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Examining the Role of HREs on the Regulation of Opioid Receptor Gene Expression in Neuronal Cells undergoing Hypoxic Mimic Condition

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Abstract

Hypoxia is a condition of inadequate oxygen supply, which can induce cell death. Using human neuronal cells treated with a hypoxia memetic compound, desferoxamine (DFO), a hypoxic cell model system was created. Our lab reported previously that treatment with DFO resulted in the decrease of cell viability. However, there were still surviving neurons. The surviving cells did not exhibit significant morphological changes, as compared to the control cells, under confocal microscopy analysis using annexin-V-FLUOS and propidium iodide staining, indicating that they were not at apoptotic or necrotic stages. These surviving neurons, therefore, developed adaptive responses under hypoxic challenge. Several changes were observed, including an increase of cellular glutathione level and the increase of hypoxia inducible factor 1-α (HIF-1α) mRNA level, a known marker of hypoxia, in surviving cells when compared to those of control cells. The increase of human kappa opioid receptor (hKOR) expression was also observed in surviving neurons when compared to the control cells. Clinically, opioids are used to modulate pain sensation, which can result from events such as stroke, trauma and cardiac arrest that lead to hypoxic conditions. There are 4 potential HIF response elements found in the 5' upstream region of this gene. Two of the four elements have shown a significant increase of reporter activity in cells undergoing DFO-induced hypoxic conditions. To determine if these two elements displayed an interactive effect under hypoxic challenge, they were simultaneously cloned into a reporter plasmid containing the luciferase gene, and resulting plasmids were tested using NMB neuronal cells. Results showed a significant increase of promoter activity under hypoxic challenge. Mutation analysis further confirmed the important role of these elements. Taken together, this study showed the functional roles of two HIF response elements mediating the kappa opioid receptor expression under hypoxic condition.

Introduction

In order to maintain normal cellular metabolism and normal tissue/organ function, oxygen needs to be efficiently delivered to the cells of the body. To such end, human anatomy and physiological infrastructure has evolved to optimize oxygen delivery. This includes an entryway through the conducting and respiratory zones, and a way of transport in the cardiovascular system. Through the oxygenation of blood at capillaries surrounding alveoli in the pulmonary circuit and its delivery to target tissues at capillary beds of the systemic circuit, the cardiovascular system aims to provide appropriate oxygenation to meet each cell's metabolic demands. There are several medical conditions, however, that can lead to low oxygen (hypoxic) conditions. These include cardiac arrest, stroke, diabetes, and cancer; conditions which cause reduced blood circulation to affected regions. This is seen in cancer as the center of growing tumors outstrip their blood supply, leading to reduced oxygen bioavailability in the tumor microenvironment (Vaupel and Mayer 2007). Even medical procedures such as surgery can have a similar effect in increasing the oxygen demand to tissues as a result of reduced cardiac output due to the use of anesthesia.

These events of compromised oxygen availability are sensed in the body, and allow for systemic and cellular responses. Systemically, sensing of blood oxygenation though central chemoreceptors (located in the brain stem) and peripheral chemoreceptors, such as carotid bodies found in arterial circulation, as well as the direct response of vascular smooth muscle cells, control alveolar ventilation and blood circulation to optimize oxygen delivery to metabolically active tissues (Michiels, 2004). Intracellularly, however, mitochondria act as oxygen sensors and consume most of the oxygen in cells to allow for oxidative phosphorylation, the primary and most efficient metabolic pathway for ATP production (Guzy et al., 2005; Solaini et al., 2010). It

follows then that oxygen deficiency can lead to cell death as not enough ATP is produced to meet cellular demands.

Highly metabolic cells, such as neuronal cells, are particularly sensitive to hypoxic conditions since their Na^{2+}/K^{+} pump ATPase requires a steady production of ATP to maintain membrane potential (Mark et al., 1995). Without sufficient ATP to meet their metabolic demands, neuronal cells undergo membrane depolarization, uncontrolled Ca^{2+} influx, and activation of phospholipases and proteases leading to cell death (Michiels, 2004). In addition, hypoxic conditions allow for the accumulation of reactive oxygen species (ROS) through the functionality of mitochondrial complex III (Guzy et al., 2005) and can lead to cell death through damage to lipids, proteins and DNA (Uttara et al., 2009). Some neurons, however, are able to survive hypoxic conditions through cellular adaptations that include an increase in the expression and the stabilization of hypoxia inducible factor 1-α (HIF-1α) (López-Hernández et al., 2012; Guzy and Shumaker, 2006).

