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Hypoxia-Induced Differential Effects on Opioid Receptor Gene Expression in Human Cell Lines

Christopher P. Karch

Submitted in partial fulfillment of the requirements for the
Degree of Masters of Science in Microbiology from the
Department of Biology of Seton Hall University
May 2006

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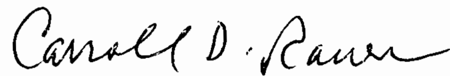

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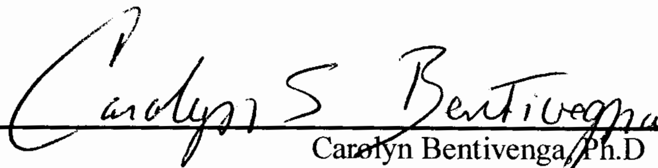

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Abstract

Hypoxia is known clinically as ischemia. Ischemic events such as strokes and cardiac arrest cause hypoxia in the human central nervous system, which leads to many detrimental effects including cell death. The functional role of the opioid receptors in neuronal hypoxia is unclear; therefore the effect of hypoxia on the opioid receptor gene expression was examined using human cell cultures as model systems, including neuronal (NMB), glial (U87), and nonneuronal/nonglial (HeLa) cell lines. Hypoxic conditions were generated by treating the cells with a hypoxia mimic, desferoxamine (DFO), for 24 hours. RT-PCR results demonstrated an upregulation of HIF- 1α in all three cell lines, suggesting that these cell lines were functional model systems for studying hypoxia's effects. In neuronal cells, no change of δ Opioid Receptor (DOR) expression was noted, κ Opioid Receptor (KOR) expression was upregulated, and μ Opioid Receptor (MOR) expression was downregulated. In glial cells KOR expression was upregulated, while there was no change in DOR or MOR expression. Finally, there was no detectable change in any of the opioid receptors in the nonneuronal/nonglial cell lines. These results showed that a differential expression of opioid receptor genes occurs under hypoxia in human neuronal and glial cells, but not in the nonneuronal/nonglial cells.

Introduction

Hypoxia and Ischemia

Hypoxia is the condition of low oxygen in any system. When hypoxia occurs in animals it is generally caused by a lack of blood flow to a particular tissue. Clinically this condition is known as ischemia. Low glucose availability is also a consequence of ischemia. Many pathological conditions lead to ischemia, including stroke, global ischemia (cardiac arrest), atherosclerosis, physical trauma, tachycardia, and hypotension. These conditions are some of the leading causes of death in the United States (Anderson et al., 2005). In order to alleviate and prevent the damage caused by hypoxic conditions, a better understanding of the hypoxia's effects at the cellular and molecular level is needed.

In vivo, neurons are known to be highly sensitive to low oxygen levels due to their high level of metabolic activity. Neurons are unable to generate enough ATP to operate the Na^+/K^+ pumps to keep their membranes polarized using anaerobic metabolism for extended periods of time (Hansen, 1985). With decreased oxygen availability and subsequent fall in ATP levels, Na^+ enters the neuron causing depolarization (Won et al., 2002). Once depolarized, an influx of Ca^{2+} occurs, causing glutamate to be released into the synaptic cleft. The glutamate binds to receptors on the postsynaptic neuron leading to Ca^{2+} influx. This process is known as excitotoxcity (Jabaudon et al., 2000).

Ca^{2+} influx causes many different effects inside the cell including swelling of organelles (Hayashi and Abe, 2004), inhibition of electron transport in the mitochondria (Ward et al., 2000), reduction in ATP transport from the mitochondria (Vesce et al., 2005), generation of reactive oxygen species (ROS) (Won et al., 2002), and activation of proteases (Liebetrau et al., 1999). Eventually, all of the cellular changes that have occurred in the neuron lead to necrosis or apoptosis. Which death pathway occurs depends on the intensity of the ischemic insult, age of the neuron, and what model system is being examined (Ankarcrona et al., 1995).

Hypoxia, Opioids, and Opioid Receptors

Many different mammals are known to hibernate during colder winter months, surviving, but decreasing respiratory and metabolic rates (Drew et al., 2004). With the decrease in respiration (Buck and Barnes, 2000) and a reduction in circulating glucose in the blood stream of these mammals (Osborne et al., 1999), conditions similar to ischemia are created, but somehow these animals are able to survive without any long-term damage to their nervous systems (Drew et al., 2004). Evidence has been accumulating over the past twenty years that opioids increase both in the blood stream and in nervous tissue in many different species of hibernating mammals (Romano et al., 2004; Benedict et al., 1999; Bolling et al., 1997; Oeltgen et al., 1988; Cui et al., 1996).

