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Chemical Induced Hypoxia Differentially Affects Gene Expression in Human Neuronal Cells

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Abstract

Previously, our laboratory used desferoxamine (DFO), to generate hypoxic conditions (a low oxygen condition) which decreased human neuronal cell viability, but some cells still survived. These surviving neurons showed no morphological changes when compared to the non-treated group. However, the alteration of several intracellular events were detected, such as an increase of hypoxia inducible factor 1 (HIF-1) mRNA levels, a decrease of human mu opioid receptor (hMOR) message, and no change of human delta opioid receptor (hDOR) receptor message. Western blot analysis showed the Janus kinase (JAK)/ Signal Transducers and Activators of Transcription (STAT) pathway was activated and an increase in STAT3 (P-STAT3) levels at was observed at 12 and 24 hour treatment. Heavy metal compounds such as cobalt chloride ($CoCl_2$) can also cause hypoxia in cells. To investigate adaptive responses of human neuronal cells under $CoCl_2$ versus DFO induced hypoxic conditions, we examined the expression of several opioid receptor genes. Preliminary results of RT-PCR showed a decrease in hMOR message levels, no change in hDOR message levels and a significant increase in hKOR message levels. Taken together, these results showed that similar adaptive responses were developed under DFO or $CoCl_2$ induced hypoxia, suggesting that opioid receptor expression may be linked to neuronal survival. The JAK/STAT pathway was also examined using confocal analysis. DFO treated cells showed an increase in P-STAT3 levels in the nucleus after 24 hours, while $CoCl_2$ treated cells showed a decrease in P-STAT3 levels after 24 hours. Collectively, these two compounds activated the JAK/STAT pathway at different times, implicating that JAK/STAT may have a differential role when different compounds (DFO or $CoCl_2$) are used to induce hypoxic conditions. Further understanding these mechanisms will assist to identify potential therapeutic targets in the future.

Introduction

Heavy Metals:

Heavy metals are present throughout the environment and can cause exposures from mining activities, lead, toxic wastes, emissions, and can be used in technology such as nanomaterials (Sears, 2013; Alarfi et al., 2013). The human body has a variety of different processes which rely on certain heavy metals as cofactors in order to perform normal biological functions such as maintaining tissue integrity, biological mobility, metabolism, and transport proteins (Sears, 2013). The use of heavy metals has both benefits and consequences for biological systems. An overabundance of heavy metals within the body due to a detoxification failure can potentially cause serious harm to the cells and tissues, especially to the central nervous system (Briner, 2012). The CNS has a sensitive susceptibility to heavy metal stress or ionic storm, due to the high metabolic activity of neuronal cells. The body has different measures and many regulatory proteins to prevent these scenarios from occurring (Briner, 2012).

Albumin, an aqueous protein found in the blood is used to regulate the osmotic pressure of blood, and also contains many binding sites for bilirubin, fatty acids, drugs and heavy metals such as aluminum, cadmium, cobalt and others (Briner, 2012). Proteins, like albumin, found throughout tissues of the body first interact with a wide variety of heavy metals. When it is saturated, it can become over run or unable to rid the body of heavy metals (Briner, 2012). Iron is the most abundant transition metal present within the human body, but the majority is inaccessible in the bound form present in hemoglobin (Samanovic et al., 2012). Iron is required for a number of functions such as cell multiplication, has redox activity, and acts as an important cofactor for proteins (Bianchi et al., 1999; Briner, 2012; Smirnova et al., 2012). Unbound iron

can induce free radical production through a Fenton reaction, further increasing reactive oxygen species which can be damaging to cells and it has also been shown that iron accumulation after an ischemic event is linked to regional oxidative stress (Goldsmith Hamrick et al., 2005; Briner, 2012).

Like iron, other heavy metals such as copper, zinc, and magnesium also play critical roles in enzymatic function and maintaining homeostasis. An overabundance of many of the aforementioned metals can lead to toxicity. Copper for example, can become lethal when dealing with phagocytic cells which use hydrogen peroxide to eliminate invaders (Samanovic, et al.,2012). This heavy metal is involved in a reaction which breaks down hydrogen peroxide into the hydroxyl radical and the hydroxyl anion, which can damage lipids, proteins, and nucleic acids which can lead to cell death (Samanovic et al., 2012)

Cobalt is a metal which can contribute to toxicity along with a variety of different diseases, such as interstitial pneumonitis, asthma and fibrosis (Alarfi et al., 2013). Cobalt can cause a heightened production of reactive oxygen species causing DNA damage (Alarfi et al., 2013; Jin et al.,2012). Some heavy metals can induce specific cellular conditions, such as hypoxia. Chelation of heavy metals along with small complex formation with the use of glutathione is part of the natural detoxification process (Sears, 2013). Desferrioxamine and cobalt chloride ($CoCl_2$) have been used in order to mimic hypoxic conditions for research.

Hypoxia is a low oxygen condition present in a cell or tissue. Homeostasis of oxygen is essential for growth, development and preservation of cells and tissues (Uchida et.al., 2004). Oxygen plays a critical role in energy production as the final electron acceptor in redox reactions in cells (Wang, et al., 2012). Oxygen deprivation is also a significant contributor to neurological

conditions such as spinal cord injury, traumatic brain injury and stroke (Siddiq et al., 2007). However other recreational activities such as scuba diving, mountain climbing and other rigorous physical activity can also induce hypoxia. Low oxygen levels may also increase free radicals, damage DNA, and lipid membranes, as well as cause cell death (Alarfi et al., 2013; Monroe et al., 2006).

Hypoxia Inducible Factor-1

Hypoxia Inducible Factor-1 (HIF-1) is a transcriptional complex which is induced under hypoxic conditions. This factor is made up of two different subunits, the alpha subunit and the beta subunit (Cook et al., 2010). The alpha subunit contains an oxygen-dependent degradation domain (ODDD) which plays a role in oxygen stability (Zhang et al., 2011) and the beta subunit is known as the aryl-hydrocarbon receptor and nuclear translocator (ARNT)(Liu, 2012; Zhang et al., 2011; Smirnowa et al., 2012).

