

Dexmedetomidine protects against oxygen-glucose deprivation/reoxygenation injury-induced apoptosis via the p38 MAPK/ERK signalling pathway

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Abstract

Objective: To investigate the protective effects of dexmedetomidine (DEX) in oxygen-glucose deprivation/reoxygenation (OGD/R) injury, which is involved in a number of ischaemic diseases.

Methods: An *in vitro* OGD/R injury model was generated using mouse Neuro 2A neuroblastoma (N2A) cells. Different concentrations of DEX were administered to OGD/R cells. CV-65 was used to inhibit p38 microtubule associated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signalling. Cell proliferation, cell cycle, apoptosis, and the levels of proteins related to p38 MAPK/ERK signalling and apoptosis were evaluated using Cell Counting Kit-8, flow cytometry, TdT-UTP nick end labelling and Western blot analysis, respectively.

Results: DEX treatment of OGD/R cells promoted cell survival and attenuated OGD/R-induced cell apoptosis. It also activated the p38 MAPK/ERK signalling pathway, increased the levels of Bcl-2, and decreased the levels of Bax and cleaved caspase-3. Treatment with the p38 MAPK/ERK inhibitor CV-65 inhibited the activation of p38 MAPK/ERK and abrogated the DEX-induced effects on cell survival and apoptosis.

Conclusions: DEX protects N2A cells from OGD/R-induced apoptosis via the activation of the p38 MAPK/ERK signalling pathway. DEX might be an effective agent for the treatment of ischaemic diseases.

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Keywords

Dexmedetomidine, oxygen-glucose deprivation/reoxygenation (OGD/R) injury, apoptosis, p38 MAPK/ERK

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Introduction

Oxygen-glucose deprivation/reoxygenation (OGD/R) injury is one of the critical pathological processes, which is involved in a series of ischaemic diseases, such as ischaemic heart disease, kidney ischaemic injury, and ischaemic stroke.¹⁻³ Currently, because of limited treatments for ischaemic diseases, many patients still suffer from mortality as well as a poor prognosis.⁴ Although much progress has been made, understanding of the mechanisms involved in OGD/R injury remains largely limited. Thus, it is of vital importance to explore the pivotal mediators that participate in the OGD/R process and to develop targeted agents for effective treatment.

Dexmedetomidine (DEX) is a highly selective agonist of α_2 -adrenoceptors on autoreceptors, presynaptic receptors and postsynaptic receptors (Figure 1a).⁵ Therefore, DEX has been extensively applied in sedation and anaesthesia.^{6,7} More interestingly, numerous studies indicate that DEX exhibits distinct neuroprotective effects against cerebral ischaemia.⁸⁻¹⁰ One theory is that DEX reduces inflammation by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling and suppressing the levels of inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6.¹¹⁻¹³ However, the underlying precise mechanisms are not well established. Moreover, it is still unclear whether DEX has any other impact on the OGD/R process by acting through other pathways.

This present study investigated the protective effects of DEX in an *in vitro* OGD/R

injury model using mouse Neuro 2A neuroblastoma (N2A) cells as previously described.¹⁴

Materials and methods

Cell culture and treatment

The mouse N2A cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies Inc.). The culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. In order to generate the *in vitro* OGD/R injury model as previously described,¹⁴ N2A cells were cultured in serum/glucose-free DMEM medium in a humidified atmosphere containing 5% CO₂ and 95% N₂ at 37°C for 4 h, followed by their return to DMEM supplemented with 10% fetal bovine serum for a 12-h recovery in normoxic conditions. Then, Non-OGD or OGD/R N2A cells were treated with dexmedetomidine solutions (Abbott Laboratories, Worcester, MA, USA) at 50 ng/ml, 100 ng/ml and 500 ng/ml for 60 min at 37°C for subsequent experiments. In addition, for p38 microtubule associated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signalling inhibition, cells were treated with the inhibitor CV-65 (Abcam®, Cambridge, MA, USA) at 20 μ M for 60 min at 37°C as previously described.¹⁵