Opioids are compounds that elicit numerous pharmacological effects. These compounds can exist either endogenously, produced inside the organism, or exogenous, produced outside of the organism and are taken in either by ingestion, absorption, or intravenously. Chemically, these compounds fall into two major classes, peptide and

alkaloid opioids. Peptide opioids include the endogenous opioids as well as the synthetic derivatives of the naturally occurring peptide opioids. Known peptide opioids exist in three classes: enkephalins, dynorphins, and endorphins. Alkaloids are plant-derived compounds such as morphine (Janecka et al., 2004).

Opioids bind to membrane bound opioid receptors and mediate many known pharmacological effects such as analgesia, respiratory depression, euphoria, effects on feeding, hormone release, neurotransmitter release, decrease of gastrointestinal motility, and cause various effects on anxiety. Opioid receptors are 7 transmembrane receptors, also known as G protein-coupled receptors (Chen, 1993). Three major types of opioid receptors are found in mammals, δ (DOR), κ (KOR), and μ (MOR). For each receptor type there are different subtypes (Waldhoer et al., 2004). While there are some differences in agonist specificity, generally it has been found that enkephalin has the highest affinity for DOR, dynorphin has the highest affinity for KOR, and endorphin has the highest affinity for MOR (Janecka et al., 2004).

In hibernating animals it has been observed that DOR agonists play a primary role in initiating, sustaining, and ending hibernation in mammals by differential expression in different regions of the brain (Oeltgen et al., 1988, Cui et al., 1996). It has also been demonstrated that DOR and KOR, but not MOR agonists circulate in the blood stream and prevent ischemic injury to the heart during hibernation (Romano et al., 2004 Benedict et al., 1999 Bolling et al., 1997). DOR agonists have also been shown to be neuroprotective in hibernating mammals (Borlongan et al., 2004).

Considering these protective effects of opioids, studies have begun in non-hibernating mammals to see what role opioids play in cytoprotection. Studies have

shown that when neurons and myocytes are pretreated with opioids a higher survival rate during hypoxic conditions is observed. Preconditioning with a DOR agonist leads to neuroprotection in murine studies (Ma et al., 2005; Zhang et al., 2006). It has been reported that agonists for KOR and MOR have no effect on neuroprotection (Zhang et al., 2000). However, in another study a KOR agonist was able to offer neuroprotection, when mice were exposed before or after the ischemic insult (Goyagi et al., 2003). The MOR agonist, morphine, was also shown to provide neuroprotection when given prior to hypoxic treatment, but when given concurrently there was an increase in the neurotoxicity of hypoxic event (Ammon-Trieber et al., 2005). All studies looking at opioid receptor's role in hypoxia have used animal models (Barry and Zuo, 2005); to date no study has used a human model to determine the role of hypoxia on the gene expression of the opioid receptors.

Hypoxia Inducible Factor 1 (HIF-1)

Gene expression during hypoxic conditions in any mammalian cell is mainly under the control of the transcriptional factor hypoxia inducible factor-1 (HIF-1). HIF-1 exists as a heterodimer made up of a 120 kDa HIF-1α subunit and 91-94 kDa HIF-1β subunit. Both subunits are members of the basic-helix–loop-helix (bHLH)-PAS family of transcriptional factors (Wang et al., 1995). bHLH family of transcription factors must dimerize before they are able to affect transcription. The basic region of the bHLH domain binds to DNA, while the rest of the protein interacts with the transcriptional machinery (Kewly et al., 2004).

It was originally thought that low pO_2 controlled transcription of HIF-1α (Wang et al., 1995), but subsequent studies showed that the gene is constitutively expressed and translated inside the cell. However, under normoxia the half-life of the HIF-1α protein is less than 5 minutes (Wang et al., 1995). Under hypoxic conditions, most of the regulation of HIF-1α occurs on the protein level (Huang et al., 1996; Kallio et al., 1997). Some studies have noted an upregulation of HIF-1α mRNA as well an increase in protein stabilization in a rat's brain undergoing hypoxia (Royer et al., 2000)

From studies using inhibitors and temperature sensitive mutants of the ubiquitin-proteasome pathway it was determined that HIF-1α is degraded by the ubiquitin-26S proteasome pathway under normoxic conditions (Salceda and Caro, 1997). During normoxia proline residues are hydroxylated by a family of 3 different prolyl-4-hydroxylases that use oxygen as a substrate and Fe^{2+} as a cofactor (Bruick and McKnight, 2001; Masson et al., 2001). The hydroxyprolines interact with E3 containing von Hippel Linadau tumor suppressor protein and initiate proteolysis of HIF-1α by the 26S proteasome (Ivan et al., 2001; Jaakkola et al., 2001).

HIF-1β was first identified as aryl hydrocarbon receptor nuclear translocator (ARNT). It was originally observed acting as a transcriptional factor upon exposure to compounds such dioxin; it has since been established that it binds to many basic-helix-loop-helix transcription factors (Kewley, 2004). Like HIF-1α it was originally believed that the level of HIF-1β gene expression was controlled by pO_2 (Wang et al., 1995). However, it has been determined that the protein is not oxygen sensitive and thus, not degraded like HIF-1α (Huang et al., 1996; Kallio et al., 1997). It has also been observed

that there is a higher affinity for HIF-β to bind with HIF-1α then with its other partners (Gradin et al., 1996).