HIF-1 Transcription factor Regulates Genes in the Cellular Hypoxic Response

HIF-1α is a known cellular marker of hypoxia, and along with HIF 1-β (aryl hydrocarbon receptor nuclear translocator), makes up the HIF-1 heterodimeric complex that is an important transcription factor in the cellular hypoxic response (Semenza, 2002). While HIF 1-β is constitutively present and constitutively expressed in cells under normoxic conditions (Guzy and Shumaker, 2006), HIF-1α levels are only elevated in hypoxic conditions (Hou et al., 2013). Under normoxic conditions, HIF-1α is hydroxylated by closely related enzymes known as prolyl hydroxylases (PHDs) at conserved proline residues Pro-402 and Pro-564 in its oxygen degradation domain (ODD) (Snell et al., 2014). Prolyl hydroxylases need oxygen, Fe (II), and 2-oxyglutarate for their catalytic activity (Trollmann, et al 2014). Hydroxylation of HIF-1α makes it a suitable substrate for polyubiquitylation by Von Hippel Lindau (VHL) tumor suppressor

protein, a component of the E3 ubiquitin ligase complex, which marks it for ubiquitin proteasome degradation (Warnecke et al., 2003; Kim and Kaelin, 2004). Under hypoxic conditions, however, prolyl hydroxylase activity is inhibited, due to decreased O_2 bioavailability to target tissues, resulting in strong upregulation of HIF-1α protein, as its hydroxylation is suppressed. Stabilization of HIF-1α allows it to accumulate and translocate to the nucleus where it heterodimerizes with HIF 1β to act as a transcription factor by binding to hypoxia response elements (HREs), cis-acting factors, to enhance the transcription of numerous target genes (Keith, Johnson and Simon, 2011). Among these targets are genes such as vascular endothelial growth factor (VEGF), involved in enhancing oxygen delivery to tissues (angiogenesis in tumors), erythropoietin (epo) which promotes cell survival and proliferation, and neuronal nitric oxide synthase (Koshikawa, 2003; Rössler et al., 2004; Li et al., 2015).

Treatment of Neuronal cells with Desferoxamine Results in Hypoxic Mimic condition

While hypoxic conditions can be generated through the use of hypoxia incubation chambers, compounds such as desferoxamine (DFO) and cobalt chloride ($CoCl_2$) are able to produce hypoxic mimic conditions in treated cells (Wang and Semenza., 2003). DFO functions as a strong iron chelator that inhibits prolyl hydroxylases, as they require Fe (II) for their catalytic activity, preventing HIF-1α degradation (Siddiq, 2005). This stabilization allows for the dimerization of HIF-1α with HIF-1β, and the consequent regulation of genes involved in the cellular hypoxic response by HIF-1. Treatment of neuronal cells with increasing DFO concentrations has previously shown a decrease in cell viability and an increase in cellular glutathione, an antioxidant produced by cells to cope with ROS production (Cook et al., 2010; Schulz et al., 2000). A significant decrease in cell viability and a significant increase in cellular glutathione level were also observed in neuronal cells treated with DFO for 24 hours when compared to vehicle treated cells (Cook et al., 2010). The surviving/attached cells were also

confirmed to be viable cells through confocal microscopy analysis using Annexin V FLUOS and Propidium Iodide staining as they did not exhibit significant morphological changes when compared to vehicle treated cells (Cook et al., 2010). RT-PCR analysis, however, did show an increase of endogenous HIF-1α mRNA levels in cells treated with DFO for 24 hours when compared to control, suggesting that surviving cells were undergoing cellular adaptation to the chemically induced hypoxic challenge (Cook et al., 2010).

Function of Opioid Receptors

There are 3 major types of opioid receptors, delta-opioid receptor (DOR), mu-opioid receptor (MOR), and kappa-opioid receptor (KOR) found in humans (Feng et al., 2012). They are 7 transmembrane G-protein coupled receptors that activate inhibitory G-proteins and are involved in the regulation of pain, emotional tone, stress and reward pathways (Al-Hasani and Bruchas, 2011; Kieffer and Gaveriaux Ruff., 2002). They also regulate a number of peripheral physiological functions, including respiratory, cardiovascular and immune functions (Feng et al., 2012). Clinically, opioids are used to modulate pain sensation which can arise in patients undergoing hypoxia from post-operative events or cancer (Al-Hasani and Bruchas, 2011). MOR has been shown to have an essential role in the analgesic effects of known opioid agonist morphine, the most widely used painkiller in contemporary medicine (Kieffer and Gaveriaux Ruff., 2002). This receptor also plays a central role in reward processing, the initiation of addictive behaviors, and along with DOR positively modulates hedonic state (Pradhan et al., 2011; Cahill et al., 2014). While KOR activation, on the other hand, has been associated with dysphoric effects and the stress response, systemic KOR agonists also produce robust analgesia (Lalanne et al., 2014; Cahill et al., 2014).