Under normal oxygen conditions or normoxia, the alpha subunit is targeted for degradation while the beta subunit remains unaffected (Uchida et al., 2004; Smirnova et al., 2012). Prolyl-4 hydroxylases (PHD's) are part of a 2-oxoglutarate dependent hydroxylase super family which use a non-heme catalytic moiety (Heyman, et al., 2011; Siddiq et al., 2007). These enzymes hydroxylate HIF-1α on amino acid proline residues at the 402 and 564 positions and are regulated through the use oxygen, 2-oxoglutarate and iron (Siddiq et al., 2007; Jin et al., 2012). This allows for the alpha subunit to bind to the von Hippel-Lindau tumor suppressor protein (vPHL) ubiquitin ligase complex allowing proteostomal degradation of the alpha subunit (Siddiq et al., 2007; Jin et al., 2012). Normoxic conditions allow for a production rate of HIF which is

equivalent to the breakdown of the alpha subunit, maintaining low amounts of HIF within the cell (Siddiq et al., 2007)

Under hypoxic conditions, the vPHL does not recognize HIF-1 alpha due to the decreased amount of oxygen needed to create the hydroxylation used to signal ubiquitin ligase and bind to HIF-1 alpha, allowing it to increase within the cell during hypoxia (Siddiq et al., 2007, Uchida et al., 2004). The alpha subunit becomes stabilized and dimerizes with HIF-1 beta and translocate into the nucleus (Siddiq et al, 2007). The dimer is a basic helix-loop-helix (bHLH), per,ARNT,SIM (PAS) structure, which allows it to bind the pentanucleotide hypoxia response element (HRE) sequence (RCGTC) (Siddiq et al., 2007; Van de Sluis et al., 2010) The binding of the HIF-1 αβ dimer to the HRE's allows adaptations such as vascular endothelial growth factor (VEGF) and erythropoietin expression, increased glucose-1 transporter activity, glycolysis up regulation, and scavenging of free radicals (Siddiq et al., 2007; Heyman et al., 2011).

Signal Transduction

Cells surviving hypoxic insults may trigger signal transduction pathways to promote cell survival. Extracellular ligands bind to surface receptors of the cell causing signal transduction cascades to occur. One of the signal transduction pathways, Janus kinases (JAKs)/Signal Tranducers and Activators of Transcription (STATs), are important for the developing and maintaining tissues and the nervous system (Kaur et al., 2005; Monroe et al., 2006). Dysregulation of signaling can lead to fetal death, cancers, inadequate immune responses and developmental disorders (Kaur et al., 2005). JAKs become activated through autophosphorylation of the cytoplasmic tails of receptors and serve to recruit and bind STATs which are phosphorylated on amino acid residues (Garcia, 1998; Scott et al., 2002). STATs then

form homo or heterodimers using interactions through the Src-homology 2 domain (SH2) and the phosphorylated tyrosine residues and translocate to the nucleus in order to activate a variety of different STAT-associated genes (Takeda et al., 1997; Bromberg et al., 1999; Kaur et al., 2005; Monroe et al., 2006).

Different proteins of the STAT family can activate different cellular responses (Takeda et al., 1997; Scott et al., 2002). For example, STAT -1, -4, -6, are activated through different ligands biding to cytokine receptors such as interleukins, interferons, growth hormone, prolactin, and leptin binding to a surface receptor which then triggers a signaling cascade (Takeda et al., 1997; Kaur et al., 2005; Scott et al., 2002). STAT3 has been identified as an acute phase response factor and has also been shown to provide protection against neuroinflammation in the CNS (Takeda et al., 1997; Qin et al., 2012).

Opioid Receptors

Our lab recently found that expressions of opioid receptor genes were altered during hypoxic responses (Cook et al., 2010). The opioid receptors, are part of a superfamily of 7 transmembrane G-protein coupled receptors and are expressed in the nervous system (Al-hasini and Bruchas, 2011; Chao and Xia, 2010). These receptors can mediate pain sensation (Al-hasani and Bruchas, 2011). There are at least three types of opioid receptors in neuronal cells: Kappa opioid receptors (KOR), mu opioid receptors (MOR) and delta opioid receptors (DOR) (Chao and Xia, 2010). Previous studies reported that opioid receptors may or may not provide neuroprotection against hypoxia using animal models (Feng et al., 2012; Feng et al., 2009; Zhang et al., 2000, Yang et al., 2012, Zhang et al., 2002, Wang et al., 2012).

Therefore, to investigate the functional role of human opioid receptors under hypoxic conditions in human neuronal cells, our lab created a hypoxic neuronal cell model using DFO (Cook et al., 2010). Results showed that DFO inducing hypoxia decreased hMOR mRNA expression and did not alter hDOR mRNA expression (Cook et al., 2010). In this study, we therefore are interested to determine if similar outcomes can also be detected under different hypoxic conditions using a different compound such as $CoCl_2$. Similar results could suggest the importance of these cellular responses under a hypoxic challenge.

Hypoxic Mimicking Compounds

PHD's use a non-heme iron in the catalytic portion of the enzyme, therefore a shortage of intracellular iron can inhibit their overall activity (Zhang et al., 2011). CoCl2 can also stabilize the alpha subunit and up regulate HRE within the nucleus (Siddiq et al., 2013). There are three possible mechanisms which can be used in order to create the hypoxic response in manipulating the normal degradation pathway (Siddiq et al., 2013).

The other chemical compound desferroxamine (DFO), can target the non-heme iron of PHD's (Zhang et al, 2011). Iron is a cofactor of the PHD enzyme to determine oxygen levels within the cell (Heyman et al., 2011). Therefore, DFO, the iron chelator, can mimic the hypoxic condition by increasing HIFD-1 alpha expression (Zhang et al., 2011).

Neuronal Cell Model

To understand how human neuronal cells survive under hypoxia, our lab created a hypoxic human neuronal cell model (Cook et al., 2010). Human neuronal cells exposed to DFO showed a decrease in cell viability (Figure 1 A and B, Cook et al., 2010). Cell viability decreased in a dose and time dependent manner. However, a fraction of the neurons were able to

survive the hypoxic insult (Cook et al., 2010). Increase of cellular glutathione can combat free radicals and supports cell survival. These surviving neuronal cells showed that there was no morphology difference between DFO treated cells and non-treated cells (control) using annexin fluorescine and propidium iodide staining, (Cook et al., 2010). (Figure 1 C and D). The positive control using hydrogen peroxide showed that cells underwent necrosis (Cook et al., 2010). Although no morphological changes between control and DFO treatment groups were detected, surviving neurons developed several adaptive responses (Cook et al., 2010). First, an increase of HIF mRNA levels was found by RT-PCR, which corroborated with a notion that up regulation of HIF-1 alpha may mediate cell survival (Cook et al., 2010). MOR mRNA levels were decreased after DFO exposure at 12 and 24 hours (Figure 2 B, E), while DOR levels remained the same (Figure 2 C) (Cook et al., 2010). Can these changes also be found under different hypoxic conditions? Do cells use the same or different mechanisms to promote survival under a hypoxia challenge?