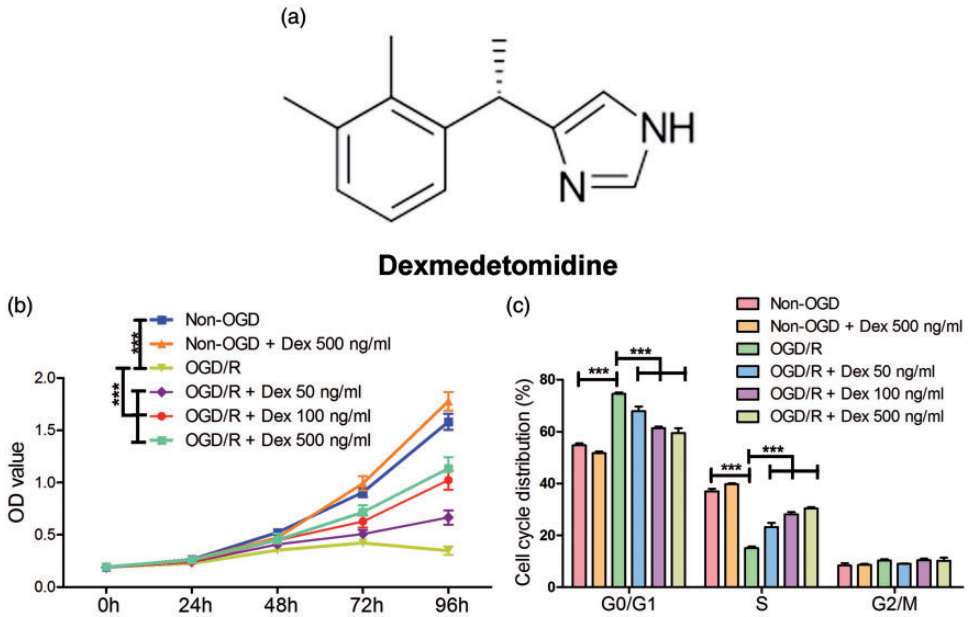


Figure 1. Effects of dexmedetomidine (DEX) on cell viability and cell cycle in an *in vitro* oxygen-glucose deprivation/reoxygenation (OGD/R) injury model. (a) Structure of DEX. (b) Cell growth curves of Non-OGD or OGD/R N2A cells treated with DEX at the indicated concentrations measured using a Cell Counting Kit-8 assay ($n = 3$). (c) Cell cycle distribution of cell groups detected by flow cytometry ($n = 3$). Data are presented as mean \pm SD. *** $P < 0.001$; Non-OGD versus OGD/R; Student's *t*-test. *** $P < 0.001$; OGD/R, OGD/R+Dex 50 ng/ml, OGD/R+Dex 100 ng/ml, and OGD/R+Dex 500 ng/ml; one-way analysis of variance. OD, optical density. The colour version of this figure is available at: <http://imr.sagepub.com>.

CCK-8 assay and cell growth curves

After corresponding treatment, cells were suspended and counted using an automated cell counter (CountessTM II Automated Cell Counter; Invitrogen, Carlsbad, CA, USA). Approximately 3.0×10^3 cells per well were seeded into 96-well plates in triplicate. Then, cell viability was examined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) according to manufacturer's protocol. Briefly, 10 μ l of CCK-8 solution was added to each well at the time-points of 0, 24, 48, 72, and 96 h. After 2 h of culture at 37°C, the optical density (OD) value was monitored with a plate reader at 450 nm (xMarkTM Microplate Absorbance Spectrophotometer; Bio-Rad, Hercules, CA, USA). The cell

growth curves were drawn based on OD values every 24 h.

Flow cytometry analysis of cell cycle and apoptosis

For cell cycle analysis, approximately 1.0×10^6 cells were collected and treated with 70% ethanol for fixing at 4°C for 12 h. The cells were washed twice in 0.01 M phosphate-buffered saline (PBS; pH 7.4) at room temperature followed by staining with 10 μ g/ml propidium iodide (PI) for 10 min at room temperature (Beckman Coulter, Brea, CA, USA). Then, the stained cells were analysed using a flow cytometer (AttuneTM NxT Acoustic Focusing Cytometer; Thermo Fisher Scientific Inc., Rockford, IL, USA). For cell apoptosis

analysis, approximately 1.0×10^6 cells were collected and double stained with fluorescein isothiocyanate (FITC)-labelled Annexin V and PI using an FITC Annexin V Apoptosis Detection Kit according to the manufacturer's instructions (TransGen Biotech, Beijing, China); and then the stained cells were analysed using a flow cytometer (Attune™ NxT Acoustic Focusing Cytometer; Thermo Fisher Scientific Inc.) to calculate the percentage of early apoptotic cells. Experiments were repeated in triplicate.