Goal: To Examine the Regulation of Kappa Opioid Receptor Expression

Using a DFO-induced hypoxic cell model with human neuronal cells, our lab previously discovered a significant increase in endogenous human KOR (hKOR) mRNA levels in cells treated with DFO for 24 hours when compared to non-treated cells. There are 4 potential HIF-1 HREs found on the 5' flanking region of the hKOR gene start codon. This study, therefore, aimed to examine the functional role of these HREs in directing promoter activity under hypoxic conditions using neuronal NMB cells.

Materials and Methods

Cell Culture:

Neuroblastoma cells (NMB) were cultured in 5% Carbon Dioxide, 37 degrees Celsius environment with Roswell Park Memorial Institute Media (RPMI) 1640 infused with 10% Fetal Bovine Serum and antibiotics streptomycin and penicillin.

Plasmid Construction and Cloning:

The potential HIF-1 response elements were generated by PCR. The PCR products were cloned into pCR2.1 vector (Life technologies), containing ampicillin resistance gene, and then transformed by heat shock method into E.coli host cells. Miniprep DNA products (QIAGEN) of bacterial cultures, which survived under ampicillin selection, were subjected to restriction enzyme digest analysis on 2% agarose gel and to DNA sequencing. The plasmids containing the correct DNA sequences were prepared by maxiprep (QIAGEN), subjected to gel extraction (QIAGEN) using glass milk and eluted with sterile autoclaved H_2O. Gel extraction products were further subcloned into pGL3 promoter vector (Promega) that contains a luciferase reporter gene and SV40 promoter, and then transformed into E.coli host cells using heat shock. After selection of cultures surviving ampicillin selection, miniprep DNA were further subjected to restriction enzyme digest analysis and DNA sequencing. The plasmids containing the correct sequences were selected and prepared using Maxiprep (QIAGEN).

Transient Transfection:

Cells were transiently transfected using Effectene (QIAGEN). The pCH110 plasmid that contains β-galactosidase gene was used for normalization purposes. Twenty-four hours after transfection, cells were treated with 300 μM DFO (Sigma-Aldrich, St. Louis, MO) and incubated for 24 hours. Cells were then rinsed with cold phosphate buffered saline, harvested and lysed

with diluted 5X lysis buffer (1X) (Promega). Luciferase activity was determined using luciferase substrate (Promega) and a luminometer for measurement.

Statistical Analysis

All data acquired were normalized and error bars were presented as +/- SEM. The student T-test was used for statistical comparison between treated samples and between the control and treatment groups. Statistical significance was defined as $P<0.01$. All experiments were repeated at least 4 times.

Results:

Regulation of Promoter Activity by Two copies of the D response element upon DFO challenge in Neuronal Cells

Since hypoxia can arise as a consequence of various traumatic events, diseases, and operative and post-operative events, the mechanism by which cells are able to enhance the expression of pro-survival genes and down-regulate the expression of pro-apoptotic genes, is of high clinical relevance. Previously, our lab developed a chemically induced hypoxia cell model to investigate the effects of hypoxia on key signaling mediators in the cellular hypoxic response and on the expression of opioid receptors which are involved in the clinical treatment of hypoxia-related pain sensation (Cook et al., 2010).

