Spring 5-2012

Alteration of Protein Kinases' Activities by Hypoxic Mimic Compounds

Lawerence Rasmussen
Seton Hall University

Alteration of protein kinases' activities by hypoxic mimic compounds

Lawrence Rasmussen

Submitted in partial fulfillment of the requirement for the

Degree of Master of Science in Biology from the

Department of Biological Sciences of Seton Hall University

May 2012

Approved By

Jane L Ko, PhD

Mentor

Carolyn Bentivegna, PhD

Committee Member

Allan Blake, PhD

Director of Graduate Studies

Committee Member

Jane L Ko, PhD

Chairperson, Department of Biological Sciences

Acknowledgements

I would like to express my deepest thanks to my mentor Dr. Jane Ko for allowing me the opportunity to work with her. I greatly appreciate the knowledge she has imparted upon me in the areas of signal transduction and molecular biology. Also, I am grateful for her assistance with my research, and her patience and fortitude in explaining the process of writing for my Master thesis. Through diligent guidance and pushing me to troubleshoot various conundrums that might arise in a lab setting, Dr. Ko has given me the confidence to rise above any challenges that I may face in future endeavors involving the pursuit of knowledge in the scientific realms.

I also would like to thank my committee members, Dr. Allan Blake, and Dr. Carolyn Bentivegna for their comments and suggestions towards my thesis as well as their whimsical yet sagacious phrases in the lab.

In addition, I would like to thank my fellow Dr. Ko lab mates. A special thanks to Pranjal Nahar who greatly helped me with the background knowledge of lab procedures and experiments, in addition to bolstering my mathematical skills. Thank you to George Coricor for his levity in maintaining my sanity in the lab as well as his assistance in cell culture, and Natasha Pavlichko who greatly helped me with my longer time frame treatments.

Finally, I want to thank my parents and sister as well as all my other peers at Seton Hall University Biology Department.

Table of Content

List of Figures

Abstract

Our lab previously used the hypoxic mimic compound, DFO, to simulate hypoxic conditions within neuronal cells, NMB, model system, and found that treatment decreased cell viability in a dose- and time-dependent manner. In the surviving cells there was an observed increase of glutathione levels and an increase of hypoxia inducible factor-1α mRNA. Although adaptive responses were identified in this cell model, how cells convey the message of chemical compound treatment into the cells is still unknown. Thus, investigation into the potential signal transduction pathways was performed in this study using NMB cells treated with DFO and $CoCl_2$ individually to simulate hypoxia. Members of the MAP kinase family, ERK1/2 and JNK, and transcription factors, STAT1 and STAT3, are known to initiate the signaling cascades that mediate gene expressions for cell survival in low oxygen conditions and were thus chosen for examination in this study. Western blot analysis revealed that DFO treatment increased the activity of STAT3 and decreased activity for ERK1/2 (Thr 160, Thr 177) and JNK. Additional Western blot analysis revealed that $CoCl_2$ treatment increased activity for ERK1/2 (Thr 160, Thr 177) and decreased activity of JNK. Results for p-STAT3 revealed an initial increase then a decrease in activity after 24 hours. Western blot analysis revealed that, ERK1/2 (Thr 202, Tyr 204) and STAT1 had no observable change in activity regardless of which chemical mimicker was used. Above data suggested that various signal transductions pathways were altered simultaneously in response to the chemical simulated hypoxia in NMB cells. The activities of MAP kinase and STAT family fluctuated positively or negatively in response to chemical hypoxic mimic compounds implicating that the development of different adaptive responses related to DFO and $CoCl_2$ treatments.

Introduction

Oxygen is one of most abundant elements in Earth, and it greatly impacts life of both prokaryotics and eukaryotics. Life has evolved and adapted to be able to utilize oxygen to fulfill many different redox reactions allowing for various cellular functions to be performed. The metabolism of humans, in particular, utilizes oxygen from the air to extract daily 2550 calories (based on a 70 kg 20 yr old male) from food in order to perform necessary metabolic functions (Wagner et al., 2011).

Oxygen is utilized in various parts of mammalian cells, most importantly as the final electron acceptor in the mitochondria's electron transport chain reaction where the vast majority of adenosine tri-phosphate (ATP) energy is generated. Without this massive reservoir of ATP, most cellular tasks necessary for growth and survival could not be completed. Some cells, such as human erythrocytes, do not possess organelles such as mitochondria and rely solely on glycolysis to generate ATP to fuel cellular functions (Markuszewski et al., 2005). Thus, any change to oxygen levels would not greatly impact the overall production of ATP in these types of cells. However, most human cells would be affected by a change in oxygen and would be forced to respond to the insult.

Neuronal cells of the nervous system (for both PNS and CNS) are highly active and therefore require large quantities of ATP (Ames, 2000). Thus, neurons are sensitive to the fluctuations of oxygen level and the decrease of ATP production as compared to the other cell types. For example, the ATP generated in the mitochondria of neurons is used to maintain the stability of the Na+/K+ pumps along the cellular membrane in order to maintain the membrane

potential (Hansen, 1985). With low oxygen levels, anaerobic metabolic pathways do not generate enough energy to sustain this function, resulting in an extra intracellular Na+, and causing depolarization of neuronal cells (Won et al., 2002). This depolarization may induce apoptotic pathways and leads to cell death.

When oxygen levels for an organism are lower than the normal concentrations, the environment is defined as hypoxic. Although there is no set value in literature for the concentration of oxygen to be used as a determining factor for hypoxia, values of 0 to 1% oxygen concentration, as compared to an organism's normal oxygen concentration, are widely accepted to be in the realm of hypoxia (Sørensen et al., 2009). Hypoxic conditions force cells to adapt or initiate apoptosis. Apoptosis initiator molecules include caspases, such as caspase 9, which signals the cell to begin the steps necessary for programmed cell death. Caspase 9 is activated when damage occurs to the mitochondria, disrupting ATP generation, and dislodging cytochrome C which in turn activates apoptosome that cleaves pro-enzyme caspase 9 to its active form (Delivoria-Papadopoulos et al., 2007). This molecule consequently cleaves procaspase 3 and 7 allowing the active forms of the aforementioned caspases to activate such molecules as poly-ADP ribose polymerase (PARP). PARP actively works on depleting ATP in the cell as well as starting the pathway for release of apoptosis inducible factor (AIF) from the mitochondrial membrane (Delivoria-Papadopoulos et al., 2007).

As seen with the caspase 9, apoptosis may occur due to the dislodging of vital flavoproteins or molecules such as cytochrome C from the mitochondrial membrane, which are integral to the production of ATP. Without these components, the electron transport chain fails to deliver the passing electron to the final electron acceptor, oxygen. Less ATP generation occurs, and the cell lacks the capacity to continue normal cellular functions ultimately leading to

programmed cell death. Not all forms of stress to a cell or to the mitochondria results in the immediate activation of molecules designated to apoptosis such as the aforementioned caspases. A mechanism has evolved in mammalian cells that allows for a temporary respite against hypoxic stress conditions that could disrupt the mitochondria's integrity.

Under low oxygen conditions, the hetero-dimeric compound hypoxia inducible factor (HIF) is activated, which may lead to promotion of key genes for cell survival (Manolescu et al., 2009). HIF is a transcription factor, and is composed of two subunits, HIF-1 alpha, an oxygen sensitive factor, and HIF-1 beta, which is oxygen independent protein (Rahman and Thomas, 2007). HIF-1 alpha may be referred to as an oxygen sensor for a mammalian cell, although this molecule does not directly interact with the oxygen molecule. HIF-1 alpha is regulated by prolyly hydroxylases (PHDs) that utilize oxygen, iron, and ascrobate as cofactors (Millonig et al., 2009). When oxygen levels are normal, PHD hydroxylation permits binding of von Hippel-Lindau tumor suppressor protein (pVHL) to HIF-1 alpha thus beginning polyubiquitination of HIF-1 alpha, targeting the subunit for proteasomal degradation (Millonig et al., 2009). However, during hypoxia, PHD activity decreases in correlation to the reduce levels of oxygen. Thus, degradation of HIF-1 alpha is decreased, resulting in the accumulation of the alpha portion of HIF. HIF-1 alpha is then able to translocate to the nucleus and heterodimerize with HIF-1 beta which promotes the transcription of HIF regulated genes (Millonig et al., 2009). These known genes may be grouped by function that includes the following: increase of anerobic metabolism (i.e., glyceraldehydes-3-phosphate dehydrogenase), vascular regulation (i.e., vascular endothelial growth factor (VEGF)), and erythropoiesis (i.e., erythropoietin) (Komatsu and Hadjiargyrou, 2003). The products of these genes allow for the cell to operate and maintain homeostasis under the adverse low oxygen conditions.