Research Goal

The goal of this study is to investigate how neuronal cells develop adaptive responses under $CoCl_2$ induced hypoxic conditions.

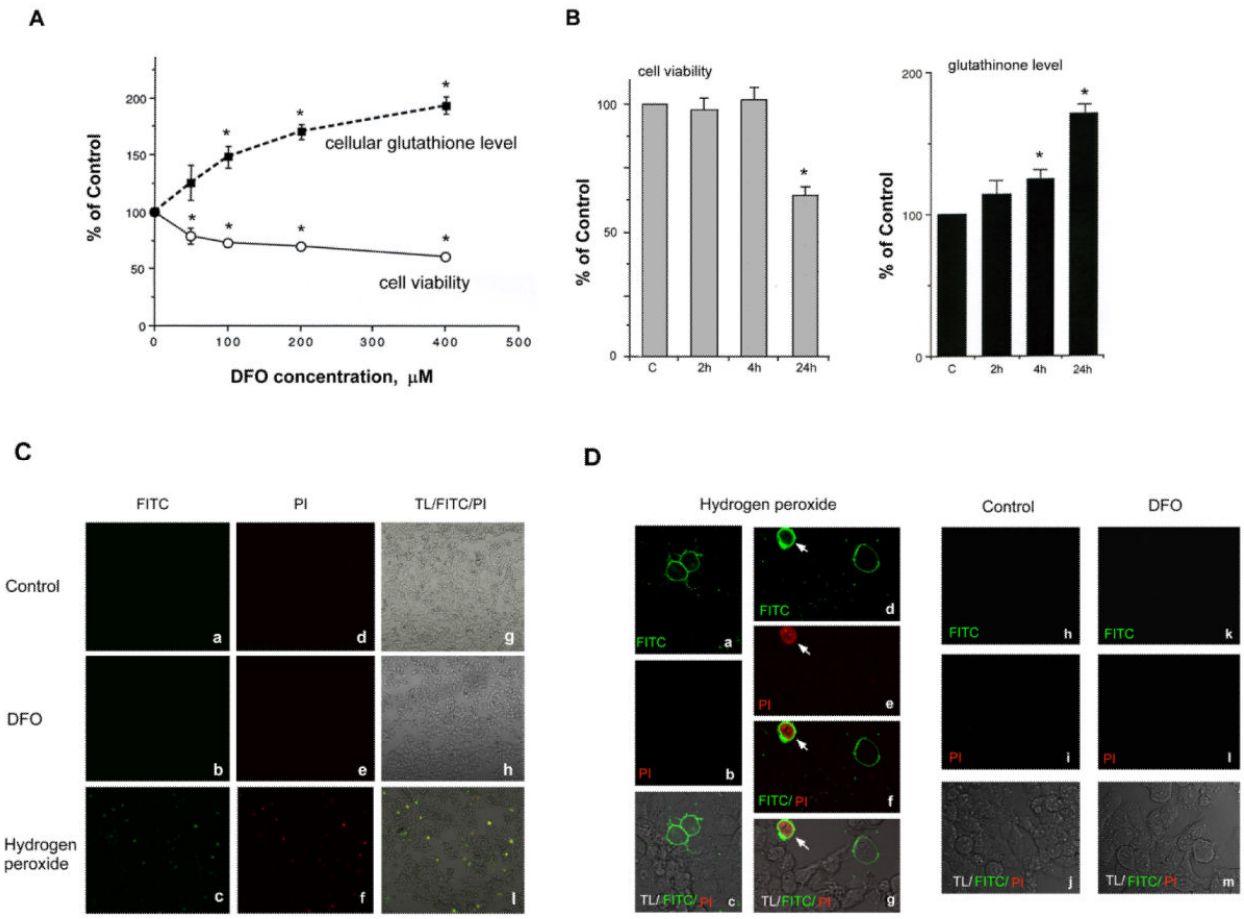


Fig. 1.

Effect of DFO on NMB cells. **A-B,** Cells were treated without (control) or with different concentrations of DFO for 24 hrs (**A**), or 200 μ M DFO for 2, 4 or 24 hrs (**B**). Total number of attached cells was determined. Cell viability (open circles in **A**; gray bars in **B**) is presented as a percentage of total cell number from DFO-treated group divided by the number from control (as 100%). Cellular glutathione level (closed squares in **A**; black bars in **B**) is present as the percentage of the average amount of glutathione per cell from DFO-treated group divided by control (as 100%). Data is present as mean± S.E. "*" indicates $p < 0.01$ (student paired t-test). **C and D,** Surviving/attached cells under DFO, no treatment (Control), or H2O2 treatment (positive control) were stained using annexin-V-FLUOS (as FITC in green color) and propidium iodide (as PI in red color). Cells were imaged using confocal microscope under 10x in **C** or 40x magnification of object lens in **D**. Merged images are overlapped images of transmitted light (TL) with FITC and PI images, or an overlapped image (panel f in **D**) of FITC and PI images. An arrow indicates the cell staining by both annexin-V and PI (Cook et al., 2010).