Our lab previously identified 4 potential HIF-1 HREs on the 5' flanking region of the hKOR gene start codon. These were labeled as A, B, C and D (A is the farthest from the start codon and D is the closest). The 4 HREs were cloned into a reporter vector, transfected into NMB neuronal cells, and a luciferase reporter assay was performed after treatment of cells with DFO for 24 hours. Only the two HREs closest to the hKOR start codon (C and D) showed a significant increase in promoter activity when compared to non-treated cells, and DFO-treated cells with an empty vector (1.3-1.4-fold increase) (data not shown). To determine the possible interactive role of having multiple copies of the same HRE in regulating hKOR expression, 2 copies of the D HRE were cloned into the pGL3 promoter luciferase vector and transfected into NMB neuronal cells. Their effect on promoter activity in DFO-treated cells was compared to that of an insert containing the nitric oxide synthase (NOS) hypoxia response element consensus sequence, as a positive control, which has been previously shown to have enhanced promoter activity in neuronal cells upon hypoxic challenge, and that of PGL-3 promoter vector without an insert (P) (Coulet et al., 2003). As can be seen in figure 1, there was a drastic increase in the

promoter activity of cells transfected with two copies of the D response element when compared to both, cells transfected with an empty vector and cells transfected with the NOS response element positive control after 24 hour DFO treatment. These results suggest an important role of the D response element and a synergistic effect of 2 copies of the D response element in enhancing promoter activity under hypoxic conditions.

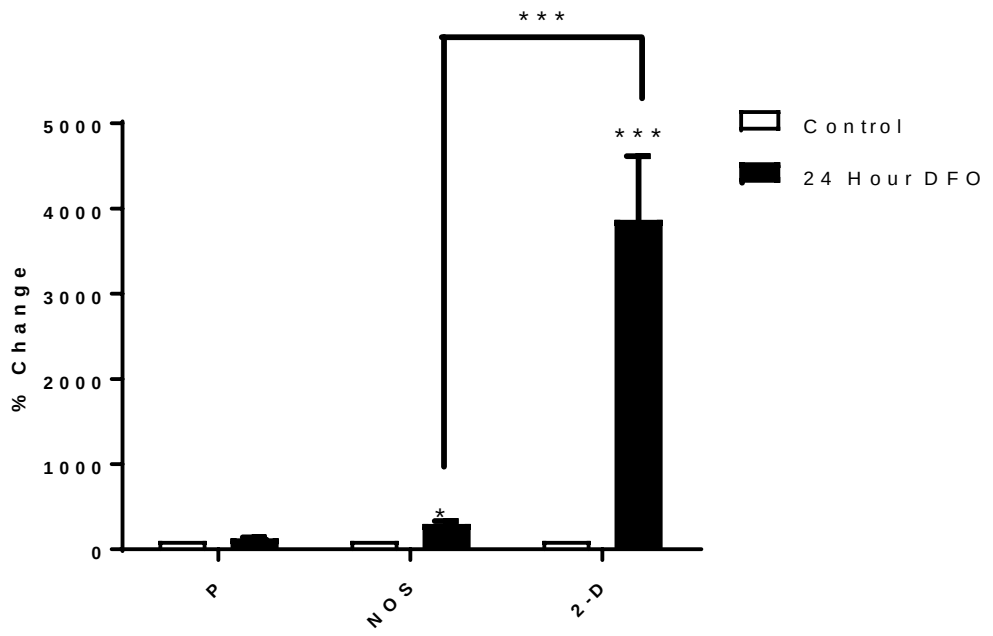

Figure 1. Effect of Hypoxia Response Elements on Luciferase Reporter Activity upon DFO Treatment.

NMB cells were transfected with P (pGL-3 vector without insert), NOS (vector with the response element consensus sequence for nitric oxide synthase, previously shown to increase reporter activity), and 2D (vector containing an insert with 2 copies of HIF-1 D response element). Cells were treated with DFO for 24 hours, harvested, and analyzed by measuring luciferase activity. The reporter activity of non-treated cells was arbitrarily defined as 100 % (control). A significant increase in reporter activity was seen in cells transfected with 2D insert when compared to cells transfected with NOS insert and P insert. Statistical analysis is presented as +/- SEM, with "*" as $P<0.01$ and "***" as $P<0.001$.

Combinatory effect of C and D HIF Response Elements on Promoter Activity in Neuronal Cells

After observing a drastic enhancement of promoter activity using two copies of the HIF-1 D response element in neuronal cells undergoing hypoxic conditions, the focus shifted towards examining what role a combination of the C and the D response elements would serve in regulating promoter activity. Thus, a vector containing a copy of the C and the D HIF-1 response elements consensus sequences separated by 11 nucleotides (CD-11; spacer of approximately 1 turn of DNA using the B form DNA model) was constructed, cloned into the reporter vector, and transfected into NMB cells. DFO-treated cells transfected with this combination of response elements were then compared to those transfected with NOS and P. As seen in figure 2, there was a significantly stronger enhancement of promoter activity in cells transfected with CD-11, as compared to cells transfected with P and NOS. These results serve to further validate the role of the C and the D response elements in enhancing promoter activity during the cellular hypoxic response. In addition, the strong enhancement of promoter activity seen with this combination of response elements when compared to NOS positive control also suggests a synergistic combinatory effect between the C and D response elements and a possible role of transactivation in this strong enhancement of promoter activity.

