Original article

Morphological changes induced by opioid receptor agonist treatment of B50 neuronal cells cultured in hypoxia

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Abstract

Introduction: Hypoxia has been implicated in nerve cell deaths that occur in a variety of neurological disorders. Opioid receptor agonists have been shown to have some positive benefits on the nervous system. The aim of the present work was to investigate the effects of hypoxia and opioid receptor agonists' treatment on the morphology of B50 neuronal cell lines cultured in hypoxia. **Materials and Methods:** The B50 cells were cultured under normoxic conditions (21%O_2; 5% CO_2) as the control group and under hypoxic conditions (5%O_2; 5% CO_2) as the experimental group. Three opioid receptor agonists namely DAMGO (μ) DSLET (δ) and ICI-199,441 (κ) were administered to the cells for 48 hours as treatment against hypoxia after 48 hours of culture at doses of 10 μM, 50 μM and 100 μM respectively. Neuronal morphology, viability, proliferation and differentiation were assessed using same field morphological assessment. In addition lactate dehydrogenase (LDH) leakage, cellular proliferation and DbcAMP induced differentiation were also assessed. μ opioid receptor mRNA was assessed using RT-PCR. **Results:** The results showed groups of dead and degenerating B50 neuronal cells and some significant changes ($P<0.05$) in cellular proliferation, viability cellular differentiation. The levels of LDH leakage showed normal B50 cells (100%), hypoxic cells (587%), and treated cells with 100 μM DAMGO (μ) (143%), 50 μM DSLET (δ) (140%) and 50 μM ICI-199,441 (κ) (109%). The changes in morphology, LDH release, neuronal viability, proliferation and differentiation were shown to be dose-dependent between treated hypoxic B50 neurons in culture. **Conclusion:** The results indicate that opioid agonists have some potential benefits in the treatment of hypoxia-induced changes in neuronal B50 cells in culture.

Keywords: opioid receptor agonists, hypoxia, B50 Neuronal cells, neuromorphology, receptors, neurodegeneration.

1 Introduction

CNS neurons have been shown to be extremely sensitive to oxygen deprivation and indeed begin to die when oxygen supply is reduced or completely eliminated (ZHANG, QIAN, ZHAO et al., 2006). Decrease in the exchange of respiratory gases in the lungs or in their transport in the blood can prevent the supply of oxygen from meeting the metabolic demands of neuronal cells. Hypoxia can lead to oxidative stress which has been implicated in nerve cell death that occur in a variety of neurological disorders like stroke, dementia, multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD) (MAHER, 2001). Neuronal loss, and neuritic and cytoskeletal lesions represent the major dementia-associated abnormalities in Alzheimer's disease (DE LA MONTE, NEELY, CANNON et al., 2000). Hypoxia leads to metabolic cellular processes with the resultant oxidative species such as superoxide radical anion, hydrogen peroxide and lipid peroxides generated intracellularly (BENZI, GORINI, GHIGINI et al., 1994; CHEN and BUCK, 2000).

It is evident that hypoxia threatens brain function throughout the entire life span starting from early foetal age until death although, the molecular mechanisms involved in these processes are still not very well understood. Gibson and Huang (2002) used the oxidative processes in the brain as a biomarker of Alzheimer's disease and showed that diminished metabolism and excessive oxidative stress occurs in the brains of patients with this condition. It has been observed that oxidative stress can cause neurodegeneration associated with enhanced susceptibility to apoptosis due to activation of proapoptotosis genes, neurite retraction while hypoxia-like injury causes neuronal loss (BOSSENMEYER-POURIE, LIEVRE, GROJEAN et al., 2002). Both oxidative stress and hypoxic injury could contribute to neurodegeneration similar to that found in Alzheimer's disease (DE LA MONTE, NEELY, CANNON et al., 2000).

Rodrigo, Fernandez, Serrano et al. (2005), have shown that a reduction of oxygen supply to the brain may provoke, global brain ischemia or in defined cerebral territories, focal ischemia, depending on the area of the brain affected. It has been shown that ischemia results in severe focal and global damage of the brain tissues accompanied by biochemical and molecular alterations while hypoxia results in depletion of cellular and tissue energy and consequent death of the cells involved (FAHRIG and SOMMERMEYER, 1993; RODRIGO, FERNANDEZ, SERRANO et al., 2005).

