Expression of estrogen receptor α and β in rat astrocytes in primary culture: effects of hypoxia and glucose deprivation

Maie D. Al-Bader, Slava A. Malatiali, Zoran B. Redzic

Department of Physiology, Faculty of Medicine, Kuwait University, Kuwait.

Corresponding author:

Dr. Zoran Redzic

Department of Physiology, Faculty of Medicine, Kuwait University, P.O. Box 24923 Safat, 13110, Kuwait.

Fax number: +965 25338937

Telephone number: +965 25319593

E-mail: redzic@hse.edu.kw

Short title:

Effects of hypoxia on estrogen receptor expression in astrocytes
Summary
Estrogen replacement therapy could play a role in the reduction of injury associated with cerebral ischemia in vivo, which could be, at least partially, a consequence of estrogen influence of glutamate buffering by astrocytes during hypoxia/ischemia. Estrogen exerts biological effects through interaction with its two receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which are both expressed in astrocytes. This study explored effects of hypoxia and glucose deprivation (HGD), alone or followed by 1h recovery, on ERα and ERβ expression in primary rat astrocyte cultures following 1h exposure to: a) 5%CO₂ in air (control group-CG); b) 2%O₂ / 5%CO₂ in N₂ with glucose deprivation (HGD group-HGDG); or c) the HGDG protocol followed by 1h CG protocol (recovery group-RG). ERα mRNA expression decreased in HGDG. At the protein level, full-length ERα (67 kDa) and three ERα-immunoreactive protein bands (63, 60 and 52 kDa) were detected. A significant decrease in the 52 kDa band was seen in HGDG, while a significant decrease in expression of the full length ERα was seen in the RG. ERβ mRNA and protein expression (a 54 kDa single band) did not change. The observed decrease in ERα protein may limit estrogen-mediated signalling in astrocytes during hypoxia and recovery.

Keywords: Astrocytes; Estrogen receptor; Hypoxia; Glucose Deprivation; Ischemia; Brain
Introduction

Mild to moderate reduction in cerebral blood flow, as seen in the penumbra during transient focal cerebral ischemia or in the whole brain during hypoperfusion, could trigger events through several signaling pathways, including activation of AMP-activated protein kinases (Li and McCullough, 2010), which could lead to apoptotic cell death (Hossmann, 2008). A well established theory suggests a role for glutamate accumulation in the interstitial fluid (Mergenthaler et al., 2004), which binds to ionotropic receptors, causing an increase in Ca\(^{++}\) entry into the cells (Broughton et al., 2009) and facilitating an accumulation of intracellular Ca\(^{++}\) that triggers the intrinsic apoptotic pathway (Dirnagl et al., 1999). Thus, the amount of glutamate released in the early stages of an ischemic event could be of considerable importance in determining the extent of apoptotic cell death in the penumbra (Hertz, 2008). Astrocytes play a key role in buffering extracellular glutamate during hypoxia / ischemia (Hertz, 2008) because these cells have a considerable potential to take up glutamate from the extracellular space through the action of glutamate transporters (Chao et al., 2010) and can accumulate glutamate for a considerable time during an ischemic event (Haberg et al., 1998, Haberg et al., 2001).

Estrogen can influence glutamate buffering by astrocytes during brain ischemia but the results are conflicting: administration of estrogen to rat astrocytes in primary culture significantly increased expression of glutamate transporters at mRNA and protein levels and this was accompanied by elevated l-glutamate uptake (Sato et al., 2003). However, another study revealed that estrogens down-regulate l-glutamate uptake activity of astrocytes via membrane ER\(\alpha\) (Billeci et al., 2008). Most studies support a role for estrogen replacement therapy in the reduction of ischemic brain injury (Billeci et al., 2008). Female gerbils experienced less severe
brain damage following severe incomplete hemispheric ischemia produced by unilateral carotid occlusion than did male gerbils (Hall et al., 1991). Exogenous administration of 17β-estradiol reduces infarct volume following middle cerebral artery occlusion (MCAO) in ovariectomized female rats (Dubal et al., 1998, Rusa et al., 1999). Exogenous estradiol, when administered at the onset of MCAO, also showed to be protective in male rats (Toung et al., 1998). The doses of 17β-E2 used in these studies did not influence cerebral blood flow, implying that the neuroprotective effect of 17β-E2 occurs directly at the level of the brain rather than involving the vasculature (Rusa et al., 1999).