Western blot analysis

Western blot analysis was performed as previously described.¹⁶ Briefly, total proteins were isolated from cells lysed with RIPA reagent (Beyotime, Jiangsu, China) supplemented with 1 mM phenylmethylsulphonyl fluoride (Roche Diagnostics, Mannheim, Germany). Then, the protein concentration was measured using a BCA protein assay kit (Sigma-Aldrich, St Louis, MO, USA). Thereafter, equal quantities of proteins were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Bio-Rad) and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% fat-free milk for 2 h at room temperature, the membranes were incubated with primary antibodies against p-p38 MAPK (1:500 dilution), p38 MAPK (1:500 dilution), p-ERK1/2 (1:500 dilution), ERK1/2 (1:500 dilution), Bax (1:500 dilution), Bcl-2 (1:500 dilution), caspase-3 (1:500 dilution) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000 dilution) (Cell Signaling Technology®, Danvers, MA, USA) at 4°C overnight. The membranes were washed three times for 10 min each time in $1 \times$ Tris buffered saline/0.1% Tween-20 (TBST; pH 7.4) at room temperature. Then, the membranes were incubated with horseradish peroxidase-conjugated

anti-rabbit immunoglobulin G secondary antibodies (1:2000 dilution; Bioworld Technology, St Louis Park, MN, USA) for 1 h at room temperature. The membranes were washed three times for 10 min each time in $1 \times$ TBST (pH 7.4) at room temperature. The proteins were visualized using the ChemiDoc™ MP Imaging System (Bio-Rad). Experiments were repeated in triplicate.

TUNEL staining

Cell apoptosis were detected by TdT-UTP nick end labelling (TUNEL) staining using a one-step TUNEL kit (Beyotime) according to manufacturer's instructions. Briefly, after attaching to the slides, cells were fixed with 4% paraformaldehyde, rinsed with 0.01 M PBS (pH 7.4), and treated with 0.1% Triton X-100 treatment for 3 min on ice. Then, cells were stained with FITC-labelled TUNEL for 1 h at 37°C. The nucleus was stained with 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min (Beyotime). The apoptotic cells were identified by green fluorescence and were imaged under a fluorescence microscope (Leica TCS SP5; Leica Microsystems, Mannheim, Germany). Experiments were repeated in triplicate.

Apoptosis ELISA assay

This assay was performed as previously described.¹⁷ The Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) was used to detect cell apoptosis after indicated treatments according to the manufacturer's instructions. In brief, the cytoplasmic histone/DNA fragments were extracted from the cells. Subsequently, the immobilized anti-histone antibody was used to bind DNA associated with nucleosomal histones. The peroxidase-labelled anti-DNA antibody was then added to detect the

immobilized histone/DNA fragments. After the reaction with substrate for peroxidase, the spectrophotometric absorbance and the OD values of the samples were determined by a plate reader at 405 nm (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad). Experiments were repeated in triplicate.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows®. All data are shown as mean \pm SD. Student's *t*-test was applied to show the differences between the two groups. One-way analysis of variance was performed to show the differences between multiple groups. A *P*-value < 0.05 was considered to be statistically significant.

Results

In order to investigate the role of DEX in OGD/R injury, an *in vitro* OGD/R injury model was established using N2A cells. Cells were treated with DEX at different concentrations (50 ng/ml, 100 ng/ml and 500 ng/ml), followed by the analysis of cell viability using a CCK-8 assay kit. The results showed that OGD/R injury significantly inhibited cell viability and cell proliferation compared with the Non-OGD control ($P < 0.001$) (Figure 1b). The treatment with DEX significantly abolished the suppression of cell proliferation caused by OGD/R ($P < 0.001$ for all comparisons); and the effect was correlated with DEX dose. Cells were then subjected to cell cycle analyses and the mean \pm SD S phase percentages of Non-OGD/R, OGD/R and OGD/R+Dex 500 ng/ml were 36.97 ± 1.73 , 15.09 ± 1.12 and 30.34 ± 0.90 , respectively (Figure 1c); and the G0/G1 phase percentages of Non-OGD/R, OGD/R and OGD/R+Dex 500ng/ml were 54.68 ± 1.47 , 74.58 ± 0.89