The dimerized HIF-1 binds to a region flanking the 3' region of inducible genes known as the hypoxia response element (Semenza and Wang, 1992). In neurons, previous studies have shown that HIF-1 that can lead to up-regulation of genes that are neuroprotective such as erythropoietin (Liu et al., 2005). Genes important in glycolysis and glucose transport are also upregulated in neurons (Zaman et al., 1999). It has also been demonstrated that vascular endothelial growth factor (VEGF) is upregulated in neurons after HIF induction (Althaus et al., 2006; Siddiq et al., 2005). HIF-1 also can induce transcription of pyruvate dehydrogenase kinase, which prevents pyruvate from entering the Krebs cycle, forcing fermentation (Papandreou et al., 2006).

HIF-1 has been shown to downregulates genes. In human endothelial cells HIF-1, Carbamoylphosphate synthetase-aspartate carbamoyltransferase-dihydroorotase (CAD), which encodes the first enzymatic steps in pyrimidine biosynthesis, is downregulated and thus, inhibits DNA and RNA synthesis, preventing cell growth and cell division (Chen et al., 2005).

Mimicked Hypoxia

In order to study the effect of hypoxia on gene expression, cells can be grown in low oxygen, treated with glutamate, or treated with a hypoxia mimic. Deferoxamine methanesulfonate (DFO) also known as desferrioxamine mesylate (DFX) is a bacterial siderophore that functions as a hypoxia mimic. DFO was shown to increase the expression of erythropoietin and increase the concentration of the dimerized HIF-1 at

similar rates as 1% oxygen in Hep3B cells as well as in cultured Chinese Hampster Ovary cells (Wang and Semenza, 1993). Similar results were later obtained both in primary neuronal cultures and brains from animal models (Zaman et al., 1999; Bernaudin et al., 2000; Hamrick et al., 2005). DFO mimics hypoxia by increasing cellular concentration of HIF-1 leading to gene expression that would occur during hypoxia without generating any of the other effects of hypoxia (Jaakkola et al., 2001).

Goal

No previous study has examined the expression of the opioid receptor genes in humans under hypoxia. Therefore, in this study we determined whether a change of opioid receptor gene expression occurred under hypoxia by using human neuronal and nonneuronal cell model systems.

Materials and Methods

Cell culture

NMB neuronal cells were grown in Roswell Park Memorial Institute Medium (RPMI) containing 10% heat inactivated Fetal Calf Serum (FCS). U87 glial and HeLa nonneuronal/nonglial (American Tissue Culture Collection, Manassas, VA) cells were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat inactivated (FCS). All cells were cultured in T-75 cm^2 flasks (Fischer Scientific, Tunstin, CA) at 37°C and 5% CO_2 . Once confluency was reached as determined by visual observation using a microscope, cells were passed into new T-75 cm^2 flasks.

DFO Treatments

NMB, U87, and HeLa cells were counted using the trypan blue exclusion method and then seeded into 6 well plates at 0.8 x 10^6, 0.65 x 10^6,0.21 x 10^6 cells per well respectively. Cells were treated with 200µM of DFO (Sigma, St. Louis, MO) for 24 hours, and control cells were left untreated.

RNA Extraction

Cells were incubated with Trizol reagent (NMR) for 5 minutes at room temperature, and cell lysates were transferred to a centrifguge tube. Phase separation reagent was added to

each tube. The tubes were incubated at room temperature for 8 minutes, and were then centrifuged at 12,000 rpm for 15 minutes at 4°C. Isopropanol was added to the aqueous phase. The tubes were incubated at room temperature for 8 minutes. RNA pellets were obtained by centrifugation at 12,000 rpm for 8 minutes at 4°C. The RNA pellet was washed with 70% ethanol and dissolved in Diethyl Pyrocarbonate (DEPC) treated water. RNA concentration was determined by using the UV spectrophotometer and calculated by the formula of OD_{260} x 50 μg/ mL.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase reactions were set up using 5 μg of RNA, random primers, dNTPs, 5x buffer, DTT, RNase inhibitor, and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison WI). Reactions were carried out in a thermocycler for 50 minutes at 37°C followed by 15 minutes at 70°C.