Estrogen regulates gene expression through interaction with estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which are both expressed in the brain (Shughrue et al., 1997). ERs are selectively expressed in some types of neurons, like Purkinje cells (Ikeda and Nagai, 2006) and inhibitory interneurons in hippocampus, but not in the pyramidal neurons or in granule cells (Prewitt and Wilson, 2008). Estrogen interacts directly with apoptotic signalling processes in neurons (Meda et al., 2000). Astrocytes express both ERα (Huppmann et al., 2008, Bondar et al., 2009, Yi et al., 2009) and ERβ (Huppmann et al., 2008) receptors. However, no data exist so far regarding the responses of these receptors upon the conditions that mimic conditions during short cerebral ischemia and recovery. The aim of our study was to assess the expression of ERα and ERβ in rat astrocytes in primary culture at the transcript level, by real time-polymerase chain reaction (ReT-PCR), and at the protein level by immunoblotting and to explore changes in the expression of ERα and ERβ after 1h HGD alone or followed by 1 hour recovery.
Methods

This study was approved by the Medical Research Council of the Faculty of Medicine, Kuwait University. Animals were maintained and handled in the Animal House, Health Science Centre, and all procedures were conducted in accordance with the animal care guidelines in this institution and in accordance with the Guidelines on Laboratory Animal Care.

Chemicals and solutions

Two days old female Sprague Dawley pups were used to initiate primary cultures of astrocytes; cortices from four pups were used to initiate primary cultures in four 25cm² flasks or in four 6-well plates. The following solutions were used: (a) phosphate buffered saline (PBS), containing (mmol/L): NaCl 136.9, KCl 2.7, KH₂PO₄ 1.5, Na₂HPO₄ 7.7, pH 7.4; (b) tissue buffer, which was Ca²⁺ and Mg²⁺ free Hank’s Balanced Salt Solution (GIBCO, Carlsbad, USA) containing (mmol/L) NaCl 140, KCl 5.4, Na₂HPO₄ 0.67, KH₂PO₄ 0.5, glucose 17, sucrose 12, HEPES 20 and penicillin/gentamicin 100 U/ml; pH 7.4; (c) Dulbecco’s modified Eagle’s medium (DMEM) containing 7 mM D-glucose, L-glutamine and Na⁺-pyruvate. This medium was prepared by adding an appropriate amount of a high glucose DMEM (GIBCO, catalogue number 11995-040) and 10% (v/v) Fetal Calf Serum (FCS) (Invitrogen) to a low glucose DMEM (GIBCO, 11885-084); (d) low glucose, pyruvate-free DMEM, containing L-glutamine and 1.5 mM D-glucose, which was prepared by adding appropriate amount of a high glucose, pyruvate-free DMEM (Gibco, 31053-028) and 10% FCS to a glucose and pyruvate free DMEM (Gibco, 11966-025). It also contained 10 mM 2-deoxy-glucose (2-DG) (Sigma), an analogue of D-glucose that cannot be metabolized beyond the 2-deoxy-glucose 6-phosphate, thereby competitively inhibiting glycolysis (e) homogenisation buffer, containing 10 mM Tris, 1.5mM EDTA, 10% (v/v) glycerol, 1 µg/ml leupeptin, 100 µg/ml bacitracin, and 1 µg/ml pepstatin.
Reverse-transcription PCR buffers, enzymes and reagents were supplied by Invitrogen. Real time PCR (ReT-PCR) reagents were purchased from Applied Biosystems, USA. A rabbit polyclonal antibody that recognized a peptide corresponding to amino acids 1-150 of human ERβ (H-150; Santa Cruz Biotechnology, Inc., CA, U.S.A), which cross-reacts with rat ERβ (Qiu eta l., 2005) and a mouse monoclonal anti-ERα raised against the amino acid residues 151-165 of human ERα (MAB463, Millipore, Ann Arbor, MI, U.S.A.) that cross-reacts with rat ERα (Ali et al., 1993, Jesmin et al., 2002) were used. For the detection of actin, a mouse monoclonal IgG1 anti-human actin antibody was used (Millipore, USA). PVDF membranes were obtained from Amersham Biotech (Buckinghamshire, UK). General laboratory chemicals were purchased from Merck (Dagenham, U.K.) and all fine chemicals were obtained from Sigma (Poole, U.K.).