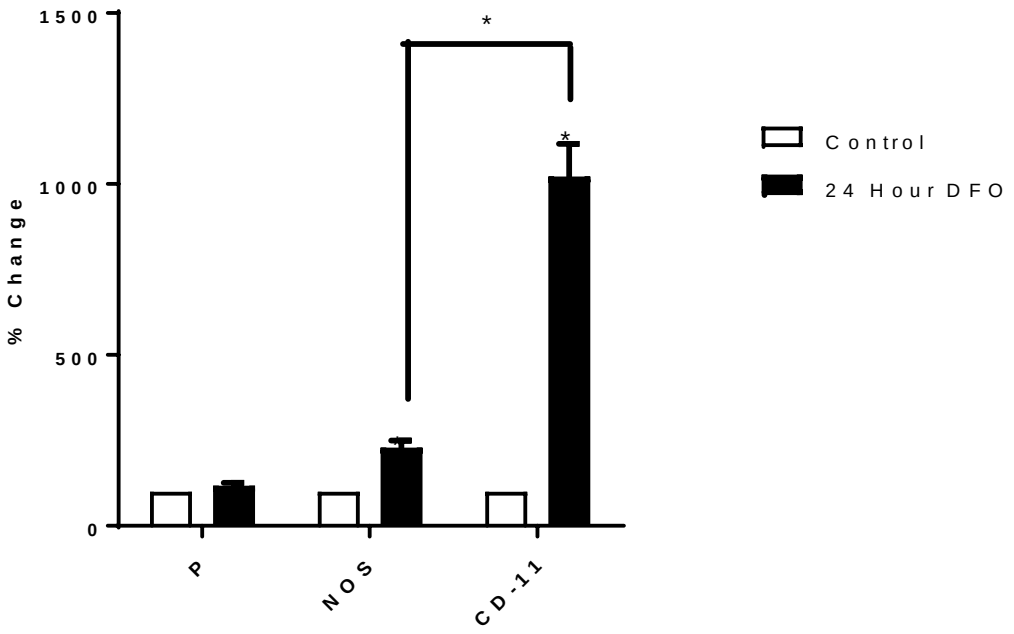

Figure 2. Effect of a Combination of HIF Response Elements on Reporter Activity after DFO Treatment.

NMB cells were transfected with P (vector without insert), NOS (positive control; vector with the response element consensus sequence for nitric oxide synthase, previously shown to increase reporter activity), and CD-11 (a vector containing an insert with C and D responsive elements separated by 11 nucleotides). Cells were treated with DFO for 24 hours, harvested and analyzed by measuring luciferase activity. A significant increase in reporter activity was observed in DFO-treated cells transfected with CD-11 when compared to cells transfected with NOS and cells transfected with P. Statistical analysis is presented as +/- SEM, with "*" as $P<0.01$.

Role of Nucleotide Spacing between C and D Response Elements in their Regulation of Promoter Activity

In order to further examine the interactive role that the combination of C and D response elements has in enhancing target gene promoter activity during the cellular hypoxic response, different spatial arrangements were used between the combination of response elements. The model of 10.5 (approximated to 11) base pairs per turn of the B form DNA (the normal form present in most DNA stretches in cells) was used to analyze how the physical arrangement of these response elements may have an effect in dictating the strength of promoter functionality. Thus, an insert containing a combination of the C and D response element consensus sequences separated by 17 nucleotides (CD-17) was also constructed to analyze the effect of differing HRE orientations on the DNA helix. CD-11 and CD-17 inserts were transfected into NMB cells, and their effect on promoter activity was analyzed with a luciferase reported assay after DFO treatment. As can be seen in figure 3, there was a stronger enhancement of promoter activity in cells transfected with the CD-11 insert in comparison to cells transfected with the CD-17 insert. These results indicate that the physical arrangement of the response elements can dictate their synergism/the strength of possible transactivation in enhancing promoter power and target gene expression.