The signal transduction pathway that allows for the stabilization of HIF-1 alpha and consequently the promotion of expression of survival genes during hypoxic events is still being explored in many cell lines. However, a constant component of the identification of potential pathways that allow for the adaption to stress, such as low oxygen, is likely related to the activity of kinases (Nakabayashi and Sasaki, 2004), which are responsible for conveying a signal in the form of a phosphate group and leading to the eventual activation or inhibition of the targeted genes related to the initial signal (Nakabayashi and Sasaki, 2004). Kinases are categorized by similar chemical structures and response to similar stimuli. In the literatures, a variety of kinases within a myriad of signal transduction pathways are associated with responding to low oxygen environments (Yang et al., 2005).

In particular, the family of mitogen activated proteins (MAP) has been reported to be active under hypoxic conditions, and the MAP kinase family may be responsible for cell adaptation and survival under unfavorable conditions (Yang et al., 2005). Currently, three kinases, extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 group of protein kinases are known to belong to the MAP kinase family (Zarubin and Han, 2005). ERKs have been reported to regulate and respond to growth factors and monitor cellular division as witnessed by observation of concentrations of VEGF in primary cortical neurons under adverse conditions (Ma et al., 2011). In regards to low oxygen environments, an increase of activity of ERK1/2 was found in human microvascular endothelial cells-1(HMEC-1) (Minet et al., 2000). JNK is a stress kinase that functions primarily with mitigating responses to external stimuli such as cytokines and heat shock (Weston and Davis, 2002), but has also displayed activity in hypoxic environments. For example, an increase of JNK activity in a hypoxia

environment, such as a hypoxic gas chamber, has also been found in cerebral cortical neuronal nuclei of newborn piglets (Mishra et al., 2004)

Similarly, Signal Transducer and Activator of Transcription (STAT) may also be activated either by stress or cytokine stimulation (Takeda and Akira, 2000; Horvath, 2000). Up to now, seven STAT proteins are identified in mammals: STAT1, STAT2, STAT3, STAT4, STAT5, STAT6, and STAT7 (Lee et al., 2006). All STATs have conserved domains, including a SH2 domain near the carboxy-terminus, and located here is a tyrosine residue that is the target of phosphorylation by such molecules as Janus kinase (JAK) (Takeda and Akira, 2000). Once phosphorylated STATs either homo- or hetero-dimerize through interactions of one STAT to another's SH2 domain, allowing for translocation into the nucleus to alter the targeted gene expressions (Takeda and Akira, 2000). Several known STAT target genes have been identified, and these genes are involved in embryonic development, programmed cell death, organogenesis, innate immunity, adaptive immunity, and/or cellular growth (Horvath, 2000). Previously, increases of STAT1 and STAT3 activities under hypoxic conditions have been reported using human breast cancer cell line (MCF-7) (Lee et al., 2006).

The potential involvement of the MAP kinase subfamily and STATs in response to low oxygen environments in human neuronal cells (neuroblastoma, NMB) was therefore examined. The chemical mimickers, cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) were used to simulate hypoxia. Previously, our lab reported that when administering DFO in a dose and time dependent manner, there was an increase in levels of glutathione and higher concentrations of HIF (Cook et al., 2010). HIF is a strong promoter in mammalian's adaptation to low oxygen insults. Glutathione is responsible for negating damaging free radicals and preserving and regulating DNA synthesis. Using confocal analysis with annexin-V-fluorescein and propidium

iodide staining revealed that surviving/attached cells under DFO challenge were morphologically similar to control (vehicle-treated) cells (Cook et al, 2010). Thus, this cell model system permits investigation into potential pathways that allow for adaptation of NMB cells to hypoxic mimickers.

Two hypoxic mimics, DFO and $CoCl_2$, were chosen for this study. Either chemical is able to produce a mimic hypoxic condition in cells by acting as an iron chelator that affects the binding of the hydroxyl group to HIF1-alpha by PHD (Semenza, 2004). The structure of PHD contains an Fe(++) at its catalytic site, and unlike a red blood cell's heme group, PHD's iron component may be easily chelated out of the molecule (Semenza, 2004). With the removal of the iron by either DFO or $CoCl_2$, PHD is no longer stable and is unable to hydroxylize HIF1-alpha, thus the signal for pVHL to add an ubiquitone to HIF1-alpha is stopped. Although DFO chelates the iron component through a hydroxamic group (R-CO-NH-OH) (Harmatz et al., 2007) and $CoCl_2$ binds the iron molecule via the strong negative charge of cobalt, the end result is the same. PHD is destabilized and HIF-1 alpha is not targeted for proteasome degradation; instead, HIF-1 alpha concentration accumulates and translocates to the nucleus to dimerize with the HIF-1 beta.

Therefore, the objective of this thesis is to investigate prospective adaptive signal transduction pathways of NMB cells in response to hypoxic stress simulated by chemical mimickers, DFO and $CoCl_2$. The potential kinases and transcription factors, ERK1/2, JNK, STAT1, and STAT3, were examined in response to either DFO or $CoCl_2$ in this study.

Material and Methods

Cell Culture:

Human neuroblastoma (NMB) cells were grown in Roswell Park Memorial Institute Medium (RPMI) with the addition of 10% Fetal Bovine Serum at 37 degrees Celsius and 5% CO_2 incubator. Cells were treated with either a single dose of 0.3mM desferrioxamine (DFO) or 0.3mM cobalt chloride ($CoCl_2$) and were incubated for various times as indicated in the results. Both DFO and $CoCl_2$ were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Lysis:

NMB cells were harvested by removal of media, washed with cold PBS, and disassociated from the flask using PBS-EDTA. The cells were then lysed by adding lysing buffer containing 1% SDS, PBS, and the following protease inhibitors with a uniform concentration of 1.0 mM: sodium orthovandate (Na3VO4), pepstatin, aprotinin, phenylmethylsulphonyl fluoride (PMSF). The cell mixture was then sonicated. The resulting lysates were stored at -80 degrees celsius.

Lowry Assay:

Protein concentration of cell lysate was determined by a Lowry Assay (Lowry et al., 1951). Bovine serum albumin (BSA) was used to as the protein standard for generating the standard curve. Briefly, protein samples were incubated with solution C (1.8mM Na_2Co_3, 98mM NaOH, 0.95mM sodium potassium tartrate, and 0.4mM $CuSO_4$ $5H_2O$) at room temperature for 10

minutes, and then further incubated with Folin-Ciocalteau reagent (Lowry et al., 1951). Protein concentration was determined using spectrophometer at 660 nm.

Western Blotting:

Sample protiens were first separated using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then electically transferred onto phenylmethylsulfonyl fluoride (PVDF) membranes from GE Healthcare. The membrane was then blocked using blocking solution, containing a mixture of evaporated milk and 0.3% Tween Twenty-Buffer Solution, probed with JNK, ERK1/2 primary antibodies from Santa Cruz Bio-Technology (Santa Cruz, CA) and STAT1 and STAT 3 primary antibodies from Cell Signalling Technology (Danvers, M.A.), and subsequently probed with secondary antibodies from Jackson Immuno Research (West Grove, PA). The signals were detected with chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Results

Previously, the human neuronal hypoxic model was generated by treating human neuroblastoma, NMB, cells with the hypoxic chemical mimicker DFO (Cook et al, 2010). An increase, as depicted in a concentration and time dependent manner, of HIF and cellular glutathione levels were found in surviving cells under such a treatment (Cook et al, 2010). Although the increase of both HIF and glutathione levels has been reported in this cell model, the pathways that convey activation and/or may pertain to the increase of these two critical components were not identified. Thus, to investigate the potential signal transduction pathways that respond to such a hypoxia simulated condition in the cell model system, the chemical hypoxic mimickers, DFO and $CoCl_2$, were used and the obtained results were compared in this study.

