

Sufentanil ameliorates oxygen-glucose deprivation/reoxygenation-induced endothelial barrier dysfunction in HCMECs via the PI3K/Akt signaling pathway

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Abstract. Ischemic heart disease, a chronic myocardial damage disease caused by coronary artery ischemia, is the leading cause of death worldwide. The aim of the present study was to explore the efficacy of sufentanil in myocardial ischemia/reperfusion (I/R) injury. Oxygen and glucose deprivation/reoxygenation (OGD/R) was utilized to induce human cardiac microvascular endothelial cells (HCMECs) to simulate myocardial I/R injury *in vitro*. The Cell Counting Kit-8 assay was used to detect the effects of sufentanil on HCMECs and OGD/R-induced HCMECs. The TUNEL, lactate dehydrogenase (LDH) activity, immunofluorescence and *in vitro* permeability assays, were used to assess apoptosis, LDH activity, VE-cadherin protein expression levels and endothelial barrier function in OGD/R-induced HCMECs, respectively. Moreover, western blotting was performed to assess the protein expression levels of apoptosis, endothelial barrier function and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)-related proteins. The results demonstrated that sufentanil had no significant influence on the viability of HCMECs but increased the viability of OGD/R-induced HCMECs in a dose-dependent manner. Furthermore, sufentanil inhibited cell apoptosis and permeability of OGD/R-induced HCMECs but enhanced the protein expression levels of tight junction proteins, including ZO-1, Occludin, VE-cadherin and Claudin-5. Sufentanil was also demonstrated to activate the PI3K/Akt signaling pathway. In addition, the use of LY294002, an inhibitor of the PI3K/Akt signaling pathway, partially abolished the protective effects of sufentanil on apoptosis, permeability and tight junction protein expression levels. These results indicated that sufentanil ameliorated OGD/R-induced endothelial barrier dysfunction in HCMECs, potentially via the PI3K/Akt signaling pathway.

The present study therefore suggested that sufentanil may serve as a novel therapeutic option for the improvement of myocardial I/R injury.

Introduction

Coronary artery disease (CAD) is a common health problem and is the main cause of mortality globally (1). CAD is predominantly established following myocardial ischemia/reperfusion (I/R) injury, which refers to myocardial dysfunction and injury following the reperfusion of previously viable ischemic cardiac tissues (2). It has previously been reported that myocardial I/R injury disrupts endothelial barrier function, enhances endothelial permeability and contributes to cellular swelling, which results in microthrombosis and microvascular obstruction, and therefore blocks blood supply to the heart (3,4). Furthermore, myocardial I/R injury involves numerous pathophysiological responses, including calcium overload, oxygen radical production, endothelial dysfunction, immune response, mitochondrial dysfunction, cardiomyocyte apoptosis and autophagy and platelet aggregation (5-7).

Sufentanil is a lipophilic opioid agonist that is selective for μ -opioid receptors and can therefore exert analgesic and sedative effects (8). Compared with fentanyl and remifentanyl, sufentanil not only reduces the incidence of postoperative nausea and vomiting, but also maintains stable hemodynamics (9,10). As a derivative of fentanyl, sufentanil is widely used for the control of tracheal intubation-induced cardiovascular responses due to its significant μ -receptor affinity (11). Moreover, the continuous administration of sufentanil exhibits cardioprotective effects on the human myocardium against hypoxia/reoxygenation (H/R) *in vitro* (12). Furthermore, sufentanil has been reported to activate the PI3K/Akt signaling pathway (13).

Phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt) serve as the two most vital proteins in the PI3K/Akt signaling pathway (14). As an intracellular phosphatidylinositol kinase, PI3K serves a vital role in various aspects of cardiology, including cell survival, myocardial hypertrophy and myocardial contractility (15,16). Being a serine/threonine tyrosine kinase, Akt acts as a downstream effector molecule of PI3K and promotes numerous biological mechanisms, such as cell proliferation and apoptosis (17,18). Furthermore, the PI3K/Akt signaling pathway acts as a

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crucial cardioprotective contributor against myocardial infarction and I/R injury (19).

In the present study, the oxygen-glucose deprivation/reoxygenation (OGD/R) model was established to simulate myocardial I/R injury *in vitro*. The aims of the present study were to investigate the efficacy of sufentanil in myocardial I/R injury as well as to explore its relationship with the PI3K/Akt signaling pathway.

Materials and methods

Cell culture and treatment. Immortalized human cardiac microvascular endothelial cells (HCMECs), without mycoplasma contamination, were purchased from ScienCell Research Laboratories, Inc. HCMECs were cultured in DMEM (Wisent, Inc.), which was supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

Establishment of the OGD/R model. The OGD/R model was established to simulate myocardial I/R injury *in vitro*. HCMECs were cultured in glucose-free DMEM for 2, 4, 6 or 8 h and were then incubated at 37°C in hypoxic conditions (1% O₂, 5% CO₂ and 94% N₂). Subsequently, the cells were added to normal DMEM with continuous reoxygenation (21% O₂, 5% CO₂ and 74% N₂). During the process of reoxygenation, sufentanil at different concentrations (5, 10 and 20 μ M) was administered to the HCMECs. To explore the relationship between sufentanil and the PI3K/Akt signaling pathway, LY294002 (10 μ M; Sigma-Aldrich; Merck KGaA), an inhibitor of the PI3K/Akt signaling pathway, was used to treat HCMECs for 1 h prior to sufentanil treatment.