Opioid receptors belong to the large super family of G protein-coupled receptors (GPCRs). The GPCRs have very important physiological roles in that they mediate the

physiological actions of majority of known neurotransmitters and hormones (HE, FONG, VON ZASTROW et al., 2002). Opioid receptors are important because they are activated by both endogenous opioid peptides and exogenous opiate drugs (HUGHES and KOSTERLITZ, 1983; HE, FONG, VON ZASTROW et al., 2002) and are regulated by multiple mechanisms including a well-characterised and highly conserved process involving receptor phosphorylation by G protein receptor kinase (FERGUSON, 2001; HE, FONG, VON ZASTROW et al., 2002). Delta opioid receptors have been shown to play an important role in delayed hypoxia preconditioning-induced neuroprotection against severe hypoxic injury (ZHANG, QIAN, ZHAO et al., 2006).

The aim of this study was to investigate the effect of hypoxia on the morphology of B50 neuronal cells and to evaluate the effect of opioid receptor agonists on neuronal morphology and viability during hypoxia using B50 neuronal cell lines in culture.

2 Materials and Methods

2.1 Neuronal culture

B50 cells were cultured and maintained in a normoxic incubator (21%O_2; 5% CO_2) and were designated as control cells. A second batch of cells were cultured under hypoxic condition (5%O_2; 5% CO_2) and designated as hypoxic experimental cells. All cells were cultured in 12-well culture plates for 48 hours and three highly potent opioid receptor agonists {DAMGO(μ), DSLET(δ) and ICI--199,441(κ)}, were administered to the cells as treatment against hypoxia for 48 hours at a concentration of 10 μM, 50 μM and 100 μM respectively. The cells were also treated with opioid antagonists (CTAP for μ, ICI-174,864 for δ and NOR for κ) to evaluate the effect of the antagonists on the action of the agonists.

2.2 Cellular viability, proliferation and differentiation

B50 cells were cultured in 12-well culture plates under normoxic and hypoxic conditions and were harvested after 0, 24, 48, 72, 96, 120 and 144 hours respectively with the 0 hour was regarded as the starting point of the culture. The cells were harvested by detaching them from the culture plates using trypsin-EDTA solution and were centrifuged at 2500xg for 5 minutes. The supernatants were decanted and cellular pellets collected. The cells were resuspended in the culture medium and examined using the Trypan Blue exclusion method. The total number of cells, viable cells and percentage viability were calculated.

Neuronal proliferation was studied using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay reagent obtained from Promega. The assay method was used according to the manufacturer's instruction. Briefly, 100 μl of the culture media containing the cells was pipetted into each well of the 96-well assay plate and then 20 μl of the Cell Titer 96® One Solution reagent was added into each well. The solutions were incubated for 2 hours at 37 °C. The absorbance was recorded at 490 nm using the Dynex MRX Model, 96 well microplate reader. The mean absorbance was then used to determine the number of viable cells in proliferation in normoxic and hypoxic B50 cells.

B50 cells were grown in culture media containing 1mM DbcAMP. The degree of neuronal differentiation was assessed after 96hrs in culture. The cells were assessed by random field strip counting of the number of differentiated cells under the microscope at 200 times magnification. The counted differentiated cells contained two neuritic processes (axon and dendrite), which were longer than cell body diameter according to the method of Richter-Landsberg and Jastorff (1986) and Oda, Kume, Katsuki et al. (2007). Each count was repeated two times and all the experiments were performed on three separate occasions.

2.3 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release which has been shown to be a reliable index of cellular injury (ZHANG, QIAN, ZHAO et al., 2006), was used to assess the level of neuronal injury in normal, hypoxic and treated cells, using a LDH kit and procedure from Sigma. The working solution of LDH assay cofactor was prepared by adding 25 ml of deionized, sterilized tissue culture water to bottle of lyophilized cofactors. The lactate dehydrogenase assay mixture was prepared by mixing equal amounts of LDH assay substrate, LDH assay cofactor and LDH assay dye solution. The LDH assay mixture was added at double the volume of the supernatant medium removed for assaying. The plates were covered with aluminium foil to protect it from light and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of one tenth volume of 1N HCL solution to each well. The absorbance was measured at a wavelength of 490 nm using Dynex MRX model of microplate reader and the result was calculated.

2.4 Data analysis

The parameters were assayed two times in triplicate in the normal, hypoxic and treated experimental groups of cultured B50 neuronal cells and the results are presented as mean ± standard deviation (SD). The Student's one tailed, paired t-test was used to test the level of significance and a P-value less than 0.05 was considered to be significant. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

3 Results

3.1 Morphological changes

Morphological changes were observed between the normal, hypoxic and treated B50 cells (Figures 1-4).The normoxic cells showed normal neuronal cell morphology (Figures 1 and 3a), while the hypoxic non-treated cells showed groups of dead and degenerating cells (Figures 2, 3b and 3c). The hypoxic, drug-treated B50 cells showed reduced cell death and healthier neuronal cells (Figures 4a-c).

