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Original Article

The Effect of Remifentanil Preconditioning on Injured Keratinocyte

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Background: Incisional site of surgical operation become transient ischemic state and then occur reoxygenation due to vasodilatation by inflammatory reaction, the productive reactive oxygen species (ROS) give rise to many physiologic results. Apoptosis have major role on elimination of inflammatory cell and formation of granulation tissue in normal wound healing process. Remifentanil can prevent the inflammatory response and can suppress inducible nitric oxide synthase expression in a septic mouse model. After cardiopulmonary bypass for coronary artery surgery, remifentanil can also inhibit the release of biomarkers of myocardial damage. Here we investigated whether remifentanil pretreatment has cellular protective effect against hypoxia-reoxygenation in HaCaT human keratinocytes, if so, the role of apoptosis and autophagy on this phenomenon.
Methods: The HaCaT human keratinocytes were exposed to various concentrations of remifentanil (0.01, 0.05, 0.1, 0.5 and 1 ng/ml) for 2 h before hypoxia (RPC/HR group). These cells were cultured under 1% oxygen tension for 24h at 37°C. After hypoxia, to simulate reoxygenation and recovery, the cells were reoxygenated for 12 h at 37°C. 3-MA/RPC/HR group was treated 3-methyladenine (3-MA), autophagy inhibitor for 1h before remifentanil treatment. Cell viability was measured using a quantitative colorimetric assay with thiazolyl blue tetrazoliumbromide (MTT, amresco), showing the mitochondrial activity of living cells. To investigate whether the occurrence of autophagy and apoptosis, we used fluorescence microscopy and Western blot analysis.
Results: The viability against hypoxia-reoxygenation injury in remifentanil preconditioning keratinocytes were increased, and these cells were showed stimulated expression of autophagy 3-MA suppressed the induction of autophagy effectively and the protective effects on apoptosis. Atg5, Beclin-1, LC3-II and p62 were elevated in RPC/HR group. But they were decreased when autophagy was suppressed by 3-MA.
Conclusions: Remifentanil preconditioning showed the protective effect in human keratinocytes, and we concluded that autophagy may take the major role in the recovery of wound from hypoxia-reoxygenation injury. We suggest that further research is needed about the cell protective effects of autophagy.

Key Words: Remifentanil; Keratinocyte

INTRODUCTION

Remifentanil, an ultra-short-acting mu-opioid receptor agonist, is unique from other opioids because of its esterase-based metabolism, minimal accumulation, and very rapid onset and offset of clinical action [1]. Recently, remifentanil is used as adjuvant drug for general anesthesia. Remifentanil can prevent the inflammatory response and can suppress inducible nitric oxide synthase expression in a septic mouse model [2]. After cardiopulmonary bypass for coronary artery surgery, remifentanil can also inhibit the release of biomarkers of myocardial damage [3].

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Skin incision is essential part of surgical operation which needed general anesthesia, following wound healing process [4]. Incisional site become transient ischemic state and then occur reoxygenation due to vasodilatation by inflammatory reaction, the productive reactive oxygen species (ROS) give rise to many physiologic results [5].

Apoptosis have major role on elimination of inflammatory cell and formation of granulation tissue in normal wound healing process. Keratinocyte apoptosis and autophagy is an inevitable process during skin tissue ischemia-reperfusion induced injury [6].

Autophagy is the major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome [7]. However, the purpose of autophagy is not the simple elimination of materials; but instead, autophagy serves as a dynamic recycling system that produces new building blocks and energy for cellular renovation and homeostasis. Cells routinely replace their contents to stay healthy but also to make morphological and functional changes. The physiological role of autophagy still remains unknown in some key organs, such as in the bone, skin, and blood vessels [8-10].

Here we investigated whether remifentanil pretreatment has cellular protective effect against hypoxia-reoxygenation in HaCaT human keratinocytes, if so, the role of apoptosis and autophagy on this phenomenon.