PCR reactions were set up in 0.5mL PCR tubes with 10x buffer, $MgCl_2$, dNTPs, and TAQ polymerase. The reactions were run in the thermocycler for 1 minute at 95°C, 35 seconds at 68 °C, and 35 seconds at 72°C. For HIF-1α, 35 PCR cycles were run with 4 μL of the RT product, and the primers for an internal standard, β-actin, was added at cycle 21. The HIF-1α primers were 5'-CCAGCAGACTCAAATACAAGAACC-3' and 5'-GTATGTGGGTAGGAGATGGAGAT-3'. The β-actin primers were, 5'-CCTTCCTGGGCATGGAGTCCTG-3' and 5'-TACAGCGAGGCCAGGATGG-3'. For DOR 25 cycles of PCR was used with 3 μL of RT product. The DOR primers were 5'-GTTCACCAGCATCTTCACGCTC-3' and 5'-CGGTCCTTCTCCTTGGAGCCC-3'. For KOR 40 cylces of PCR were used with 4 μL of RT product. The KOR primers were 5'-

CCTTCCTGGGCATGGAGTCCTG-3' and 5'-TACAGCGAGGCCAGGATGG-3'. For MOR 35 cycles of PCR were run with 3 μL of RT product. The MOR primers were 5'-CTGGAAGGGCAGGGTACTGGTG-3' and 5'-CTGCCCCCACGAACGCCAGCAAT-

Data Analysis

The PCR product was run on a 2% agarose gel, and the gel image was quantized using AlphaEaseFC software (AlphaInnotech Corporation, San Leandro, CA).

Quantification data are expressed as mean +/- standard error. Experimental groups were compared by using the student-paired t-test. All data were analyzed and graphed using Prism (Graphpad). $P<0.05$ was considered statistically significant.

Results

Gene Expression in Neuronal (NMB) Cells

NMB neuronal cells treated with 200μM of the hypoxia mimic DFO for 24 hours were used as a cell model system. In order to determine if a hypoxic human neuronal model system was successfully created, RNA was extracted and RT-PCR was run with primers specific for HIF-1α. In lane 3 of Figure 1.a, an increase in HIF-1α expression was observed after 24 hours of hypoxia. These HIF-1α signals (lanes 2 and 3) were then normalized using the internal standard β-actin. From the normalized pixel density it was determined that the upregulation of HIF-1α gene expression was significant ($p= 0.0297$) (Figure 1.b). The upregulation of HIF-1α gene expression suggested that a model neuronal hypoxic system was created. This cell model system was then used to study the effects of hypoxia on the gene expression of the opioid receptors.

No significant ($p=0.4317$) change of DOR expression was observed using the non-treated cells or the cell with 24 hours of hypoxic mimic treatment (Figure 1.c and Figure 1.d). KOR expression was significantly ($p=0.0013$) upregulated after 24 hours of hypoxic treatment (Figure 1.e and Figure 1.f). Finally, MOR expression was significantly ($p=0.0088$) downregulated after 24 hours of hypoxic treatment (Figure 1.g and Figure 1.h). These results suggest that there is a differential expression of opioid receptors under hypoxia in a neuronal cell model.