Cell counting kit-8 (CCK-8) assay. To assess the effects of sufentanil on HCMEC viability and OGD/R-induced HCMECs a CCK-8 assay was performed at 37°C. Following OGD/R induction, 10 μ l CCK-8 reagent per well was added to the HCMECs seeded into a 96-well plate at a density of 1x10⁵ cells/well for 3 h. A wavelength of 450 nm was used to determine the absorbance using a microplate reader (Bio-Rad Laboratories, Inc.).

Lactate dehydrogenase (LDH) activity assay. LDH activity was analyzed to assess cell death. A loss of plasma membrane integrity is demonstrated by the release of LDH. Therefore, 100- μ l cell supernatant was added to 100 μ l cytotoxicity detection reagent (cat. no. C0016; Beyotime Institute of Biotechnology) in a 96-well plate. Colorimetric analysis of sodium pyruvate reduction in the presence of oxycodone was used to determine LDH activity as previously described (20).

TUNEL assay. A TUNEL kit (cat. no. C1086; Beyotime Institute of Biotechnology) was used to assess the effects of sufentanil on the apoptosis of OGD/R-induced HCMECs. Briefly, the cells were fixed using 4% paraformaldehyde for 15 min, after which the cells were permeabilized using 0.25% Triton X-100 for 20 min at room temperature. Subsequently, the cells were stained using TUNEL reagent for 1 h and with 1 μ g/ml DAPI (cat. no. C1005; Beyotime Institute of Biotechnology) in the

dark. In total, 10 fields were randomly selected and observed using a fluorescent microscope (magnification, x200; Olympus Corporation).

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined using a BCA Protein Assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology). An equal amount of protein (30 μ g) was loaded into each lane and separated on a 10% gel using SDS-PAGE and separated proteins were subsequently transferred onto a PVDF membrane. Membranes were blocked with 5% non-fat milk for 2 h at room temperature and were then incubated with primary antibodies against the following: Bcl-2 (1:1,000; cat. no. ab32124), Bax (1:1,000; cat. no. ab32503), cleaved (c)-caspase-3 (1:500; cat. no. ab32042), cytochrome c (1:5,000; cat. no. ab133504), Zonula occludens-1 (ZO-1; 1:1,000; cat. no. ab216880), Occludin (1:1,000; cat. no. ab216327), vascular endothelial (VE)-cadherin (1:2,000; cat. no. ab33168), Claudin-5 (1:1,000; cat. no. ab131259), phosphorylated (p)-PI3K (1:1,000; cat. no. ab278545), p-Akt (1:1,000; cat. no. ab38449), PI3K (1:1,000; cat. no. ab32089), Akt (1:500; cat. no. ab8805) or GAPDH (1:2,500; cat. no. ab9485; all from Abcam) at 4°C overnight. Following the primary incubation, cells were incubated with an HRP-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6759; Abcam) at room temperature for 2 h. Protein bands were visualized using ECL (MilliporeSigma) and subsequently analyzed with ImageJ software v1.8.0 (National Institutes of Health).

In vitro permeability assay. Using the *In Vitro* Permeability Assay kit (MilliporeSigma), the effects of sufentanil on endothelial barrier function in OGD/R-induced HCMECs were investigated. Briefly, in order to form a tight monolayer, 5x10³ HCMECs were inoculated onto collagen-coated inserts and cultured for 72 h. After treating cells for 1 h, OGD/R induction was performed. Each receiver plate contained 500 μ l glucose-free DMEM. To each insert 150 μ l 2.5% FITC-dextran (40 kDa) solution was added for 20 min at 37°C in the dark. The extent of endothelial permeability was quantified using the fluorescence intensity of the bottom plate, which had been permeated by FITC-dextran. Cells were observed using wavelengths of 485 nm (excitation) and 535 nm (emission) using a fluorescence spectrophotometer (Tecan Group, Ltd.) as previously described (21).

Immunofluorescence. HCMECs were cultured as a confluent monolayer on glass slides. Cells were fixed with 2% paraformaldehyde in PBS at 4°C for 3 h and permeabilized in 0.5% Triton X-100 in 1X PBS at room temperature for 1 h. Subsequently, blocking was performed at 37°C using 1% BSA (Beijing Solarbio Science & Technology Co., Ltd.) in 0.2% Triton X-100 for 30 min. Then, cells were incubated with rabbit anti-VE-cadherin antibody (1:200; cat. no. ab33168; Abcam) overnight at 4°C. Cells were rinsed in PBS three times and were subsequently incubated with a goat anti-rabbit IgG Alexa Fluor 555-conjugated secondary antibody (1:100; cat. no. A27017; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 1 h and 1 mg/ml DAPI. A confocal microscope was used to examine the slides.