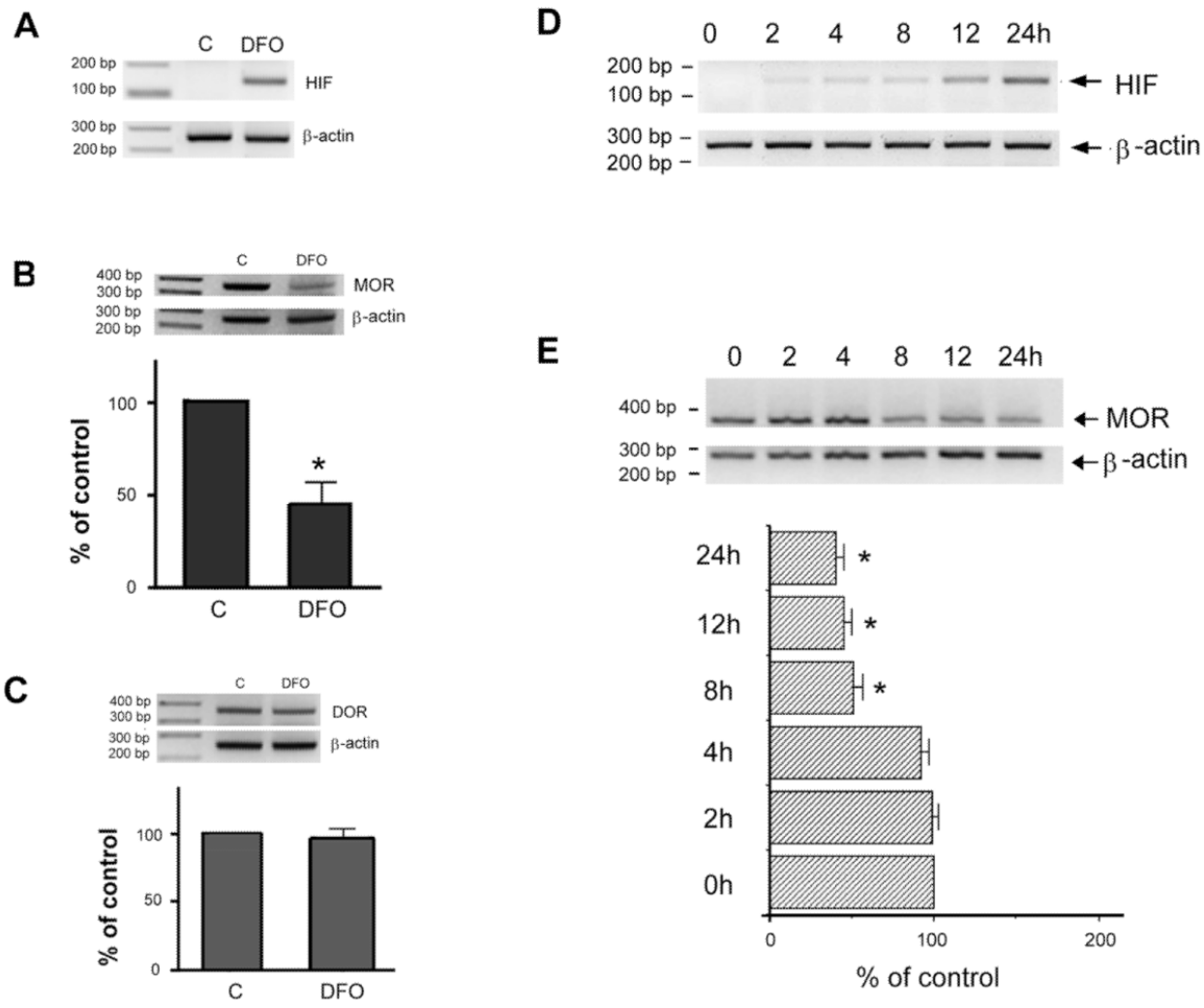


Fig. 2.

DFO alters endogenous HIF-1 α and hMOR message levels RNA was extracted from cells treated without (control, C) or with DFO (**A-C**) for 24 hrs, or with DFO for 0, 2, 4, 8, 12 or 24 hrs (**D-E**). RT-PCR was performed using human HIF-1 α (**A, D**), MOR (**B, E**) or DOR (**C**)-specific primers. Human β-actin specific primers were included in every PCR reaction (added at the cycle 19) as an internal control for normalization purpose. The normalized message from control, or from cells at time zero, was arbitrarily defined as 100%. Quantitative analysis of message levels are presented as mean ± S.E. "*" indicates $p < 0.01$. (Student's paired t-test) (Cook et al., 2010).

Materials and Methods:

Cell Culture:

Human Neuroblastoma cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 containing 10% heat inactivated fetal bovine serum (FBS). Cells were grown in tissue culture flasks and incubated at 37˚ Celsius with 5% carbon dioxide and 95% air. Cells were grown to confluence using 6 well plates. Cells were treated with 300 µM cobalt chloride (CoCl2) and incubated at various times as indicated by the results.

RNA Extraction:

Cells were harvested by removing the media and washed with ice cold phosphate buffer saline (PBS). Cells were lysed using Tri-reagent (MRC). Chloroform was then added to samples and incubated for 8 minutes at room temperature. Samples were centrifuged at 12,000 RPM for 10 minutes at 4°C. RNAs from the aqueous phase were collected and further precipitated by adding isopropanol. The RNA pellets were then washed with 70% ethanol and re-suspended using diethyl polycarbonate (DEPC) treated water. The RNA concentration was determined using a UV spectrometer.

RT-PCR:

RNAs from cells were processed to cDNAs using reverse transcriptase (RT) from Invitrogen. The reaction mixture contained dithiothreitol (DTT), random hexamer, RNase inhibitors, dNTP's, and buffer. First strand cDNA was synthesized a thermocycler and amplification of cDNA was performed for 50 minutes at 37˚C and 15 minutes at 70˚C. PCR amplification was then performed using $MgCl_2$, PCR buffer, dNTP's, a specific primer (listed

below), and Taq polymerase enzyme. An internal standard (β-actin) was also included in every reaction for normalization. KOR cDNA amplification was performed for 1 minute at 95˚C, 35 seconds at 68˚C and 40 seconds at 72˚C for 43 cycles. KOR primers were 5'-CCTTCCTGGGATGGAGTCCTG-3' and 5'TACACGCAGGCCAGGATGG-3'. MOR cDNA amplification was performed for 1minute at 95˚C, 35 seconds at 68˚C and 40 seconds at 72˚C for 32 cycles. MOR primers were 5'-CTGGAAGGGCAGGGTACTGGTG-3'and 5'-CTGCCCCCACGAACGCCAGCAAT-3'. DOR amplification was performed for 1minute at 95˚C, 35 seconds at 67˚C and 40 seconds at 72˚C for 25 cycles. DOR primers were 5'-GTTCACCAGCATCTTCACGCTC-3' and 5'-CGGTCCTTCTCCTTGGAGCCC-3'. β-actin was used as an internal standard and was added 19 cycles before the end of each reaction. β-actin primers were 5'-CCTTCCTGGGCATGGAGTCCTG-3' and TACACGCAGGCCAGGATGG-3'.

Agarose Gel Electrophoresis:

PCR products were analyzed using a 1.5% agarose gel. The DNA markers were also used simultaneously for size comparison. Gels were imaged using Alpha Imager and band intensities were quantitized using Image Quant Software. The data is presented at the percentage of control groups. The control is arbitrarily defined as 100%.

