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Study of Cellular Responses under Chemically Induced Hypoxia

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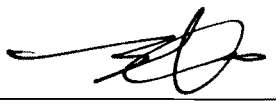
Approved By



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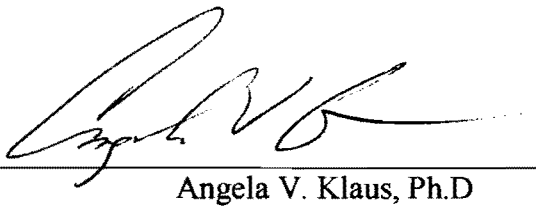

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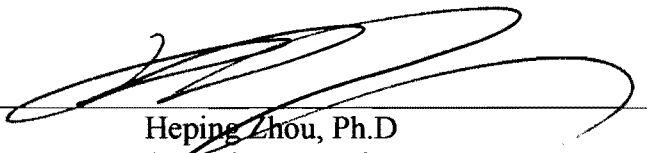

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Abstract:

Hypoxia is a condition of inadequate oxygen supply. Adaptive responses can be developed by cells that undergo hypoxic conditions. To understand human hypoxic responses, our lab generated a human hypoxic neuronal cell model system using the hypoxic mimicking compound, Desferoxamine (DFO), to simulate hypoxic condition. We found that DFO treatment decreased cell viability. However, some cells still survived. Using confocal image analysis the surviving cells were morphologically similar to control (vehicle-treated) cells. These surviving cells developed the adaptive responses, such as the decrease of human mu opioid receptor (hMOR) message level and the increase of hypoxia inducible factor 1 (HIF-1) mRNA level by RT-PCR analysis. Additionally the level of human delta opioid receptor (hDOR) message was not changed, whereas, the human kappa opioid receptor (hKOR) message level was up regulated in these surviving neurons. Therefore, this study was to first investigate if hKOR protein levels increased under hypoxia. The results of radioisotope ligand binding-assay showed that 48 hour DFO treatment resulted in the increase of hKOR protein levels. To further examine if the HIF response element located at the hKOR promoter region was responsible for the increase of hKOR message level, four putative HIF-1 response elements from the hKOR gene were cloned into the pGL3-promoter vector, a plasmid containing the luciferase reporter gene. Using transient transfection with human Neuorblastoma (NMB) cells, the preliminary results showed that several HIF responsive elements are involved in the up-regulation of hKOR message under DFO simulated hypoxic condition.

Introduction

Hypoxia:

Oxygen is essential for cell survival; therefore, tissue oxygenation is essential for all normal physiological functions, including but not limited to, cell growth, respiration, and metabolism. However, medical conditions such as cardiac arrest, asthma, pneumonia, and stroke cause the body to be deprived of adequate oxygen or a condition called hypoxia. Additionally, hiking at high altitudes, diving underwater, and extensive athletic training can cause hypoxic conditions in the body. In turn, hypoxia can be defined as the deficiency in the bioavailability of oxygen to the tissues of the body (Loiacono, et al., 2010). Connett and colleagues define in theory three thresholds of cell hypoxia (Connett et al., 1990).

1. The first threshold is when cellular oxygen decreases but adenosine triphosphate (ATP) production is maintained at level sufficient to match ATP demand by metabolic adaptation.
2. The second occurs when steady state ATP turnover can be maintained only by the production of ATP from anaerobic glycolysis by the Embden-Meyerhof pathway. This pathway generates only 2 molecules of ATP per 1 molecule of glucose metabolized. For highly metabolic tissues such as the kidney, liver, and brain, anaerobic glycolysis is too cumbersome to be effective and these organs develop ATP depletion rapidly under hypoxic conditions.
3. The last threshold is when glycolysis becomes insufficient to produce enough ATP to maintain cell function and structural integrity.

Many tissues after the second threshold develop ATP depletion. One of these tissues is the brain; nerve cells in the brain require high levels of ATP to operate the sodium-potassium

pump. When 50-60% of ATP is lost due the decrease levels of oxygen, depolarization of the membrane and uptake of sodium and water occurs (Loiacono et al., 2010). Depolarization results in an influx in calcium through voltage-gated calcium channels, and leads to glutamate neurotransmitter release (Cook et al, 2010). Glutamate binds to glutamate receptors, initiating a process of calcium influx and excitatory injury called the glutamate cascade (Dendorfer et al., 2005). The death of neurons from these insults can follow quickly in by swelling and lysing, or through a complex process resembling apoptosis (Dendorfer et al., 2005). However, many studies have shown that some neuronal cells under hypoxic conditions do survive (Cook et al., 2010). These surviving cells develop adaptive responses, and express high levels of hypoxia inducible factor 1α (HIF-1α) (Chavez et al., 2006), which is a transcription factor and a major regulator of the cellular response to hypoxia (Semenza, 2000).