Several key enzymes are known to be involved in the potential signal transduction pathways (Lee et al., 2006; Wang et al., 2005; Minet et al., 2000; Chen et al., 2010; Tanga et al., 2009; Yang et al., 2005; Vacotto et al., 2008). For example, the activities of members of MAP kinase subfamily, extracellular signal-regulated kinase (ERK1/2) and c-jun N-terminal kinase (JNK) and signal transducer proteins such as Signal Transducer and Activator of Transcription 1 (STAT1) and Signal Transducer and Activator of Transcription (STAT3) were shown to be changed under a low oxygen environment. Most often this type of signal resided in the form of a phosphorylated kinase/protein; thereby, the analysis of the altered level/amount of phosphorylated proteins will allow examination of changes in their active status. In addition, the fluctuations of phosphorylation levels of a protein were mostly observed in a short time period (Lee et al., 2006; Wang et al., 2005; Minet et al., 2000; Chen et al., 2010; Vacotto et al., 2008).

Therefore, in this study, neuronal cells, NMB, were exposed to 0.3 mM of DFO or 0.3 mM of $CoCl_2$ for a short period of time, 5 minute (min), 10 min, 15 min, as well as for a longer time period, such as1 hour (hr), and 24 hr. The protein kinases' activities were then monitored by observing changes in their phosphorylation levels detected by Western Blot analysis that utilized specific phosphorylated antibodies to probe for the corresponding investigated protein kinase.

Investigation of p-ERK1/2 activity using neuronal cells treated with DFO

Measurement of ERK1/2 activity was achieved through Western blot analysis using p-ERK1/2 (Thr 160, Thr 177) antibody. Beta tubulin is a cytoskeleton house-keeping protein and consequently its signal was utilized as an internal standard for normalization.

Western blot analysis (Fig. 1A) showed that activity of p-ERK1/2 (Thr 160, Thr 177) decreased from 5 min to 1 hr. Quantitative data was shown in Fig. 1B, revealing that the decrease of activity is significantly observed at 1 hr treatment ($p < 0.005$). Upon the 24 hr treatment, the activity of p-ERK1/2 (Thr 160, Thr 177) resumed to normal level. These results suggested that DFO causes a decrease in p-ERK1/2 (Thr 160, Thr 177) activity with the 1 hr time point displaying a significant reduction.

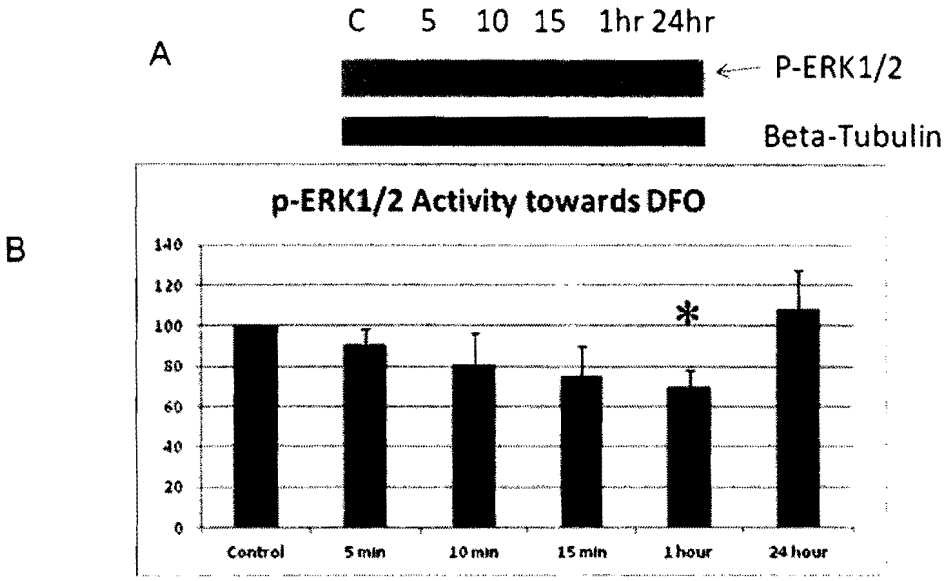

Figure 1. ERK1/2(Thr 160, Thr 177) activity in human neuronal cells exposed to DFO at various time points. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-ERK1/2 was examined using Western blot analysis. (A) Representative western blot image of p-ERK1/2 on top, with Beta-Tubulin on bottom. (B) Quantification of p-ERK1/2(Thr 160, Thr177) normalized to Beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05.

Investigation of p-ERK1/2 activity in the cells treated with $CoCl_2$

Similarly, Western blot analysis was performed using p-ERK1/2 (Thr 160, Thr 177) antibody with the same cell line. The cells were treated with $CoCl_2$ for 5 min, 10 min, 15 min, 1 hr, and 24 hr. Beta tubulin is utilized as an internal standard for normalization.

Western blot analysis (Fig. 2A) demonstrated that activity of p-ERK1/2 (Thr 160, Thr 177) showed no significant decrease within 15 min exposure to $CoCl_2$. However, there was a noted increase of p-ERK1/2 (Thr 160, Thr 177) activity after 1 hr and 24 hr of $CoCl_2$ simulated hypoxia. Quantitative data was shown in Fig. 2B revealing that the increase of activity at 1 hr is significantly observed ($p < 0.05$). These results suggested that $CoCl_2$ causes an increase of activity of ERK1/2 (Thr 160, Thr 177) at the 1 hr and 24 hr treatment.

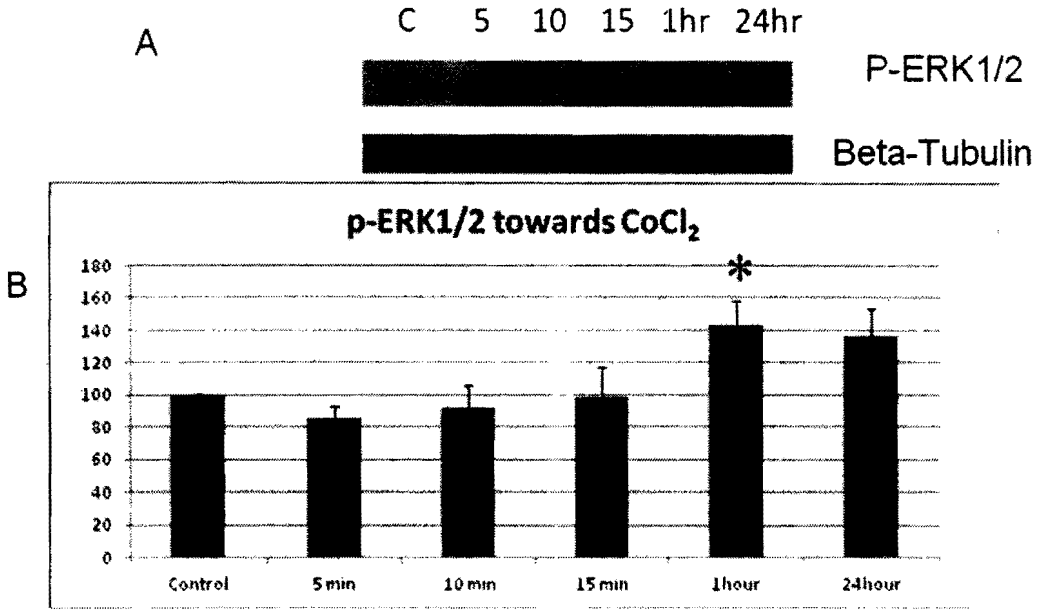

Figure 2. ERK1/2(Thr 160, Thr 177) activity in neuronal cells exposed to $CoCl_2$ at various time points. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-ERK1/2 was examined using Western blot analysis. (A) Representative western blot image of p-ERK1/2 on top, with Beta-Tubulin on bottom. (B) Quantification of p-ERK1/2(Thr 160, Thr177) normalized to Beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05.

Investigation of p-ERK1/2 activity in cells treated with DFO and $CoCl_2$

Measurement of ERK1/2 activity was achieved through Western blot analysis using p-ERK1/2(Thr 202, Tyr 204) antibody, and beta tubulin was utilized as an internal standard. Western blot analysis (Fig. 3) revealed that there was no detectable activity of p-ERK1/2 (Thr 202, Tyr 204) regardless of which chemical mimicker was used, DFO or $CoCl_2$. These results showed that the activity of ERK1/2 (Thr 202, Tyr 204) was not altered in the presence of any of the chemical treatments.

