JPP JOURNAL OF Pharmacy and Pharmacology

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Research Paper

Dexmedetomidine protects against oxygen–glucose deprivation-induced injury through the I2 imidazoline receptor-PI3K/AKT pathway in rat C6 glioma cells

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Keywords
dexmedetomidine; hypoxia-inducible factor (HIF)-1α; oxygen–glucose deprivation; RTP801; vascular endothelial growth factor (VEGF)

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Received March 22, 2011
Accepted September 22, 2011

doi: 10.1111/j.2042-7158.2011.01382.x

Abstract

Objectives To explore the protection and the mechanism of dexmedetomidine on the oxygen–glucose deprivation (OGD) insults in rat C6 glioma cells.
Methods Cells were subjected to OGD then assessed by viability studies. After dexmedetomidine treatment, p-AKT, hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF) and RTP801 expression were measured.
Key findings Three hours of OGD decreased cell viability to 48.8%, which was reversed to 67.4% by 1 μM dexmedetomidine. Hoechst 33342 and propidium iodide double stains showed that the protection of dexmedetomidine was mainly by an anti-apoptosis effect, which was also strengthened by decreasing caspase-3 expression. Dexmedetomidine protection was mainly blocked by the I2 imidazoline receptor antagonist idazoxan and BU 224, but not by the α_1-adrenoceptor antagonist prazosin, the α_2-adrenoceptor antagonist yohimbine and RX 821002, or the I1 imidazoline receptor antagonist efaroxan. On the other hand, dexmedetomidine enhanced AKT phosphorylation. Furthermore, the protection of dexmedetomidine was blocked by the PI3K/AKT inhibitor wortmannin. The proteins of HIF-1α, VEGF and RTP801 were significantly increased by dexmedetomidine treatment.
Conclusions Dexmedetomidine activated the I2 imidazoline receptor-PI3K/AKT pathway, and up-regulated HIF-1α, VEGF and RTP801 expression to protect against OGD-induced injury in rat C6 cells.

Introduction

Dexmedetomidine is a potent and highly selective agonist of α_2-adrenergic receptors with many actions on the central nervous system (CNS), including clinical sedation, anaesthetic-sparing effects, and analgesia. Recently, it was shown that dexmedetomidine could improve the histomorphological and neurological outcome after cerebral ischaemia.[1] Many mechanisms have been suggested for this, such as the modulation of central catecholamine and glutamate release, hypothermia, and inhibition of apoptosis and neural cell death.[2] However, the mechanisms by which dexmedetomidine exerts its protective effect are unclear.

Hypoxic/ischaemic insults activate adaptive mechanisms and alter gene expression within the injured areas to combat the progression of pathological events.[3] A well-characterized molecular pathway that mediates oxygen sensing occurs through the induction of the transcription factor hypoxia-inducible factor (HIF-1). HIF-1α expression is exponentially induced as the oxygen concentration of cells decreases, which promotes several dozen genes expression, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and regulated in development and DNA damage responses 1 (REDD1, RTP801).[4–6] We have investigated the protective effects of dexmedetomidine in an in-vitro model of ischaemia based on oxygen–glucose deprivation (OGD) in rat C6 glioma cells. Our data demonstrated that dexmedetomidine activated the I2 imidazoline receptor-PI3K/AKT pathway, and up-regulated HIF-1α, VEGF and RTP801 expression to protect against OGD-induced injury in rat C6 cells.

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Materials and Methods

Materials

Dexmedetomidine was obtained from Santa Cruz Laboratories (Santa Cruz, CA, USA). Prazosin, yohimbine, idazoxan, efaroxan and wortmannin were purchased from Sigma-Aldrich (St Louis, MO, USA). As a general rule, drugs were dissolved in sterile purified water before dilution into culture medium. Primary antibodies against caspase-3, cleaved caspase-3 (Asp175), AKT, phospho-AKT (Ser473) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), primary antibody against VEGF antibody was obtained from Boster Biotech. (Wuhan, China), primary antibody against HIF-1α was obtained from Santa Cruz Biotech (Santa Cruz, CA, USA) and primary antibody against RTP801 was obtained from Sigma (St Louis, MO, USA).

Cell culture

Rat glioma C6 cells were obtained from the Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York, NY, USA) supplemented with 10% heated inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every three days. For each experiment, C6 cells were plated to different plates in standard DMEM culture media at a specified density so that cells grew to confluence on the next day when experiments were performed.