Hypoxia Inducible Factor 1 (HIF-1)

In mammalian cells, HIF-1 has been shown to play an essential role in the cellular and systemic oxygen homeostasis (Lee et al., 2003). HIF-1 has been considered to be a master transcriptional regulator in hypoxia (Yan et al., 2011) and be a key modulator for the induction of genes that facilitate adaption and survival of cells (Ke et al., 2006). The genes targeted by HIF-1 include vascular endothelial growth factor (VEGF) that is involved in angiogenesis (Conway et al., 2001), insulin-like growth factors (IGF) (Krishnamachary et al., 2003), and transforming growth factors (TGF) (Semenza, 2003). These genes can activate signal transduction pathways, which lead to cell proliferation and survival (Lee et al., 2003).

HIF-1 is a heterodimer complex that consists of a hypoxic inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Ke et al., 2006, Lee et al., 2003). These proteins belong to the basic helix-loop-helix- Per-ARNT-Sim (bHLH-PAS) protein family (Wang et al., 1995). HIF-1α subunit stability can be affected by oxygen levels. The α subunit contains two PAS and bHLH domains; which are required for heterodimerzing of alpha and beta subunits and for binding to DNA (Lee et al., 2003). It also contains two transactivation domains N-terminal (N-TAD) and C-terminal (C-TAD). The C-TAD terminal has been shown to interact with CBP/p300 coactivators to activate gene transcription (Ke et al., 2006, Le et al., 2003).

The other important domain that mediates oxygen regulated stability is the oxygen-dependent degradation domain (ODDD) (Pugh et al., 1997). During normal conditions, HIF-1α has a short half-life of 5 minutes. In normoxia, hydroxylation of two proline residues Pro 402, and Pro 564 within the ODDD regions mediated by prolyl-4-hydroxylase (PHD) (Masson et al., 2001), and the acetylation of lysine residue Lys 532 by acetyl transferase arrest-defective-1 (ARD1) promote the interaction with an ubiquitin E3 ligase complex Von Hippel-Lindau (pVHL) (Jeong et al., 2002). This in-turn marks HIF-1α for degradation by the 26S proteasome. At the same time, the hydroxylation of asparagine residue Asn 803 in the C-TAD of HIF-1α by FIH-1 prevents the interaction of HIF-1α to CBP/p300 (Lando et al., 2002).

However, during a hypoxic condition, activities of PHD and FIH-1 are inhibited, which results in no proline and asparagine hydoroxylation. Thus, there is no VHL binding and HIF-1α is stabilized. HIF-1α translocates to the nucleus and forms a heterodimer with HIF-1β, which then recruits CBP/p300 co-activators and enhances the gene transcription. These targets genes are related to erythropoiesis/iron metabolism, angiogenesis, vascular tone, glucose metabolism,

cell proliferation/survival, and apoptosis (Lee et al., 2003; Ke et al., 2006; Jeong et al., 2002). HIF-1 plays a major role in oxygen sensing system that governs cellular response to decrease levels of oxygen (Schoolmeesters et al., 2012).

In previous studies, HIF-1 has been shown to offer protection to neuronal cells under hypoxic insult using animal model systems. In the ischemic rat brain, HIF-1 increased VEGF gene expression, which then increased blood-brain barrier permeability, and may offer neuroprotection (Yan et al., 2011). In astrocytes of rats, an increase of HIF-1 increased glucose uptake (Valle-Casuso et al., 2012). By exposing 14 day old embryonic mice to hyoxia-reperfusion injury, HIF-1α provided neuronal protection (Vangeison et al., 2008). Moreover, using the hypoxic pre-conditional mouse model, the induction of HIF-1 and its target genes offered therapeutic potential (Ferriero, 2005).

Therefore, to study the hypoxic response of human neurons, human neuroblastoma cell lines are commonly used as models (Jogi et al., 2004). In the human NMB cells, hypoxia stabilizes HIF-1α proteins and activates expression of known hypoxic induced genes, such as erythropoietin, VEGF, IGF, TGF which are associated with cell metabolism or cell proliferation/survival (Mannello et al., 2011; Krishnamachary et al., 2003; LeCouter et al., 2001).

Previously, we generated a hypoxic cell model system, using human neuronal NMB cells with the hypoxic mimicking compound desferoxamine (DFO) (Cook et al., 2010). Approximately 60% of neuronal cells survived under chemically induced hypoxic conditions (Figure 1) (Cook et al., 2010). Using annexin-V-FLUOS and propidium iodide staining (Figure 2), the surviving cells did not show any significant morphological change as compared to the control cells using confocal microscopy analysis. This proves that cells were not at the apoptotic

or necrotic stages (Cook et al., 2010). Furthermore, using RT-PCR, HIF-1 mRNA levels increased after 24 hours (Figure 3) (Cook et al., 2010). These data demonstrated that surviving neuronal cells did develop the adaptive responses, and can be used as a human hypoxic cell model system.

Opioid Receptors:

The NMB cell line also contains various types of opioid receptors, which have been suggested to play a role in neuronal cell death and survival (Tegeder et al., 2004). In mammalian cells there are three types of opioid receptors Mu, Kappa, and Delta (Waldhower et al., 2004). They are G protein coupled receptors embedded in the plasma membrane of neurons (Waldhower et al., 2004). When receptors are bound by a ligand, the associated G protein is activated and further activates the 2nd messenger systems (Minami et al., 1995), which then can alter protein phosphorylation via the kinases and/or alter gene transcription (Waldhower et al., 2004).