Treatment of animals, tissue extraction and initiation of culture

A total of thirty 1-2 day old Sprague-Dawley rats were used in this study. Efforts were made to reduce the number of animals used and to minimize animal suffering. Rats were sacrificed by cervical dislocation, skull was opened, dura mater was dissected and brain immersed in ice-cold tissue buffer. The remaining meninges and choroid plexuses were carefully removed from brain surface under a magnifying glass, cerebral cortices were separated and the white matter carefully removed. The remaining cortices were kept in tissue buffer on ice.

Primary cultures were produced as described earlier (Dolman et al., 2005). Briefly, the isolated tissue was incubated into warm (37°C) tissue buffer containing 0.25% trypsin (Gibco) for 30 min. Trypsin was inactivated by adding a serum-containing DMEM, tissue was triturated through a pipette tip for 1 min and the cell suspension was filtered through 80 micron cell strainer (BD Falcon, San Jose, USA). The cell suspension was centrifuged at 600 x g for 5 min, the pellet was re-suspended in DMEM and placed in 25 cm² cell culture flasks (Nunc, Rochester,
NY, USA) pre-treated with poly-l-lysine (0.01%) for 15 min. The cell cultures were incubated at 37°C in water saturated air with 5% CO₂ and medium was changed every 2 days.

Primary cultures were purified as described by Rist et al. (1997). Briefly, at sub-confluence, cell contaminants on the top of the monolayer were separated from astrocytes by shaking for 24h at 110 rpm at 37°C on a Stuart mini orbital shaker (Stuart Scientific, Stone, U.K.) at 37°C. Further purification of the cell cultures was achieved at confluence (day 11-12 after plating) by adding cytosine arabinoside (0.01 mM) to the culture medium for 3-4 days. Since at this stage astrocytes reach confluence, they do not divide further, so cytosine arabinoside affects only contaminating dividing cells and no effects of this treatment on astrocytes morphology could be observed under the phase contrast microscopy, which was in agreement with the results reported before (Rist et al., 1997). Cells were used for experiments 18-20 days after seeding. It has been shown earlier that this procedure produces astrocytes primary cultures, with reported purity of >90% (Rist et al., 1997) and >95% (Romero et al., 1995), which was estimated counting cells that were stained with antibodies for glial fibrillary acidic protein (GFAP) (Rist et al., 1997, Romero et al., 1995) and high expression of GFAP mRNA (Dolman et al., 2005).

**Treatment of the cells and experimental groups**

In this study primary cultures were divided into three experimental groups: control group (CG): primary cultures were incubated in DMEM and exposed to 5% CO₂ in air during the course of experiment; hypoxia and glucose deprivation (HGDG) group: primary cultures were transferred into a handmade transparent glove-box chamber (manufactured in the Health Science Centre workshop) with 2% O₂, 5% CO₂ in N₂; the composition of gases was constantly monitored by a NormocapOxy gas analyzer (Datex-Ohmeda, GE Health Care); cultures were
washed with PBS and low glucose, pyruvate-free DMEM with 10 mM 2-DG was added to primary cultures. Cells were kept under these conditions for 1h. All solutions were kept in the chamber for 12h prior to experiments in order to equilibrate gases; recovery groups (RG): primary cultures were exposed to the same protocol as described for the HGDG and then were exposed to the same conditions as flasks from the CG for 1h.