Oxygen and glucose deprivation

On the day of the experiment, the culture medium was removed, the cells were washed with warm phosphate buffered saline (PBS, pH 7.4), and then the experimental medium was added. For experiments under normal culture conditions (21% oxygen with glucose), the experimental medium was DMEM (glucose concentration 5.5 mM). To achieve OGD, a technique similar to that described by Newcomb-Fernandez *et al*.[7] was used. Briefly, the OGD experimental medium (DMEM without glucose) was previously gassed with 95%N_2/5%CO_2 for 30 min, and was added to cell culture wells, which had been washed three times with PBS. OGD was induced by incubating cells in a humidified airtight chamber (Billups-Rothberg Inc., Del Mar, CA, USA) equipped with an air lock and continuously flushed with 95%N_2/5%CO_2 for 15 min at 37°C. The airtight chamber was then sealed and kept in a 37°C incubator for the appropriate time periods. Oxygen concentration was below 0.2%, as monitored by an oxygen analyser (Sable Systems, Las Vegas, NV, USA). Dexmedetomidine or vehicle was added 30 min before OGD, and the antagonists or inhibitors were added 15 min before adding dexmedetomidine. During OGD, drugs were always added in the culture media.

Viability studies

For cell viability assays, C6 cells were seeded on 96-well plates at 7.5×10^3 cells per well. After the treatments, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/ml, and the cells were incubated for 3 h at 37°C. The media were removed and 100 μl dimethyl sulfoxide (DMSO) was added into each well. The absorbance at 490 nm was measured on a plate-reader (ELX800, BIO-TEK Instruments Inc., Winooski, VT, USA). The cell viability was determined as the percentage absorbance relative to untreated controls.

Apoptosis and necrosis assays

Cells grown on coverslips were stained with Hoechst 33342 (10 μg/ml) and propidium iodide (PI, 10 μg/ml, Sigma-Aldrich) for 10 min at 37°C. The stained cells were photographed under a fluorescence microscope (Olympus BX51, Olympus, Japan) with emission at 460 nm and excitation at 350 nm (for Hoechst 33342) or at 620 and 536 nm (for PI). The apoptotic cells were determined by condensed or fragmented nuclei with strong bright Hoechst 33342 staining, whereas the necrotic cells were identified by red PI staining.

Western blots

The C6 cells were homogenized and the total proteins were purified using cell and tissue protein extraction reagents according to the manufacturer's instructions (KC-415; Kangchen, Shanghai, China). Forty micrograms protein equivalent of each sample was electrophoresed on polyacrylamide gel, transferred onto nitrocellulose, and probed with primary antibodies followed by secondary antibodies. Anti-β-actin antibody (1 : 2000; Proteintech, Chicago, IL, USA) was used as the control. Blots were visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and analysed using Odyssey software. Relative optical densities were obtained by comparing measured values with the mean values from the control group.

Statistics

Data are given as mean ± SD of 4–6 experiments. Drug induced percentage inhibition of protection was calculated from the values for percentage MTT metabolism and determined by the formula:

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$$\frac{(protective\ drug + OGD) - (inhibitory\ drug + protective\ drug + OGD)}{(protective\ drug + OGD) - (OGD\ alone)} \times 100\%*$$

(*In this case, 'protective drug' means dexmedetomidine and 'inhibitory drug' means α-adrenoceptor antagonist, or imidazoline receptor antagonist.)

Statistical analysis was performed by one-way analysis of variance followed by the Newman–Keuls test for multiple comparison tests (performed with SPSS software). *P*-values lower than 0.05 were considered to be statistically significant.

Results

Oxygen–glucose deprivation induced injury in rat glioma C6 cells

Exposure of C6 cells to OGD insult decreased cell viability in a time-dependent manner. Under our experimental conditions, the cell viability was approximate 81% or 49% after incubation for 2 or 3 h OGD insult, respectively (Figure 1). In this experiment, 3-h OGD was chosen for further study.

Effects of dexmedetomidine on oxygen–glucose deprivation-induced injury

When C6 cells were exposed to 3-h OGD, the mean viability rate significantly decreased to 48.8 ± 8.3% (Figure 2). Perfusion with dexmedetomidine (0.01–10 μM) alone for 3 h did not alter viability (data not shown), whereas dexmedetomidine applied in combination with OGD significantly increased viability rates. At a concentration of 1 μM, dexmedetomidine reversed the OGD-induced injury by 67.4 ± 7.5% (Figure 2).

Oxygen–glucose deprivation induced cell death

To verify the type of cell death, Hoechst 33342 and PI stain were used to identify apoptosis and necrosis. Without OGD, the nuclei of control cells showed uniform blue fluorescence, indicating that they were healthy (Figure 3a). In contrast, after treatment with OGD, a few nuclei were swollen and stained red by the membrane-impermeable dye PI, indicating necrotic cell death. Many displayed apoptotic characteristics with fragmented nuclei stained by Hoechst 33342 (Figure 3b). These data showed that OGD-induced cell injury mostly occurred via apoptosis. Treatment with 1 μM dexmedetomidine decreased this injury (Figure 3c).