Previous studies have shown that treatment with Delta opioid receptor (DOR) agonist enkephalin led to neuroprotection in hypoxic conditions (Ma et al., 2005). Treatment with Kappa opioid receptor (KOR) agonist U-50488 offered neuroprotective effects to mice in ischemic environment (Schunk et al., 2011), and preconditioning with Mu opioid receptor (MOR) agonist morphine reduced neuronal death of rat cells in ischemic condition (Zhao et al., 2006). However, there are also opposite results found in the literature. It has been shown that DOR agonist did not offer protection against moderate and mild ischemia in rat brain (Iwata et al., 2007).

Furthermore, MOR and KOR did not protect neocortical neurons against ischemic injury (Zhang 2000).

In addition, opioid receptors are also known to modulate the sense of pain (Schunk et al., 2011; Zhao et al., 2006). Stroke, trauma, and cardiac arrest cause hypoxic conditions and can also cause pain in the body (Schunk et al., 2011). Therefore, using the human neuronal NMB hypoxic model system, our lab also found that there was a reduction of MOR mRNA message compared to the control (Figure 4A), and no significant changes were observed for DOR mRNA expression (Figure 4B) using RT-PCR (Cook et al., 2010). In addition, KOR mRNA expression showed a significant increase in the surviving cells (Karch et al., lab unpublished data).These results indicated; that opioid receptors may have a role in cell survival.

Hypoxic mimicking compound

To generate hypoxic condition, cells can be incubated in a hypoxic chambers or the usage of chemical compounds that induces hypoxic mimic conditions. These compounds include ophenanthroline, iodochlorohydroxyquinoline, cobalt sulfate heptahydrate, and DFO (Xia et al., 2009; Ryter et al., 2000; Saini et al., 2009). DFO has been commonly used in hypoxia related research, and it is an iron chelator; it chelates the iron in the PHD region of HIF-1α that is loosely bound by 2-histidine-1-carboxylate coordination motif. By diminishing the availability of iron, HIF-1α is stabilized because the prolyl-4-hydorxylase requires oxygen, iron and ascoarbate as cofactors (Ke et al., 2006); therefore, DFO can activate downstream responses similar to hypoxic condition (Bartolome et al., 2008). In animal models, DFO induced hypoxia-like adaptive changes in the host (Dendorfer et al., 2005; Hamrick et al., 2005; Prass K et al., 2002).

DFO has been shown to increase HIF-1α expression in mice and rats neuronal cells (Jaakkola et al., 2001; Pichiule et al., 2007).

Goal

Our previous studies using NMB hypoxic model system have shown an increase of HIF-1α mRNA levels, and an increase in hKOR messenger level using RT-PCR analysis. This study is therefore to investigate if (1) the change of mRNA level resulted in the changes of protein levels of opioid receptors, and (2) if HIF-1α mediated the mRNA change of hKOR in the same cell model system.

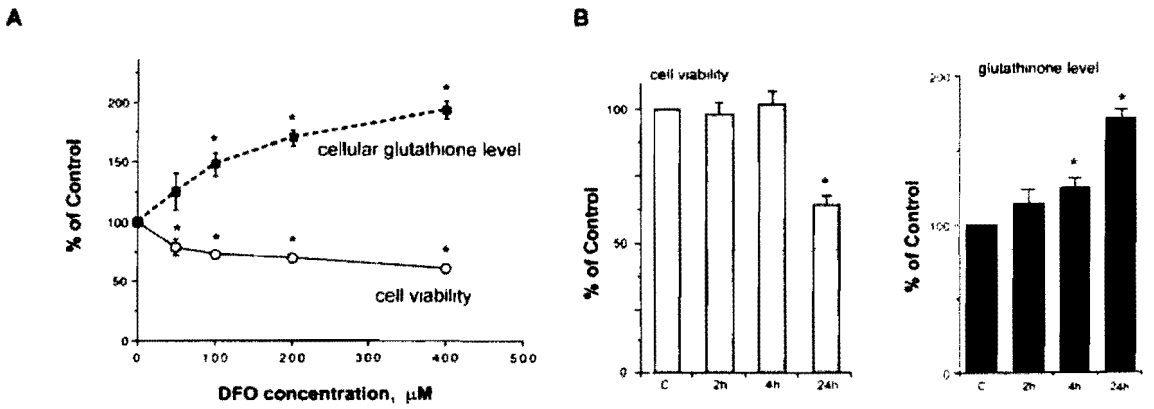


Figure 1: The effect of DFO NMB cells, cells were treated with DFO in 2, 4, and 24hrs, and cell viability and GST levels were measured (Cook et al., 2010).