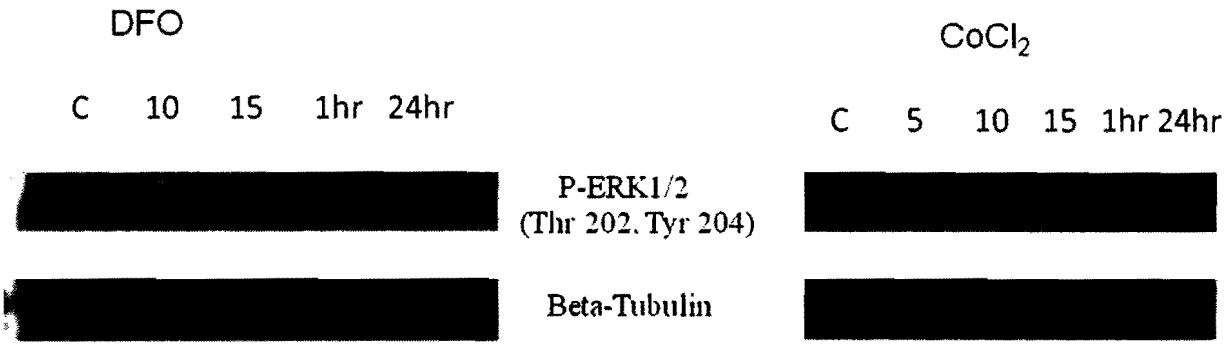

Figure 3. Time course of ERK1/2(thr 202, tyr 204) activity in cells exposed to DFO or $CoCl_2$. Cells were treated in one dose at 10 min, 15 min, 1 hr, and 24 hr and p-ERK1/2 was examined by Western blot. Representative western blot image of p-ERK1/2 (thr 202, tyr 204) on top, with Beta-Tubulin on bottom. N=4 for both DFO and $CoCl_2$ trials. Little observed activity of p-ERK1/2 (thr 202, tyr 204) regardless if DFO or $CoCl_2$ was used to simulate hypoxia in neuronal cells.

Investigation of p-JNK activity using cells treated with DFO

Measurement of JNK activity was achieved through Western blot analysis using p-JNK antibody and beta tubulin as an internal standard.

Western blot analysis (Fig. 4A) revealed that activity of p-JNK decreased from 5 min to 15 min. Quantitative data was shown in Fig. 4B revealing that the decrease of activity is significantly observed at 5, 10, and 15 min. treatments ($p < 0.05$). Activity of p-JNK began to gradually resume to normal level after 1 hr and 24 hr exposure. These results showed that DFO treatment caused the decrease of JNK activity starting at the first 15 min and then gradually returned to normal levels starting at 1hr. No significant difference was found at the 24 hr treatment.

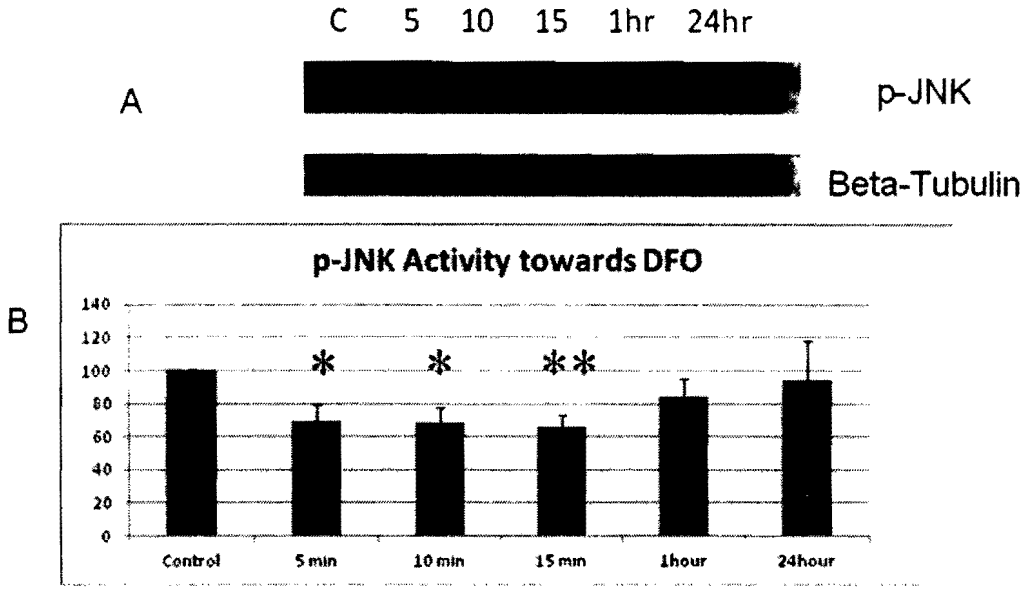

Figure 4. Time course of JNK activity in cells exposed to DFO. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-JNK was examined using Western blot analysis. (A) Representative western blot image of p-JNK on top, with Beta-Tubulin on bottom. (B) Quantification of p-JNK normalized to beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05. ** Indicates p-value less than 0.005.

Investigation of p-JNK activity using cells treated with $CoCl_2$

Similarly, Western blot analysis was performed using p-JNK antibody with cells treated with $CoCl_2$ for 5 min, 10 min, 15 min, 1 hr, and 24 hr. Beta tubulin was again utilized as an internal standard.

Western blot analysis (Fig. 5A) revealed that activity of p-JNK decreases from 5 min through 24 hr. Quantitative data was shown in Fig. 5B revealing that the decrease of activity is significantly observed at 10 min, 1 hr, and 24 hr. treatment ($p < 0.05$). This data showed that $CoCl_2$ treatment caused a continual decrease of activity of JNK starting at 5 min.

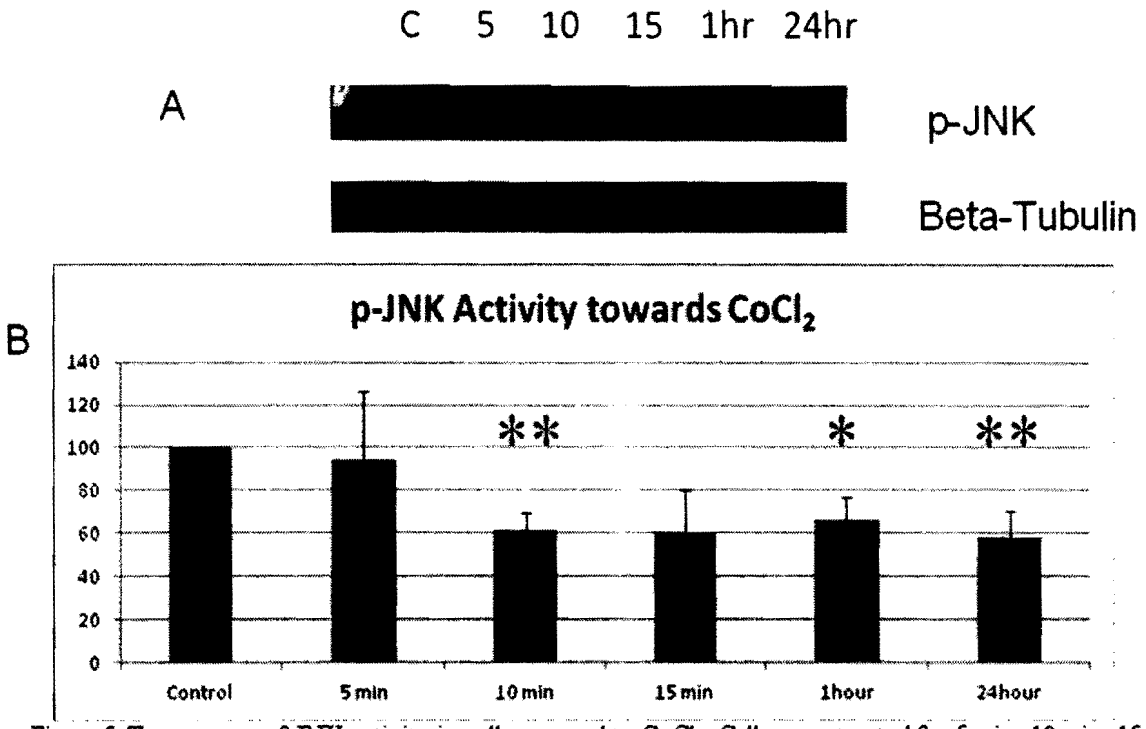

Figure 5. Time course of JNK activity in cells exposed to $CoCl_2$. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-JNK was examined using Western blot analysis. (A) Representative western blot image of p-JNK on top, with beta-Tubulin on bottom. (B) Quantification of p-JNK normalized to beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05. ** Indicates p-value less than 0.005.