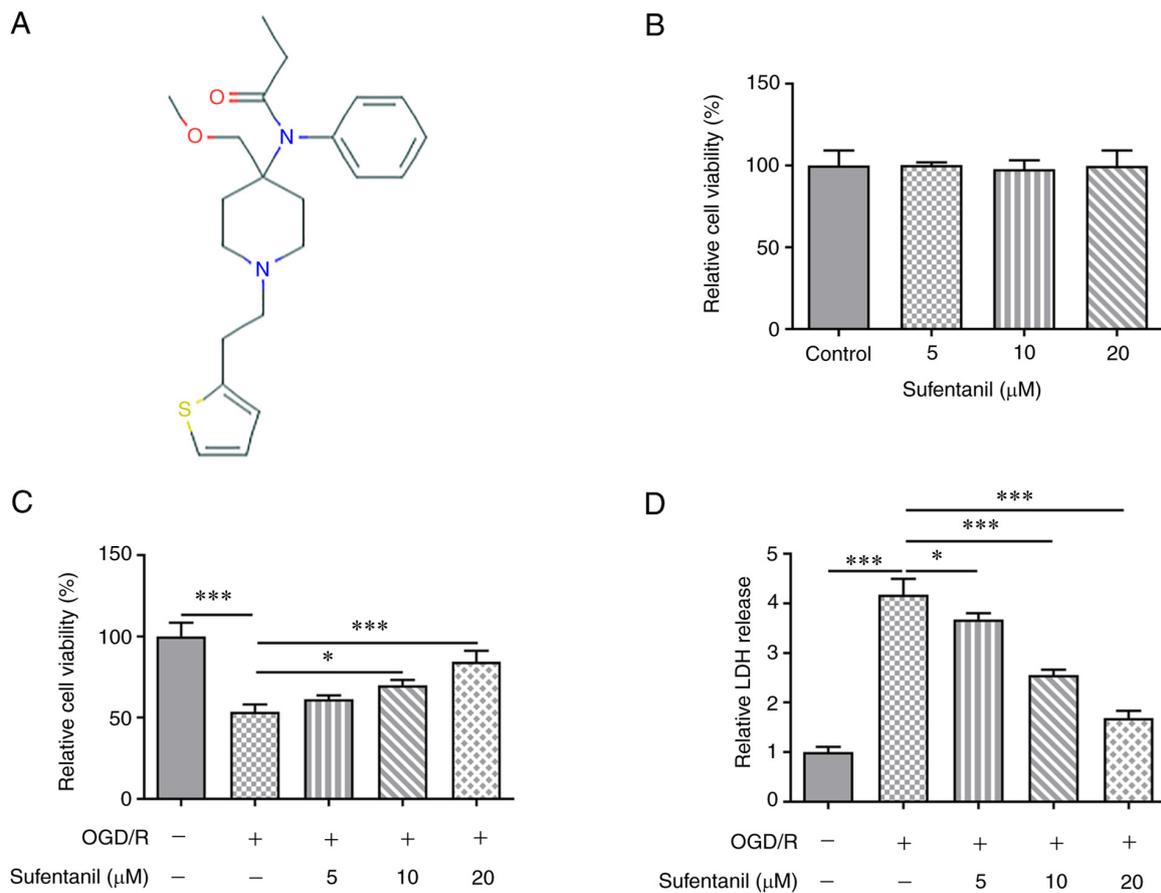


Figure 1. Sufentanil enhances the viability of OGD/R-induced HCMECs. (A) The chemical structure formula of sufentanil. (B) The viability of HCMECs was detected using CCK-8. (C) The viability of OGD/R-induced HCMECs was detected using CCK-8. (D) The LDH activity was detected using LDH assay. Data are expressed as the mean \pm SD. * $P < 0.05$ and *** $P < 0.001$. OGD/R, oxygen and glucose deprivation/reoxygenation; HCMECs, human cardiac microvascular endothelial cells; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase.

Statistical analysis. Data were analyzed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). All data are presented as the mean \pm SD. A one-way ANOVA was used to statistically compare differences among more than two groups, followed by Tukey's post-hoc test. An unpaired Student's t-test was used to compare means between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Sufentanil enhances the viability of OGD/R-induced HCMECs. The chemical structure of sufentanil is presented in Fig. 1A. Sufentanil was demonstrated to have no significant effect on the viability of HCMECs (Fig. 1B). Compared with the control group, OGD/R induction significantly decreased HCMEC viability, which was partially enhanced by sufentanil treatment (Fig. 1C). Furthermore, it was determined that sufentanil exhibited promotive effects on the viability of OGD/R-induced HCMECs in a dose-dependent manner. OGD/R induction increased LDH activity, which was then gradually decreased by sufentanil treatment (Fig. 1D). These results indicated the inhibitory effects of sufentanil on LDH activity in OGD/R-induced HCMECs.

Sufentanil inhibits the apoptosis of OGD/R-induced HCMECs. Compared with the control group, HCMEC

apoptosis was significantly increased by OGD/R induction. However, the enhanced apoptosis in OGD/R-induced HCMECs was suppressed by sufentanil treatment in a dose-dependent manner. Furthermore, 20 μ M sufentanil had optimal suppressive effects on the apoptosis of HCMECs with OGD/R induction (Fig. 2A and B). Moreover, OGD/R induction downregulated Bcl-2 protein expression levels but upregulated the protein expression levels of Bax, c-caspase-3 and cytochrome *c*. However, sufentanil treatment reversed the effects of OGD/R induction on these proteins. This was demonstrated by the upregulated Bcl-2 protein expression levels, as well as the downregulated protein expression levels of Bax, c-caspase-3 and cytochrome *c* in the OGD/R + 5 μ M, OGD/R + 10 μ M and OGD/R + 20 μ M groups (Fig. 2C).