and 59.50 ± 3.08 , respectively. OGD/R injury of N2A cells led to a significant decrease of S phase as well as a significant increase of G0/G1 phase compared with the Non-OGD control ($P < 0.001$ for both comparisons), which could be gradually attenuated by the treatment of DEX (Figure 1c). However, the treatment with DEX had a smaller effect on Non-OGD cells.

In terms of the effect of DEX on cell apoptosis in OGD/R injury, TUNEL staining was used to identify apoptotic cells under the fluorescence microscope. The results showed that more TUNEL-stained cells were detected in the OGD/R group compared with the Non-OGD group; whereas less TUNEL-stained cells were observed in the three DEX treatment groups compared with the OGD/R group (Figure 2a). Quantitative analysis of cell apoptosis using flow cytometry generated data that were consistent with the TUNEL staining assay (Figure 2b): the percentages of early apoptotic cells for Non-OGD/R, OGD/R and OGD/R+Dex 500 ng/ml were 5.15 ± 0.24 , 37.31 ± 1.01 and 21.77 ± 1.51 respectively. The OGD/R injury significantly induced cell apoptosis compared with the Non-OGD control cells ($P < 0.001$). DEX treatment significantly attenuated cell apoptosis caused by OGD/R injury ($P < 0.001$ for all comparisons). Moreover, the Cell Death Detection ELISA assay showed that treatment with DEX significantly reduced the level of cell apoptosis caused by OGD/R injury ($P < 0.001$ for all comparisons) (Figure 2c). TUNEL staining, flow cytometry analysis, and the Cell Death Detection ELISA assay showed that DEX treatment had little effect on Non-OGD cells.

Experiments were then undertaken to explore the mechanisms underlying the significant protective role of DEX against OGD/R-induced cell apoptosis. Western blot analysis showed that the OGD/R injury significantly reduced the