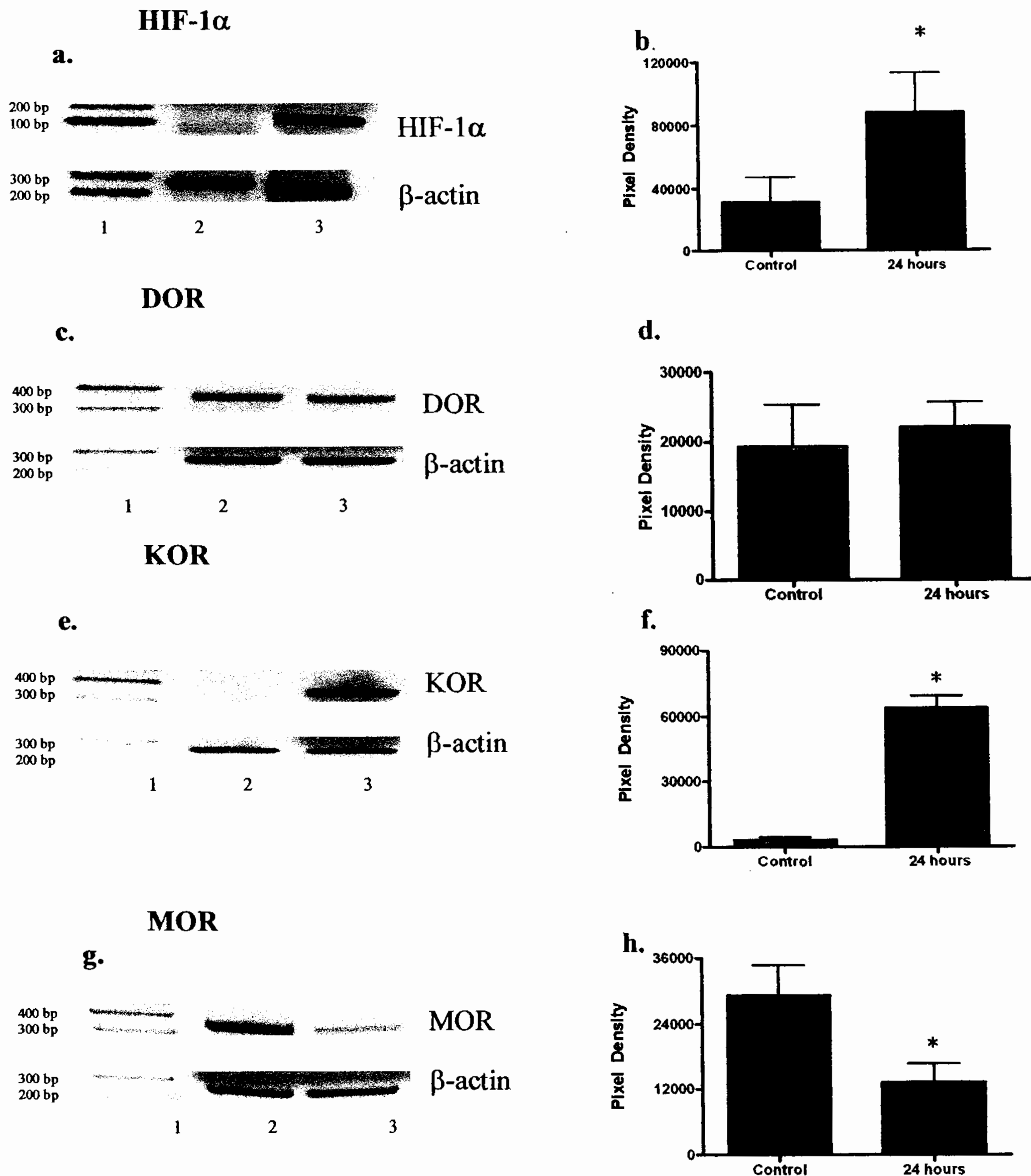

Figure 1: Gene expression in NMB neuronal cells. For all gels lane 1 ladder, lane 2 control (untreated), lane 3 24 hours of DFO treatment. β-actin was used as an internal standard for normalization. a. Electrophoretic analysis of RT-PCR product of HIF-1α. b. Quantification data based on pixel density of HIF-1α bands $p= 0.0297$. c. Electrophoretic analysis of RT-PCR product of DOR. d. Quantification data based on pixel density of DOR bands $p=0.4317$. e. Electrophoretic analysis of RT-PCR product of KOR. f. Quantification data based on pixel density of KOR bands $p=0.0013$. g. Electrophoretic analysis of RT-PCR product of MOR. h. Quantification data based on pixel density of MOR bands $p=0.0088$. Graphed Data expressed +/- standard error. (n=4) Paried T-test was used to determine significance ($p<0.05$) *.

Gene expression in Glial (U87) Cells

U87 glial cells treated with 200μM of the hypoxia mimic DFO for 24 hours were used as a cell model system. In order to determine if a hypoxic human glial model system was created, RNA was extracted and RT-PCR was run with primers specific for HIF-1α. In lane 3 of Figure 2.a an increase in HIF-1α expression was observed after 24 hours of hypoxia. These HIF-1α signals (lanes 2 and 3) were then normalized using the internal standard β-actin. From the normalized pixel density it was determined that the upregulation of HIF-1α gene expression was significant ($p= 0.0297$) (Figure 2.b). The upregulation of HIF-1α gene expression suggested that a model glial hypoxic system was created. This cell model system was then used study the effects of hypoxia on the gene expression of the opioid receptors.

No significant ($p=0.6536$) change of DOR expression was observed using the non-treated cells or cells with 24 hours of hypoxic mimic treatment (Figure 2.c and Figure 2.d). KOR expression was significantly ($p=0.0324$) upregulated after 24 hours of hypoxic treatment (Figure 2.e and Figure 2.f). Finally, there was no significant ($p=0.1877$) change in MOR expression after 24 hours of mimicked hypoxic treatment (Figure 2.g and Figure 2.h). These results suggested that there was a differential expression of opioid receptors under hypoxia in a glial cell model.