Sufentanil reduces the improved cell permeability of OGD/R-induced HCMECs and upregulates the protein expression levels of tight junction proteins. The results demonstrated that OGD/R induction significantly enhanced the relative fluorescence intensity of HCMECs, which was subsequently reduced following sufentanil treatment (Fig. 3A). In addition, OGD/R induction contributed to decreased protein expression levels of ZO-1, Occludin, VE-cadherin and Claudin-5. However, sufentanil exhibited the opposite effect, whereby ZO-1, Occludin, VE-cadherin and Claudin-5 protein expression levels were increased in the OGD/R + 5 μ M,

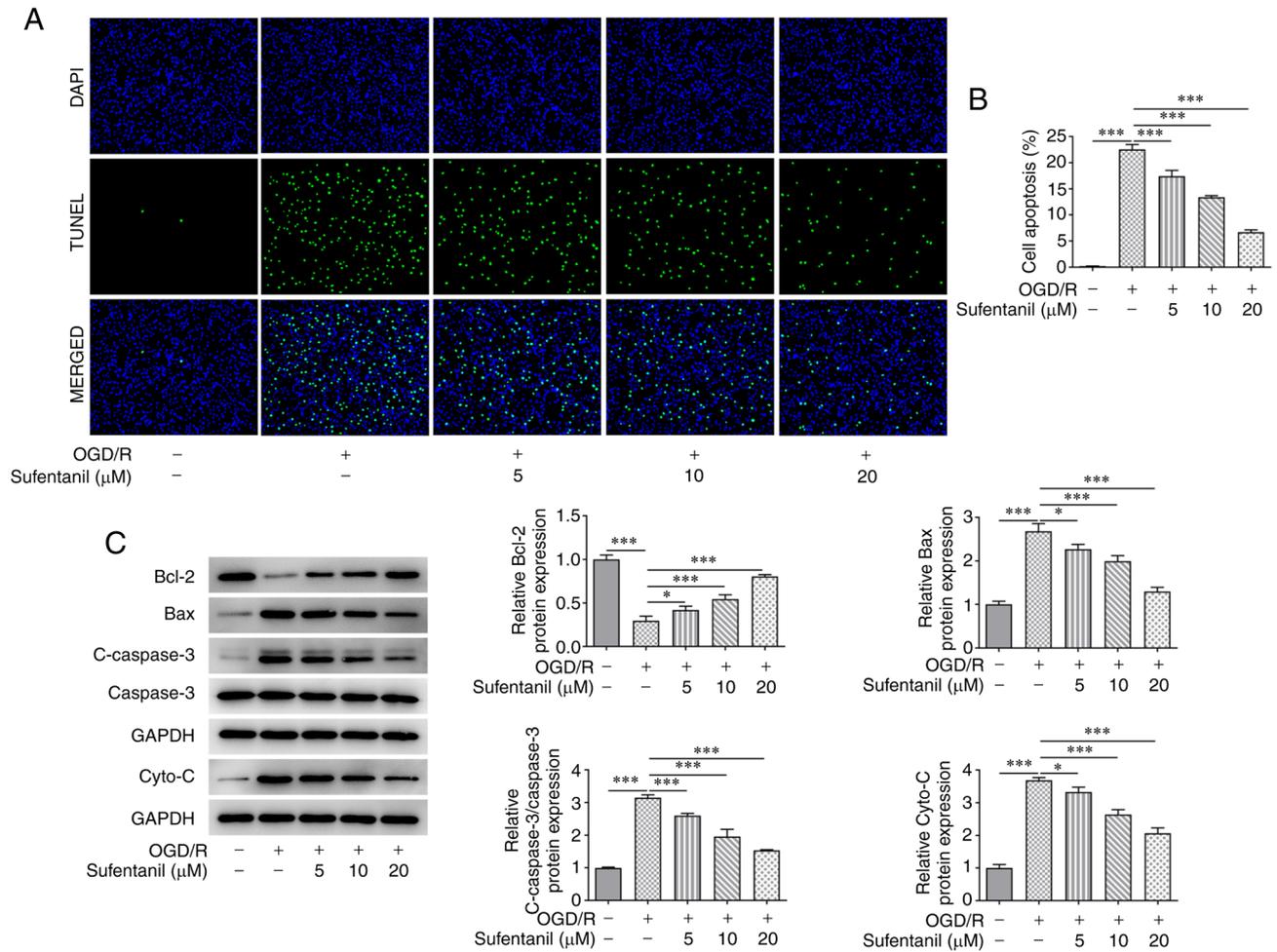


Figure 2. Sufentanil inhibits the apoptosis of OGD/R-induced human cardiac microvascular endothelial cells. (A and B) The apoptosis was detected using TUNEL. (C) The expression levels of apoptosis-related proteins were detected using western blotting. Data are expressed as the mean \pm SD. * $P < 0.05$ and *** $P < 0.001$. OGD/R, oxygen and glucose deprivation/reoxygenation.