**Expression of ERα and ERβ at the transcript level**

*RNA extraction and reverse transcription*

At the end of each protocol, total RNA was extracted with Trizol (Invitrogen) and dissolved in diethylpyrocarbonate-treated water at 55°C. RNA concentrations were determined by absorbance at 260 nm and 2 μg portions were used for reverse transcription. Intron-spanning primers were used in the study that excluded interference from genomic DNA in the results; however, all RNA samples were treated with DNAase, 4 units/sample (Invitrogen, Carlsbad, USA) prior to reverse transcription. Two μg portions were used for first-strand cDNA synthesis, which was performed with MuMLV reverse transcriptase (Invitrogen) using random hexamers, according to the manufacturer’s instructions (RT+ samples). Negative controls (RT- samples) were processed under the same conditions, with the reverse transcriptase replaced with water.

*Real-time PCR*

The ReT-PCR reaction was carried out in a ReT-PCR system (Applied Biosystems, model 7500), using the following gene expression assays, which had intron-spanning primers and hydrolysis probes labeled with 6–carboxyfluorescein (FAM) as a reporter dye and 6-carboxy-tetramethyl-rhodamine (TAMRA) as a quencher dye: assay ID Rn01430446_m1, expected amplicon length 73 bp for ERα, assay ID Rn00562610_m1, expected amplicon length 89 bp for ERβ and assay ID Hs99999901-sl, expected amplicon length 187 bp for 18S ribosomal
RNA. The PCR reactions were prepared using the TaqMan universal master mix (Applied Biosystems) and were carried out in a 96-well plate that was sealed with an adhesive transparent foil, employing the thermal profile that was suggested by the manufacturer: 2 min at 50°C (1 cycle); 10 min at 95°C (1 cycle) and then 15 s at 95°C and 1 min at 60°C for 40 cycles. In some cases, a 10 μL sample of the PCR product was analyzed by electrophoresis on a 2% agarose gel that contained ethidium bromide in order to verify that product sizes were as expected.

In some cases, a series of dilutions of cDNA was made (2-64 folds) and then ReT-PCR reactions were carried out as outlined above. The obtained Ct values for ERα, ERβ and 18S were then plotted against the log dilution and the coefficient of linearity and the slope were calculated and efficiencies of the PCR were estimated using the following equation (Pfaffl, 2001):

$$E = 10^{-1/slope} \quad (1)$$

Under theoretically ideal circumstances, in every PCR cycle the amount of product should be doubled giving the ideal efficiency of 2.00.

The relative gene expression was calculated using the efficiency corrected calculation model for multiple samples and based on one reference gene (Pfaffl, 2001). All samples were done in duplicates and the ΔCt value for each sample was determined by subtracting the average 18S Ct value from the average ERα or ERβ Ct value. The fold change was then calculated as:

$$Ratio = \left( \frac{E_{target}}{E_{reference}} \right)^{\Delta Ct_{(control-sample)}}$$  \quad (2)

This equation expresses fold change (ratio) of ER genes in the HGDG and RG versus the CG in comparison to the 18S gene. $E_{target}$ and $E_{reference}$ are the efficiencies of target and reference
gene transcripts, respectively. In every experiment, 3 flasks for the same batch were exposed to experimental protocols (one in each group: CG, HGDG, RG); the ratio was calculated for the HGDG, RG; finally, the average fold change ±SD in the HGDG and the fold change in the RG (both relative to the CG) were calculated from 3-4 different batches using the equation 2.