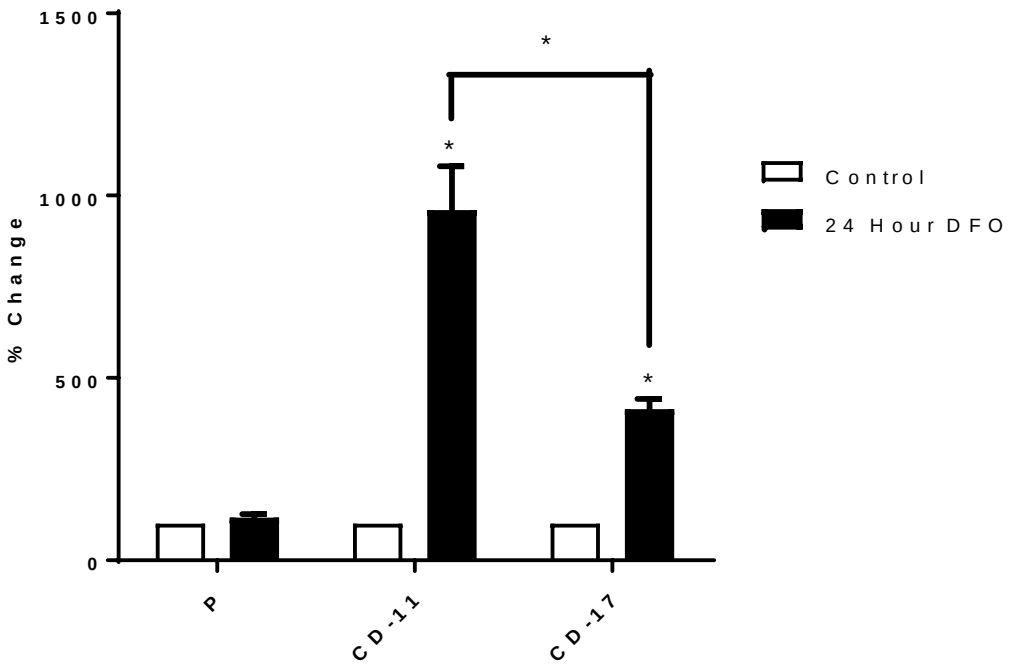

Figure 3. Role of Spacing between Two HIF Response Elements in the Regulation of Promoter activity.

NMB Cells were transfected with P (vector without insert), CD-11 (a vector containing an insert with C and D response elements separated by 11 nucleotides), and CD-17 (a vector containing an insert with C and D response elements separated by 17 nucleotides). Cells were treated with DFO for 24 hours, harvested and then analyzed by measuring luciferase activity. A significant difference in the enhancement of promoter activity was observed in DFO-treated neuronal cells transfected with a combination of the C and D response elements separated by 11 nucleotides when compared to cells transfected the C and D response elements combination separated by 17 nucleotides. Statistical analysis is presented as +/- SEM, with "*" as $P<0.01$.

Mutation of the C and D Response Element Consensus Sequence Abrogates Enhancement of Promoter activity

To confirm the functional role of the C and D HREs in directing promoter activity and expression of target gene, two point mutations were introduced at the consensus sequences of the C and D (CD-11) response elements (CG was mutated to AA in the conserved "ACGTG" sequence). The mutated CD-11 insert, labeled as "MD" (figure 4), was cloned into the reporter vector and transfected into NMB cells. After 24 hour DFO treatment, cells were rinsed with PBS, harvested, lysed and a luciferase reporter assay was used to compare the reporter activity of cells transfected with MD and cells transfected with CD-11. The results, portrayed in figure 4, showed a complete abrogation of the enhancement of reporter activity, previously seen with the combination of C and D response elements, after the introduction of the two point mutations. Cells transfected with mutated CD-11 had promoter activity levels similar to those of cells transfected with the empty reporter vector. This mutation analysis served to confirm the importance of the C and D response elements in regulating promoter activity and target gene expression under the cellular hypoxic response.