OGD/R + 10 μ M and OGD/R + 20 μ M groups, compared with the OGD/R group (Fig. 3B). Furthermore, the decreased VE-cadherin protein expression levels in OGD/R-induced HCMECs were subsequently increased by sufentanil treatment, which suggested that sufentanil treatment had a promotive effect on tight junction proteins (Fig. 3C). Based on the aforementioned results, the dose of 20 μ M sufentanil was selected for the following experiments.

LY294002 reverses the protective effects of sufentanil on OGD/R-induced HCMEC apoptosis. The results demonstrated OGD/R induction reduced the protein expression levels of p-PI3K and p-Akt compared with the control group. However, this was reversed by sufentanil treatment, as demonstrated by the increased protein expression levels of p-PI3K and p-Akt in the OGD/R + sufentanil group (Fig. 4A). Moreover, the decreased cell viability in HCMECs caused by OGD/R induction was improved following sufentanil treatment. These results indicated that sufentanil promoted the viability of OGD/R-induced HCMECs. However, LY294002, an inhibitor of the PI3K/Akt signaling pathway, partially abolished the protective effects of sufentanil, which was demonstrated by the decreased viability in the LY294002 + OGD/R + sufentanil group compared with the OGD/R + sufentanil group (Fig. 4B).

Moreover, LDH activity was significantly enhanced by OGD/R induction compared with the control group. However, compared with the OGD/R + sufentanil group, LDH activity was increased by LY294002 treatment (Fig. 4C).

The results also demonstrated that the decreased apoptotic rate in OGD/R-induced HCMECs, as a result of sufentanil treatment, was increased by LY294002 treatment compared with the OGD/R + sufentanil group (Fig. 4D and E). Moreover, sufentanil upregulated Bcl-2 protein expression levels but downregulated the protein expression levels of Bax, c-caspase-3 and cytochrome *c* compared with the OGD/R group. However, LY294002 had the opposite effect on the expression levels of these proteins, which was demonstrated by the decreased Bcl-2, as well as increased Bax, c-caspase-3 and cytochrome *c* protein expression levels in the LY294002 + OGD/R + sufentanil group, compared with the OGD/R + sufentanil group (Fig. 4F).

LY294002 reverses the protective effects of sufentanil on the cell permeability and tight junction proteins of OGD/R-induced HCMECs. The results demonstrated that the enhanced fluorescence intensity caused by OGD/R induction was significantly reduced following sufentanil treatment compared with the OGD/R group (Fig. 5A). However, compared with the