MATERIALS AND METHODS

1. Reagents

The following reagents were obtained commercially: 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), acridine orange, monodansyl cadaverine (MDC), 3-methyladenine (3-MA, class III PI3K inhibitor) was obtained from Calbiochem (La Jolla, CA, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Biovision (Milpitas, CA, USA). Antibodies used in the study were as follows; caspase-3 (1:1,000), Bcl-xl (1:1,000), Bax (1:1,000), from ENZO, LC3-II (1:3,000) and Beclin-1 (1:1,000) from Abcam, p62 (1:1,000) and Atg5 (1:500) from Santa Cruz. Secondary antibodies against rabbit (1:3,000), and mouse (1:3,000), immunoglobulins were purchased from Bio-Rad.

2. Cell culture

Human keratinocytes (HaCaT) were obtained from the American Type Culture Collection (ATCC, manassas, USA). Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% inactivated fetal bovine serum (FBS, GIBCO) containing 500 μg/mL penicillin and 500 μg/mL streptomycin (GIBCO), and cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Media were changed every 3 days.

3. Hypoxia/reoxygenation (H/R) of cultured human keratinocytes and drug treatment

The HaCaT human keratinocytes were cultured under 1% oxygen tension. Cells were seeded in a 96-well plate (1×10^4 cells) before exposure to hypoxic conditions. The cells were gassed with 94% N_2, and 5% CO_2 (Anaerobic System PROOX model 110; BioSpherix, USA) and incubated for 24 h at 37°C. After hypoxia, to simulate reoxygenation and recovery, the cells were reoxygenated for 12 h at 37°C in H/R group. To determine whether the remifentanil (Ultiva; GlaxoSmithKline Pharmaceuticals, Uxbridge, UK) affects H/R injured human keratinocytes, cells were exposed to various concentrations of remifentanil (0.01, 0.05, 0.1, 0.5 and 1 ng/ml) for 2 h before hypoxia (RPC/HR group). Control group did not receive hypoxia and remifentanil treatment for 36 h. 3-MA/RPC/HR group was treated 3-methyladenine (3-MA) for 1h before remifentanil treatment (Fig. 1).

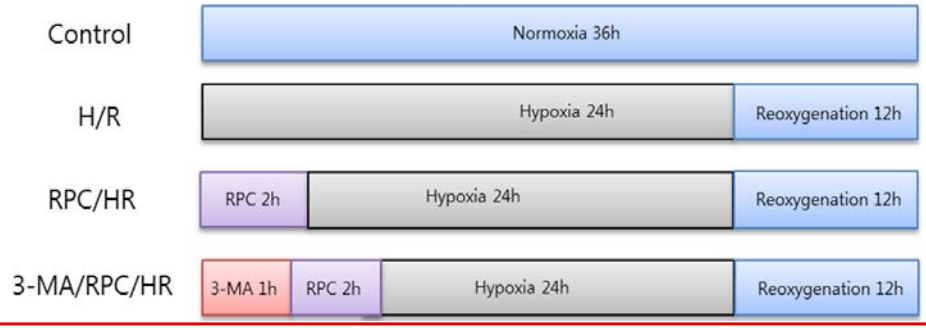

Fig. 1. The experiment protocols. In vitro experiment are shown. Control = normoxia group, Hypoxia-reoxygenation (H/R) = no remifentanil treatment group, RPC/HR = remifentanil preconditioning group, 3MA/RPC/HR = Both 3-MA and remifentanil treatment group.

4. MTT assay

Cell viability was measured using a quantitative colorimetric assay with thiazolyl blue tetrazoliumbromide (MTT, amresco), showing the mitochondrial activity of living cells. HaCaT cells (3×10^4) were seed in 96-well plates. After drug treatment as indicated, cells were incubated with 300 ㎕ MTT (final concentration 0.5 mg/mL) for 1.5 h at 37℃. The reaction was terminated by addition of 200 ㎕ DMSO. Cell viability was measured by an ELISA reader (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength.