Confocal Microscopy:

Cells were grown to confluence and seeded onto coverslips coated with poly-L lysine. Cells were treated with 300μM DFO or 300μM $CoCl_2$ for 24 hours. Cells were fixed in 4% Paraformaldehyde, perforated with 0.3% Triton and then blocked with 2% BSA. Cells were further incubated with P-STAT3 primary antibody (Cell Signaling), and then incubated with a

Cy3 labeled secondary antibody (Cell Signaling). Cells were imaged using laser scanning confocal microscopy (Olympus Fluoview).

Results

Opioid receptors are known to alleviate pain sensation of an injury or trauma. Therefore, it is important to understand if there is an alteration of the opioid receptor expression occurring under hypoxic conditions. Previously, our laboratory created a human hypoxic cell model system using DFO compound and found a decrease of MOR mRNA levels and no change of DOR mRNA levels after 24 hour treatment (Cook et al., 2010). The chemical compound, $CoCl_2$, is also known to mimic the hypoxia condition. Questions were therefore raised. For example, can opioid receptor expression be altered by CoCl2 treatment? Are the changes of opioid receptor expressions specific to DFO treatment only?

Examining the effect of $CoCl_2$ on mu-Opioid Receptor Expressions using NMB cells

To investigate the effect of cobalt on mu-opioid receptor expression, neuronal NMB cells were treated with 300μM $CoCl_2$ for a period of 2, 4, 8, 12 and 24 hours, respectively. The RNAs were extracted from treated cells. The human MOR (hMOR) expression level was then examined using RT-PCR with a specific-hMOR primer set. The specific β-actin primer set was also used as an internal standard for the normalization purpose. The PCR products were then analyzed using electrophoresis. The results (Fig. 3, right panel) showed that MOR expression levels remained constant for 2, 4, and 8 hour treatment groups but a decrease was detected at 12 hours and also 24 hour treatments as compared to control. The quantitative data is shown in Fig 1, left panel, demonstrating a decrease in MOR expression to an average of 60% of control. This data showed that a decrease of mu-Opioid receptor mRNA expression levels after $CoCl_2$ exposure for 12 and 24 hours in surviving neurons.

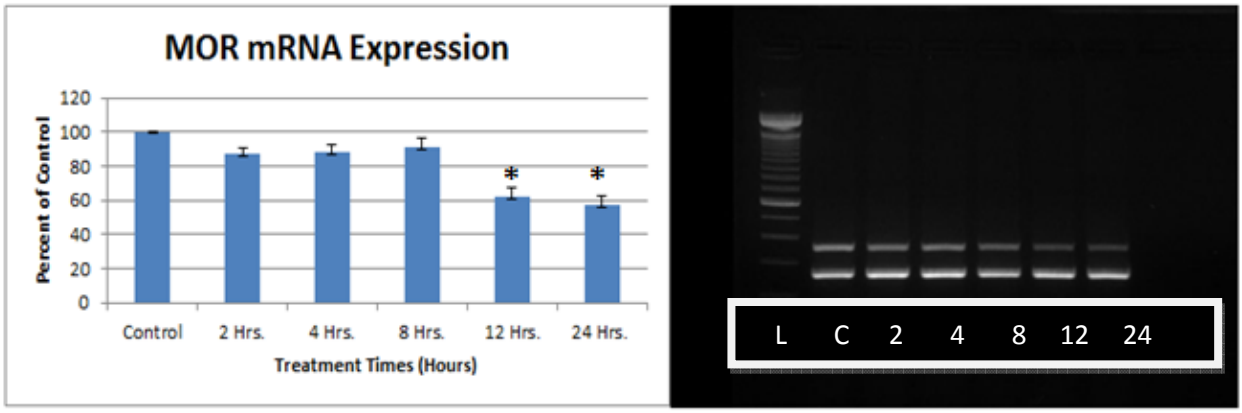


Figure 3. Time course of MOR expression in human neuronal cells exposed to $CoCl_2$. Cells were treated for 2, 4, 8, 12, and 24 hours with 300µM $CoCl_2$. RNA was extracted and RT-PCR was performed using the hMOR specific primers (top band). The β actin was also used as the internal standard (bottom band). PCR product was examined using agarose gel electrophoresis and analyzed using the image quant software. Signal was calculated (left panel) as a percentage of the Control group (no treatment as 100%). Values are representative with +/- SE. N= 6. It is shown that the 12 and 24 hour treatments have weaker signal than the control, 2, 4, and 8 hour treatments**. "*"** Indicates $p < 0.0001$ (Student's paired T-test).

Examining the Effect of $CoCl_2$ on delta-Opioid Receptor Expressions

To investigate the effect of $CoCl_2$ on human delta-opioid receptor (hDOR) in NMB cells, hDOR expression levels were also examined using RT-PCR with a specific hDOR primer set. The β-actin primer set was also included as an internal standard. The PCR products were then analyzed using electrophoresis. Results from RT-PCR and gel electrophoresis showed no significant changes among treatment groups as compared to the control group. The quantitative data (Fig 4, left panel) further validated that DOR expression levels remained constant during $CoCl_2$ treatments. Therefore, these results suggested that $CoCl_2$ exposure resulted in no significant change in hDOR expression levels in NMB cells.

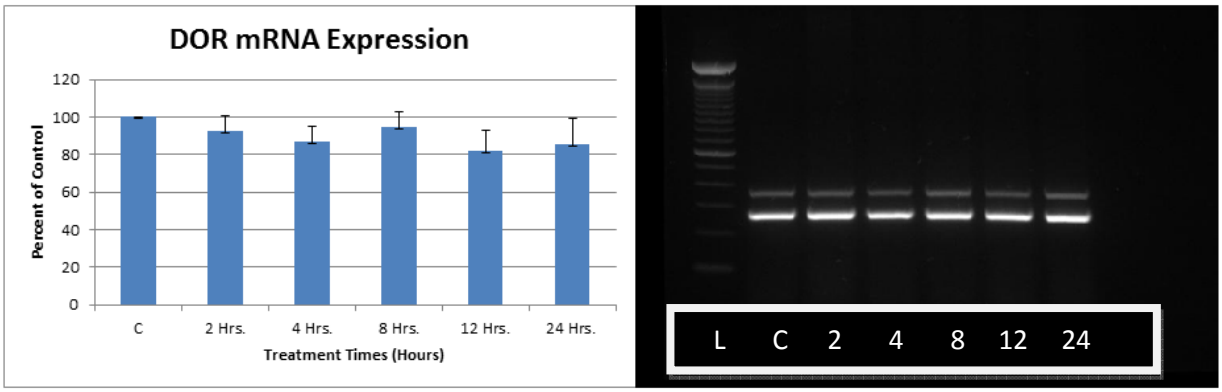


Figure 4: Time course of DOR expression in human neuronal cells exposed to $CoCl_2$. Cells were treated for 2, 4, 8, 12, and 24 with 300µM $CoCl_2$. RNA was extracted and RT-PCR was performed using the hDOR specific primers (top band) and beta actin was used as the internal standard (bottom band). PCR product was examined using agarose gel electrophoresis (right panel) and analyzed using the image quant software (left panel). Signal was calculated as a percentage of the Control group (no treatment as 100%). Values are representative with +/- SE. N= 6.