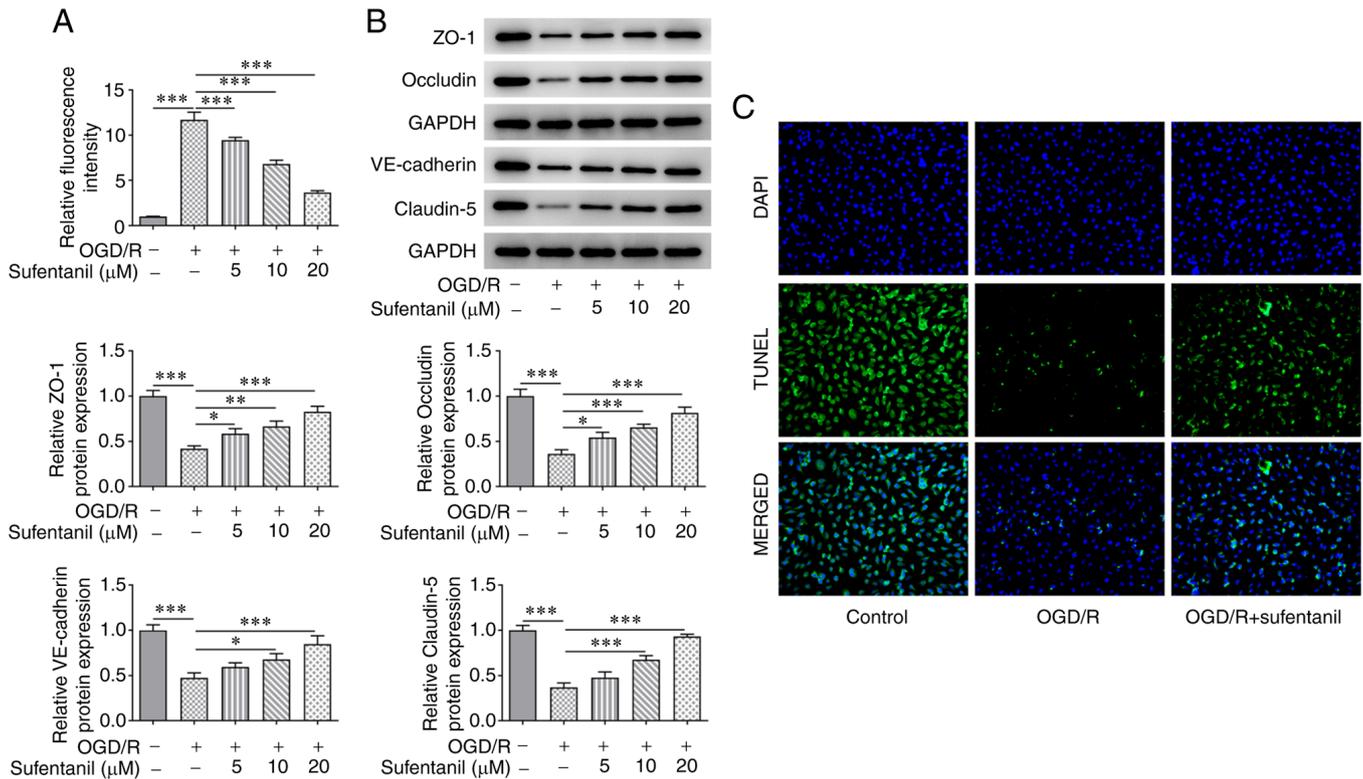


Figure 3. Sufentanil reduces the improved cell permeability of OGD/R-induced human cardiac microvascular endothelial cells and upregulates the expression levels of tight junction proteins. (A) The endothelial barrier function was detected using *in vitro* permeability assay. (B) The expression levels of endothelial barrier function-related proteins were detected using western blot analysis. (C) The level of VE-cadherin was detected using immunofluorescence. Magnification, x200. Data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. OGD/R, oxygen and glucose deprivation/reoxygenation; VE, vascular endothelial.

OGD/R + sufentanil group, LY294002 reversed the inhibitory effects of sufentanil, which was demonstrated by the increased fluorescence intensity in the LY294002 + OGD/R + sufentanil group. Furthermore, the upregulated protein expression levels of ZO-1, Occludin, VE-cadherin and Claudin-5 in OGD/R-induced HCMECs with sufentanil treatment, were partially reduced by LY294002 administration. These results suggested that LY294002 may reverse the protective effects of sufentanil on endothelial barrier function of OGD/R-induced HCMECs (Fig. 5B). Moreover, LY294002 treatment had the opposite effect on VE-cadherin protein expression levels, which was demonstrated by decreased expression of VE-cadherin in the LY294002 + OGD/R + sufentanil group compared with the OGD/R + sufentanil group (Fig. 5C).

Discussion

In the present study, it was demonstrated that sufentanil had no significant influence on the viability of HCMECs but promoted the viability of OGD/R-induced HCMECs in a dose-dependent manner. To determine the effects of sufentanil on OGD/R-induced HCMECs, a series of cellular experiments were performed. The results demonstrated that sufentanil inhibited the apoptosis and cell permeability of OGD/R-induced HCMECs, but enhanced the protein expression levels of the tight junction protein VE-cadherin. Furthermore, the protein expression levels of p-PI3K and p-Akt in OGD/R-induced HCMECs were significantly upregulated

by sufentanil treatment, which revealed that sufentanil could potentially activate the PI3K/Akt signaling pathway. To further investigate the relationship among sufentanil, myocardial I/R injury and the PI3K/Akt signaling pathway, LY294002, an inhibitor of the PI3K/Akt signaling pathway, was used to treat HCMECs. The results demonstrated that LY294002 partially abolished the protective effects of sufentanil on the apoptosis, cell permeability and on tight junction protein expression of OGD/R-induced HCMECs.

Being a selective μ-opioid receptor agonist, sufentanil is commonly used in the clinic and exerts a protective effect on myocardial I/R injury (22). For example, sufentanil protects against myocardial I/R injury in rats via activation of the ERK1/2 signaling pathway (23). Moreover, sufentanil has been reported to reduce myocardial infarct size, preserve phosphorylation of connexin 43 and confer cardioprotective effects (24). Previous studies have demonstrated that microvascular dysfunction in cardiac I/R injury is typically characterized by inflammation, reduced microvascular flow, and impaired angiogenesis and self-repairing capacity (25-28). Among these, endothelial apoptosis is closely associated with the initiation of angiogenesis (29). In the present study, it was demonstrated that an increased apoptotic rate in OGD/R-induced HCMECs was suppressed by sufentanil in a dose-dependent manner. Furthermore, sufentanil treatment increased Bcl-2 protein expression levels but decreased the protein expression levels of Bax, c-caspase-3 and cytochrome c in OGD/R-induced HCMECs, compared with cells without sufentanil treatment.