Investigation of p-STAT3 activity using cells treated with DFO

Measurement of STAT3 activity was achieved through Western blot analysis using p-STAT3 antibody. Beta tubulin is a cytoskeleton house-keeping protein and consequently was utilized as an internal standard.

Western blot analysis (Fig. 6A) revealed that activity of p-STAT3 increases slightly from 5 min and to the 24 hr treatment. Quantitative data was shown in Fig. 6B revealing that the increase of activity was significantly observed at 5 min and 15 min ($p< 0.05$), and at 24 hr treatment ($p<0.001$). These results showed that an increase of STAT3 activity of is significantly observed at 24 hr treatment.

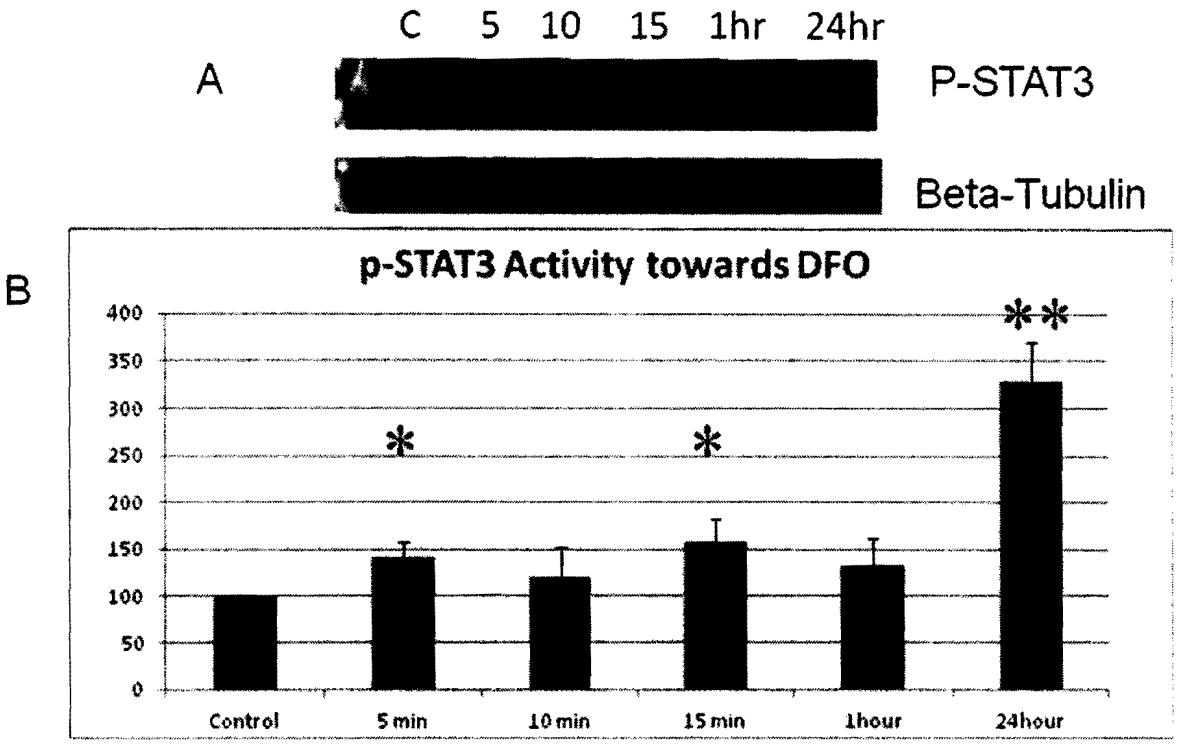

Figure 6. Time course of STAT3 activity in cells exposed to DFO. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-STAT3 was examined using Western blot analysis. (A) Representative western blot image of p-STAT3 on top, with beta-Tubulin on bottom. (B) Quantification of p-STAT3 normalized to beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05. ** Indicates p-value less than 0.005.

Further investigation of p-STAT3 activity using cells treated with DFO

Measurement of STAT3 activity was further investigated using 4 hr, 6 hr, 8 hr, 12 hr, and 24 hr treatment by Western blot analysis using p-STAT3 antibody and beta tubulin was again utilized as an internal standard.

Western blot analysis (Fig. 7A) revealed that activity of p-STAT3 increases at 12 hr and 24 hr. Quantitative data was shown in Fig. 7B revealing that the increase of activity is significantly observed at both 12 hr and 24 hr treatment ($p < 0.005$). These results indicated that DFO causes a significant increase of activity at the 12 hr and 24 hr time intervals.

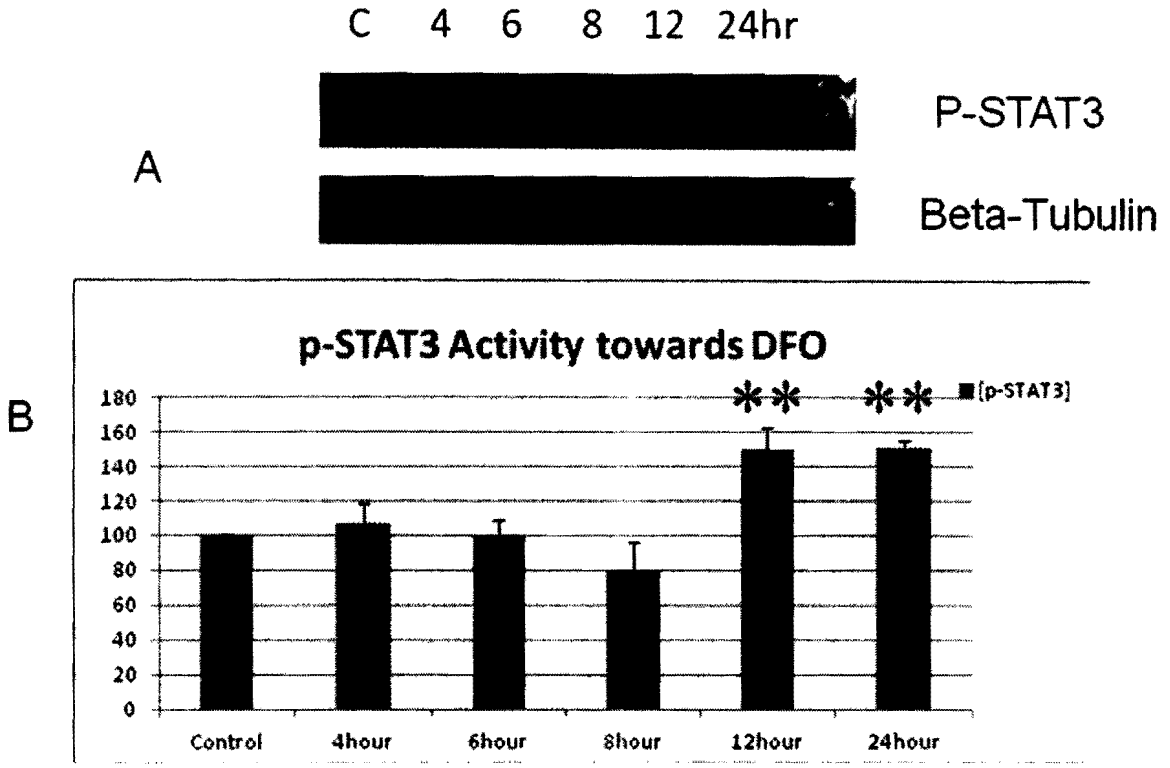

Figure 7. Time course of STAT3 activity in human neuronal cells exposed to $CoCl_2$. Cells were treated for 4 hr, 6 hr, 8 hr, 12 hr, and 24 hr , and p-STAT3 was examined using Western blot analysis. (A) Representative western blot image of p-STAT3 on top, with beta-Tubulin on bottom. (B) Quantification of p-STAT3 normalized to beta-Tubulin. Values are representative of the means with +/- SE. N=6 * Indicates the significant change with a p-value less than 0.05. ** Indicates p-value less than 0.005.

Investigation of p-STAT3 activity using cells treated with $CoCl_2$

Similarly, Western blot analysis was performed using p-STAT3 antibody with the cells treated with $CoCl_2$ for 5 min, 10 min, 15 min, 1 hr, and 24 hr. Beta tubulin was utilized as an internal standard for normalization.