5. Fluorescence microscopy (with 1 ng/ml remifentanil treatment)

Cells were grown on coverslips and treated with HS-1200. After 24 h, cells were stained with 0.05 mM MDC, a selective fluorescent marker for autophagic vacuoles, at 37℃ for 1 h. The cellular fluorescence changes were observed using a fluorescence microscope (Axioskop, Carl Zeiss, Germany). For further detection of the acidic cellular compartment, we used acridine orange, which emits bright red fluorescence in acidic vesicles but fluoresces green in the cytoplasm and nucleus. Cells were stained with 1 ㎍/mL acridine orange for 15 min and washed with PBS. AVOs formation was obtained under a confocal microscope LSM 700 (Carl Zeiss, Germany).

6. Western blot analysis (with 1 ng/ml remifentanil treatment)

Cells (2×10^6) were washed twice in ice-cold PBS, resuspended in 200 ㎕ ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 ㎕/ml aprotinin and 2 ㎕/ml leupeptin] and incubated at 4℃ for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4℃. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 20 ㎍ of proteins were resolved by 10% SDS/PAGE. The gels were transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and reacted with appropriate primary antibodies. Immunostaining with secondary antibodies was detected using SuperSignal West Femto (Pierce, Rockford, IL, USA) enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA). Antibodies used in the study were as follows: LC3-II (1:3,000), Beclin-1 (1:1,000) from Abcam, p62 (1:1,000), Atg5 (1:500), from Santa Cruz.

7. Statistical analysis

All experiments were repeated five times. Multiple groups were compared using one-way analysis of variance

(ANOVA) followed by a post hoc Turkey's test. The data were expressed as the mean ± standard deviation (SD). Values of $P < 0.05$ were considered significant (SPSS 13.0 Software, SPSS Inc., Chicago, IL, USA).

RESULTS

1. Effect of remifentanil treatment on cell viability

The cell viability assay was performed 36 h after hypoxia. Various concentrations of remifentanil did not show any toxic effect on HaCaT human keratinocytes, and the cell proliferation rate was higher in the 0.5 and 1 ng/ml groups compared to the control group (Fig. 2). The cell viability was decreased in H/R group and remifentanil increased the cell viability dose-dependent manner (Fig. 3A). In 3-MA/RPC/HR group, similar to the results, the cell viability was lower than RPC/HR groups (Fig. 3B). Among all of the concentrations, 1 ng/ml remifentanil represented the highest cell viability. Based on this result, all subsequent experiments were performed with 1 ng/ml remifentanil.

2. Effect of remifentanil preconditioning on autophagy activation

Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in RPC/HR

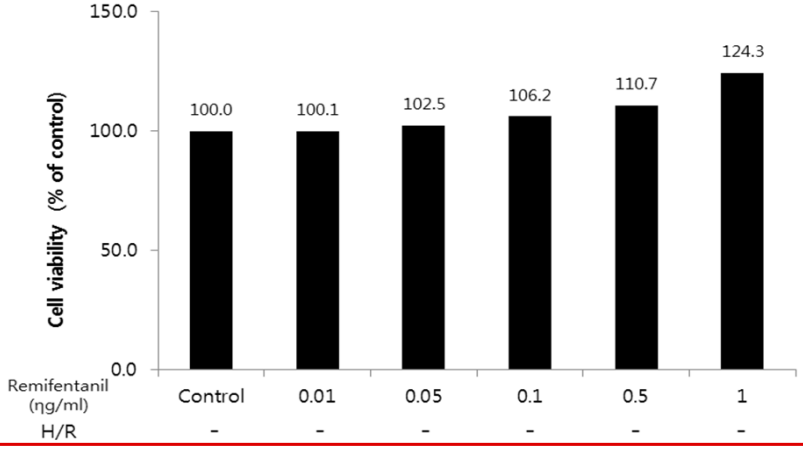

Fig. 2. The effect of remifentanil on HaCaT cells by MTT assay. Various concentrations of remifentanil did not show any toxic effect on HaCaT human keratinocytes, and the cell proliferation rate was higher in the 0.5 and 1 ng/ml groups compared to the control group.