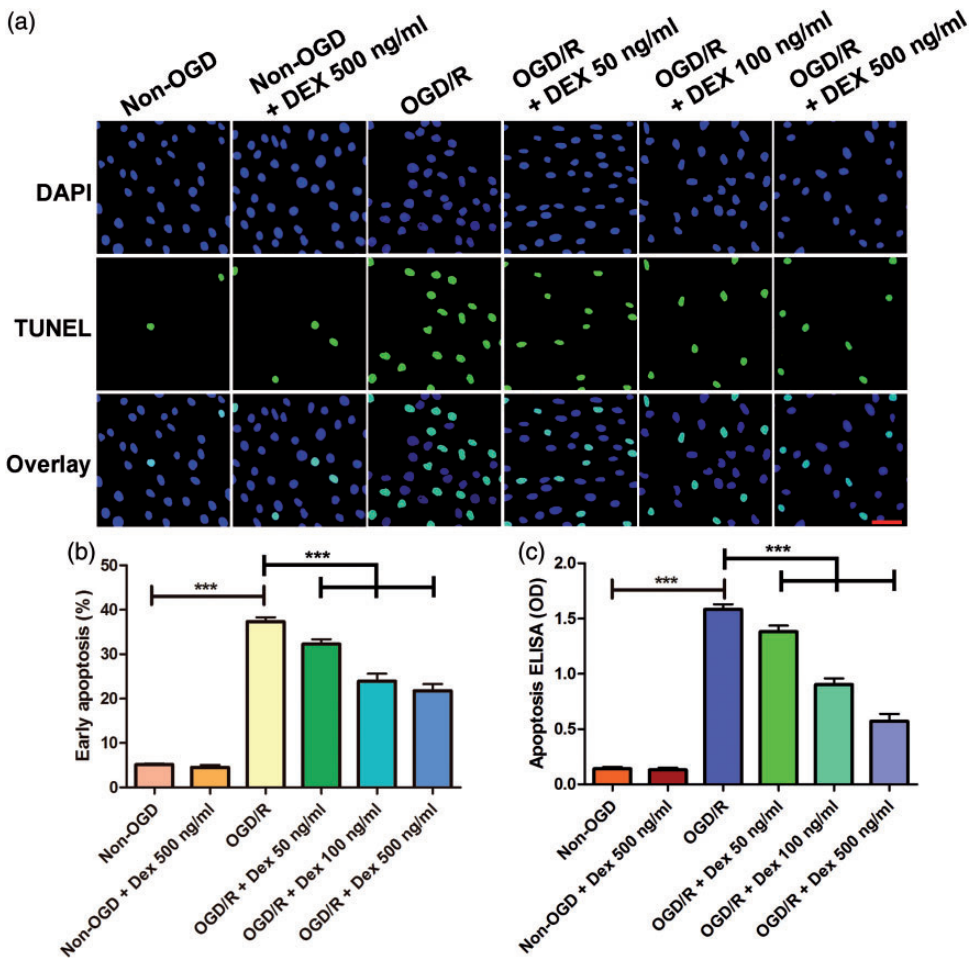


Figure 2. Effect of dexmedetomidine (DEX) on cell apoptosis during oxygen-glucose deprivation/reoxygenation (OGD/R) injury. (a) TdT-UTP nick end labelling (TUNEL) staining of cell groups showing apoptotic cells (green fluorescence). 4',6-diamidino-2-phenylindole (DAPI) stained the nuclei blue. Scale bar, 100 μ m. (b) Early apoptotic cell counts of cell groups analysed with flow cytometry by measuring the percentage of Annexin V-stained cells ($n = 3$). (c) Cell apoptosis was measured using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) ($n = 3$). Data are presented as mean \pm SD. *** $P < 0.001$; Non-OGD versus OGD/R; Student's t -test. *** $P < 0.001$; OGD/R, OGD/R+Dex 50 ng/ml, OGD/R+Dex 100 ng/ml, and OGD/R+Dex 500 ng/ml; one-way analysis of variance. OD, optical density. The colour version of this figure is available at: <http://imr.sagepub.com>.

phosphorylation levels of p38 MAPK and ERK1/2 ($P < 0.001$ for all comparisons) (Figures 3a, 3b). DEX treatment counteracted OGD/R injury-induced inhibition of p38 MAPK/ERK signalling and elevated the phosphorylation levels of p38 MAPK and ERK1/2. Western blot analysis of the

levels of apoptosis-related proteins showed that OGD/R cells had significantly higher levels of Bax and activated caspase-3 and lower levels of Bcl-2 compared with the Non-OGD/R cells ($P < 0.001$ for all comparisons) (Figures 3c and 3d). DEX treatment decreased Bax and cleaved caspase-3