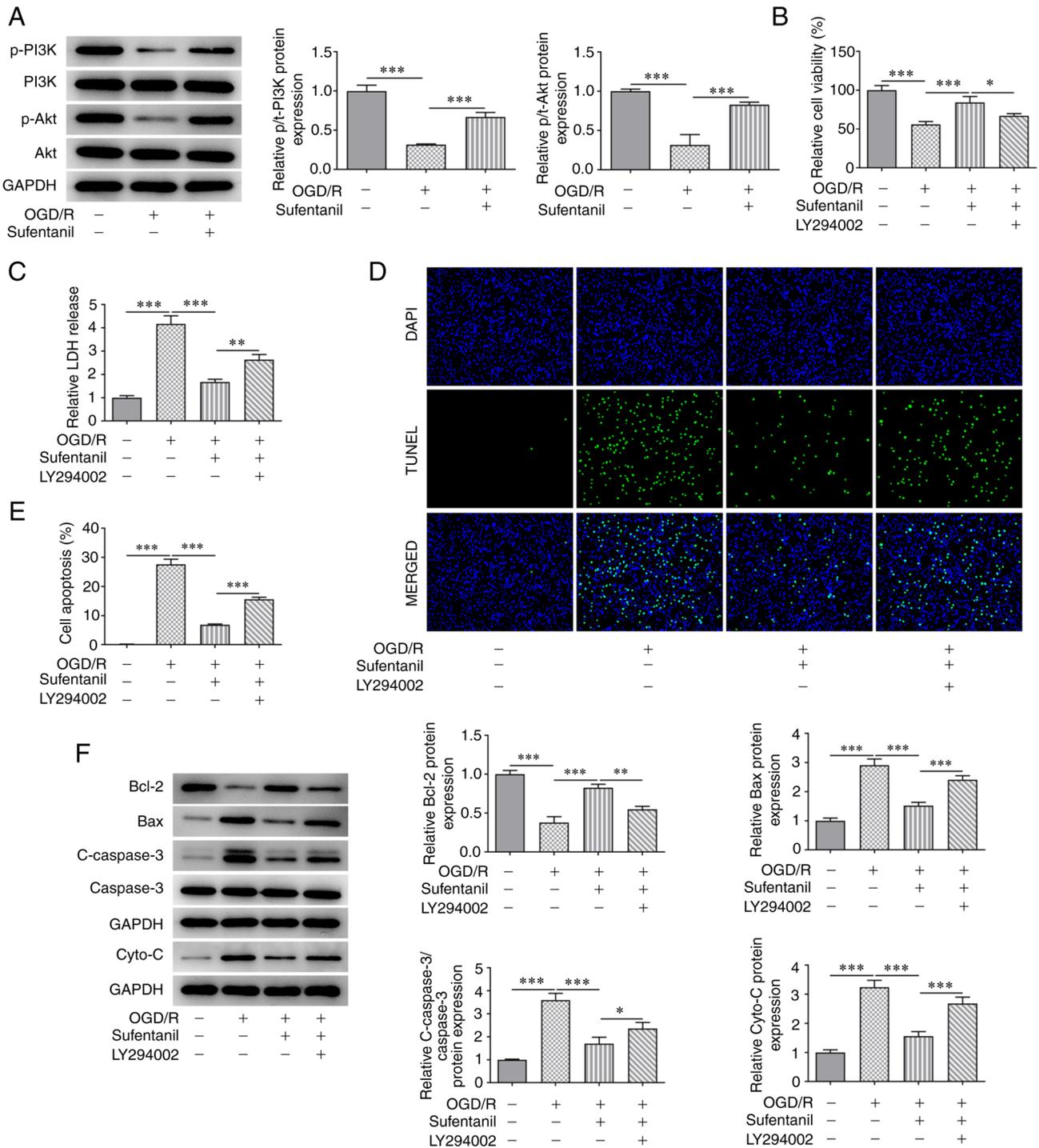


Figure 4. LY294002 reverses the protective effects of sufentanil on the apoptosis of OGD/R-induced HCMECs. HCMECs were treated with 10 μ M LY294002 for 1 h before 20- μ M sufentanil treatment. (A) The expression levels of PI3K/Akt signaling pathway-related proteins were detected using western blotting. (B) The viability was detected using Cell Counting Kit-8. (C) The LDH activity was detected using LDH assay. (D and E) The apoptosis was detected using TUNEL. (F) The expression levels of apoptosis-related proteins were detected using western blotting. Data are expressed as the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001. OGD/R, oxygen and glucose deprivation/reoxygenation; HCMECs, human cardiac microvascular endothelial cells; LDH, lactate dehydrogenase.

These results suggested that sufentanil potentially inhibits apoptosis in myocardial I/R injury.

The insufficient secretion of endothelium-derived diastolic factor and the excessive secretion of endothelin-1 in myocardial microvascular endothelial cells results in increased cell permeability (30). ZO-1, VE-cadherin and Claudin-5, which are important mediators of endothelial adherence junctions,

serve critical roles in maintaining the blood-brain barrier balance in ischemic stroke (31,32). In the present study, it was determined that the increased fluorescence intensity of OGD/R-induced HCMECs was markedly decreased in a dose-dependent manner following treatment with sufentanil. Furthermore, sufentanil treatment enhanced the protein expression levels of ZO-1, Occludin, VE-cadherin and Claudin-5

