtaact gcccacactgc cagactgtt
661 attttttttt cttgcagtctg tttcttctat gttactacgt gcagctttttttt cagatgtctg
721 aacaagctgt tttcttggag cggctgtaaat tctggtactcg ctgttttctc tttcttcttg
781 ggacaaaaag gcctgtatcc tttgaaacgc cggcactgggt gatcctcctgg agagcatgaga
841 gactcaagtg ctctctttttc gatgtgagttt accatgcgct gttcctctcttt ctactgtctg
901 aacctctgat taggaactga taaaatcgct gcggaaatgct ccaaaatgctt ccaaaatgctt
961 actgcaactct gctatcgagat tttaaatgct gcggaaatgtt gcttcctggtgc gtttgctca
1021 tctgtgagcctg atttacaagga aatttattttt aagatctattt gccaggtcctt cccagtttcc
1081 accataactctt gcgggctctct tggaaatagat gaaagatgttg tctcgtatag tctttctgatc
1141 cttcgggagag gctgtagttt ccaacgttata aagataaacc tgcaccctctg agaaagagcc
1201 ctaatggtggaa aagattgaaag cacaatatgtt gaaattgac aaatactttt gtttataaatg
1261 tggcatttac cttcctctcc aatatattttg agatatcttata gatagcctttt tttaaatgctt
1321 aatattctctg aactatcttt ttctctgttact tggaaaagtg agatggatag ttagttttttt
1381 cctatcattt ttagctcaca gcttttttatt tgaacgatctg cttgagtgac gataattttt
1441 cacaaatcct gtttattttt tttcttgatgc agcgtcctctt cggcggatcc acgttttctt tttatatagctt
1501 gtaagttgct cttgggctttt caaaaatgtc ttagttcatt ttattttttag cttggaatttttcc
1561 cggctcttttt cttcatcatttt atgtctttct tccctcttctct gctgctttat cccatgtttct
1621 atttatgtg cetctcttttt gtagctacag tttcctacagt taaaaatctttt gagaacttac
1681 aaaaaaaaaa aaaaaaaa

Appendix 7.5 - cDNA Sequence for LDHA-like 6B. The following cDNA sequence was utilized to perform qPCR analysis on control and H₂O₂ treated CCF-STTG1 cells for LDHA-like 6B.
Appendix 7.6: Viable cell count of CCF-STTG1 cells. CCF-STTG1 cells were grown in α-MEM + 10% FBS for up to 10 days. Viable cells were counted by the TBEA. n=5 ± SD (dotted lines). P ≤ 0.05.
Appendix 7.7: Non-viable cell count for CCF-STTG1 cells. CCF-STTG1 cells were treated with A) control and B) 0.1mM Al for 24h. Non-viable cells were indentified using the TBEA. n=5 ± SD (dotted lines). P ≤ 0.05
Appendix 7.8 – Caspase 3 cleavage in astrocytes. An immunoblot was performed on the CFE from astrocytes treated with A) control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for caspase 3 cleavage, indicative of apoptosis (**Note: the lack of cleavage products)
Appendix 7.9 - In-gel activity stain for SDH. CCF-STTG1 cells were incubated in A) Control B) 0.1 mM Al for 24 h and then an in-gel activity stain for SDH was performed on the mitochondrial protein.
Appendix 7.10 - Activity of LDH in HepG2 cytoplasm and mitochondria. HepG2 cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. I) In-gel activity stain for LDH in the cytoplasm. II) In-gel activity stain for LDH in the mitochondria.
Appendix 7.11 – Lactate consumption and monocarboxylate production in HepG2 mitochondria. Control, Al (0.01mM and 0.1mM), and 40 μM H₂O₂ mitochondria (0.2mg/mL) from HepG2 cells were incubated with 5mM lactate and 0.5mM NAD for 1h. The reaction mixture was then subjected to HPLC analysis. Values are expressed as percentage compared to control. n=3 ± SD; p ≤ 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5.9%</td>
<td>100 ± 6.6%</td>
<td>100 ± 2.1%</td>
</tr>
<tr>
<td>0.01 mM Al</td>
<td>86.1 ± 3.9%</td>
<td>134.7 ± 3.3%</td>
<td>299.5 ± 3.2%</td>
</tr>
<tr>
<td>0.1 mM Al</td>
<td>41.2 ± 1.6%</td>
<td>340.2 ± 9.2%</td>
<td>716.3 ± 3.7%</td>
</tr>
<tr>
<td>40 μM H₂O₂</td>
<td>41.3 ± 4.2%</td>
<td>327.2 ± 4.3%</td>
<td>858.9 ± 1.4%</td>
</tr>
</tbody>
</table>
Appendix 7.12 - Activity of PDH in HepG2 mitochondria. HepG2 cells were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. An In-gel activity stain for PDH was performed. 1mM Br-pyruvate was used as an inhibitor to demonstrate band specificity.
Appendix 7.13 - Fluorescence microscopy of triglyceride levels in HepG2 cells. HepG2 were incubated in A) Control B) 0.01mM Al C) 0.1mM Al and D) 40μM H₂O₂ for 24 h. Post-treatment, the cells were treated with Hoechst (nucleus) = blue and Oil Red O (triglycerides) = red. Scale bar = 10 μm.
Appendix 7.14 - KG and Succinate levels in HepG2 cells. CFE was obtained from HepG2 cells and A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H2O2 containing media. HPLC analysis was performed to ascertain □ = KG levels (open bar) and ■ = succinate levels (closed bar) in the CFE. n=3 ± SD. P ≤ 0.05. (*Indicates a significant change KG levels, whereas ** is indicative of a significant change in succinate levels).
Appendix 7.15 - Carnitine homeostasis in HepG2 cells. HPLC analysis was done to measure free carnitine levels in the CFE from A) Control B) 0.01 mM Al C) 0.1 mM Al and D) 40 μM H2O2 treated HepG2 cells. Where the open bar □ is non-recovered cells and the closed bar ■ are cells recovered with 5mM KG for 8h. n=3 ± SD. P ≤ 0.05. (*Indicates a significant change in metabolite levels).
Appendix 7.16 - Expression levels of BBDOX in HepG2 cells. Immunoblot analysis of CFE for BBDOX was performed in A) Control, B) 0.01mM Al, C) 0.1mM Al, and D) 40 μM H₂O₂ stressed HepG2 cells. VDAC served as a loading control for these experiments.
Appendix 7.17 - Palmitate metabolism in Al treated HepG2 cells. Cells were treated with A) Control and B) 0.25 mM Al. Following a 24h treatment period, the cells were subsequently given I) BSA for 24h or II) 2 mM palmitate for 24h or III) 2 mM palmitate for 24h followed by a 5 mM KG recovery for 8h. HPLC analysis was performed to measure intracellular palmitate. n=4 ± SD. P ≤ 0.05. (*Indicates a significant lack of consumption of palmitate in the Al stressed conditions).