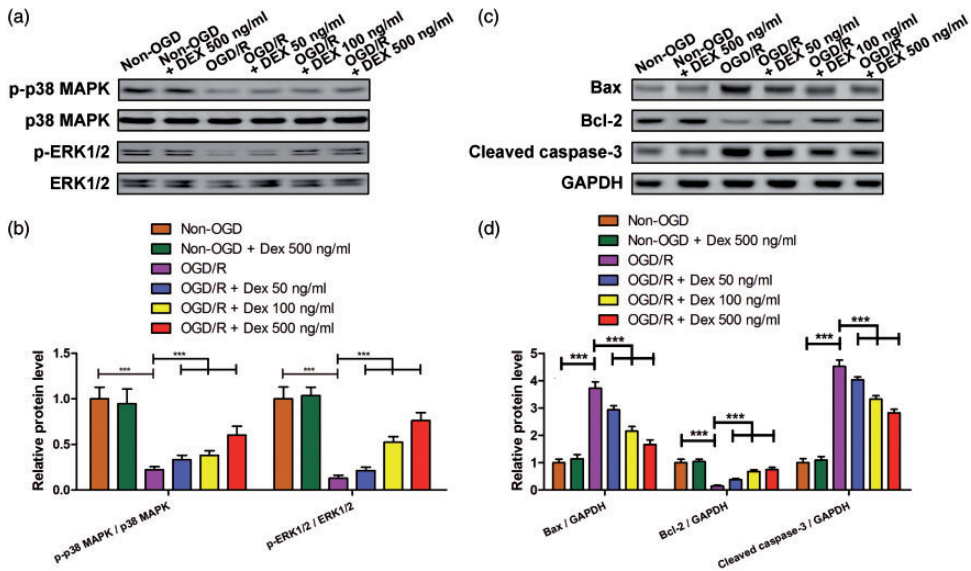


Figure 3. Effect of dexmedetomidine (DEX) on p38 microtubule associated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signalling and apoptosis-related molecules in an *in vitro* oxygen-glucose deprivation/reoxygenation (OGD/R) injury model. (a) Western blot analysis of p38 MAPK/ERK signalling proteins in cell groups. (b) Quantitative analysis of protein levels shown in Figure 3a ($n = 3$). (c) Western blot analysis showing levels of Bax, Bcl-2 and cleaved caspase-3 in cell groups. (d) Quantitative analysis of protein levels shown in Figure 3c ($n = 3$). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control. Data are presented as mean \pm SD. *** $P < 0.001$; Non-OGD versus OGD/R; Student's *t*-test. *** $P < 0.001$; OGD/R, OGD/R+Dex 50 ng/ml, OGD/R+Dex 100 ng/ml, and OGD/R+Dex 500 ng/ml; one-way analysis of variance. p, phosphorylated. The colour version of this figure is available at: <http://imr.sagepub.com>.

levels and increased Bcl-2 levels, which therefore increased the ratio of Bcl-2/Bax.

In order to examine whether p38 MAPK/ERK signalling is essential for the DEX-mediated protective effects in OGD/R damage, the p38 MAPK/ERK inhibitor CV-65 was used in the following experiments. Western blot analysis showed that co-treatment of DEX and CV-65 significantly inhibited the activation of p38 MAPK/ERK compared with the single treatment of OGD/R cells with DEX ($P < 0.001$ for both comparisons) (Figures 4a and 4b). Treatment with DEX decreased Bax and cleaved caspase-3 levels, increased Bcl-2 levels and elevated the ratio of Bcl-2/Bax in OGD/R cells; effects that were abolished by co-treatment with

CV-65 (Figures 4c and 4d). The CCK-8 cell viability assay showed that the additional treatment of CV-65 counteracted the DEX-induced enhancement of cell proliferation and survival during OGD/R (Figure 4e). Flow cytometric analysis showed that the additional treatment with CV-65 abrogated the DEX-induced decrease of cell apoptosis and restored apoptotic cell numbers to a high level (Figure 4f).

Discussion

Dexmedetomidine is a selective agonist of α_2 -adrenoceptors, which is widely applied in sedation and anaesthesia. Accumulating evidence has shown that DEX plays other complex roles, especially protective roles in