3.2 Total cell count and viability study

The time course effect of hypoxia on total cell count and viability showed that after 48 hours of culture, the normal cultured B50 cells had $6.50\pm0.84 \times 10^6$; $6.01\pm0.85 \times 10^6$; 92.25±1.17; 90-93%, as total cell count, total viable cells, percentage viability and range of percentage viability

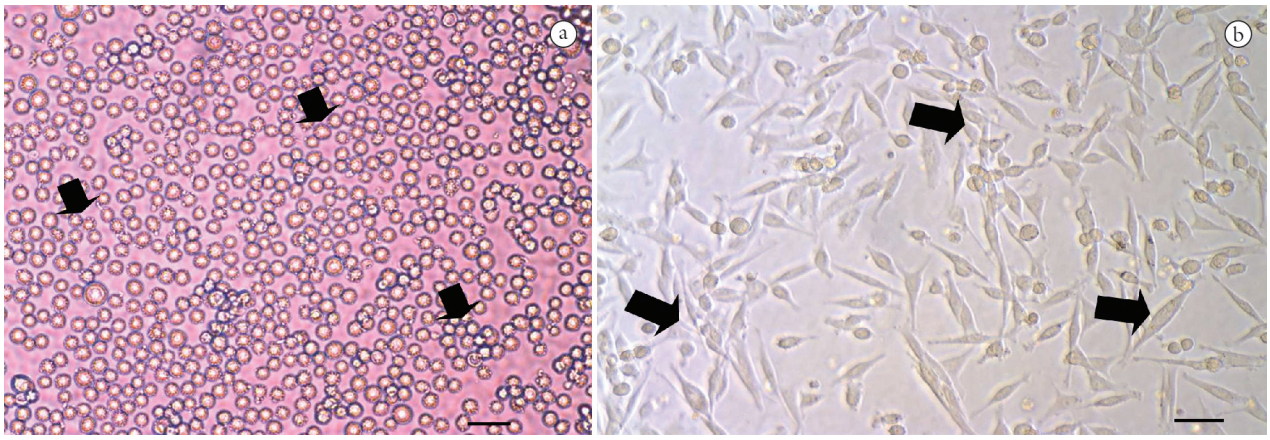

Figure 1. a) Representative of normal B50 cells at 0hrs of culture (21%O_2 and 5% CO_2) with normal cells (arrow). B50 cells was observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5 mm × 40 magnification. b) Representative of normal B50 cells at 24hrs of culture (21%O_2 and 5% CO_2) with normal cells (arrow). B50 cells was observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5 mm × 40 magnification.

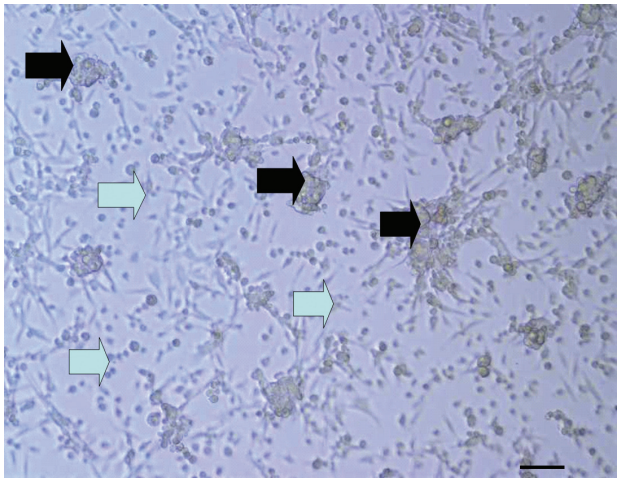

Figure 2. Representative of B50 cells in hypoxia at 24hrs of culture (5%O2 and 5% CO2) with some groups of degenerating cells (Black arrow) and some normal cells (Blue arrow). Cells was observed in six different plates with same field morphological method in a quadripoint analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mm × 20 magnification.

respectively, while the hypoxic cells had 5.34±1.45 × 10^6, 3.89±1.22 × 10^6, 72.01±3.39 and 67-76% as total cells count, total viable cells, percentage viability and range of percentage viability respectively (Table 1). The difference in the total cell count and total viable cells between the normoxic and hypoxic cells was statistically significant ($P<0.05$) and demonstrated that the longer the cells were cultured, the greater was the effect on the total cell count and viability of the B50 cells.

3.3 Neuronal proliferation

The result of the effect of opioid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia, showed the same pattern of proliferation between the normoxic cells and those cells treated with 100 µM DAMGO or 10 µM DSLET. There was a non-significant increase in cellular proliferation in the cells treated with 100 µM ICI-199,441, while the decrease in neuronal proliferation with 10 µM DAMGO, 100 µM DSLET and 10 µM ICI-199441 was not significant when compared with the normoxic cells (Table 2).