When the cells were exposed to OGD for 3 h, a rapid impairment of mitochondrial function (as assessed by the MTT test) and loss of cell viability were observed (Figure 3d). On the basis of nuclear morphology by Hoechst 33342 and PI staining, this early neuronal death occurred mainly by apoptosis (55%) and not by necrosis (only 7%).

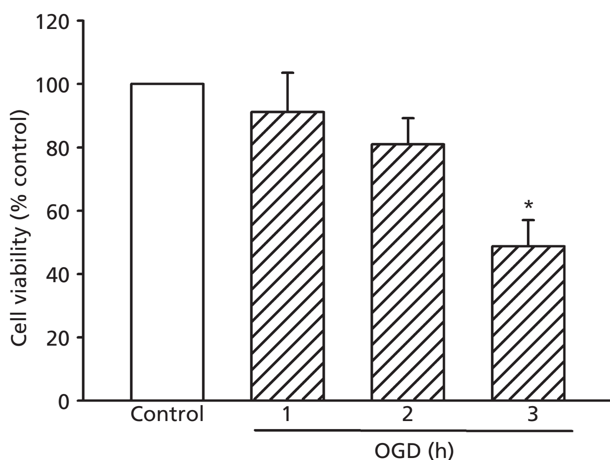

Figure 1 Effects of oxygen–glucose deprivation on the cell viability in rat C6 glioma cells. Cells were exposed to oxygen–glucose deprivation (OGD) and recovery in the normal culture medium for various durations. Cell viability was assayed after recovery in normal culture medium using the MTT reduction assay. Results expressed as percentage of control. Data are mean ± SD of 4–6 independent experiments with three replicates for each condition. *$P < 0.05$ compared with control group (analysis of variance followed by Student–Newman–Keuls test).

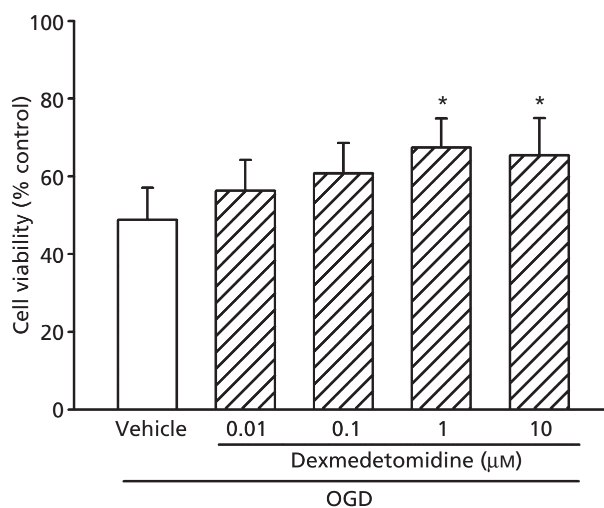

Figure 2 Effects of dexmedetomidine on oxygen–glucose deprivation-induced injury. Rat C6 glioma cells were treated with dexmedetomidine during 3-h oxygen–glucose deprivation (OGD) and 2-h recovery in normal culture medium. Cell viability was determined by MTT assay. Results expressed as percentage of control values, and are from four to six independent experiments with three replicates for each condition. *$P < 0.05$ compared with OGD group (analysis of variance followed by Student–Newman–Keuls test).