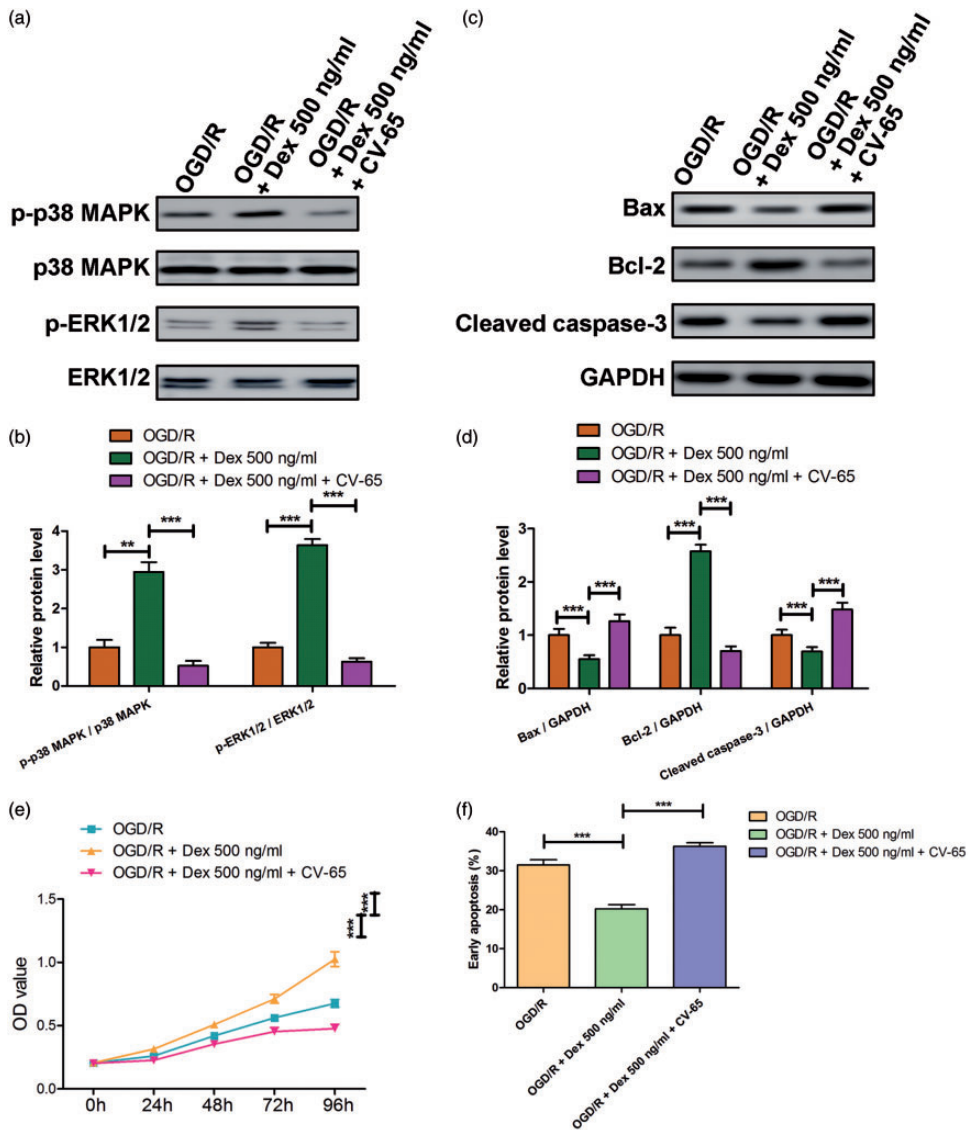


Figure 4. Effect of the inhibition of p38 microtubule associated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signalling on the protective effects of dexmedetomidine (DEX) in an *in vitro* oxygen-glucose deprivation/reoxygenation (OGD/R) injury model. (a) Western blot analysis of p38 MAPK/ERK signalling proteins in OGD/R N2A cells, OGD/R N2A cells treated with DEX 500 ng/ml, and OGD/R N2A cells co-treated with DEX 500 ng/ml and the p38 MAPK/ERK signalling inhibitor CV-65 20 μ M. (b) Quantitative analysis of protein levels shown in Figure 4a ($n = 3$). (c) Western blot analysis showing levels of Bax, Bcl-2 and cleaved caspase-3 in cell groups. (d) Quantitative analysis of protein levels shown in Figure 4c ($n = 3$). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control. (e) Cell growth curves of cell groups measured using a Cell Counting Kit-8 assay ($n = 3$). (f) Early apoptotic cell counts of cell groups analysed with flow cytometry by measuring the percentage of Annexin V-stained cells ($n = 3$). Data are presented as mean \pm SD. *** $P < 0.001$; OGD/R versus OGD/R+Dex 500 ng/ml by Student's *t*-test. *** $P < 0.001$; OGD/R+Dex 500 ng/ml vs OGD/R+Dex 500 ng/ml+CV-65 by Student's *t*-test. p, phosphorylated; OD, optical density. The colour version of this figure is available at: <http://imr.sagepub.com>.