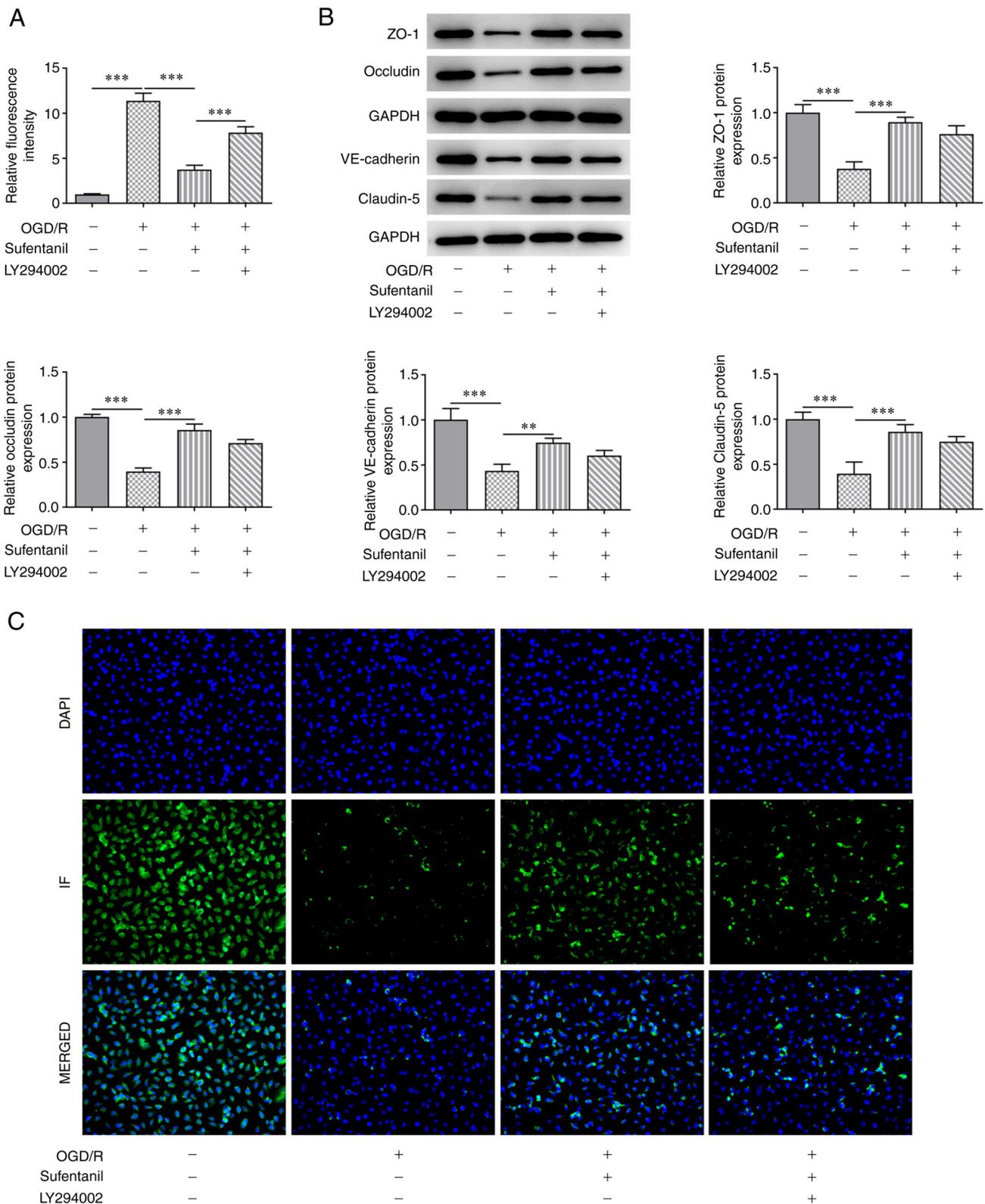


Figure 5. LY294002 reverses the protective effects of sufentanil on the cell permeability and tight junction proteins of OGD/R-induced HCMECs. HCMECs were treated with 10 μ M LY294002 for 1 h before 20 μ M sufentanil treatment. (A) The endothelial barrier function was detected using *in vitro* permeability assay. (B) The expression levels of endothelial barrier function-related proteins were detected using western blot analysis. (C) The level of VE-cadherin was detected using IF. Magnification, x200. Data are expressed as the mean \pm SD. **P<0.01 and ***P<0.001. OGD/R, oxygen and glucose deprivation/reoxygenation; HCMECs, human cardiac microvascular endothelial cells; IF, immunofluorescence; VE, vascular endothelial.

in OGD/R-induced HCMECs. These results indicated that sufentanil may have had a suppressive effect on the enhanced cell permeability of OGD/R-induced HCMECs. Furthermore,

the decreased protein expression levels of the tight junction protein VE-cadherin, caused by OGD/R induction, were also improved following sufentanil treatment.

It has previously been reported that the activation of the PI3K/Akt signaling pathway contributes to the inhibition of apoptosis and endothelial dysfunction of H/R-induced HCMECs (33). Moreover, an increasing number of studies have demonstrated that the regulation of the PI3K/Akt signaling pathway alleviates apoptosis and endothelial dysfunction in myocardial I/R injury (34,35), and sufentanil activates the PI3K/Akt signaling pathway (36). In the present study, it was demonstrated that the decreased protein expression levels of p-PI3K and p-Akt in OGD/R-induced HCMECs were significantly upregulated by sufentanil treatment. These results suggested that sufentanil may activate the PI3K/Akt signaling pathway, which is consistent with the results from a previous study (36). However, further experiments performed in the present study demonstrated that LY294002, an inhibitor of the PI3K/Akt signaling pathway, reversed the protective effects of sufentanil on apoptosis, cell permeability and tight junction proteins in OGD/R-induced HCMECs. Therefore, these results indicated that sufentanil may ameliorate OGD/R-induced endothelial barrier dysfunction in HCMECs via activating the PI3K/Akt signaling pathway.

In conclusion, the present study highlighted the potential therapeutic application of sufentanil in myocardial I/R injury via the activation of the PI3K/Akt signaling pathway. However, further studies are needed to examine the effects of sufentanil on myocardial I/R injury *in vivo*.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LW and XZ designed the study, drafted and revised the manuscript. LW, CG and XZ analyzed the data and searched the literature. LW and CG performed the experiments. All authors read and approved the final manuscript. LW and CG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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