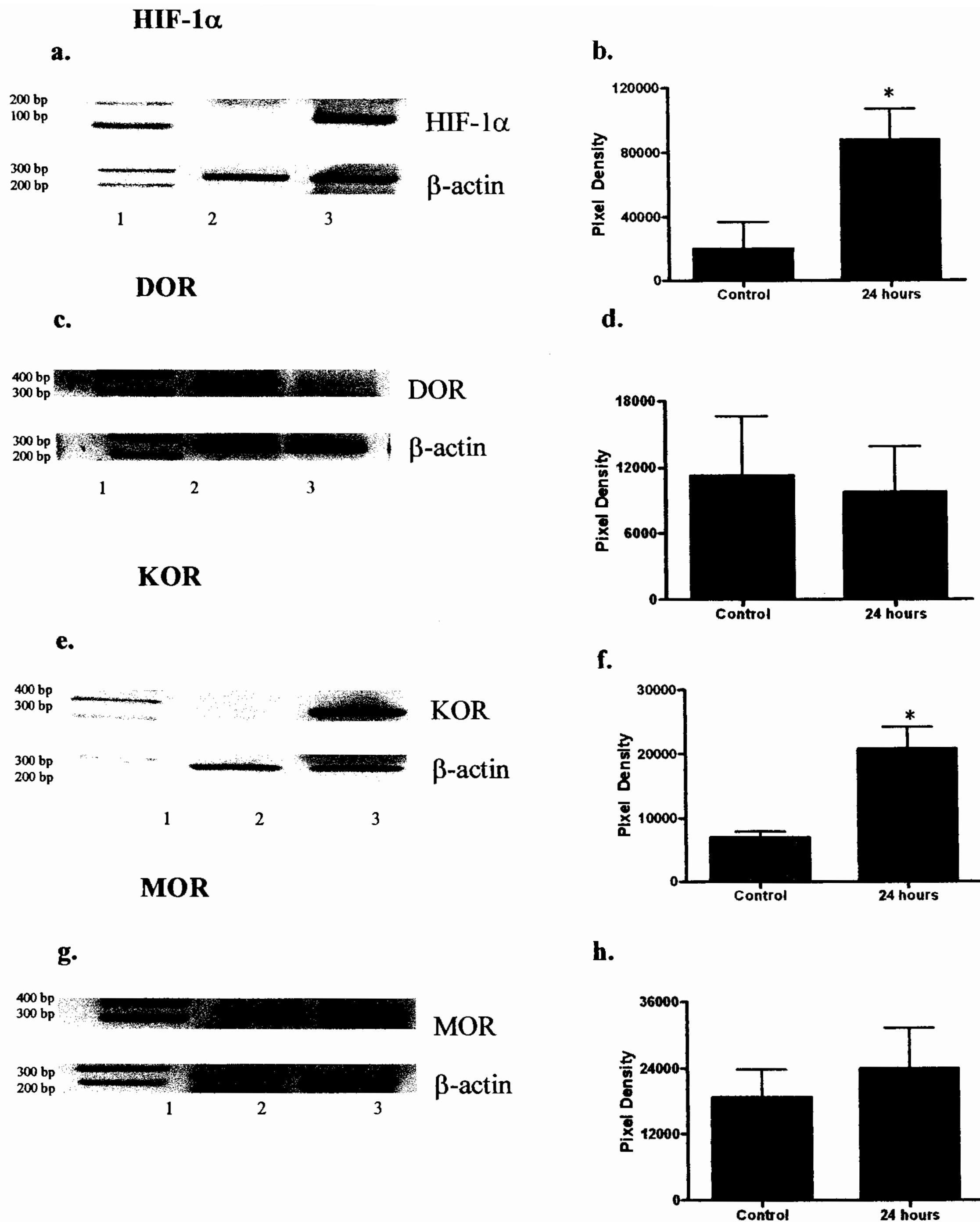

Figure 2: Gene expression in U87 glial cells. For all gels lane 1 ladder, lane 2 control (untreated), lane 3 24 hours of DFO treatment. β-actin was used as an internal standard for normalization. a. Electrophoretic analysis of RT-PCR product of HIF-1α. b. Quantification data based on pixel density of HIF-1α bands $p= 0.0034$. c. Electrophoretic analysis of RT-PCR product of DOR. d. Quantification data based on pixel density of DOR bands $p=0.6536$. e. Electrophoretic analysis of RT-PCR product of KOR. f. Quantification data based on pixel density of KOR bands $p=0.0324$. g. Electrophoretic analysis of RT-PCR product of MOR. h. Quantification data based on pixel density of MOR bands ($p=0.1877$). Graphed Data expressed +/- standard error. ($n=3$) Paried T-test was used to determine statistical significance ($p<0.05$)*.

Gene expression in Nonneuronal-Nonglial (HeLa) Cells

HeLa nonneuronal/nonglial cells treated with 200μM of the hypoxia mimic DFO for 24 hours were used as a cell model system. In order to determine if a hypoxic human nonneuronal/nonglial cells model system was created, RNA was extracted and RT-PCR was run with primers specific for HIF-1α. In lane 3 of Figure 2.a an increase in HIF-1α expression was observed after 24 hours of hypoxia. These HIF-1α signals (lanes 2 and 3) were then normalized using the internal standard β-actin. From the normalized pixel density it was determined that the upregulation of HIF-1α gene expression was significant ($p = 0.0297$) (Figure 2.b). The upregulation of HIF-1α gene expression suggested that a model nonneuronal/nonglial cells hypoxic system was created. This cell model system was then used study the effects of hypoxia on the gene expression of the opioid receptors.

No significant ($p=0.4628$) change in DOR expression was observed using the nontreated, or cells with after 24 hours of hypoxic mimic treatment (Figure 3.c and Figure 3.d). There was no significant ($p=0.3351$) change in KOR expression after 24 hours of hypoxic treatment (Figure 3.e and Figure 3.f). Finally, there was no significant ($p=0.2260$) change in MOR expression after 24 hours of mimicked hypoxic treatment (Figure 2.g and Figure 2.h). These results suggested that there was no differential expression of opioid receptors under hypoxia in a nonneuronal/nonglial cell model.