Western blot analysis (Fig. 8A) revealed that activity of p-STAT3 increased from 5 min to 1 hr, and decreased at 24 hr treatment. Quantitative data was shown in Fig. 8B revealing that the increase of activity was significantly observed at the 1 hr treatment ($p < 0.005$) and a decrease of activity was significantly observed at 24 hr treatment ($p < 0.001$). Thus, the data showed that when $CoCl_2$ caused an increase of activity of STAT3 up to 1 hr, whereas there was a drastic decrease of activity observed at the 24 hr treatment.

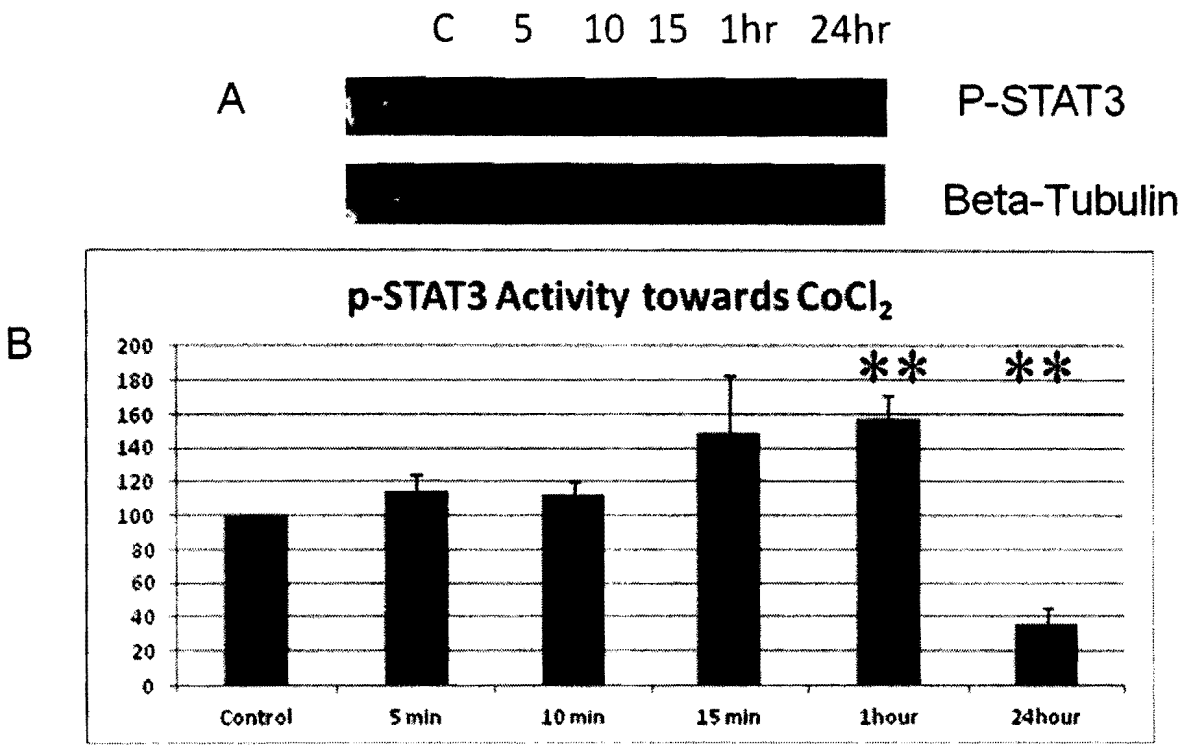

Figure 8. Time course of STAT3 activity in cells exposed to DFO. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hours, and p-STAT3 was examined using Western blot analysis. (A) Representative western blot image of p-STAT3 on top, with Beta-Tubulin on bottom. (B) Quantification of p-STAT3 normalized to Beta-Tubulin. Values are representative of the means with +/- SE. N=6. * Indicates the significant change with a p-value less than 0.05. ** Indicates p-value less than 0.005.

Investigation of p-STAT1 activity using cells treated with DFO and $CoCl_2$

Measurement of STAT1 activity was achieved through Western blot analysis using p-STAT1 antibody and beta tubulin was utilized as an internal standard. Western blot analysis (Fig. 9) revealed that activity of p-STAT1 was not detectable regardless of which chemical mimicker was used, DFO or $CoCl_2$. These results showed that the activity of STAT1 was not altered in the presence of either of the chemical treatments.

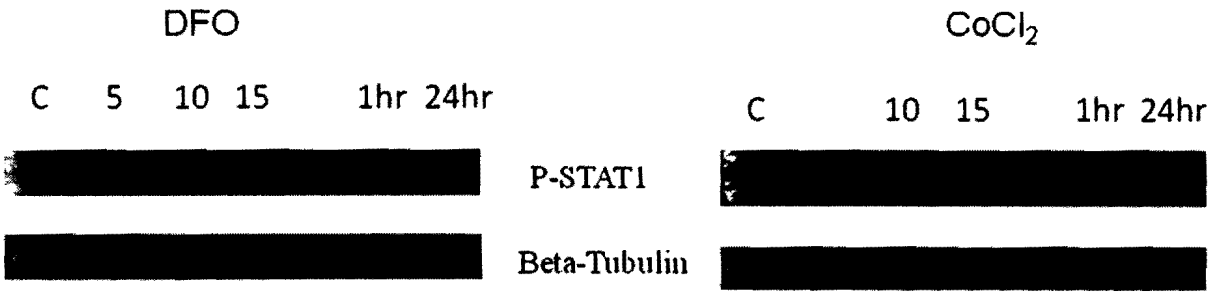

Figure 9. Time course of STAT1 activity in cells exposed to DFO or $CoCl_2$. Cells were treated for 5 min, 10 min, 15 min, 1 hr, and 24 hr, and p-STAT1 was examined using Western blot analysis. Representative western blot image of p-STAT1 on top, with beta-Tubulin on bottom. N=4 for both DFO and $CoCl_2$ trials. Little observed activity of p-STAT1 regardless if DFO or CoCl2 was used to simulate hypoxia in cells.

Discussion

Previously, a cell model system was used which exhibited several adaptive responses upon DFO treatment (Cook et al., 2010). However, the signal pathways that may be related to the adaptive responses in this cell model system were still unknown. This research was to identify signal transduction pathways in response to simulated hypoxia by chemical mimickers, DFO and $CoCl_2$, by examining the activity changes in MAP kinase subfamily via ERK1/2 and JNK as well as transcription factors STAT1 and STAT3.

In this study, $CoCl_2$ treatment caused the activity of ERK1/2 (Thr 160, Thr 177) to increase at the treatments of 1 hr and 24 hr (Fig. 2). This increase of ERK1/2 (Thr160, Thr 177) activity witnessed in $CoCl_2$ treatments concurs with other findings of p-ERK1/2 (Thr 160, Thr 177) in human microvascular endothelial cells-1 (HMEC-1) cells (Minet et. al., 2000), human placental artery endothelial (HPAE) cells (Wang et al., 2009), and in primary cultured SD rat cortical neurons (Tanga et al., 2009), all of which used gas chambers to create a low oxygen environment. In HMEC-1 cells the increase of activity of the monitored kinase was observed at 30 min, 1hr, and 2 hr of hypoxic exposure (Minet et. al., 2000) and primary cortical neurons showed an increase of kinase activity after 90 min (Tanga et al., 2009); however, in HPAE cells the increase of activity of ERK1/2 (Thr160, Thr177) was noted at an early 5 min time point (Wang et al., 2009). On the other hand, DFO treatment revealed that there is a decrease of ERK1/2 (Thr 160, Thr 177) activity between 5 min to 1 hr as seen in Figure 1, and activity resumed to normal levels after 24 hr exposure. In human monocytic leukemia THP-1 cells, DFO resulted in an increase of p-ERK1/2 from 1 hr to 12 hr then a decrease to normal

levels after 24 hr (Seo et al., 2006). Different responses were observed which may be a result of different cell types as well as different treatments.

There was no significant ERK1/2 (Thr 202, Tyr 204) activity detected (Fig 3) under either DFO or $CoCl_2$ treatment. This observation corroborates with the report of p-ERK1/2 (Thr 202, Tyr 204) in rat neural stem cells undergoing 0.3% oxygen levels (Chen et al. 2010). However, in human HMEC-1 (Minet et al., 2000) an increase of activity of ERK1/2 (Thr 202, Tyr 204) from 30 min to 2 hr was reported, which may be due to the different responses of cell types.