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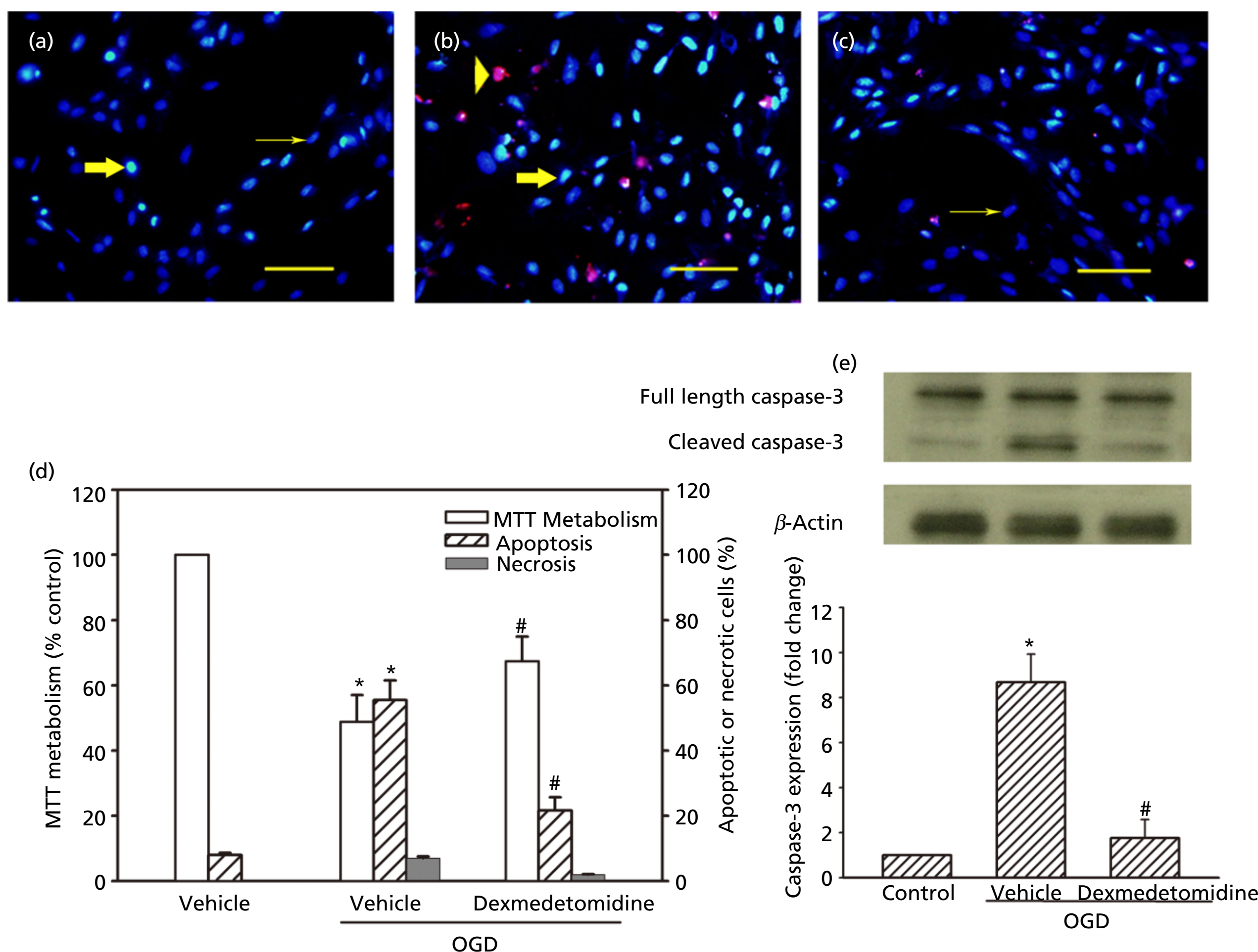

Figure 3 Effects of dexmedetomidine on oxygen–glucose deprivation-induced apoptotic cell death in rat C6 glioma cells. Cells were exposed to either (a) normal condition (control) or (b) 3-h oxygen–glucose deprivation (OGD) or (c) pre-incubation with 1 μmol/l dexmedetomidine followed immediately by double-staining with cell membrane-permeable (Hoechst 33342; blue) and -impermeable (propidium iodide; red) DNA labelling fluorochromes. Hoechst 33342 stained the nuclei of normal (small arrows) and apoptotic (arrowhead) cells with intact plasma membrane. Propidium iodide stained the nuclei of cells with damaged plasma membrane, i.e. necrotic cells (large arrows). Scale bars, 50 μm. (d) Mitochondrial function, apoptosis and necrosis in cultured C6 cells exposed to 6-h OGD. Apoptotic nuclei were detected by nuclear staining with Hoechst 33342 and propidium iodide. Mitochondrial activity was assessed by measuring MTT metabolism and data were expressed as percentage of respective control. (e) Western blot was showed that OGD enhanced cleaved caspase-3 expression, which was inhibited by pretreatment 1 μM dexmedetomidine. Data represent the mean ± SD of at least three independent experiments. $^{*}P < 0.05$ compared with respective controls (vehicle); $^{\#}P < 0.05$ compared with respective OGD groups (analysis of variance followed by Student–Newman–Keuls test).

Dexmedetomidine at 1 μM reduced the OGD-induced apoptosis and necrosis to 22% and 2%, respectively. Thus the decrease in MTT metabolism almost paralleled the appearance of nuclear morphological changes, and MTT metabolism was chosen to indicate cell viability.

To further confer dexmedetomidine protected OGD-induced injury by its anti-apoptosis effect, caspase-3 expression was explored. OGD insult caused robust caspase-3 activation by 8.7-fold compared with the control group. Pretreatment with dexmedetomidine significantly decreased caspase-3 expression to 1.8-fold (Figure 3e).