Expression of ERα and ERβ at the protein level

Cellular monolayers from CG, HGDG and RG were washed twice with ice-cold saline, detached from the surface by Trypsin-EDTA solution and then homogenized in ice-cold homogenization buffer using a Polytron homogenizer, to yield a 5% (w/v) homogenate.

Twenty µg of protein was loaded per lane, and separated using 7.5% SDS-PAGE. The monoclonal ERα antibody was used at a dilution of 1:500 in 10% non-fat dry milk in Tris Buffer Saline-Tween20 (TBS-T, containing 20 mM Tris, 137 mM NaCl, pH 7.6 and 0.1% v/v Tween20), the polyclonal ERβ antibody was used at a dilution of 1:100 in 10% non-fat dry milk in TBS-T and the anti-actin antibody was used at a dilution of 1:50,000 in 5% non-fat dry milk in TBS-T. Western blot analysis and immunodetection of total ER proteins together with analysis of protein sizes was performed as described earlier (Al-Bader et al., 2008). In preliminary experiments, the primary antibody was omitted and filters were incubated with secondary antibody only. No bands were detected with this antibody. Once membranes were probed with the ER antibodies, they were stripped and re-probed with anti-actin antibody.

Statistical Analysis

Data was tested for statistical significance using unpaired T-test (Epicalc 2000). In some cases, as indicated in the Results section, a difference between the obtained Ct values in experimental groups was tested by ANOVA.
Results

Effect of hypoxia and glucose deprivation, and recovery on the expression of ERs at the transcript level

A plot of log dilution against the Ct values for each pair of primers is shown in Figure 1. Those data points revealed a linear increase of Ct values with the Pearson’s coefficient r being 0.81, 0.85 and 0.82 for ERα, ERβ and 18S ribosomal RNA, respectively. The slopes of the corresponding lines were estimated and the PCR efficiencies, calculated using equation 1, were 2.48, 2.03 and 1.93 for ERα, ERβ and 18S ribosomal RNA respectively.

Table 1 shows the estimated average Ct values of ERs in rat astrocytes in the CG, HGDG group and RG. The Ct values in all negative controls (RT- samples) were above 40 (not shown). Initially, three housekeeping genes were used: glyceraldehyde 3-phosphate dehydrogenase, beta actin and 18S ribosomal RNA. There was no significant difference in the obtained Ct values between the CG, HGDG and RG for either of those genes (p>0.05 by ANOVA); however, the intra-essay and inter-essay variability, calculated for each gene as explained by Pfaffl (2001), revealed that 18S ribosomal RNA had the most consistent expression in all experimental groups. Therefore, this RNA was used for calculations of the relative expression of ER mRNAs and the Ct values in RT+ samples are shown in Table 1.

There are several splice variants of ERα mRNA in primates and mice, but no known splice variants in the rat brain; there are four known splice variants of ERβ in the rat brain: ERβ1, ERβ2, ERβ1δ3, and ERβ2δ3 (Petersen et al., 1998, Price et al., 2000). However, there were no splice variants or single-nucleotide polymorphism positions documented in transcript and single-nucleotide polymorphism databases in the regions of the genes corresponding to the
portions of the cDNA to be amplified in this study according to the ENSEMBL entries. Thus, use of intron-spanning primers could not affect the quantification data in this study.

The obtained data from the CG indicated that mRNAs encoding ERα and ERβ were $10^4$ and $10^6$ fold less abundant than 18S ribosomal RNA, respectively and that mRNA encoding ERβ was $\sim10^2$ fold more abundant than mRNA encoding ERα. The obtained fold-change values indicated that HGD protocol caused a 50% decrease in the amount of mRNA for ERα, when compared to the CG, while the amount of mRNA for ERβ did not change. In the RG, the amount of mRNAs for ERα and for ERβ did not change when compared to the CG.