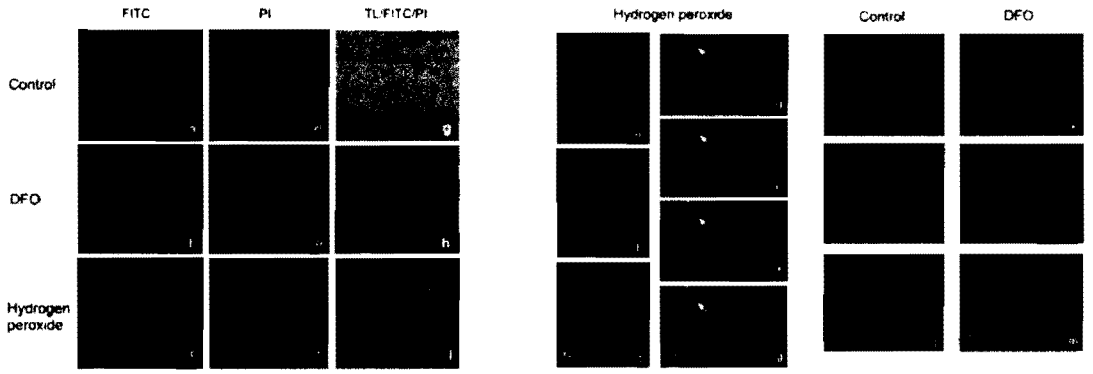


Figure 2: Surviving cells were stained with annexin V-FLUOS and propidium iodide; the cell images were taken using confocal microscope under 10X or 40X magnification (Cook et al., 2010).

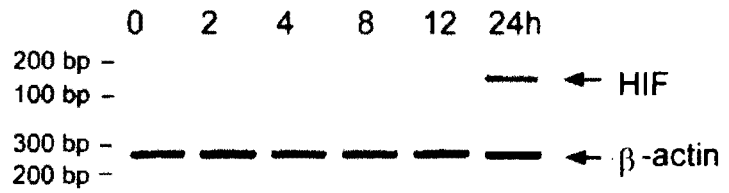


Figure 3: RT-PCR analysis of HIF-1, expression of HIF-1 mRNA is increased under DFO treatment (Cook et al., 2010).

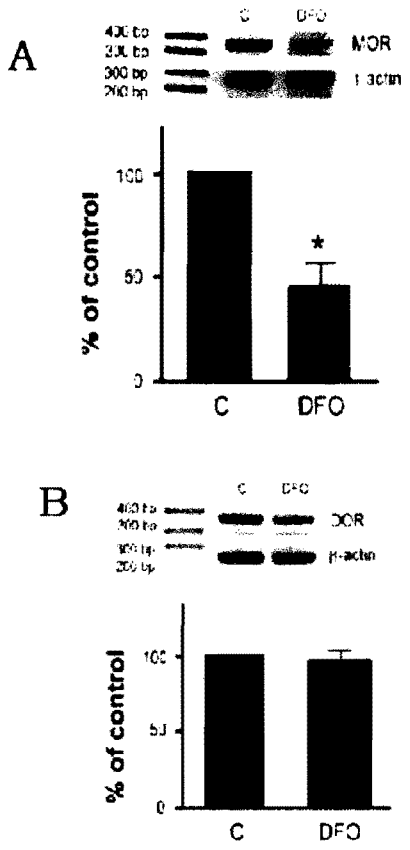


Figure 4: RT-PCR analysis of the three human opioid receptors. A. Mu opioid receptor mRNA expression, B. Delta opioid receptor mRNA expression. Quantitative analysis of opioid expression is presented as mean ± S. E (Cook et al., 2010).

Methods and Materials:

Cell Culture:

Human Neuroblastoma cells were grown in Roswell Park Memorial Institute Medium (RPMI) 1640 containing 10% heat inactivated Fetal Bovine Serum (FBS). The cells were cultivated in tissue culture flasks (Fisher Scientific, Tunsitn, CA) in an atmosphere of 5% CO_2 and 95% air at 37°C incubator.

Plasmid Construction:

The region containing the potential HIF response element were generated by PCR using 1min at 95°C, 30 sec at 60°C and 30 sec at 72°C. The PCR products were then cloned into pCR2.1 vector (Life Technologies, Grand Island, NY). The inserts with correct sequences were then sublconed into pGL3 promoter vector (Promega, Madison, WI) that contains a luciferase reporter gene. The subcloned plasmids were then digested using Xho1 and Asp718 (Promega, Madison, WI) to verify the presence of the insert. The plasmids containing correct sequences were then used for further study.

Transient Transfection:

Cells were plated in 6 well plates and then were transiently transfected using Effectene Transfection reagent (QIAGEN, Valencia, CA). Twenty-four hours after transfection, cells were treated with 300 μM DFO for 24 hours treatments. After treatment cells harvested and lysed with lysis buffer (Promega, Madison, WI). Luciferase activity was determined following manufacturer procedure (Promega, Madison, WI) using luminometer (Berthold).

Membrane Preparation:

Cells were harvested with PBS/EDTA and then homogenized in 25 mM HEPES, pH 7.4, containing 0.32 M sucrose, and protease inhibitors phenylmethylsulfonylflurodie (PMSF), sodium orthovanadate, aprotinin, and pepstatin. Cell lysates were then centrifuged at 3000 rpm for 10 minutes at 4°C, and supernatant was collected. Next, supernatant was centrifuged at 24,000 rpm for 60 minutes at 4°C, The pellet was resuspended in 25mM HEPES, pH 7.4 and was ready for use. The protein concentration was determined using Lowry assay.