Taken together, the results from this study served to elucidate a mechanism used by neuronal cells surviving DFO-induced hypoxic challenge to upregulate the expression of human kappa opioid receptor. While cell viability decreased with increased DFO concentration and with increased time treatment, the surviving neuronal cells showed adaptive cellular responses through an increase in cellular glutathione levels and the upregulation of HIF-1α expression. These surviving/attached cells were not at apoptotic or necrotic stages as was seen through confocal analysis. An increase in hKOR expression was also seen in surviving cells after DFO treatment. From the 4 potential HIF-1 response elements in the 5' upstream region of the hKOR start codon, luciferase reporter assay analysis showed that 2 copies of the D response element

leads to a drastic enhancement of promoter activity after DFO treatment. In addition, using a combination of C and D response elements also leads to a strong enhancement of promoter activity suggesting a possible synergistic/trans-activating role. This synergism also seems to depend on the physical arrangement of the response element consensus sequences as the insert containing the C and D consensus sequences separated by 11 nucleotides showed a significantly stronger enhancement of promoter activity than the insert with C and D consensus sequences separated by 17 nucleotides. Finally, mutation of the C and D response element consensus sequences resulted in no enhancement of promoter activity, suggesting that these two response elements serve an important role in the regulation of gene expression during the neuronal cellular adaptive response to hypoxic conditions

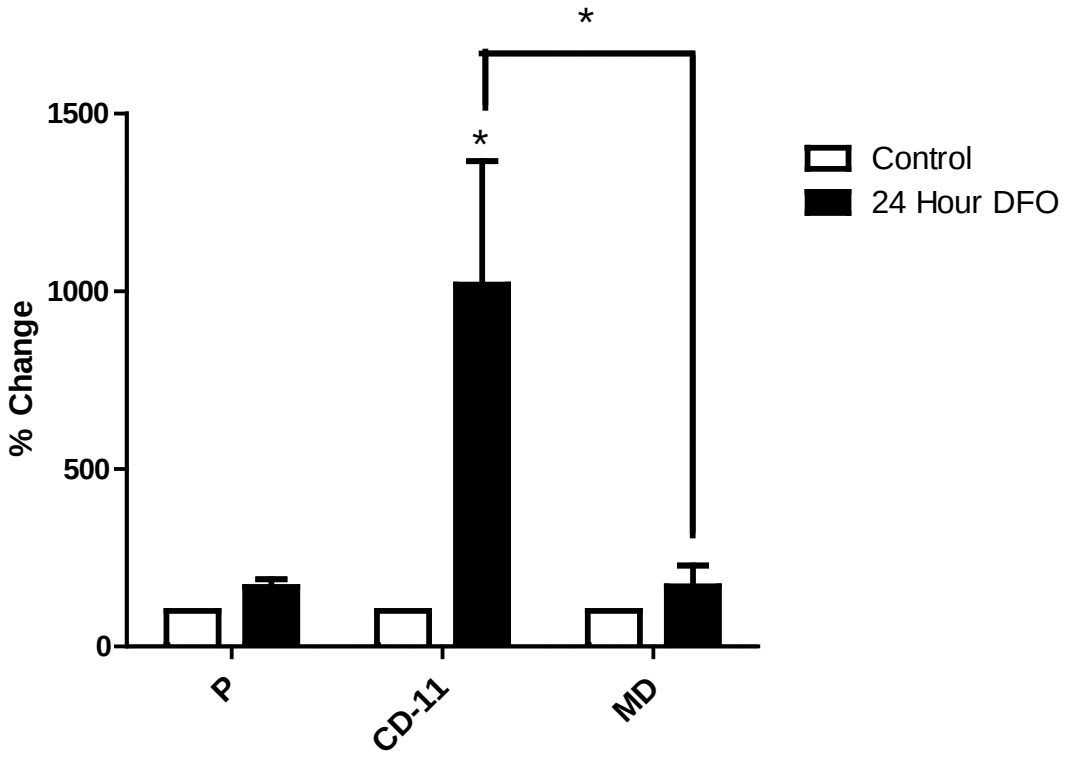

Figure 4. Effect of Mutation of Hypoxia Response Element on Regulation of Promoter Activity after DFO Treatment.

NMB cells were transfected with P (vector without insert; negative control), CD-11 (a vector containing an insert with C and D responsive elements separated by 11 nucleotides), and MD (a vector containing an insert with two point mutations at each of the C and D response element consensus sequences). Cells were treated with DFO for 24 hours, harvested and then analyzed by measuring luciferase activity. The enhancement of promoter activity by the CD-11 insert was abrogated after the introduction of two point mutations on the C and D response element consensus sequences. Statistical analysis is presented as +/- SEM, with "*" as $P<0.01$.