**Effect of hypoxia, glucose deprivation and recovery on the expression of ERs at the protein level**

Immunoblots of the astrocyte protein extracts revealed the presence of ERα and ERβ at the protein level. Rat beta actin, which was used as an internal control and to prove equal loading of protein samples, migrated with mobility comparable to an apparent size of 42kDa (Figure 2A).

In addition to the full length ERα, three splice variants (so-called ERα-inmmunoreactive proteins) have been detected in rat earlier; they migrated during electrophoresis with apparent molecular weights from $\sim50$ kDa to $\sim65$ kDa (Pasqualini et al., 2001). This finding was also confirmed in later studies that detected that ERα in the rat brain and in astrocytes existed as the full length ERα (apparent MW 66-67 kDa) and at least one ERα-inmmunoreactive protein with apparent MW of 52 kDa (Bondar et al., 2009). In our study, immunoblotting using primary antibodies against ERα, revealed presence of two major bands in astrocytes that corresponded to the full length ERα ($\sim67$ kDa band) and a 52 kDa ERα-inmmunoreactive protein (Figure 2A). In addition to those, two minor ERα-inmmunoreactive protein bands that migrated with apparent
MW of ~60 kDa and 63 kDa were also detected. HGD protocol caused a significant decrease in the expression of the 52 kDa band compared to the expression in the CG (Figure 2B), while the expression of other bands did not change significantly. In the RG 1h there was a significant decrease in the expression of 67 kDa band when compared to the expression in the CG (p<0.05), while the expression other bands did not change.

The predicted MW of a wild type ERβ was 54 kDa; when primary antibodies against ERβ were used, presence of a single band that migrated with an apparent size of 54 kDa was detected. However, it appears that HGD and recovery protocols did not affect expression of this protein, since the density of this band, relative to density of actin bands, did not differ between HGDG / RG and the CG (Figure 3B).
Discussion

Experimental conditions used in this study, hypoxia and glucose deprivation mimic, at least partially, conditions that may occur during hypoperfusion in the brain (i.e. in the penumbra during focal cerebral ischemia or during a transient global cerebral ischemia, like hypotension). Using the same in vitro model, it has been shown that there was ~50% depletion in cellular ATP in the HGDG after 1h, which partially reversed after 1h recovery; also there was an increase in ATP and adenosine concentration in the cell culture medium, while cell viability did not change and apoptosis was generally rare (Redzic et al., 2010).

This study revealed that amount of mRNA for both ERα and ERβ in astrocytes in the CG was low, giving the Ct values of ~30, with mRNA for ERβ being about $10^2$ fold more abundant than mRNA for ERα. This may indicate a low turnover rate of ERs in astrocytes and is in accordance with a finding that ERs’ mRNAs are expressed at very low abundance in rodent neurons (Wilson et al., 2008). It has been shown that deletion of ERα (in ERα knockout mice) abolished the protective actions of estradiol in the brain during permanent cerebral ischemia; while in the absence of ERβ (in ERβ knockout mice) the ability of estradiol to protect brain injury during permanent cerebral ischemia was preserved (Dubal et al., 2001). Following brain ischemia ERα was reactivated in rat neurons after 3-6 hours (Dubal et al., 2006). This expression pattern of ER gene expression could be a compensatory mechanism to prevent cell death (Wilson and Westberry, 2009). This study revealed changes in the ERα mRNA, which decreased after 1h HGD protocol, so it was less abundant in the HGDG than in the CG, while no change in the expression of ERβ was observed; after the recovery period the amount of mRNA for ERα in was not different from the amount in the CG.
For ERα and β, a number of variant transcripts have been described (Kuo et al., 2009). However, many of these transcripts are created by skipping internal exons, so they retain the same reading frame as the full-length transcript; thus, only few corresponding variant proteins have been detected (Taylor, 2010). It has been shown previously, using anti-full length ERα antibodies, that ERα protein existed in rat as full length protein and three splice variants with MW ranging from ~50 to ~65 kDa (Pasqualini et al, 2001). We found in this study that ERα protein existed in astrocytes as the full length ERα, which migrated with apparent MW 67 kDa, and an ERα-immunoreactive protein, which migrated with apparent MW of 52 kDa and as two additional splice variants, which migrated with apparent MWs of 60 and 63 kDa. It was believed initially that ERs were purely nuclear receptors, but it was found that 5-10% of total cellular ERs existed as proteins with various amount palmitoylated cysteine residues in the plasma membrane and that these proteins mediate membrane-initiated steroid signaling (MISS). MISS depends on coactivation of metabotropic glutamate receptors, causing estradiol-induced calcium influx (Kuo et al., 2009). However, only the full-length ER could bind estradiol and trigger MISS causing increased calcium influx (Bondar et al., 2009). This study showed that 1h HGD protocol did not affect the amount of the full length ERα; however, after 1h recovery, the level of this protein was significantly reduced when compared to control. Since it is believed that estrogen signaling could be protective during cerebral ischemia / hypoxia, our finding indicates a possibility that estrogen could exert limited protective effects on astrocytes during short the recovery phase because of the reduced amount of the full length ERα. HGD protocol caused significant reduction in the level of a 52 kDa ERα-immunoreactive protein, which is likely to be a splice variant of the full length ERα. Also, this protein could be a proteolytic / degradation product, although in this
study, every precaution was taken to prevent degradation of proteins, so all procedures were performed at 4°C and in the presence of several protease inhibitors.