Radioisotope Ligand Binding-Assay:

Cell membranes (400 ug) were incubated with the H3 diprenorphine in the absence or presence of 1 μM unlabeled opioid agonists- Mu-Damgo, Delta-Daddle, and Kappa-U-50 in 25 mM HEPES buffer, pH 7.4 for 60 minutes at room temperature in a final volume of 1 ml. The reaction was stopped by filtration through filters. The filters were washed two times with 1 ml of

cold 25 mM HEPES, pH 7.4. Then the filters were equilibrated with ScintiVerse-BD (Fisher, Tunsiten, CA) before assaying in the beta counter.

Results

Examining the protein expression of hKOR in NMB cells

Previously, in our lab DFO induced hypoxic mimic responses were investigated using the human neuronal NMB cell lines (Cook et al., 2010). Using this cell model system, we found that some neuronal cells survived under DFO treatment, and the level of HIF messages was increased, and the hMOR messages were also decreased in these surviving cells (Cook et al., 2010). RT-PCR analysis further showed an increase of hKOR mRNA expression in these surviving NMB cells (Karch et al., unpublished data). Therefore, the logical next step was to examine if hKOR protein levels also increased under the treatment.

The receptor-ligand binding-assay was then carried out using the membrane preparations from NMB cells with the radioisotope-labeled ligand, 2nM H^3-dipernorphine, 1μM U-50488 as the cold competitive ligand. There are three opioid receptors, mu, delta, and kappa found in NMB cells. Therefore, to block mu and delta receptor binding, 1μM of DAMGO and DADLE were also included in every reaction. In Figure 5, the results indicated that the 48 hour treated group showed a significant increase (around 2-fold), however, the 24 hour treatment did not result in a significant increase as compared to the control group, suggesting that there is a lag between the changes of mRNA level and the changes of protein level. Overall, this data suggested that hKOR protein level is significantly increased at 48 hours of DFO treatment.

Plasmid Construction of hKOR promoter region:

To further investigate if the HIF-1 is involved in the increase of hKOR mRNA level in this cell model system, the luciferase reporter assay was chosen. First the plasmid construction was performed by cloning the putative HIF responsive elements of the hKOR promoter region into pGL3-promoter vector. There are a total of 4 putative HIF responsive elements in the 5' flanking region of hKOR gene, which are designated as A (the far one from the gene), B, C and D (The closest one). Each of these DNA fragments containing the putative HIF response elements was generated by PCR method. The PCR products were then cloned into pCR2.1 vector (Fig. 6A). The E. coli competent cells were then transformed using the ligation mixture with heat-shock method. The transformed bacteria were then selected under the ampicillin containing plates. The plasmid was then purified from the individual colony, and each plasmid was verified by restriction enzymes digestion. Each digested sample was then examined using 2% agarose gel to check for the presence of the cloned insert.

The clones with the insert were then subjected to DNA sequencing. An insert with the correct sequence was then sub-cloned into pGL3-promoter vector (Fig. 6B), which contains the luciferase reporter gene. The similar screening processes for various clones were used (as described above). Figure 7A, shows a representative gel screening of clone A, Fig 7B for clone B, 7C for clone C, and 7D for clone D. The plasmids contained the inserts were subjected to sequencing again. The plasmids with the correct sequences were then subjected to large scale plasmid purification.

Examining the function of putative HIF-1 responsive elements using the luciferase reporter assay

To determine the functional role of these putative HIF responsive elements, NMB cells were transiently transfected with the pGL3 promoter-luciferase reporter gene and HIF expression vector. Each set contained a negative control plasmid, a blank pGL-3 promoter vector (P). After transfection, cells were treated with DFO for 24 hours. The cells were then harvested and lysed. The luciferase activity was then determined.

The preliminary data from the luciferase assay (Fig. 8) showed that A and C had a slight increase and B show no difference of luciferase activity after 24 hours of DFO treatment. However, D showed the strongest increase in expression among the 4 putative elements. The result indicated that A, C, and D regions increase in expression after 24 hours of DFO treatment.

In addition, we also obtained a clone plasmid containing 2 copies of HIF responsive element D (2-D). The luciferase activity of this plasmid was also examined using the same procedure as previously described. Fig. 9 showed that the 2-D plasmid had a very strong response under the treatment indicating that HIF responsive element in the D region may play an important role under hypoxic condition.