The treatment of opioid agonist and antagonist on cellular proliferation in cultured B50 cells in hypoxia showed the same trend of cellular proliferation for normal cultured cells and that of antagonist CTAP with agonist 100 µM DAMGO. The groups treated with different levels of antagonist/agonist showed different levels of decrease from the control but decrease in the proliferation was not significant (Table 3).

3.4 Neuronal differentiation

The result of the effect of hypoxia on neuronal B50 cell differentiation using DbcAMP in normal and hypoxic cultures, showed that the B50 cells in normal culture were significantly different from those differentiated under hypoxia ($p<0.05$) and from those cells raised under normoxic conditions but subjected to hypoxia after 48 hours of normal culture ($p<0.05$). The rate of neuronal differentiation was affected in B50 cells cultured in normoxic culture but transferred to hypoxia when compared with those cultured under hypoxia however the difference was not significant (Figure 5).

3.5 LDH release from opioid agonist treated B50 neuronal cells cultured in hypoxia

The LDH leakage from normal B50 cell (100%) was significantly increased ($p<0.05$) when compared with untreated hypoxic cells (587%) and treated hypoxic B50 cells with different concentration of opioid agonists. The LDH leakage from hypoxic untreated cells (587%) was 5 fold higher than that from normoxic cells, and cells treated with 100 µM DAMGO (143%); 50 µM DSLET (140%); 100 µM DSLET (143%); 10 µM ICI-199441 (113%) and

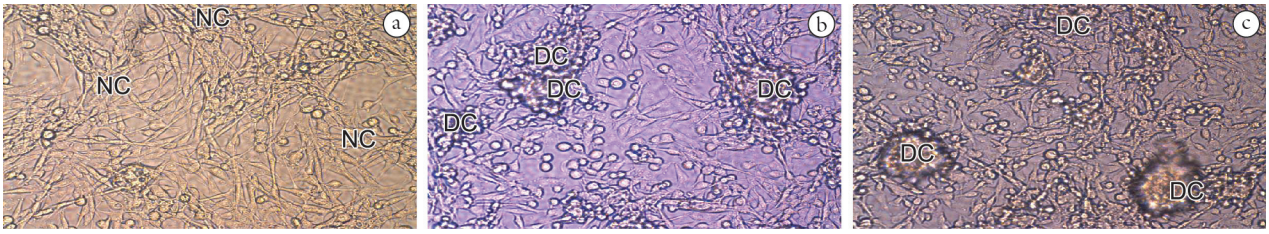

Figure 3. a) Hypoxic B50 cells treated with 10 mM ICI with many healthy cells (HC). Mg × 200. b) Hypoxic B50 cells non- treated (DC) with 50 mM DSLET with many dead and degenerating cells (DC). Mg × 200. c) Hypoxic B50 cells treated with 100 mM DAMGO with many dead and degenerating cells (DC). Mg × 200.

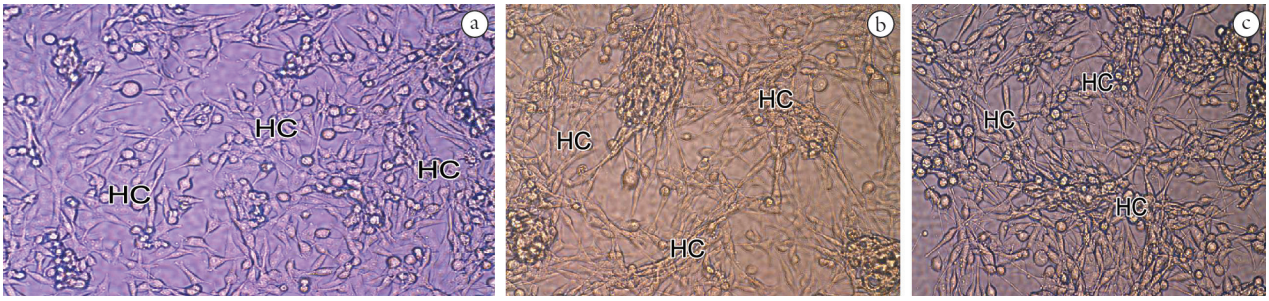

Figure 4. a) Hypoxic B50 cells treated with 10 mM ICI with many healthy cells (HC). Mg × 200. b) Hypoxic B50 cells treated with 50 mM DSLET with many healthy cells (HC). Mg × 200. c) Hypoxic B50 cells treated with 100 mM DAMGO with many healthy cells (HC). Mg × 200.