Little is known about the molecular mechanism that could be involved in a change of ERα expression during HGD and recovery treatments. Hypoxia can alter the amount of mRNA for various proteins via hypoxia inducible factor-1 (HIF-1), so a hypothesis could be established that the observed changes in ERα could be HIF-mediated. HIF-1α is not found in astrocytes under normoxic conditions due to ubiquitin-proteasomal degradation; however, it was shown that the lack of oxygen stabilizes the α-subunit of the transcription factor HIF-1 via inhibition of its degradation. Thus, the level of HIF-1α in astrocytes in culture increased after oxygen deprivation (Karovic et al., 2007). HIF-1 binds to the cis-elements within target genes that include several growth factors, such as erythropoietin (Semenza, 2007). There are two possible mechanisms that could link HIF-1 to the ERα downregulation. First, HIF-1 interacts with ERα and this interaction down-regulates the ERα transcriptional activity (Cho et al., 2005) but had no effect on ERβ transcriptional activity (Yi et al., 2009), which appears to be in accordance with the finding that there was no change in the amount of ERβ mRNA in the HGDG. Second, it has been shown that insulin receptor substrate-2 (IRS-2) is phosphorylated on tyrosine following treatment of cells with erythropoietin (Verdier et al., 1997), an action which was believed to mimic the effects of insulin. It is known that cell signalling via insulin receptor or and epidermal growth factor receptor can decrease ERα mRNA expression (Wilson and Westberry, 2009), which is consistent with findings of this study.

However, due to a short period of HGD and recovery treatments in this study, a decrease in the ERα at the protein level is likely to be due to degradation of existing ERα, rather than to a
reduction in mRNA and a consequent reduction in ERα translation. It has been shown that hypoxia induced proteasome-dependent downregulation of ERα (Cho et al., 2006) and direct repression of ER which has been shown in breast cancer cell lines following hypoxia (Cho et al., 2005). Also, it has been shown that ERα is degraded in response to hypoxia and estradiol via a proteasome pathway, which was not seen for the ERβ (Yi et al., 2009). These data support a hypothesis that a downregulation of ERα at the transcript and at the protein level, which was observed in this study, was induced by HIF-1 –mediated pathways.

Estrogen signalling increased expression of glutamate transporters GLT-1 and GLAST on the mRNA and protein level in rat astrocytes through binding to nuclear estrogen receptors, a process which was accompanied by an increase in glutamate uptake (Pawlak et al., 2005). Therefore, a downregulation in ERα during HGD protocol could be one of the possible reasons for an observed reduced ability of astrocytes to take up glutamate via a Na+ -dependent mechanism during hypoxia (Dallas et al., 2007).