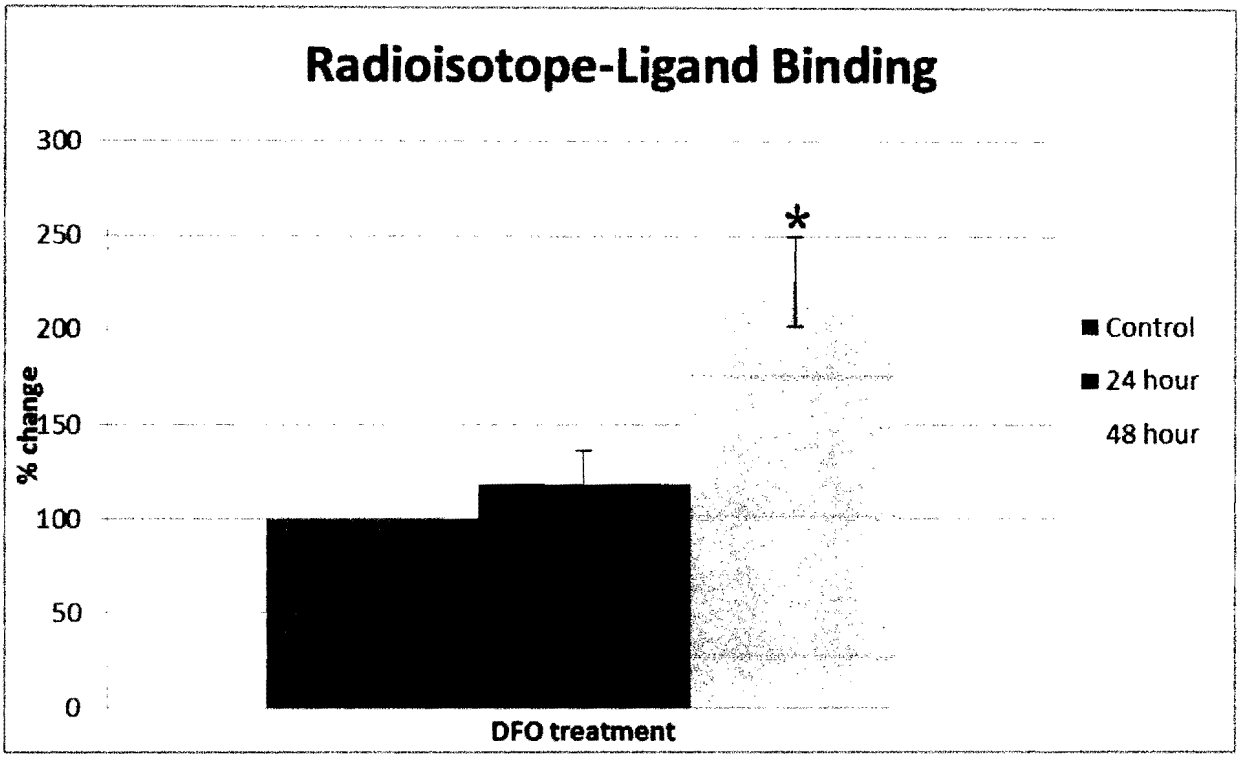
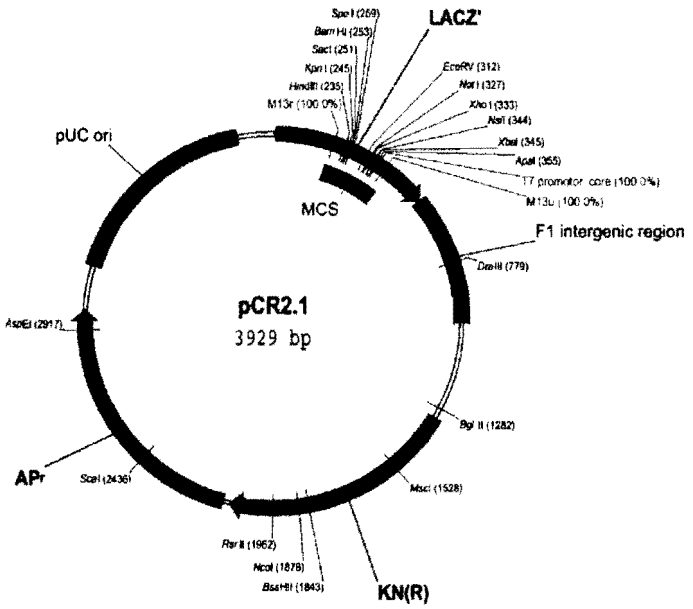
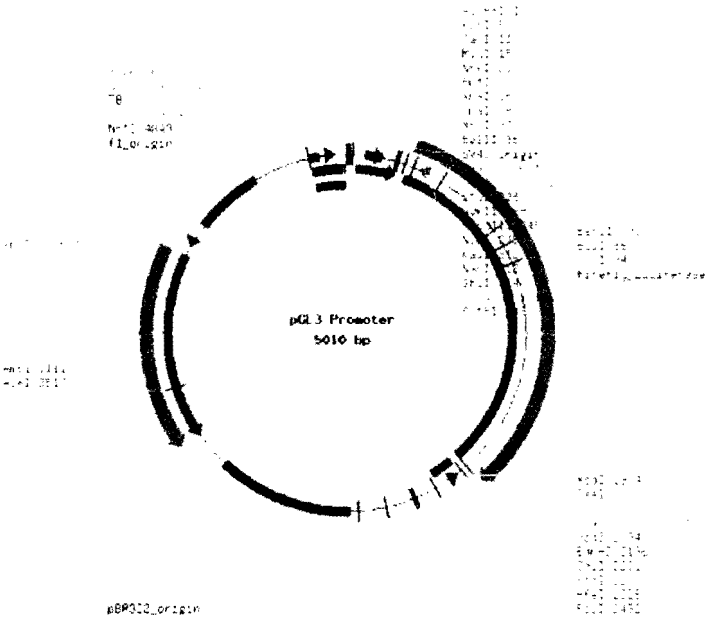


Figure 5: NMB cells were treated without or with DFO for 24 and 48 hours. Membranes were prepared and used for radioisotope-ligand binding. Each sample had the same amount of protein. The data is expressed as mean ± standard error. (n=3) * indicates $p<0.001$



A



B

Figure 6: Cloning of the region of interest. A. pCR2.1 Vector was used to clone the regions of interest (adapted from Lablife), and B. then sub-cloned into pGL3-promoter with luciferase reporter gene (adapted from Promega).