50 µM ICI-199441 (109%), and about three fold higher in cells treated with 10 µM DAMGO (338%) and 100 µM ICI-199441 (322%) (Table 4).

3.6 LDH release from opioid agonist and antagonist treated B50 cells cultured in hypoxia

The LDH release from hypoxic treated cells with different concentrations of opioid agonist in the presence of antagonist, showed that the LDH leakage from the hypoxic cells treated with opioid agonists/antagonists was increased between 2-8 folds when compared with the normal cultured B50 cells while the LDH release was about the same as in those treated with 50 µM DANGO/ICI-199441 (416%), 50 µM ICI-199441/NOR (529%) and 100 µM ICI-199441/NOR (665%) when compared with untreated hypoxic cells (Table 4).

Data show a comparative time effect of hypoxia on total cell count, total viable cells and percentage viability in B50 cells in culture using trypan blue exclusion method from three different experiments repeated twice (n=6). The normal cells cultured in (21% O_2; 5% CO_2) and hypoxic cells in (5% O_2; 5% CO_2), showed a significant increase.

Tables 2 and 3, show the effect of opioid agonist on cell proliferation of B50 cells cultured in normal (21%O_2;5%CO_2) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with the opioid agonists for 48hrs for a total of 96hrs of culture. The cells were quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 × 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) × 10^6 cells/ml and the absorbance of the CellTiter assay (n=5) was measured at 490 nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the CellTiter assay absorbance of the test groups against the known cell numbers from the standard.

4 Discussion

When grown under hypoxic conditions rat neuronal B50 cells display high levels of cell damage and death, a situation that is somewhat ameliorated upon treatment with different concentrations of opioid (mu, delta and kappa) agonists. The opioid receptor agonists (DAMGO, DSLET and ICI-199441), appear to provide protection to the B50 cells against the hypoxic damage. The observed increase in cellular proliferation could have arisen as a result of the opioid agonists mediating cellular activities such as proliferation and differentiation leading to increased cellular signalling through proliferation and differentiation activities. Tencheva, Praskova, Velichkova et al. (2005), had observed that opioid agonists acting via specific opioid receptors have been shown to influence neural cell proliferation, and Law, McGinn, Campbell et al. (1997), has shown that Chinese hamster ovary (CHO) cells transfected with δ-opioid receptor cDNA resulted in an agonist concentration-dependent potentiation of foetal calf serum-stimulated cell proliferation. The effect of µ, δ and κ opioid agonists on proliferation was abolished by the selective µ, δ and κ antagonists, suggesting that these effects were mediated through the µ, δ and κ opioid receptors in B50 cells in culture. Since this potentiation of cellular proliferation by the opioid agonists (µ, δ and κ) was antagonized by the selective antagonists, thus indicating that $G_{i/o}$ was involved in this action. However this opioid agonist potentiation of cellular growth and proliferation apparently was dependent on the level of agonist concentrations

Table 1. The time effect of hypoxia on total cell count and viability in B50 cells.

Time(hours)/Type of treatment (n=6)	Total cell count (x10^6) cells/ml	Total Viable Cells (x10^6) cells/ml	Viability (%)
0 HRS	**2.67±1.64**	**2.5±1.4**	**93.6±2.8**
Range	(1.2-5.0)	(1.1-4.7)	(89.5-97.4)
24 HRS			
NORMAL	**3.5±0.9**	**3.05±0.8**	**87.1±0.7**
Range	(2.4-4.6)	(2.1-4.0)	(86.1-88.1)
HYPOXIA	**2.8 ± 0.4**	**1.96±0.2**	**70.9±5.4**
Range	(2.1-3.1)	(1.6-2.3)	(63.3-76.2)
48HRS			
NORMAL	**6.5±0.8**	**6.0±0.9**	**92.3±1.2**
Range	(5.2-7.4)	(4.7-6.9)	(90.4-93.6)
HYPOXIA	**5.34±1.45**	**3.9±1.2**	**72.0±3.4**
Range	(3.4-7.5)	(2.3-4.7)	(67.7-76.0)
72HRS			
NORMAL	**18.8±5.1**	**17.1±4.4**	**91.2±3.8**
Range	(10.7-26.8)	(9.4-22.4)	(82.8-95.4)
HYPOXIA	**12.6±4.9***	**9.10±2.62***	**75.3±11.6**
Range	(5.3-21.2)	(4.3-12.8)	(60.4-94.4)
96HRS			
NORMAL	**35.4±12.9**	**32.9±12**	**93.3±2.3**
Range	(16.9-51.0)	(16.1-48.5)	(89.5-95.3)
HYPOXIA	**12.5±4.0***	**10.9±4.2***	**85.7±7.0**
Range	(8.19)	(6-17)	(76.1-93.3)
120HRS			
NORMAL	**6.8±2.7**	**6.3±2.6**	**92.2±2.8**
Range	(3.4-9.9)	(3.0-9.3)	(88.2-94.3)
HYPOXIA	**7.5±1.1**	**6.1±0.7**	**81.5±4.4**
Range	(6.1-8.8)	(5.3-6.7)	(76.1-86.9)
144HRS			
NORMAL	**46.5±9.3**	**44.0±9.1**	**94.5±0.8**
Range	(36.6-57.2)	(34.4-54.2)	(93.7-95.7)
HYPOXIA	**14.9±6.0***	**11.6±6.4***	**74.2±13.6**
Range	(9.1-26.4)	(5.1-24.0)	(55.6-90.9)