Discussion and Conclusions

Due to the variety of events that can trigger the development of tissue hypoxia in the body (cancer, trauma, and diseases such as diabetes), and the clinical relevance of modulating hypoxia-induced pain sensation through opioid receptor signaling, this study aimed to investigate the possible mechanisms controlling hKOR expression in neuronal cells surviving chemically-induced hypoxic conditions. Our results suggested an important role for the C and the D HIF-1 response elements, found in the 5' upstream region of the hKOR start codon, in directing hKOR expression.

KOR, through the action of KOR agonists, has been associated with pain regulation, reward and abuse circuitry, and stress behavior pathways (Cahill et al., 2014); but does KOR have a function in neuroprotection? Kappa-agonist enadiline has been shown to be neuroprotective in rat models of focal cerebral ischemia (Hayward et al; 1993). Another study, however, reports that KOR activation does not offer neuroprotection to rat cortical neurons (Zhang at al., 2002). To examine the effect of hypoxic conditions on endogenous neuronal hKOR mRNA levels, our lab previously used a DFO-induced hypoxia cell model with NMB cells. RT-PCR analysis of cell RNA extracts showed a significant increase in hKOR mRNA levels after treatments with DFO for 24 hours when compared to no treatment. HIF-1α levels were also increased in cells treated with DFO for 24 hours when compared to non-treated cells (Cook et al., 2010). HIF-1 is a known transcriptional regulator of genes involved in the hypoxic response (Ziello et al., 2007). In this study, 4 possible HIF-1 response elements were then identified on the 5' upstream region of hKOR. Two of these 4 potential response elements (C and D) were detected to have an increase in reporter activity upon DFO treatment using transfected NMB cells.

Inserts containing two copies of the D response element and combinations of the C and D response elements were constructed to study the possible interactive roles of these response elements in enhancing promoter activity. Other studies have demonstrated that the physical arrangement of response elements and the addition of non-adjacent response element multimers can have an effect on promoter activity regulation (Ponglikitmongkol, et al., 1990; Huang, et al., 2010). One study reports that ligation of 2 copies of a response element, fibroblast growth factor response element in rat bone sialoprotein gene, has led to an increase from basal promoter activity (Shimizu-Sasaki., et al 2011). In addition, specifically altering the spacing between hypoxia response element consensus sequences has been shown to cause different levels of promoter induction in mammalian cells (Kaluz et al., 2008). In our study, a drastic enhancement of promoter activity was seen in cells transfected with 2D and CD-11 when compared to control and positive control NOS, suggesting a possible synergistic/trans-activating role in their arrangement (Figures 1 and 2). Synergism refers to an increase in promoter activity in which two or more activators are present at the same time, or when two or more additional binding sites for the activator are provided, greater than the additive effect of activators working independently (Hershlag and Johnson., 1993). A comparison of the combinatory effect of the C and D response elements separated by 11 and 17 nucleotides, however, showed differing levels of promoter activity enhancement (Figure 3). The 11 base pair spacing corresponds to the model of 10.5 base pairs per turn of DNA helix in B-form DNA. These results suggest that the physical arrangement of response elements affects the strength of their functional role in promoter activity regulation. One may speculate that the changing trans-acting factor interactions from differing CD arrangements induce different levels of effect on promoter activity. An effect of stereo-alignment in affecting synergism has also been reported with human estrogen receptor tandem responsive elements (Ponglikitmongkol et al.,1990).

Furthermore, the introduction of 2 point mutations to the consensus sequences of the C and the D response elements completely abolished their effect on promoter activity during hypoxic conditions (figure 4). The functional role of HREs seems to be dependent on the presence of the conserved A/GCGTG HIF-1 binding site (Kaluz et al., 2008). This result, thus, reaffirms the importance of the C and the D response elements in the augmentation of promoter activity during the cellular hypoxic response and suggests a functional role in the enhancement of endogenous neuronal hKOR mRNA levels during DFO-induced hypoxic challenge.

This study maps a mechanism for directing the observed up-regulation of hKOR expression in neuronal cells undergoing hypoxic conditions through the functionality of HIF-1 as a trans-acting factor and the HIF-1 C and D response elements as cis-acting factors. More of these experiments will be included to increase the number of repeated measurements for statistical analysis and confirmation. In addition, since divalent metals such as $CoCl_2$ and $NiCl_2$ can also produce hypoxic-like cellular responses by taking advantage of the iron dependent activity of PHD enzymes (competing with iron at binding sites) (Vengellur et al., 2005), it may be of interest to compare the levels of promoter activity that these hypoxia mimetics can induce using this hKOR HIF-1 HRE model.

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