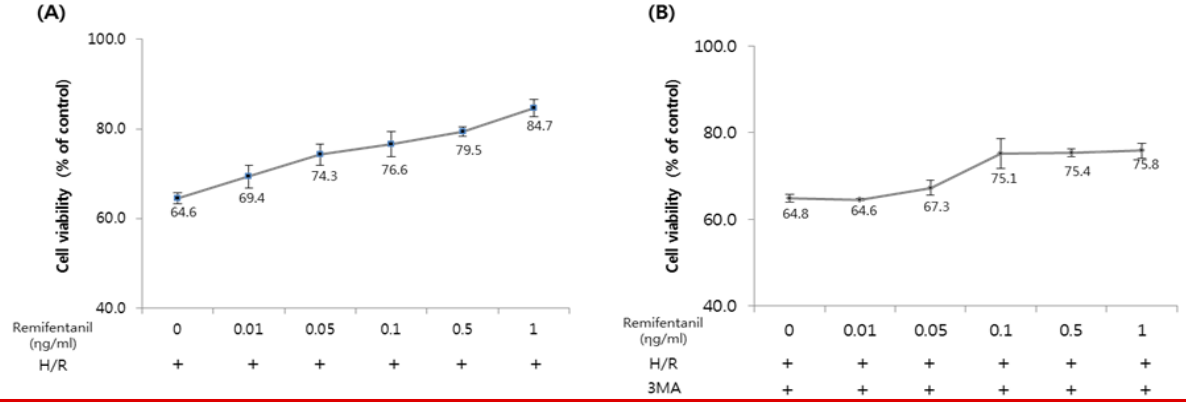

Fig. 3. The effect of remifentanil on HaCaT cells by MTT assay. (A) The cell viability was decreased in H/R group and remifentanil increased the cell viability dose-dependent manner. (B) In 3-MA/RPC/HR group, similar to the results, the cell viability was lower than RPC/HR groups.

group HaCaT cells (Fig. 4). Similarly, AO staining, red fluorescent spots appeared in RPC/HR group HaCaT cells, while the control, H/R and 3-MA/RPC/HR groups showed mainly green cytoplasmic fluorescence (Fig. 5).

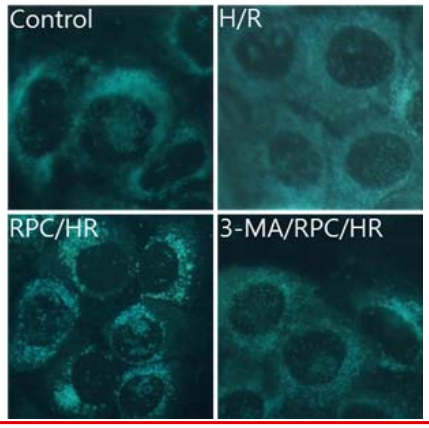

Fig. 4. MDC staining of cytoplasmic vacuoles induced by remifentanil treated in human keratinocytes. Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in RPC/HR group HaCaT cells.

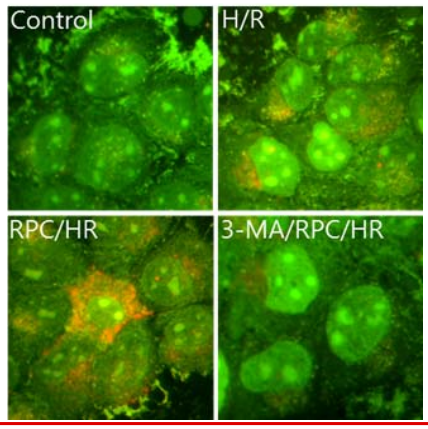

Fig. 5. AO staining of autophagosome formation induced by remifentanil treated in human keratinocytes. Red fluorescent spots appeared in RPC/HR group HaCaT cells, while the control, H/R and 3-MA/RPC/HR groups showed mainly green cytoplasmic fluorescence.