Mechanisms of the protective effects of dexmedetomidine on oxygen–glucose deprivation-induced injury

Exposure of C6 cells to the α_1-adrenoceptor antagonist prazosin or the α_2-adrenoceptor antagonist yohimbine and RX 821002 (10^{-8}–10^{-4} M) alone had no effect on cell survival (data not shown). Pretreatment with prazosin did not inhibit the protection induced by 1 μM dexmedetomidine. Further more, neither yohimbine nor RX 821002 could significantly inhibit the protection induced by 1 μM dexme-

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detomidine, only by 31% or 35% inhibition, even at the concentration of 10^{-4} M. This indicated that protection by dexmedetomidine was not mainly through α_1- or α_2-adrenoceptors (Figure 4a).

On the other hand, pretreatment with an antagonist at I2 imidazoline binding sites, idazoxan or BU 224, resulted in a concentration-related inhibition of the protection induced by 1 μM dexmedetomidine, by reaching 92.4% and 82.3% inhibition, respectively, which idazoxan or BU 224 used at concentrations of 10^{-4} M (Figure 4b). These antagonists alone had no appreciable effects on C6 cells at the concentrations used (data not shown). However, the antagonist at I1 imidazoline binding sites, efaroxan, did not block the dexmedetomidine-induced protection, only showing 31.1% inhibition, even at 10^{-4} M (Figure 4b). Thus, the protection by dexmedetomidine from OGD-induced injury in cultured C6 cells was mainly through I2 imidazoline receptors.

To investigate further the role of the I2 imidazoline receptor in the protection from OGD-induced injury, benazoline, a selective I2 imidazoline agonist was used. Benazoline (1–10 μM) reversed OGD-induced injury in a concentration-dependent manner (Figure 5a). In addition, benazoline alone did not affect C6 cells at the concentrations used (data not shown). BU 224 (10^{-8}–10^{-4} M) blocked the protection induced by benazoline (10 μM) in a concentration-dependent manner; at concentrations of 10^{-4} M 91.4% inhibition was observed (Figure 5b). In contrast, treatment with efaroxan did not alter the benazoline-induced protection.

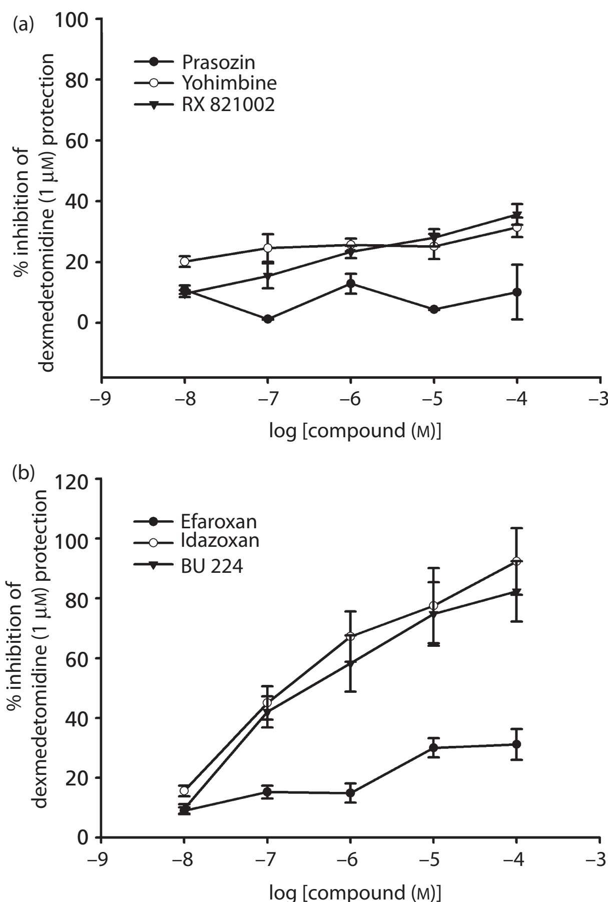

Figure 4 Effects of α-adrenoceptor antagonists (a) and imidazoline receptor antagonists (b) on the protection by dexmedetomidine. Rat C6 glioma cells were treated with vehicle or indicated drugs before dexmedetomidine treatment then exposed to 3-h oxygen–glucose deprivation (OGD). Cell viability was determined by MTT assay. Values are expressed as percentage inhibition of the protection by dexmedetomidine and are from four to six independent experiments with three replicates for each condition.