Examining the effect of $CoCl_2$ on kappa-Opioid Receptor Expressions

To investigate the effect of Cobalt on human kappa-opioid receptor (hKOR) expression, NMB cells were treated with 300μM $CoCl_2$ for a period of 2, 4, 8, 12, and 24 hours, respectively. The treated cells were harvested and RNAs were extracted. The hKOR expression level was then examined using RT-PCR with a specific hKOR primer set and β-actin as the internal standard. The results were analyzed using gel electrophoresis. The results (Fig. 5, right panel) showed similar expression levels for 2 and 4 hours. However, a significant increase in hKOR expression was detected at 8, 12 and 24 hours. The quantitative data (Fig. 3, left panel), showed an average 672.5% increase of expression level at 8 hours compared to control, 694% at 12 hours and 585.5% at 24 hours. Results therefore suggested that $CoCl_2$ exposure resulted in the increase of hKOR expression 8, 12 and 24 hour treatments.

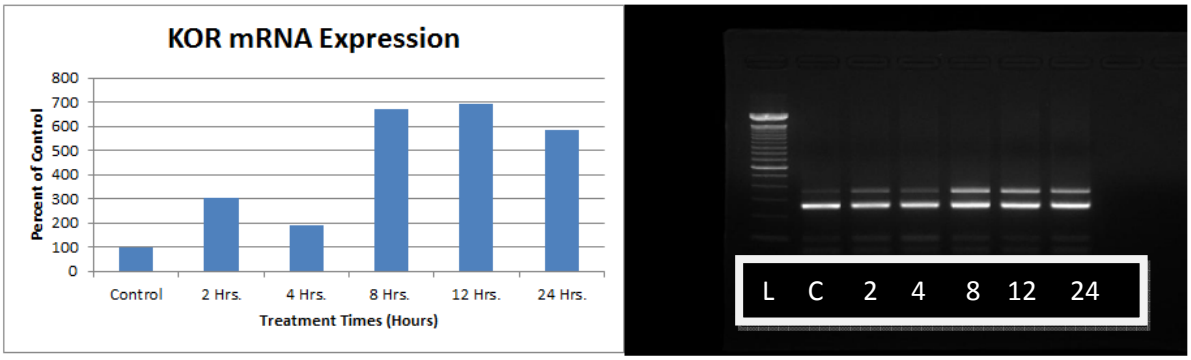


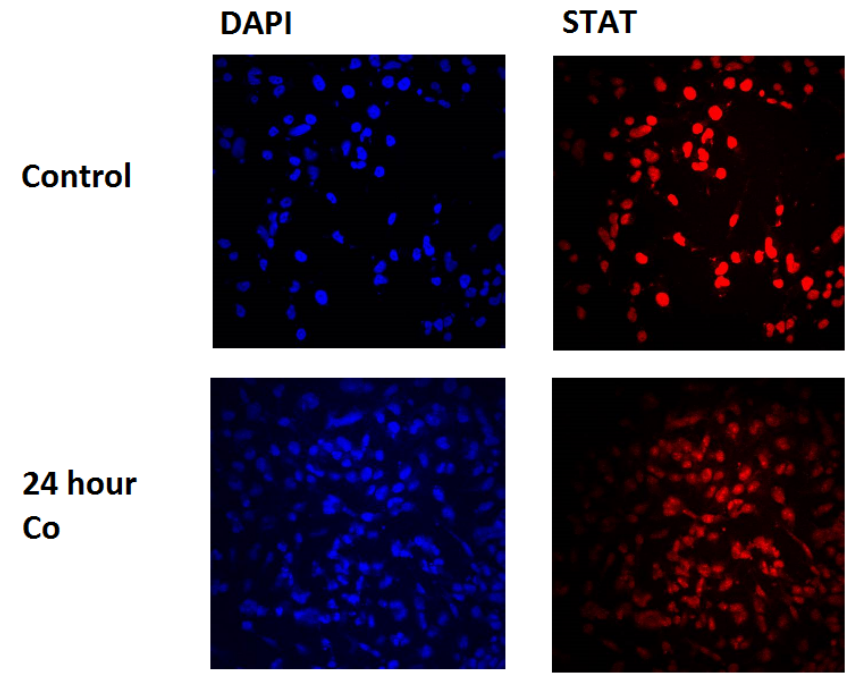
Figure 5: Time course of KOR expression in human neuronal cells exposed to $CoCl_2$. Cells were treated for 2, 4, 8, 12, and 24 hours with 300µM $CoCl_2$. RNA was extracted and RT-PCR was performed using hKOR specific primers (top bands). B actin was used as the internal standard (bottom bands). PCR product was examined using agarose gel electrophoresis and analyzed using the image quant software. Signal was calculated as a percentage of the Control group (no treatment as 100%). N=2.

Examining the Effect of $CoCl_2$ on STAT 3 Activity

Different signal transduction pathways may be involved in hypoxic condition. One of the pathways, the JAK/STAT pathway, is known to initiate transcriptional responses that regulate growth factors and cytokines of cells (Kiu and Nicholson, 2012). When the JAK/STAT pathway is activated, the STAT3 will be phosphorylated and therefore, activated. The phosphorylated STAT 3 (P-STAT 3) was therefore examined using confocal imaging analysis.