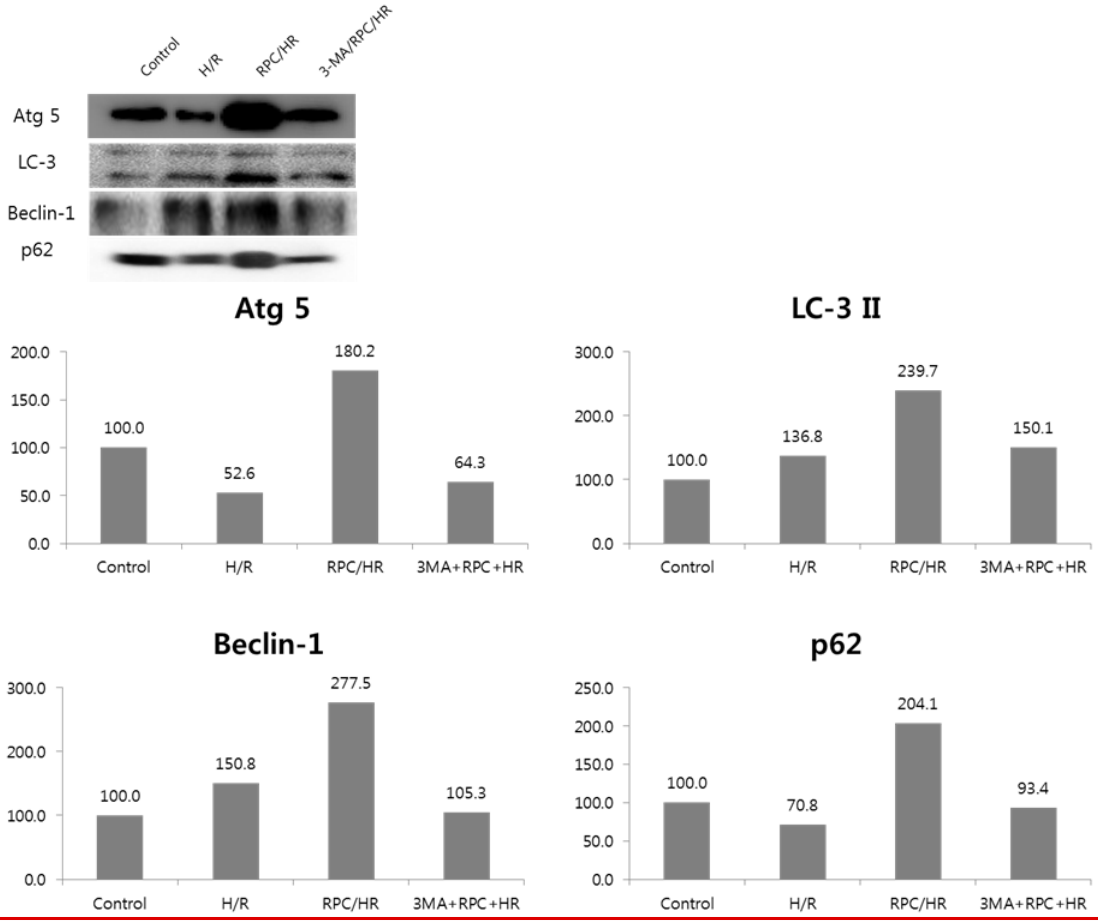

Fig. 6. Effects of remifentanil on autophagy markers in human keratinocytes. Atg5, Beclin-1, LC3-II and p62 were elevated in RPC/HR group. But they were decreased when autophagy was suppressed by 3-MA. Multiple groups were compared using one-way analysis of variance (ANOVA) followed by a post hoc Turkey's test.