(*P<0.05 versus normoxic cells; Student's t-test) in total cell count and total viable cells at 72, 96 and 144 hours. The data expressed as mean ±SD (n=6) and Range.

Table 2. The effect of opioid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density	Calculated Value(x10^6)
Normal Cells no Drug	**1.5**	**20.00±0.47**
Hypoxic cells no drug	**0.35**	**7.00±0.12***
10uM DAMGO	**1.06**	**16.25±0.53***
100uM DAMGO	**1.51**	**20.00±0.54***
10uM DSLET	**1.52**	**20.00±0.70***
100uM DSLET	**1.32**	**17.60±0.27***
10uM ICI-199441	**1.36**	**17.70±0.34***
100uM ICI-199441	**1.6**	**21.52±0.45***

Data expressed as Mean ±SD (*P<0.05).

used in that, Law, McGinn, Campbell et al. (1997), had suggested that δ- and μ-opioid receptors in transfected CHO cells activate similar but divergent second messenger pathways, resulting in differential regulation of cell growth, proliferation and differentiation. This is in agreement with the findings from the present study which show that the increase in B50 cell proliferation induced by the opioid agonist was concentration-dependent. The results show that cells treated with the agonists DAMGO and ICI-199441 had increases in cell proliferation as the concentration of the agonist increases, and while those treated with DSLET demonstrated cell proliferation decreases with increase in concentrations of the agonist. The results show some differences in the overall activity of the agonists and the

Table 3. The effect of treatment of opioid agonist/antagonist on cellular Proliferation in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density	Calculated Value (x10^6)
Normal Cells no Drug	**1.5**	**20.00±0.47**
Hypoxic cells no drug	**0.35**	**07.00±0.12***
CTAP/Mu 50 μM	**1.43**	**18.50±0.63**
CTAP/Mu 100 μM	**1.56**	**20.10±0.83***
ICI/Dt 50 μM	**1.12**	**16.25±0.37**
ICI/Dt 100 μM	**0.96**	**14.90±0.10**
NOR/KP 50 μM	**1.38**	**18.75±0.68**
NOR/KP 100 μM	**1.28**	**17.50±0.42**

Data presented as mean ±SD; n=6; *P<0.05 versus normal, untreated cells; Student's t-test.

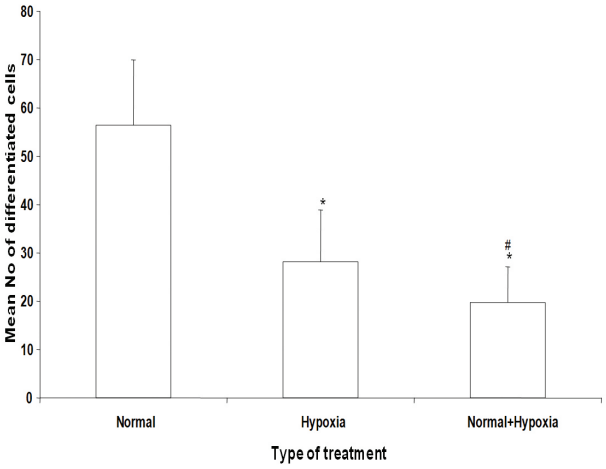

Figure 5. The effect of hypoxia on B50 cells differentiation using DbcAMP and random field assessment.

antagonists suggesting that multiple processes may be involved in the regulation of opioid receptors (TENCHEVA, PRASKOVA, VELICHKOVA et al., 2005).