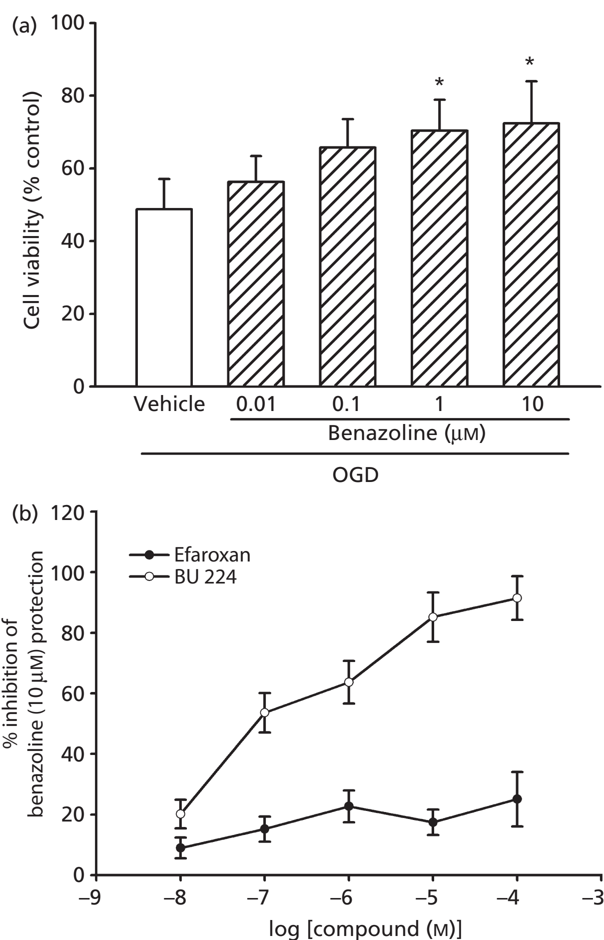

Figure 5 Effects of imidazoline receptor agonist benazoline on oxygen–glucose deprivation-induced injury (a) and imidazoline receptor antagonists (b) on the protection by dexmedetomidine. Rat C6 glioma cells were treated with benazoline during 3-h oxygen–glucose deprivation (OGD). Cell viability was determined by MTT assay. Values are expressed as percentage of control values and are from four to six independent experiments with three replicates for each condition. $*P < 0.05$ compared with OGD group (analysis of variance followed by Student–Newman–Keuls test).

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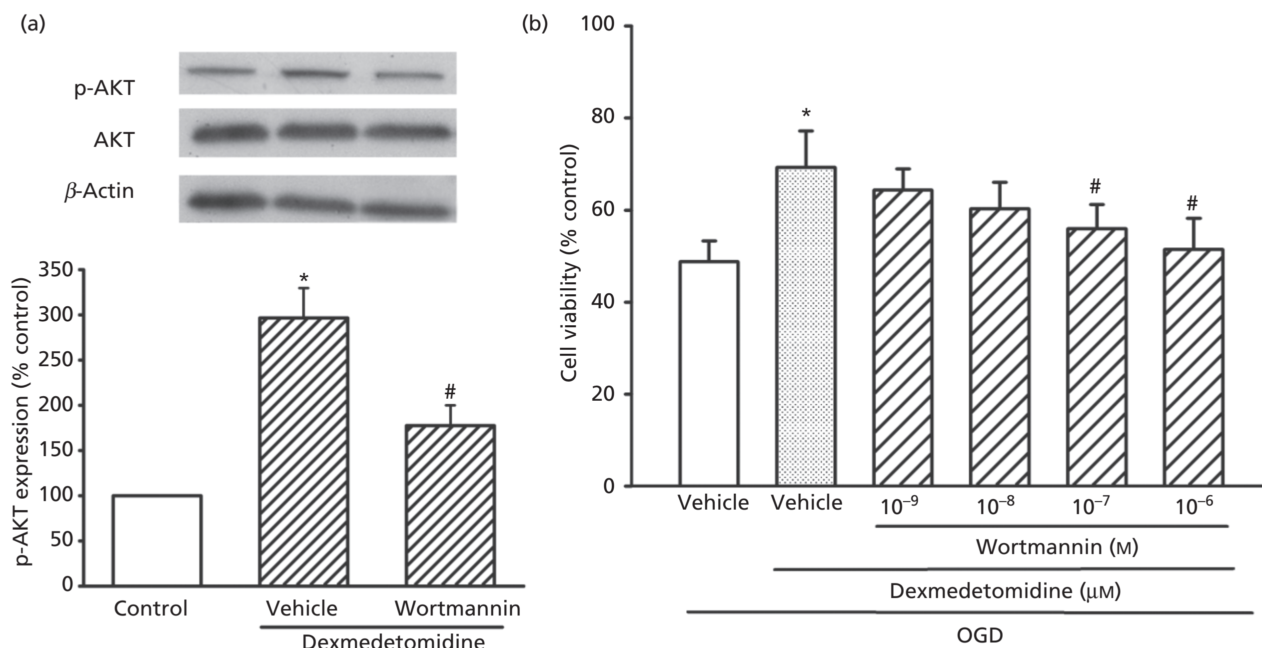

Figure 6 Effects of dexmedetomidine on the p-AKT expression (a), and effects of PI3K/AKT pathway inhibitor wortmannin on the protection by dexmedetomidine (b). (a) Rat C6 glioma cells were treated with vehicle or wortmannin (1 μM) before dexmedetomidine (1 μM) treatment, 30 min later the p-AKT expression was measured. (b) C6 cells were treated with vehicle or wortmannin before dexmedetomidine (1 μM) treatment, and then exposed to 3-h oxygen–glucose deprivation (OGD) insult. Cell viability was determined by MTT assay. Values are expressed as percentage of control values and are from four to six independent experiments with three replicates for each condition. $^{*}P < 0.05$ compared with OGD group; $^{\#}P < 0.05$ compared with dexmedetomidine treatment group (analysis of variance followed by Student–Newman–Keuls test).