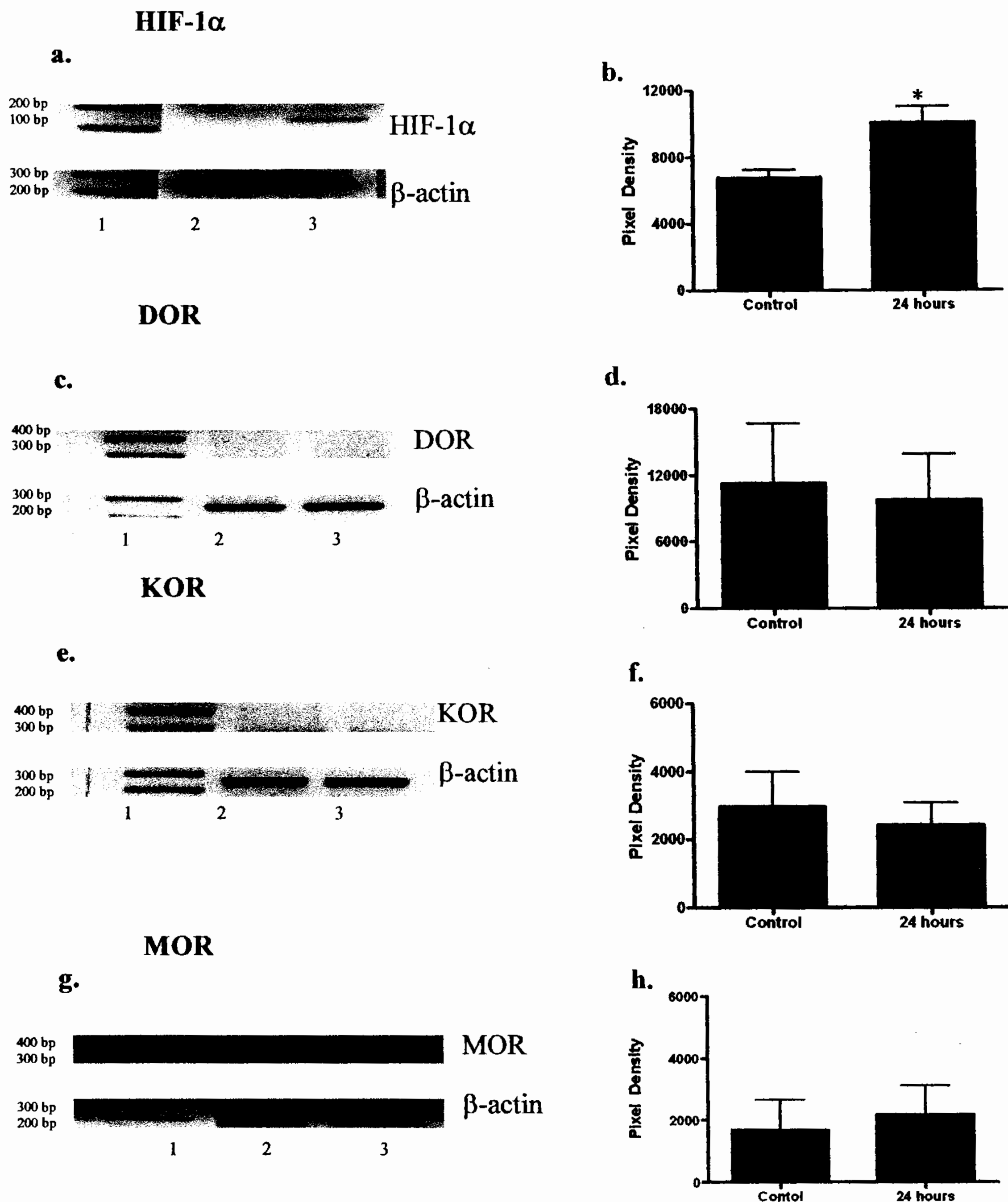

Figure 3: Gene expression in HeLa nonneuronal/nonglial cells. For all gels lane 1 ladder, lane 2 control (untreated), lane 3 24 hours of DFO treatment. β-actin was used as an internal standard for normalization. a. Electrophoretic analysis of RT-PCR product of HIF-1α. b. Quantification data based on pixel density of HIF-1α bands p= 0.0420. c. Electrophoretic analysis of RT-PCR product of DOR. d. Quantification data based on pixel density of DOR bands (p=0.4628). e. Electrophoretic analysis of RT-PCR product of KOR. f. Quantification data based on pixel density of KOR bands (p=0.3351). g. Electrophoretic analysis of RT-PCR product of MOR. h. Quantification data based on pixel density of MOR bands (p=0.2260). Graphed Data expressed +/- standard error. (n=4) Paried T-test was used to determine statistical significance ($p<0.05$)*.