Although ERK1/2 is comprised of ERK1 and ERK2 which share a 90% identical a.a. sequence to one another (Kinoshita et al., 2008), there was observed in this study a stark contrast in presence and activity of ERK1/2(Thr 160, Thr 170) and ERK1/2 (Thr 202, Tyr 204). Literature has shown that ERK1/2 needs to be phosphorylated at two sites, either Thr 202, Tyr 204 or Thr 185, Tyr 187 to achieve full catalytic function (Kinoshita et al., 2008). MAPkinase/ERK kinase (MEK) in the well studied RAS, RAF, and MEK signal transduction pathway, can dual phosphorylate ERK1/2 at either of the aforementioned two sites (Ramos, 2008). However, there exists on ERK1/2 other phosphorylation sites in a region of amino acid residues within the same Thr-Glu-Tyr motif that contains Thr 202, Tyr 204 and Thr 185, Tyr 187 (Wortzel and Seger, 2011). The phosphorylation of these nearby amino acid sites may be vital for signal transduction pathways involving ERK1/2. For example, ERK1/2 mutations in the sites of Thr 173-178 revealed that these residues are important for sub-cellular localization of ERK1/2 as well as promoting other regions of ERK for overall catalytic function of ERK1/2 (Bendetz-Nezer and Seger, 2007). Thus, differences observed between the two ERK1/2 sets in this study

may be the result of signal transduction phosphate binding preferences on ERK1/2 within NMB cells.

In addition, DFO treatment revealed that there is a decrease in JNK activity between 5 min to 15 min (Figure 4), and activity began to increase at 1 hr and return to normal levels after 24 hr exposure. $CoCl_2$ treatment revealed there is also a decrease in JNK activity starting from 5 min to 24 hr (Figure 5). As compared to the other reports, rat neural stem (Chen et al. 2010) cells showed an increase of p-JNK signal at 6 hr, 12 hr, and 24 hr at 0.3% oxygen level, and observation of cerebral cortical neuronal nuclei of newborn piglets (Mishra et al., 2004) which had undergone gas chamber hypoxia, displayed an increase of JNK activity after 1 hr exposure. Also, avian TUNEL-positive pyknotic cells (Vacotto et al., 2008) showed an increase of JNK activity after 10 and 30 min at 0.3% oxygen level. In some cell lines the role of JNK may be associated with the preservation of the cell through strong cellular survival signal transduction pathways, which would lead to an increase of p-JNK. However, there is a possibility in human neuronal cells to be more inclined to apoptosis signaling through JNK than cell survival signaling under stress conditions. Thus, if the neuronal cells are trying to survive an oxygen insult, then suppressing p-JNK apoptosis signal transduction pathway would allow a higher chance of cell survival. Therefore, such a difference between results seen here and literature may be due to the different species used and/or under different hypoxic environments.

There was no significant activity of p-STAT1 detected in this study (Fig. 9) under either DFO or $CoCl_2$ treatment. However, in different cell lines such as mouse mammary epithelial cells(HC11) and human breast cancer cell line (MCF-7) (Lee et al., 2006), and human pulmonary arterial smooth muscle cells (PASMC) (Wang et al., 2005) there was an increase of

p-STAT1 reported. Although no significant change of STAT1 activity using human neuronal cells was found in this study, there was the increase of STAT3 activities detected.

DFO treatment increased STAT3 activity from 5 min to 24 hr (Figure 6), with significant increases at 12 and 24 hr in Figure 7. $CoCl_2$ treatment also showed an increase of STAT3 activity between 5 min to 1 hr, but then a sharp decrease after 24 hr (Figure 8). Thus, both DFO and $CoCl_2$ treatments resulted in an increase of STAT3 activity with a different timetable. The increase activity of p-STAT3 was observed after 2 to 6 hr of hypoxia by gas chamber and DFO in HC11 and MCF-7 cells (Lee et al., 2006). Human ovarian cell line A2780 (Selvendiran et al., 2009) showed an increase of STAT3 activity after 24 hr and 48 hr of hypoxic gas chamber exposure, and PASMC cells displayed higher levels of p-STAT3 after 8 hr exposure to a hypoxic gas chamber (Wang et al., 2005). The consensus would indicate that across multiple species and cell types STAT3 plays an important role in response to hypoxic conditions, regardless of which hypoxic media is used.

Finally, DFO and $CoCl_2$ are chemical hypoxic mimickers and thereby the simulated hypoxia environment is not the same as a hypoxic gas chamber. Both DFO and $CoCl_2$ chelate iron from PHD (Semenza, 2004), but in addition, $CoCl_2$ can also bind directly to pVHL preventing its activity (Yuan et al., 2003). Also worth mentioning, is that 0.2-0.8mM of $CoCl_2$ has been shown in primary cultured mouse astrocytes to be toxic (Karovic et al., 2007). $CoCl_2$ used in this research with a concentration of 0.3mM falls within this parameter of $CoCl_2$ toxicity. These discrepancies can provide at least a partial explanation as to why there are divergent results in the literature, in addition to various species and cell types, which may respond differently in the same condition.

Conclusion

In summary, the objective was to investigate potential signal transduction pathways within NMB cells that convey the message from the outside to inside the cells. Members of the MAP kinase subfamily as well as the transcription factor order of STATs were chosen for examination in this study based on literature revealing the fluctuations of activities under hypoxic conditions (Chen et al., 2010; Lee et al., 2006; Minet et al., 2000; Mishra et al., 2004; Selvendiran et al., 2009; Tanga et al., 2009; Vacotto et al, 2009; Wang et al, 2005; Wang et al., 2009). Induction of hypoxia was performed with the chemical mimickers DFO and $CoCl_2$. Although both DFO and $CoCl_2$ have been shown to cause apoptosis in many cell lines, (Guo et al., 2006) they also induce hypoxic mechanisms by chelating iron from PHD allowing HIF-1 alpha to stabilize and promote adaption to low oxygen signaling.

Administration of DFO and $CoCl_2$ to NMB cells revealed fluctuations in activity in the target proteins of interest. Western blot analysis of DFO treatments using specific phosphorylated antibodies to the corresponding protein revealed increase in activity of STAT3 and decrease activity of ERK1/2 (Thr 160, Thr 170) and JNK. Further western blot analysis of $CoCl_2$ treatments revealed an increase of activity of ERK1/2 (Thr 160, Thr 170), and a decrease of activity in JNK. Results for p-STAT3 revealed an initial increase then a decrease in activity after 24 hours. Lastly, Western blot analysis of ERK1/2 (Thr 202, Tyr 204) and STAT1 revealed no observable change in activity regardless of which chemical mimicker was applied.

As observed, there were variations between DFO and $CoCl_2$ treatments. These may be due to the natures of the two compounds. As previously stated, DFO chelates the iron component

of PHD, which allows HIF-1 alpha to remain stable and begin a hypoxic signal transduction pathway. $CoCl_2$ may chelate the iron component of PHD, but $CoCl_2$ may also bind directly to the VHL (Yuan et al., 2003). Thus, $CoCl_2$ prevents VHL from binding HIF-1 alpha, allowing this molecule to remain stable in a different mechanism than DFO induced hypoxia. Finally, the concentration of $CoCl_2$ used in this research is suggested by literature to have possible cobalt toxicity to the treated cells (Karovic et al., 2007); therefore, the potential adverse effects of DFO and $CoCl_2$ should be investigated in the future.

References

Adamski J.K., Estlin E.J., Makin G.W.J. (2008). The cellular adaptations to hypoxia as novel therapeutic targets in childhood cancer. *Cancer Treatment Reviews*. 34:231-246.

Ames III A. (2000). CNS energy metabolism as related to function. *Brain Research Reviews*. 34:42-68

Hansen, A.J., (1985). Effect of anoxia on ion distribution in the brain. *Physiological Review*. 65(1):101-48.

Chen X., Tian Y., Yao L., Zhang J., and Liu Y. (2010). Hypoxia stimulates proliferation of rat neural stem cells with influence on the expression of cyclin D1 and c-Jun N-Terminal protein kinase signaling pathway *IN VITRO. Neuroscience*. 165:705-714.

Cook R. J., Karch C., Nahar P., Rivera A., Ko J. L. (2010). Effects of desferoxamine-induced hypoxia on neuronal human mu-opioid receptor gene expression. *Biochemical and Biophysical Research Communications*. 398:56-61.