Cells treated with 24 hour $CoCl_2$ or without treatment were first fixed with paraformaldehyde and then perforated with triton X-100. Cells were further incubated P-STAT3 primary antibody and then cy3-labeled secondary antibody. Results from the confocal analysis showed that P-STAT3 staining was mainly detected in the nucleus (Figure 6 A). The P-STAT3 levels were lower in the 24 hour $CoCl_2$ treated group as compared to the control group (Fig. 4 A). These results showed that surviving neurons have decreased levels of P-STAT3 compared to the control group after 24 hour $CoCl_2$ exposure, which corroborated with our Western Blot analysis (Fig. 6B from Rasmussen et al., unpublished data). In conclusion, results showed a decrease of P-STAT3 levels upon 24 hours $CoCl_2$ treatment in NMB cells. There was a transient increase of P-STAT3 levels detected using Western blot analysis (Fig. 6B, Rasmussen et al., unpublished data); therefore, this observation will need to be confirmed using confocal analysis in the future.

A.



B.

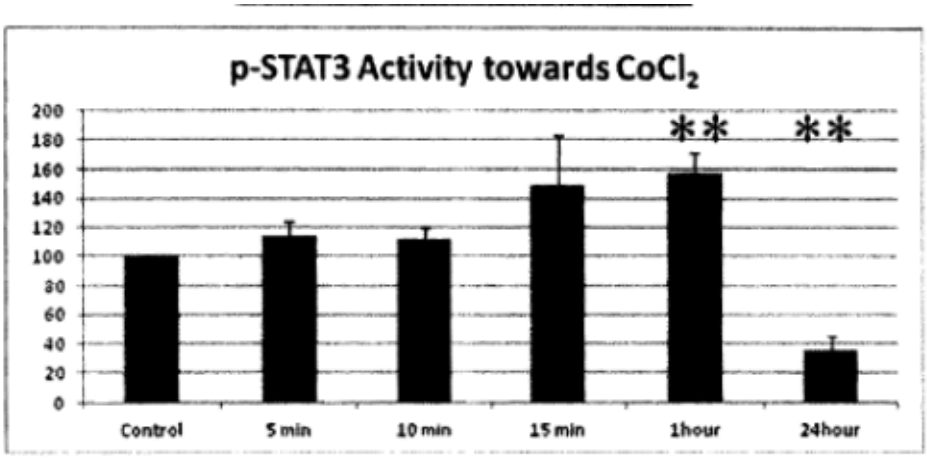


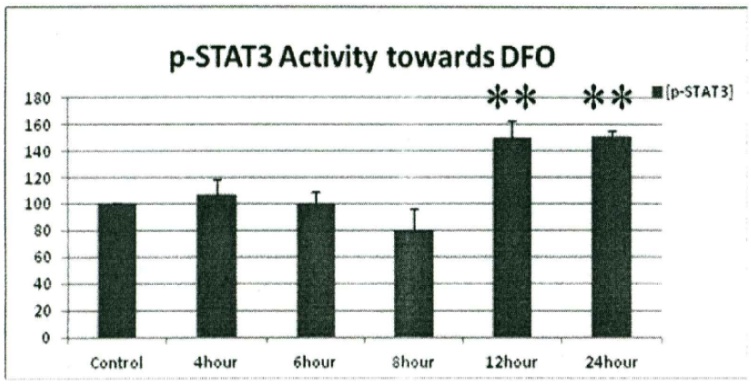
Figure 6: Examining P-STAT3 in $CoCl_2$ treated cells. A. Cells were treated with 300µM $CoCl_2$ for 24 hours. Cells were fixed in 4% paraformaldehyde and perforated with 0.3% Triton. Cells were stained for P-STAT3 and DAPI, and were then viewed under the confocal microscope. DAPI (shown in blue) was used for nuclear staining. The P-STAT3 signal was viewed using the 2nd antibody labeled with Cy3 (shown in red). **B.** Western Blot data showed a transient increase in P-STAT3 at 5, 10, 15 minutes and 1 hour, and a decrease in P-STAT 3 activity in the nucleus after 24 hour $CoCl_2$ treatment (Rasmussen et al., unpublished data).

Comparison of P-STAT 3 activity under DFO Treatment

For comparison, the activation of P-STAT3 in NMB cells under DFO treatment was also investigated. Western blot analysis showed that P-STAT 3 activity was significantly increased at 24 hour treatment and no detected change of activities during the short time treatments (Fig 7A, Rasmussen et al., unpublished data). This result was different from the results of Cobalt treatments (Fig. 6); therefore, the next logical step was to visualize/examine P-STAT3 staining using the DFO treated cells under the confocal microscope as the comparison.

Cells treated with DFO for 24 hours were fixed using paraformaldehyde and then perforated with triton X-100. Cells were then incubated P-STAT3 primary antibody and then cy3-labeled secondary antibody. DAPI was used to stain the nuclei of the cells. Confocal analysis showed that there was an increase in P-STAT3 levels after 24 hour exposure to DFO (Figure 7B, in red color). Due to the strong fluorescence intensity from the 24 hour treatment group, the laser intensity was reduced. These results demonstrated that surviving neurons have a higher level of P-STAT3 present within the nucleus, as compared to the control group. The confocal data collaborated with the Western blot data (Fig. 7A), showed an increase in P-STAT3 after 24 hours of DFO exposure. Taken together, these confocal results suggested that although both DFO and Cobalt can mimic the hypoxic condition, the response of STAT3 activities were in a compound-dependent manner.

A.



B.

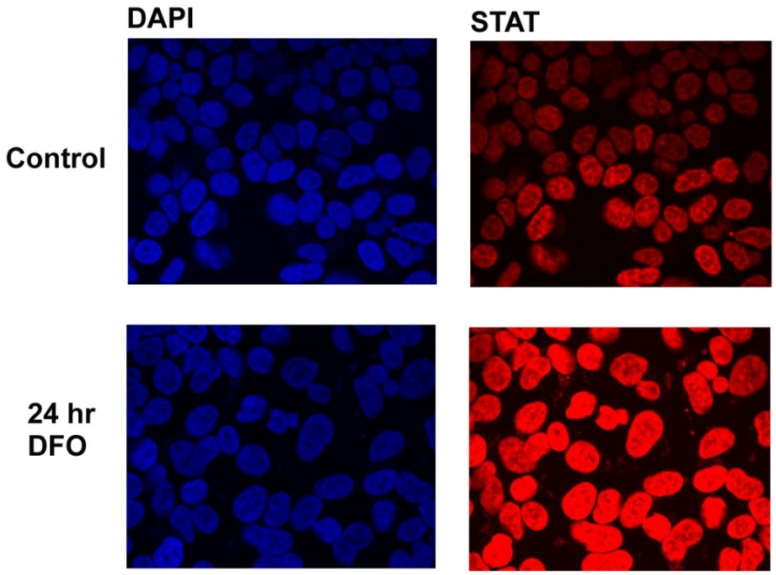


Figure 7: Examining P-STAT3 activity using DFO treated cells. A. Western blot data showed an increase in P-STAT3 activity in the nucleus after 12 and 24 hour DFO treatment (Rasmussen et al., unpublished data). **B.** Cells were treated with 300µM DFO for 24 hours. Cells were fixed in 4% paraformaldehyde and perforated with 0.3% Triton. Cells were stained for P-STAT3 and DAPI, which were then viewed under the confocal microscope/ DAPI (shown in blue) was used to stain the nucleus. The P-STAT3 signal (red color) was viewed using the 2nd antibody labeled with Cy3.