a variety of diseases and pathophysiological processes. For example, previous studies reported that DEX attenuated hyperoxia-induced pulmonary oedema,¹⁸ relieved lipopolysaccharide-induced injury in the liver,¹⁹ and protected against OGD/R damage in the myocardium, lung, kidney and brain.²⁰⁻²³ The current view is that an inflammatory response is one of the critical events during OGD/R damage,^{24,25} and that DEX plays a protective role against OGD/R injury by partly suppressing this inflammatory reaction.¹³ During transient global cerebral ischaemia, DEX reduced necrotic and apoptotic cell counts, down-regulated the levels of inflammatory cytokines including TNF- α , IL-1 β , and IL-6, and inactivated the toll-like receptor 4/NF- κ B signalling pathway.^{13,26} In addition, a previous study demonstrated that the protective role of DEX against OGD/R injury was associated with the activation of the I2 imidazoline receptor-phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B pathway and the up-regulation of hypoxia-inducible factor (HIF)-1 α , vascular endothelial growth factor and RTP801 expression.²⁷ Another study demonstrated that DEX protected mouse brain tissue from ischaemia-reperfusion injury by up-regulating HIF-1 α ; and also that it promoted the expression of Bcl-1 and p62 and decreased the expression of microtubule-associated protein 1 light chain 3 and Beclin 1 to inhibit cell apoptosis and autophagy.²⁸ DEX ameliorated ischaemia injury by inhibiting the phosphorylation of Janus kinase 2 and its downstream molecules signal transducer and activator of transcription (STAT)1 and STAT3, whilst also down-regulating the expressions of cleaved caspase 3, intercellular adhesion molecule 1 and monocyte chemoattractant protein 1.²⁹ In summary, these findings imply that DEX exhibits important effects in the recovery of OGD/R damage, which is associated with diverse activation or

inhibition of a variety of effector molecules and pathways. The precise mechanisms are still not fully understood.

This present study found that treatment with DEX following induction of OGD/R could improve cell viability and inhibit cell apoptosis. At the molecular level, DEX decreased the levels of the pro-apoptosis protein Bax, increased the levels of the anti-apoptosis protein Bcl-2, thus increasing the Bcl-2/Bax ratio, and inhibited caspase-3 activation. These findings demonstrate that DEX possesses anti-apoptosis effects against OGD/R injury, which agrees well with previous findings.^{13,26,29} In addition, the present study found that OGD/R injury led to the exacerbation of cell apoptosis along with the inhibition of p38 MAPK/ERK signalling. However, DEX treatment attenuated OGD/R-induced apoptosis and reactivated p38 MAPK/ERK signalling. This raises the question whether the activation of p38 MAPK/ERK signalling pathway is essential for the anti-apoptosis effects of DEX.

The p38 MAPK/ERK pathway belongs to the family of MAPK-activated protein kinases, which participates in a lot of crucial molecular and cellular processes including cell proliferation, survival, apoptosis, senescence and differentiation.³⁰ The ERKs have been shown to play vital roles in controlling cell proliferation and division; while, p38 MAPKs are often activated by inflammatory mediators for driving stress-related reactions.³¹ Also, accumulating evidence has shown that the activation of p38 MAPK/ERK signalling contributes to the promotion of cell survival as well as the inhibition of cell apoptosis under many physiological and pathological conditions, and that dysregulation of p38 MAPK/ERK signalling is closely associated with various diseases, such as some types of tumours, Alzheimer's disease, and HIV infection.^{32,33} In this present study, DEX protected against OGD/R-induced

apoptosis whilst activating the p38 MAPK/ERK signalling pathway. By employing the p38 MAPK/ERK inhibitor CV-65, the present study demonstrated that the activation of the p38 MAPK/ERK signalling pathway was required for the DEX-mediated protective effects against OGD/R injury. The current results showed that despite the use of DEX at a high concentration (500 ng/ml), the inhibition of p38 MAPK/ERK signalling by CV-65 could block the DEX-induced anti-apoptosis effects in the *in vitro* OGD/R injury model in N2A cells. These findings suggest that the protective role of DEX on OGD/R damage is dependent on the activation of the p38 MAPK/ERK signalling pathway.

In conclusion, this present study demonstrated that DEX was conducive to cell survival, ameliorated OGD/R-induced cell apoptosis, and possesses protective properties against OGD/R injury. In addition, DEX treatment activates the p38 MAPK/ERK signalling pathway, increases the Bcl-2/Bax ratio, and inhibits caspase-3 activation in OGD/R N2A cells. More importantly, the activation of the p38 MAPK/ERK signalling pathway was essential for DEX-mediated anti-OGD/R injury effects. Therefore, this present study increases the understandings of the mechanism underlying OGD/R damage as well as the therapeutic effects of DEX. These current findings also provide a basis for the application of DEX in the treatment of ischaemic diseases.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Funding

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