Guo G., Song L. P., Jiang Y., Liu W., Yu Y. and Chen G. Q. (2006). Hypoxia-mimetic agents desferrioxamine and cobalt chloride induce leukemic cell apoptosis through different hypoxia-inducible factor-1α independent mechanisms. *Apoptosis*. 11: 67–77

Harmatz P., Grady R. W., Dragsten P., Vichinsky E., Giardina P., Madden J., Jeng M., Miller B., Hanson G., and Hedlund B. (2007). Phase Ib clinical trial of starch-conjugated deferoxamine(40SD02): a novel long-acting iron chelator. *British Journal of Haematology*.138:374–381.

Horvath C. M. (2000) STAT proteins and transcriptional responses to extracellular signals. *Trends in Biochemical Sciences*. 25(10):496-502.

Komatsu D.E., Hadjiargyrou M. (2003) Activation of the transcription factor HIF-1 and its target genes, VEGF, HO-1, iNOS, during fracture repair. *Bone*. 34:680– 688.

Karovic O., Tonazzini I., Rebola N., Edstro¨m E., Lo¨vdahl C., Fredholm B.B., Dare E. (2007). Toxic effects of cobalt in primary cultures of mouse astrocytes similarities with hypoxia and role of HIF-1alpha. *Biochemical pharmacology*. 73:694-708.

Kinoshita T., Yoshida I., Nakae S., Okita K., Gouda M., Matsubara M., Yokota K., Ishiguro H., Tada T. (2008). Crystal structure of human mono-phosphorylated ERK1 at Tyr204. *Biochemical and Biophysical Research Communications*. 377:1123–1127.

Lee M. Y., Joung Y. H., Lim E. J., Park J. H., Ye S. K., Park T., Zhang Z, Park D. K., Lee K. J., Yang Y. M. (2006). Phosphorylation and activation of STAT proteins by hypoxia in breast cancer cells. *The Breast*. 15(2):187-195.

Lowry O. H., Rosebrough J. N., Farr A. L., Randall J. R. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry*. 193:265-275.

Ma Y., Liu W., Wang Y., Chao X., Qu Y., Wang K., Fei Z. (2011). VEGF protects rat cortical neurons from mechanical trauma injury induced apoptosis via the MEK/ERK pathway. *Brain Research Bulletin*. 86:441-446.

Manolescu B., Oprea E., Busu C., Cercasov C. (2009). Natural compounds and the hypoxia-inducible factor (HIF) signaling pathway. *Biochimie*. 91:1347–1358.

Markuszewski M. J., Szczykowska M., Siluk D., Kaliszan R. (2005). Human red blood cells targeted metabolome analysis of glycolysis cycle metabolites by capillary electrophoresis using an indirect photometric detection method. *Journal of Pharmaceutical and Biomedical Analysis*. 39: 636–642.

Millonig G., Hegedüsch S., Becker L., Seitz H.K.,
Schuppan D., Mueller S. (2009). Hypoxia-inducible factor 1α under rapid enzymatic hypoxia: Cells sense decrements of oxygen but not hypoxia per se. *Free Radical Biology & Medicine*. 46:182–191.

Minet E., Arnould T., Michel G., Roland I., Mottet D., Raes M., Remacle J., Michiels C. (2000). ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Letters*. 468:53-58.

Mishra O. P., Zubrow A. B., and Ashraf Q. M. (2004). Nitric Oxide-Mediated activation of Extracellular Signal-Regulated Kinase (ERK) and c-Jun N-Terminal Kinase (JNK) during hypoxia in cerebral cortical nuclei of newborn piglets. *Neuroscience*. 123:179-186.

Nakabayashi J., Sasaki A. (2004) Optimal phosphorylation step number of intracellular signal-transduction pathway. *Journal of Theoretical Biology*. 233:413–421.

Bendetz-Nezer S., Seger R. (2007) Role of Non-phosphorylated Activation Loop Residues in Determining ERK2 Dephosphorylation, Activity, and Subcellular Localization. *Journal of Biological Chemistry*. 282:25114-25122.

Delivoria-Papadopoulos M., Gorn M., Ashraf Q. M., Mishra O. P. (2007). ATP and cytochrome *c*-dependent activation of caspase-9 during hypoxia in the cerebral cortex of newborn piglets. *Neuroscience Letters*. 429:115–119.

Rahman Md. S., Thomas P. (2007). Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker (Micropogonias undulatus). *Gene*. 396:273–282.

Ramos W. J. (2008).The regulation of extracellular signal-regulated kinase
(ERK) in mammalian cells. *The International Journal of Biochemistry & Cell Biology*. 40:2707-2719.

Selvendiran K., Bratasz A., Kuppusamy M. L., Tazi M. F., Rivera B. K., and Kuppusamy P. (2009). Hypoxia induces chemoresistance in ovarian cancer cells by activation of signal transducer and activator of transcription 3. *Int J Cancer.* 125(9):2198-2204.

Semenza. G. L. (2004). Hydroxlyation of HIF-1: Oxygen Sensing at the Molecular Level. *Physiology.* 19:176-182.

Seo G. S., Lee S. H., Choi S. C., Choi E. Y., Oh H. M., Choi E. J., Park D. S., Kim S. W., Kim T. H., Nah Y. H., Kim S., Kim S. H., You S. H., Jun C. D. (2006). Iron chelator induces THP-1 cell differentiation potentially by modulating intracellular glutathione levels. *Free Radical Biology & Medicine.* 40:1502–1512.

Sørensen B. S., Horsman M. R., Vorum H., Honoré B., Overgaard J., Alsner J. (2009). Proteins upregulated by mild and severe hypoxia in squamous cell carcinomas in vitro identified by proteomics. *Radiotherapy and Oncology.* 92:443–449.

Takeda K., Akira S. (2000). STAT family of transcription factors in cytokine-mediated biological responses. *Cytokine & Growth Factor Reviews.* 11:199-207.

Tanga M., Cuia M., Donga Q., Rena H. M., Xiaoa B. G., Luob B. Y., Shaoa Y., Liua L., Zhoua H. G. (2009). The bradykinin B2 receptor mediates hypoxia/reoxygenation induced neuronal cell apoptosis through the ERK1/2 pathway. *Neuroscience Letters.* 450:40-44.

Vacotto M., Coso O., Fiszer de Plazas S. (2008). Programmed cell death and differential JNK, p38 and ERK response in a prenatal acute hypoxic hypoxia model. *Neurochemistry International.* 52:857–863.

Wagner B. A., Venkataraman S., Buettner G. R. (2011). The rate of oxygen utilization by cells. *Free Radical Biology and Medicine.* 51(3):700-712.

Wang G. S., Qian G. S., Zhou D. S., Zhao J. Q. (2005). JAK—STAT signaling pathway in pulmonary arterial smooth muscle cells is activated by hypoxia. *Cell Biology International.* 29:598-603.

Wang K., Jiang Y. Z., Chen D. B., Zheng J. (2009). Hypoxia Enhances FGF2- and VEGF-Stimulated Human Placental Artery Endothelial Cell Proliferation: Roles of MEK1/2/ERK1/2 and PI3K/AKT1 Pathways. *Placenta.* 30:1045–1051.

Weston C. R., Davis R. J. (2002) The JNK signal transduction pathway. *Current Opinion in Genetics & Development.* 12:14–21.

Won S.J., Kim D.Y., and Gwag B.J. (2002). Cellular and molecular pathways of ischemic neuronal death. *Journal of Biochemistry and Molecular Biology.* 35(1):67-86.

Wortzel I., Seger R. (2011). The ERK Cascade : Distinct Functions within Various Subcellular Organelles. *Genes & Cancer.* 2:195-209

Yang S. R., Cho S. D., Ahna N. S., Jung J. W., Park J. S., Jo E. H., Jae-Woong Hwang, Kim S. H., Lee B. H., Kanga K. S., Lee Y. S. (2005). The role of p38 MAP kinase and c-Jun N-terminal protein kinase signaling in the differentiation and apoptosis of immortalized neural stem cells. *Mutation Research.* 579:47-57.

Yuan Y., Hilliard G., Ferguson T., and Millhorn D. E. (2003). Cobalt Inhibits the Interaction between Hypoxia-inducible Factor-alpha and von Hippel-Lindau Protein by Direct Binding to Hypoxia-inducible Factor-alpha. *The Journal of Biological Chemistry.* 278(18): 15911–15916.

Zarubin T., Han J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Research.* 15(1):11-18.