Figure 7: Gel analysis of constructs. There are 4 putative HIF responses elements of hKOR promoter region. There are designated as A (farthest from the promoter), B, C and D (closest to the promoter).Each plasmid was digested overnight with Xho1 and Asp718, and then ran on 2% agarose gel. The plasmids with appropriate size were sequenced for the correct insert.

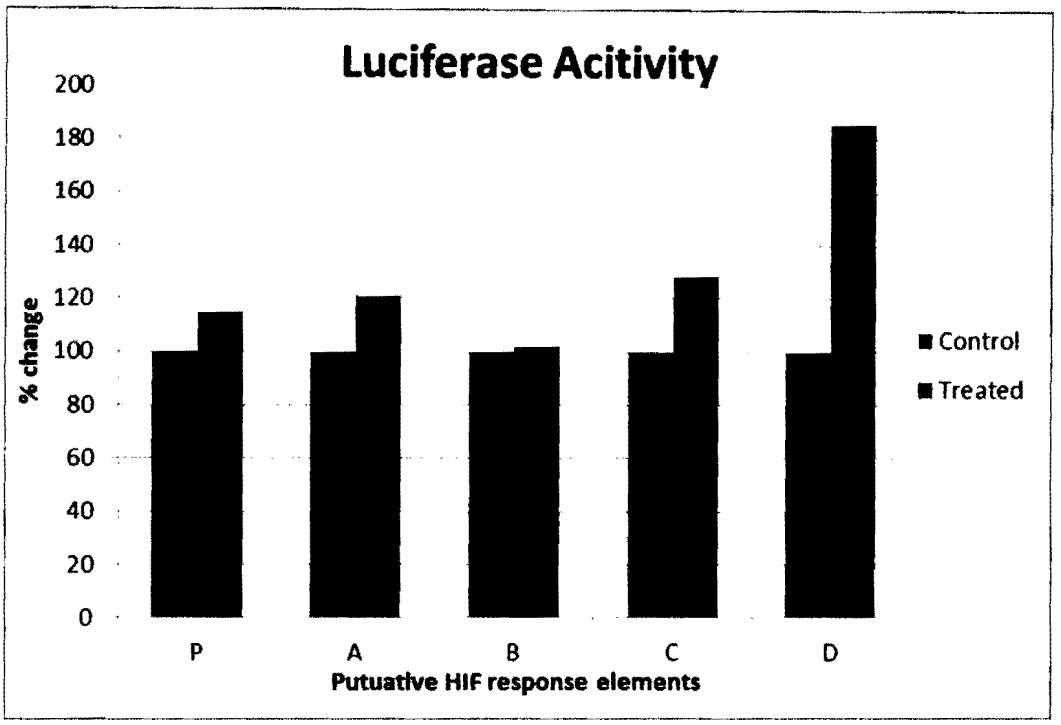


Figure 8: Preliminary resutls of luciferase acitivity of putative HIF response elements. NMB cells were plated in 6 well plates, and each group of the cells was transiently transfected with the plamsids containg the HIF putuative response elements of hKOR promoter region. P an emptry vector (negative control), A (fartherst from the promoter region) B, C, and D (closest to the promoter). Cells were analyzed 24 hours after DFO treatment, and luciferase acitivity was measured.