Acknowledgment

The authors acknowledge the technical assistance of Dr. S.S. Mohan, Mrs. L. Jacob and Mrs. A. Al-Farhan. We acknowledge help of Dr. James Craig, Department of Biochemistry, in improving manuscript style and clarity. Financial support for this study was provided by Kuwait University Grant No MY01/08.
References


Figure Captions:

**Figure 1. Validation of the method for fluorescence-based quantitative real-time PCR.** Titration curves were obtained by using cDNA to create a series of dilutions, from 1X (no dilution) to 64 X dilution. Then the quantitative real-time PCR was run and log dilutions plotted against the obtained quantification cycle (Ct) values. Each Ct value was estimated as an average from 2 replicates and each point in the Figure represents mean±SE from 3 separate cDNA samples. These data were also used to estimate the efficiency (E) of the reaction for each gene of interest.

**Figure 2. Expression of ERα protein in rat astrocytes.** A mouse monoclonal anti-ERα antibody raised against the amino acid residues 151-165 was used; 20 μg of protein was loaded on 7.5% SDS-polyacrylamide gels. (A) Representative Western blot for ERα and actin; the calculated size of the band is indicated on the left-hand side of the gel. Bands corresponding to ~67 kDa and 52 kDa molecular weight were detected for ERα. (B) Protein expression profile: ERα protein was expressed relative to actin. Results shown are mean ± S.E.M. (n ≥ 3); there was a significant decrease in the expression of the 67 kDa band between CG and RG (p=0.01) and a significant decrease in expression of the 52 kDa band between CG and HGDG (p<0.05).

**Figure 3. Expression of ERβ protein in rat astrocytes.** A rabbit polyclonal antibody corresponding to amino acids 1-150 was used; 20 μg of protein was loaded on 7.5% SDS-polyacrylamide gels. (A) Representative Western blot for ERβ and actin; the calculated size of the band is indicated on the left-hand side of the gel. One band of apparent molecular weight of 54kDa was detected for ERβ. (B) Protein expression profile: density of the ERα protein bands were expressed relative to density of the beta actin bands. Results shown are mean ± S.E.M. (n ≥ 3); no significant change in protein expression was detected.
Table 1. Expression of ERα and ERβ in rat astrocytes in primary culture and the effects of hypoxia and glucose deprivation treatment and recovery on that expression

<table>
<thead>
<tr>
<th></th>
<th>CG</th>
<th>HGDG</th>
<th>RG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ct±SE</td>
<td>E⁻Ct ± SE (x 10¹²)</td>
<td>Ct±SE</td>
</tr>
<tr>
<td>18S</td>
<td>12.99±0.03</td>
<td>195.99±3.35 x10⁶</td>
<td>13.62±0.68</td>
</tr>
<tr>
<td>ERα</td>
<td>29.63±0.43</td>
<td>2.5±0.9</td>
<td>30.69±0.33</td>
</tr>
<tr>
<td>ERβ</td>
<td>31.55±0.47</td>
<td>238±88</td>
<td>31.57±1.11</td>
</tr>
</tbody>
</table>

Data were obtained from 3-4 different samples for ERα and ERβ and from 6-8 samples for 18S RNA; each sample represented cDNA from one flask. Ct value for a sample was calculated as average from 2 replicates of one sample and ΔCt values for genes of interest and for the housekeeping gene were estimated. The linear form of the Ct values was corrected for the reaction efficiency, and is presented as E⁻Ct. The Ct values were used to estimate the fold change in the target genes for each sample (see the Method section), normalized to 18S ribosomal RNA and relative to the expression in the control group and the fold change values are presented as mean±SD.

Fig. 1
Fig. 2
Fig. 3