Discussion

This study was to examine if hypoxia induced differential expression of the human opioid receptors, which potentially could correlate with their physiological functions under hypoxia. Three human model hypoxic systems were created by treating cells with the hypoxia mimic DFO. In human neuronal (NMB), glial (U87), and nonneuronal/nonglial (HeLa) cell lines, the treatment with DFO increased HIF-1α mRNA which was associated with the increase of HIF-1 in another study (Royer et al., 2000). These findings show that all three cell lines do serve as hypoxic model systems.

This study showed that DOR gene expression remains constant during hypoxia in human neuronal cells and glial cells. A similar finding was also observed in rat cortical neurons (Zhang et al., 2006). However, in other studies a decrease in DOR expression in rat cortical neurons under hypoxia was reported (Ma et al., 2005). This study supports the conclusion that there is a constant expression of the DOR gene during hypoxia.

In small-cell lung carcinoma cells it has been observed that stimulation of DOR inhibited the influx of Ca^{2+} into cells (Sher et al., 1996). It also has been shown that in sensory neurons Ca^{2+} influx was modulated by DOR stimulation (Acosta and Lopez 1999). Considering these two findings it seems to make sense that a constant expression of the DOR gene during hypoxia would occur. Ca^{2+} influx is the first detrimental step on the pathway to a neuron's death or injury. Constant levels of DOR may function to block

excess Ca^{2+} from entering the cell, preventing the cascade leading to cell death, because in animal studies it was noted that stimulation of DOR lead to neuroprotection under hypoxia (Zhang et al., 200; Ma et al., 2005).

KOR gene expression increased in both neuronal and glial cell lines during hypoxia. This is a novel finding. No previous study using a human, rat, or murine model has demonstrated the effect of hypoxia on gene expression of KOR. It has been demonstrated that stimulation offers protection against ischemic injuries in rats (Goyagi et al., 2003; Zhang et al., 2003). An increase of the receptor message could be indicative of a method of adaptation by the cell. By having more receptors on the cell surface, there is a greater chance that a circulating opioid will bind to the cell and initiate its protective effect. The same effect could also occur if the concentration of circulating opioids themselves increased as well.

Change in MOR expression during hypoxia was only noted in the neuronal cells. In the neuronal cells there is a clear decrease in expression of MOR, while there is no change in glial or in nonneuronal/nonglial cells. These findings suggest that MOR expression is regulated by some mechanism that is neuronal specific. However, the precise mechanism is still unknown.

There is still not a clear function for MOR in hypoxia. In some previous animal studies no change in a neuron's ability to survive hypoxia was noted when a MOR agonist was used to treat cells (Zhang et al., 2000), whereas some noted detrimental effects on the neuron if the MOR agonist morphine was given at the same time as the hypoxic insult, while it was protective if given before the insult (Ammon-Treiber et al., 2005). In order to fully understand the implication of differential expression of the MOR

gene under hypoxia more research needs to be preformed to understand the physiological role of MOR under hypoxia.

There was no change in opioid receptor gene expression in the nonneuronal/nonglial cell model, which suggested that differential expression of the opioid receptors under hypoxia is a neuronal associated phenomenon. However, other opioid expressing nonneuronal cells still need to be examined.

Although the complete physiological effects of the differential expression of the human opioid receptors under hypoxia remains unclear, based on the findings of this study it appears that in human neuronal cells KOR and MOR have some roles under hypoxic conditions. In human glial cells it appears that only KOR has a function under hypoxic conditions while DOR and MOR do not appear to be involved. In order to gain a better understanding of the opioid receptors' roles under hypoxia, the physiological implications of these receptors and the signaling pathways that control the differential expression of opioid receptors under hypoxia must be further examined by using opioid agonists and antagonists as well as inhibitors of various signaling pathways.

Conclusion

In summary, in this study human neuronal, glial, and a nonneuronal/nonglial model systems were created in order to study the effects of hypoxia. After a 24 hour hypoxia mimic treatment, it was noted that in both the neuronal and the glial model systems there is a differential expression of opioid receptor genes under hypoxic treatments. This effect was not noted in the nonneuronal/nonglial cell line.

In the neuronal model system it was shown that DOR expression remains constant over 24 hrs of hypoxic treatment, KOR expression is upregulated, and MOR is downregulated. In the glial model system it was noted that there was an upregulation of KOR expression after the 24 hr hypoxic treatment while no change in DOR or MOR expression was noted. There was no change in DOR, KOR, or MOR expression in the nonneuronal/nonglial cell line.

No human model system has been used to study the effects of hypoxia on opioid gene expression previously. In the literature great variation has appeared as to what opioid receptors play what roles during hypoxia in neuronal cells. This variation may occur because of species specific responses. Based on the results of this study it appears as though in human neurons KOR and MOR play an important role during hypoxia or ischemic events. Future studies will determine the physiological implications of these

receptors and the signaling pathways that control the differential expression of opioid receptors under hypoxia.

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