There is increasing evidence that cell death after ischaemia is due to apoptosis caused by an imbalance in signalling events, among which the phosphoinositide 3′-OH kinase (PI3K) and its downstream AKT kinase pathway is believed to be an important anti-apoptotic signal.[8] In this study, dexmedetomidine-induced AKT phosphorylation increased to 296% compared with control. Pretreatment with wortmannin significantly reduced AKT phosphorylation to 177% (Figure 6a). Further more, cells exposed to dexmedetomidine and the PI3K/AKT inhibitor wortmannin had decreased viability, in a concentration-dependent manner, compared with the dexmedetomidine-treated group (Figure 6b). In our study, administration of wortmannin alone had no effect on cell survival after OGD (data not shown). These data suggested that dexmedetomidine inhibited apoptosis induced by OGD insult via the activation of the PI3K/AKT pathways.

Effects of dexmedetomidine on HIF-1α and its downstream gene expression in oxygen–glucose deprivation-induced injury

To further understand the potential mechanisms involved in the protective effects of dexmedetomidine against OGD injury, we investigated the effects of dexmedetomidine on the protein expression of HIF-1α and its downstream genes. Dexmedetomidine-induced HIF-1α expression increased to 407% compared with control. Pretreatment with BU 224 (10 μM) and wortmannin (1 μM) significantly reduced HIF-1α expression to 148% and 150%, respectively, but yohimbine (1 μM) has no effect on dexmedetomidine-induced HIF-1α expression (Figure 7a). Furthermore, dexmedetomidine also induced HIF-1α downstream genes VEGF and RTP801 expression, which was blocked by BU 224 and wortmannin but not by yohimbine (Figure 7b and 7c). Thus, dexmedetomidine may have activated the I2 imidazoline receptor-PI3K/AKT pathway, and up-regulated HIF-1α expression. Furthermore, dexmedetomidine increased VEGF and increased RTP801 expression, and then participated in the protection against OGD-induced injury in rat C6 cells.

Discussion

Cell degeneration upon ischaemia has been reported to occur either by necrosis or apoptosis.[9] In this study, we have found that within 3 h of intense OGD insult, most C6 cells displayed the morphological signs of apoptosis. Dexmedetomidine induced a marked protection against this OGD-induced apo-

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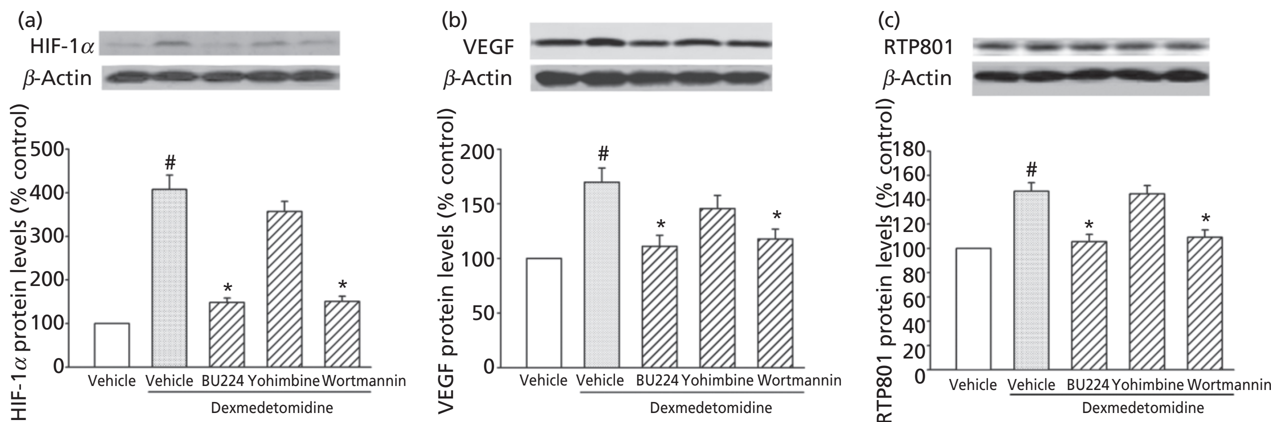

Figure 7 Effects of dexmedetomidine on HIF-1α and its downstream genes expression. Rat C6 glioma cells were treated with vehicle or indicated drugs before dexmedetomidine (1 μM) treatment, 30 min later HIF-1α, VEGF or RTP801 expression were measured. $^{\#}P < 0.05$ compared with control group; $^{*}P < 0.05$ compared with dexmedetomidine treatment group (analysis of variance followed by Student–Newman–Keuls test).

ptosis. Thus, dexmedetomidine may be a significant agent to prevent apoptosis in rat C6 glioma cells.