The effects of opioid agonists' administration on LDH release from B50 cells in hypoxia showed significant reductions in LDH leakage in hypoxia-treated B50 cells in culture. The release of intracellular LDH into the extracellular fluids has been documented as a reliable indicator of neuronal injury and damage (ZHANG, QIAN, ZHAO et al., 2006). Results showed that there was a concentration-dependent decrease in LDH release from treated B50 cells in hypoxia. The cells treated with DAMGO and DSLET had LDH release decreased with increases in the concentration of the agonists while ICI-199441, had LDH release increased as the concentration of the agonist increased. The effect of the agonists in lowering the LDH release from B50 cells was blocked by the selective μ, δ and κ (CTAP, ICI-174864 and NOR) opioid antagonists suggesting that the effect of inhibiting the LDH release from B50 cells was mediated through the (μ, δ and κ) opioid receptors, indicating the involvement of $G_{i/o}$ in the protective activities of the opioid receptor agonists. There were some relative differences in the actions of the agonists and antagonists as shown in the treatment with 100 μM DSLET and 50 μM DSLET had LDH release lower than those of the agonist involved, thus indicating that there may be other systems and processes involved in the regulation of opioid receptor activities in B50 cells in culture. These multiple processes could be as a result of interaction between the various opioid receptor subtypes involved, since it has been shown that the opioid receptor subtypes share a close homology in their sequences (MAYER, KROSLAK, TISCHMEYER et al., 2003). There may be the involvement of endogenous opioids in the system, which has been shown to be released during hypoxia (ZHANG, MYCHASKIW and COLOHAN, 2000). Also there may be the involvement of interaction between the opioid agonists, the endogenous opioid and the endogenous cannabinoid system present in the cells (SCHOFFELMEER, HOGENBOOM, WARDEH et al., 2007). This is because Zani, Braida, Capurro et al. (2007) have demonstrated that cannabinoid inhibitor (AM 404) and cannabinoid agonist (Δ^9-THC) reduced neuronal damage caused by bilateral carotid occlusion in gerbils and that this protection was mediated through an interaction between CB_1 and opioid receptors.

Hong, Gibney, Esquilin et al. (2004) have shown that although δ-opioid receptor inactivation reduces LDH activity in normoxic neurons, neither δ-opioid receptor activation nor inactivation induces any appreciable effect on LDH activity in the cortical neurons under hypoxia and as such, other signal transduction pathways such as the glycolytic pathway may play an important role in neuroprotection. Zhang, Gibney, Zhao et al. (2002) had shown clearly that the modulation of δ-opioid receptor, but not μ- and κ-opioid receptors, plays a major role in neuroprotection in both normoxic and hypoxic environments. Though the mechanism involved in this neuroprotection is not well understood, Zhang, Gibney, Zhao et al. (2002) speculated that this phenomenon may be linked to the role of δ-opioid receptors in selective regulation of G proteins, excitatory neurotransmitter release, glutamate receptor stimulation, and Ca^{2+} homeostasis. The activation of opioid receptor subtypes may have a wide range of clinical applications in treating and preventing acute and chronic hypoxia-related impairments. Further research in this area is necessary to develop a better understanding of opioid receptor activities and the pathways involved in their ability to ameliorate neuroprotection.

The use of LDH release as an index of neuronal injury does not provide direct assessment of the percentage of neurons injured and/or dead in culture. Hence the use of additional experimental approaches which include morphological assessments, live/dead trypan staining for total cell count and viability and proliferation assay was used

Table 4. The effect of opioid agonist/antagonist treatment on LDH release in cultured B50 cells in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10 μM	0.699±0.421	338±42.1*
DAMGO/CTAP10 μM	0.726±0.335	351±33.5*
DAMGO(μ)50 μM	0.344±0.210	167±21.0
DAMGO/CTAP50 μM	0.535±0.252	259±25.2*#
DAMGO(μ)100 μM	0.296±0.112	143±11.2
DAMGO/CTAP100 μM	0.752±0.305	364±30.5*#
DSLET(δ) 10 μM	0.346±0.201	167±20.1
DSLET/ICI-174864 10 μM	0.530±0.256	256±25.6*#
DSLET(δ) 50 μM	0.290±0.112	140±11.2
DSLET/ICI-174864 50 μM	0.859±0.345	416±34.5*#
DSLET(δ) 100 μM	0.296±0.221	143±22.1
DSLET/ICI-174864 100 μM	0.644±0.225	312±22.5*#
ICI-199441(κ) 10 μM	0.234±0.221	113±22.1
ICI-199441/NOR 10 μM	1.698±0.501	821±50.1*#
ICI-199441(κ) 50 μM	0.224±0.223	109±22.3
ICI-199441/NOR 50 μM	1.093±0.552	529±55.2*#
ICI-99441(κ)100 μM	0.666±0.251	322±25.1
ICI-199441/NOR 100 μM	1.375±0.446	665±44.6*#