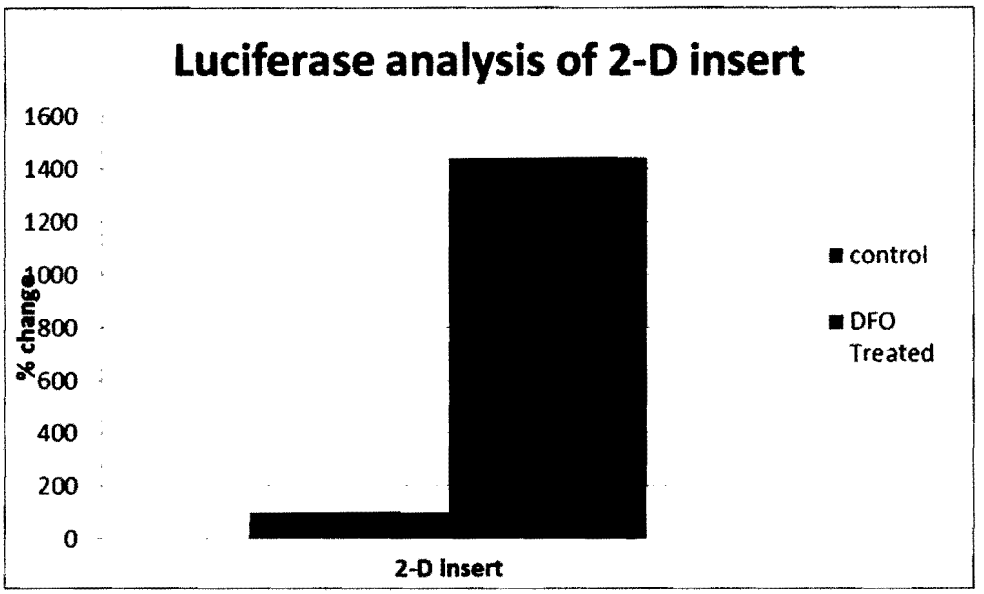


Figure 9: Prelimanry resutls of luciferase analysis of 2-D insert. The cells were treated with 2-D insert, and treated with DFO for 24 hours. Luciferase analysis was conducted and this is a representative data of the result.

Discussion

The aim of this study was first to investigate the change of opioid receptor protein levels under hypoxia, and secondly to examine if HIF mediated the mRNA change of hKOR level in NMB hypoxic cell model system. This study showed that hKOR protein levels increased at the 48 hour treatment and HIF mediated the increase of hKOR expression in this hypoxic cell model system.

The increase of hKOR mRNA level was detected after 24 hours of DFO treatment (Karch et al., unpublished data). However, the change of hKOR protein level was not detectable at 24 hour treatment using the receptor-ligand binding assay, although there was about 2-fold increase at the 48 hour treatment. One of the possibilities is the limitation of both protein and mRNA experiments, therefore proper correlation is not observed

An increase of hKOR expression was shown in this study, but do they offer neruoprotection in hypoxic condition? There are reports that have shown that hKOR offers neuroprotection in hypoxic conditions (Schunk et al., 2011; Zeynalov et al., 2000). It has been shown that the treatment with KOR agonist BRL 52537 provides a long therapeutic window against ischemia in rats (Zeynalov et al., 2006). As well as in mice brain, preconditioning with KOR agonist U-69593 has offered protection against seizures, furthermore, using a KOR antagonist nor-BNI did block the protection indicating that activation of KOR may mediate neuroprotective effect (Rubaj et al., 2000)). However, there are also opposite reports, in a study using ischemic rats; KOR did not offer any neuroprotection (Zhang et al., 2000). Whether KOR

can provide neuroprotection against hypoxia in this NMB cell model system, will be examined in the near future.

HIF-1 is a transcriptional regulator of hypoxia (Ke et al., 2006). Our preliminary results using the luciferase reporter assay showed that several HIF response regions of the hKOR gene were involved in the increase of hKOR transcription under DFO simulated hypoxia. The plasmid containing two copies of D (2-D) showed an extremely strong reporter activity, suggesting that 2 copies of the D region are acting synergistically. In summary, this study suggested that HIF may play an important role in the upregulation of hKOR message. However, we will need to repeat these experiments to confirm these preliminary results in the near future.

HIF-1 has many target genes; the most interesting would be the TGF and IGF genes which are linked to cell proliferation and survival (Semenza et al., 2003). IGF-1 has been shown to mediate neuroprotection in embryonic rat neurons, and prevents neuronal programmed cell death (Vincent et al., 2010). As stated before, the increase in glutamate in the neuronal cells causes cell death (Dendorfer et al., 2005). TGF-β has been able to protect hippocampal neurons in vitro against short term exposure from glutamate (Alzheimer et al., 2002). In addition, this study also provided evidence and suggested that hKOR is a HIF target gene.

This study indicates that hKOR may play an important role in hypoxic condition using hypoxic NMB cells model. Future experiments include the blocking of HIF-1 with siRNA strategy to observe if the downregulation of HIF will affect hKOR expression, and examining the downstream effect of opioid receptors will be useful. It is also interesting to understand the combinatory effect of putative HIF response elements on the expression of hKOR, which will provide further information about the adaptive responses of human neuronal cells under hypoxic simulated condition.

Conclusion

Using the human hypoxic neuronal cell model system we found that the treatment with DFO, a hypoxic mimicking compound, resulted in an increase of HIF-1 mRNA levels in the surviving cells. Also, there were various influences on the expression of different opioid receptor genes under hypoxia. The 24 hour treatment caused the decrease of hMOR message level in the surviving cells, but the hDOR expression showed no significant change. However, the hKOR message increased in this hypoxic model system.

This study further demonstrated that not only that hKOR mRNA level increased, but also the expression of hKOR protein increased significantly at 48 hour of treatment as compared to control and the 24 hour treatment, which can be due to the translational regulation and protein abundance. In addition, the preliminary results from the luciferase reporter assay suggested that the 5' flanking regions of the KOR gene containing the functional HIF responsive elements, which can play a role in hypoxia. Furthermore, the plasmid containing 2 copies of HIF responsive element of the D region showed an extremely strong activity, suggesting that D region has an important role in hKOR regulation under hypoxia. Further studies, such as the investigation of combinatory effect of HIF responsive elements and signaling pathways controlling the downstream effect under hypoxia, will provide the useful information to help us understand the cellular adaptive response under hypoxia.

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