Recent data supported the hypothesis that dexmedetomidine exerted its protective effects via a direct effect on the α_2-adrenergic receptor.[10] This view was supported by the demonstration of possible anti-apoptotic properties of dexmedetomidine.[11] In this study, the protective effects of dexmedetomidine were only 31% or 35% abolished by yohimbine or RX 821002. On the other hand, idazoxan or BU 224 showed 92.4% or 82.3% inhibition to the protection by dexmedetomidine, suggesting that I2 imidazoline receptors played a primary role in regulating the OGD-induced apoptosis in C6 cells. Further more, the inhibition of idazoxan was more efficient than BU 224, which may have been because idazoxan blocked not only I2 imidazoline receptors but also α_2 receptors, while BU 224 was a selective I2 imidazoline receptor antagonist. It was also shown that dexmedetomidine increased the expression of pERK1 and pERK 2, which was activated by I1-imidazoline receptors but not by α_2-adrenergic receptors in rat hippocampal slices.[12] The different effects of dexmedetomidine may have been dependent on the different cell types.

Different imidazoline receptor subtypes, including I1, I2, and non-I1/I2 (or I3) receptors, have been described.[13] I1-site-selective drugs have been used in treating hypertension, whereas I2-site receptors play a role in monoamine turnover. Cortical I2 receptors are predominantly astrocytic and their primary sub-cellular location is on the mitochondrial membrane.[14] Activation of I2 imidazoline receptors protects neurons against ischaemic damage and reduces the volume of infarction, which may be mediated through mitochondrial imidazoline receptors on astrocytes, since cortical neurons are devoid of imidazoline receptors.[15,16] In our experiment, we used C6 cells to further support the idea that activation of I2 imidazoline receptors conferred protective effects in OGD-induced apoptosis. Thus, depending on the substance used, both adrenoceptor and imidazoline receptors might be important in mediating the protective effects induced by α_2-adrenergic agonists.

The PI3K/AKT pathway has been implicated in the control of major cellular responses including cell proliferation, survival, and apoptosis.[8] For example, PI3K inhibitors inhibited carbachol-induced increases in DNA synthesis in progenitor cells.[17] Similarly, we found that dexmedetomidine increased AKT phosphorylation, which was blocked by wortmannin. These data suggested that I2 imidazoline receptor activation induced by dexmedetomidine, while simultaneously stimulating the PI3K/Akt pathway, inhibited C6 cell apoptosis and exerted protective effects in OGD injury.

In-vitro low oxygen concentrations enhanced the proliferation and reduced the cell death of CNS precursor cells.[18] This effect was attributed to the transcription factor HIF-1α, which switches on a series of genes participating in compensatory mechanisms that support cell survival in a potentially lethal microenvironment. HIF-1 regulates genes that are important in tissue survival, such as VEGF, which has been shown to induce vascular genesis and angiogenesis in many organ systems.[19] In this study, we found dexmedetomidine up-regulated the HIF-1α and VEGF protein level in rat C6 cells. RTP801 is another target gene of HIF-1 that responds to hypoxia.[6] When induced from a tetracycline-repressible promoter, RTP801 protected MCF7 and PC12 cells from hypoxia in glucose-free medium and from H_2O_2-triggered apoptosis.[6] Reduced REDD1 (RTP801) levels can sensitize cells towards apoptosis, whereas elevated levels of REDD1 induced by hypoxia or over-expression desensitize cells to apoptotic stimuli.[20] In this study, we found that dexmedetomidine could increase the expression of RTP801. Activation

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of the PI3K/Akt pathway has been reported to enhance HIF-1-activated responses and the expression of RTP801.[20] In this study, we found that dexmedetomidine up-regulated HIF-1α, VEGF and RTP801 expression by activating the I2 imidazoline receptor-PI3K/AKT pathway in rat C6 cells.

Conclusions

Dexmedetomidine activated the I2 imidazoline receptor-PI3K/AKT pathway, and up-regulated HIF-1α, VEGF and RTP801 expression to protect against OGD-induced injury in rat C6 cells.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81173040, 81170118), the Natural Science Foundation of Zhejiang Province (R2090259), and the Foundation from the Health Bureau of Zhejiang Province (2011KYA065).

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