Discussion

This study reported that $CoCl_2$ induced hypoxia resulted in differential effects on the opioid receptor genes' expressions: an increase of KOR mRNA expression, a decrease of MOR mRNA expression, and no change of DOR expression up to 24 hours of treatment. These are interesting findings, because similar results were previously observed using DFO induced hypoxia (Cook et al., 2010), indicating these responses can be important for neuronal adaptation and survival under hypoxic conditions.

The levels of MOR expression were significantly decreased at 12 and 24 hours after inducing hypoxic conditions via $CoCl_2$ treatment. Previously, a published study completed by our laboratory demonstrated a decrease of MOR expression under DFO induced hypoxia (Cook, et al., 2010). It is interesting, because two different compounds ($CoCl_2$ and DFO) mimicked hypoxic conditions resulted in a similar outcome on the decrease of hMOR expression at 12 and 24 hour treatments. These results suggested that the decrease of MOR expression may be linked to neuronal survival. MOR is found mainly in the central nervous system, and it mediates analgesic effects to alleviate severe pain (Lin et. al, 2008; Feng et al., 2012). Studies have shown that MOR may induce a toxic effect on neurons under hypoxia (Feng et al., 2012), which correlate with the decrease shown in this study. Therefore, the down regulation of MOR expression under hypoxic conditions implicates the possible reduction of MOR-mediated analgesic effects. This possibility will need to be investigated in the future.

No significant changes in DOR expression levels were detected under $CoCl_2$ induced hypoxia. Similar results were also found under DFO induced hypoxia over the same time course. DOR has been reported to provide neuroprotective effects in hypoxic or ischemic

environments using an animal model system male sprague dawley rats in combination with a wide variety of miRNAs in different tissues (Yang et al., 2012). Other studies also indicate that DOR is dependent upon protein kinase C (PKC), and that the activation of DOR increases cell proliferation and protein expression in astrocytes (Yang et al., 2012). Reports have also shown that DOR is responsible for mediating neuroptrotection and cell survival under hypoxic conditions in sprague dawley rats (Feng et al., 2012; Feng et al., 2011; Feng et al., 2009). This study showed that DOR activation during hypoxic conditions decreases neuronal injury (Zhang et al., 2002), which correlates with the levels of DOR in these neuronal cells remaining unchanged under the hypoxic conditions. The properties of DOR and possible neuroprotective effects in human neuronal cells will need to be investigated in the future.

In addition, the effect of $CoCl_2$ on the KOR mRNA levels was also examined. Preliminary data shows an increase of KOR expression $CoCl_2$ at 12 and 24 hours, indicating that neuronal survival under hypoxic conditions may be related to an increase of KOR expression levels. KOR is reported to play neuroprotective roles in animal models such as pigs and in rats (Wang et al., 2012). Studies have also shown that treating cells with KOR agonists has increased survival rates of animals after cerebral ischemia by reducing necrosis of neurons, reducing infarction of cerebral ischemia as well as improving memory and motor functions after ischemic events (Wang et al., 2012). Other compounds such as dynorphin A^3, U-62, 066E and CI 977 have been shown to reduce cortical damage in cats as well as rats and KOR agonists have been shown to improve behavioral recovery (Goyagi et al., 2003). Taken together, results found with this study implicated that the increased expression of KOR found in human neuronal cells is likely to be linked to neuronal survival.

The possible signal transduction pathway involved in acute phase responses STAT-3 has also been linked to protect against neuroinflammation (Qin et al., 2012). This study indicated that disrupting the JAK/STAT pathway has a link to diseases such as multiple sclerosis and increased STAT-3 levels protect against neuroinflammation as well as neurodegeneration in mice (Qin et al., 2012). Other studies have also indicated that the JAK/STAT pathway may be activated in order to prevent cell loss. Therefore, this study examined STAT 3 to see if there was any change of STAT 3 activity in surviving neurons. As mentioned previously, the phosphorylated form of STAT3 is the active form of the protein, therefore, P-STAT3 levels were measured in this study. Previous Western Blot analysis showed a transient increase in P-STAT 3 levels with $CoCl_2$ treatment (Rasmussen et al., unpublished data). Therefore these resulted were further investigated using confocal analysis. Data were collected based upon the western blot results. Neuronal cells were exposed to DFO or $CoCl_2$ for 24 hours and results showed an increase in P-STAT3 levels at 24 hours of DFO exposure, as well as a decrease in P-STAT 3 levels after 24 hours $CoCl_2$ exposure. These results confirm the results of Western blot analysis.

Future studies on opioid receptor function must be completed to further understand the impacts of hypoxic conditions on human neuronal cells. In addition to opioid receptor studies, the JAK/STAT pathway will also be studied. One direction for future studies would be to further examine a shorter treatment time for this pathway. Previous data showed a transient increase in P-STAT3 levels when exposed to $CoCl_2$ between 5 minutes and 1 hour of treatment. These results can be used as an example for other potential experiments with other hypoxic mimicking compounds using the same time course. Another direction would be to use a JAK blocker to prevent pathway activation. These will help us to further understand mechanisms controlling cell survival under hypoxic conditions.

Conclusion

Using the hypoxic neuronal cell model system we learned that treatment with DFO decreased cell viability, but some cells still survived. Treated cells showed an increase in HIF-1 mRNA levels, a decrease in hMOR message levels and no change in hDOR message levels. This study therefore used the cobalt chloride compound to induce hypoxia in human neuronal cells and determined cobalt-induced hypoxia could result in many differential gene expressions in human neuronal cells. This study showed that similar adaptive responses were developed under DFO or $CoCl_2$ induced hypoxia suggesting that opioid receptor expression may be linked to neuronal survival. However, the JAK/STAT signal transduction pathway may have a functional differential role under different hypoxic conditions. Further understanding these mechanisms will assist to identify key targets for neuroprotection.

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