Data presented as means ±SD; n=6; *P<0.05 versus normal B50 cells; #P<0.05 versus agonist treated hypoxic cells, Student's t-test.

to assess neuronal injury more directly. It has been shown that during hypoxia, glutamate is expelled from neurons leading to over-stimulation of glutamate receptors and subsequent injury and death of the neurons (HADDAD and JIANG, 1993; NYAKAS, BUWALDA and LUITEN, 1996). Since Zhang, Gibney, Zhao et al. (2002) had shown that because glutamate receptor expression increases during development, and sensitivity to glutamate excitotoxicity increases with neuronal maturation, the observed differences in hypoxic susceptibility between the neuronal ages in this study may be associated with the developmental increase in glutamate toxicity (CHOI and ROTHMAN, 1990).

This study has demonstrated that stimulation of opioid receptors confers some protection by reducing neuronal cell injuries and deaths after treatments in hypoxic conditions but the benefit is reduced substantially with prolonged exposure and higher concentrations of the drugs. This supports the finding that the longer the neurons stayed in the culture media both in the normal and in the experimental hypoxic groups, the greater the extent of the neuronal cell injuries and death. A possible explanation for this phenomenon is that prolonged hypoxia may cause a significant release and accumulation of endogenous glutamate which causes glutamate-induced toxicity and hence death of the cells (NYAKAS, BUWALDA and LUITEN, 1996). Another alternative explanation is that prolonged hypoxia causes the release and accumulation of endogenous opioids which saturates opioid receptors in these neurons. Hence the positive effect of the opioid agonists decreases with the increase in the time the cells stay in the medium which may result in desensitization of the receptors and the reduced effect of the action of the drugs (MAO, SUNG, JI et al., 2002; WALLACE, DODSON, NATH et al., 2006).

It has been shown that in response to short-term hypoxia, the level of enkephalins, the endogenous agonists for opioid receptors sharply increases (ZHANG, GIBNEY, ZHAO et al., 2002; KHASABOVA, SIMONE and SEYBOLD, 2002). This showed that cortical neurons may release opioids during normal function and in response to hypoxic stress as a mechanism of self-protection against injury (MA, QIAN, GHASSEMI et al., 2005; WALLACE, DODSON, NATH et al., 2006). Zhang, Gibney, Zhao et al. (2002) showed that because of high levels of endogenous opioids which may already be present in the culture media after prolonged exposure to hypoxia, adding more agonist may not increase the protection. On the other hand, desensitization of the receptors may have occurred due to prolonged treatment of the cells with the agonists in conjunction with the endogenous opioid release during chronic hypoxia. Zhang, Qian, Zhao et al. (2006) have shown that cortical neurons are highly susceptible to opioid receptor inhibition, which causes serious neuronal injury especially during hypoxic stress.

It has also been shown that opioid receptor agonists have the ability to reduce neuronal over-stimulation by blocking glutamate excitation (ZHANG, GIBNEY, ZHAO et al., 2002), and this could be the situation with the protection proffered by the μ-, δ- and κ-opioid agonists used in this study. This mechanism of cellular regulation may be utilised during normal cell functioning and in response to environmental stress like hypoxia (ANDERSEN, 2004). One possible explanation for the differences in neuroprotective abilities and actions of these three different opioid receptors is that the individual opioid receptors regulate different effectors thereby eliciting different responses. Connor and Christie (1999) had proposed that because of the common

features of opioid receptors, the selectivity of these receptors for eliciting specific pathways does not lie in the differences between each opioid receptor subtype but in their association with other divergent types of G proteins. It was observed that each opioid receptor subtype also preferentially couples to specific G proteins apart from the $G_{i/o}$ proteins that they generally couple (ZHANG, GIBNEY, ZHAO et al., 2002). Examples of this preferential coupling include as seen in δ-opioid receptors which are more efficiently coupled to $G\alpha_{16}$ protein than either μ- or κ-opioid receptors (LEE, JOSHI, CHAN et al., 1998). Also μ-receptor agonist (DAMGO) has been reported to have more selective coupling to $G\alpha_{i1}$ and $G\alpha_{oA}$ –linked opioid receptors than other opioid agonists (SAIDAK, BLAKE-PALMER, HAY et al., 2006). This shows that the opioid receptor agonists have selective activation of G-proteins in response to opioid receptor activation. This preferential coupling to other G protein subtypes may be the reason for the observed differences in the effect of the opioid agonists on the B50 cells treated in hypoxia. The activation of μ-, δ- and κ-opioid receptors by the agonists may have a wide range of clinical implications in the treatment of hypoxia-related impairments and may form the basis for the development of new neuroprotective and therapeutic drugs useful for the treatment of stroke and other neurodegenerative pathologies.

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