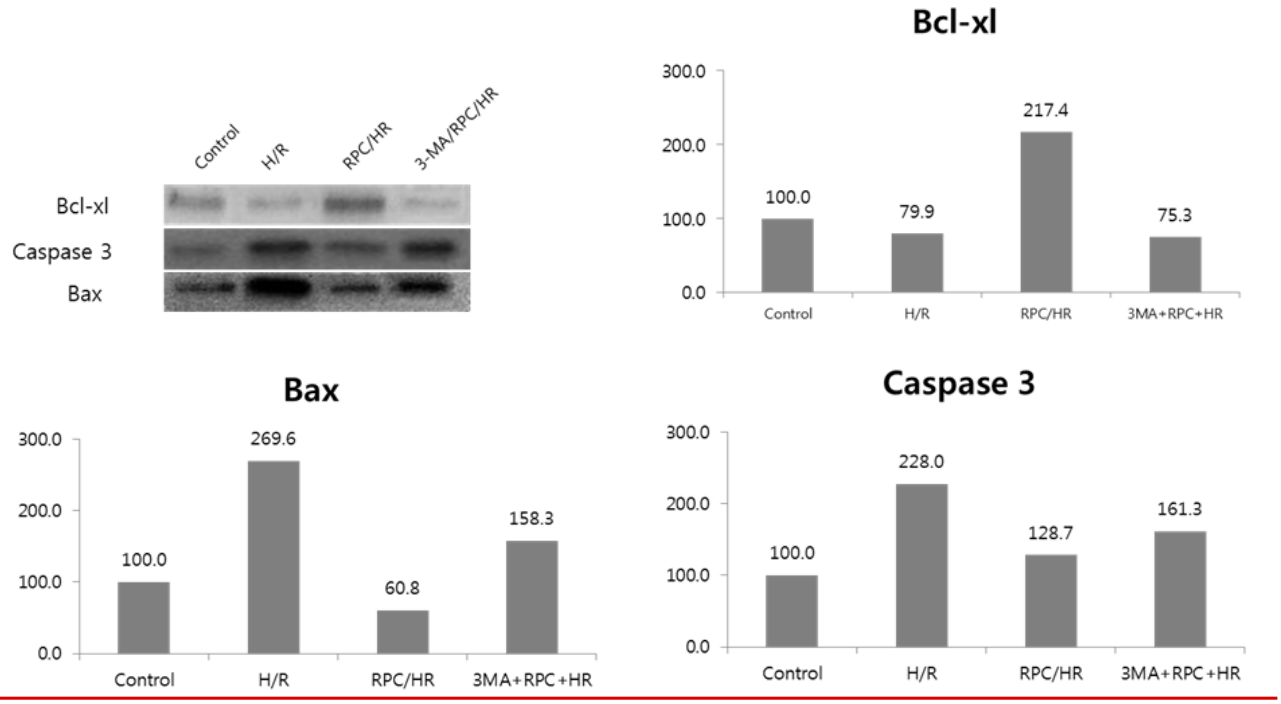

Fig. 7. The effects of remifentanil preconditioning on apoptosis activation Activation of cleaved caspase-3 is a key upstream event involved in the initiation and execution of apoptosis. Cleaved caspase-3 was up-regulated in H/R group, and decreased in RPC/HR and 3-MA/RPC/HR groups. The mitochondria-dependent pathway of apoptosis is regulated by Bcl-xl family, such as the anti-apoptotic protein Bcl-xl, pro-apoptotic protein Bax. With H/R injury, the protein level of Bax was up-regulated while the protein level of Bcl-xl was down-regulated. In RPC/HR group, the ratio was reduced and the expression of Bax decreased at the same time, indicating that remifentanil can attenuate apoptosis through mitochondrial related pathway in HaCaT human keratinocytes. Multiple groups were compared using one-way analysis of variance (ANOVA) followed by a post hoc Turkey's test.

We here examined activation of autophagy - related protein by western blotting analysis. The recruitment of LC3 to the membrane occurs via an Atg 5-dependent mechanism. Atg 5, Beclin-1, LC3-II (microtubule-associated protein 1 light chain 3 form II) and p62 were elevated in RPC/HR group cells. But they were decreased when autophagy was suppressed by 3-MA (Fig. 6).

3. Effect of remifentanil preconditioning on apoptosis activation.

Activation of cleaved caspase-3 is a key upstream event involved in the initiation and execution of apoptosis. Cleaved caspase-3 was up-regulated in H/R group, and decreased in RPC/HR and 3-MA/RPC/HR groups. The mitochondria-dependent pathway of apoptosis is regulated by Bcl-xl family, such as the anti-apoptotic protein Bcl-xl, pro-apoptotic protein Bax. With H/R injury, the protein level of Bax was up-regulated while the protein level of Bcl-xl was down-regulated. In RPC/HR group, the ratio was reduced and the expression of Bax decreased at the same time, indicating that remifentanil can attenuate apoptosis through mitochondrial related pathway in HaCaT human keratinocytes (Fig. 7).

DISCUSSION

The object of the current study was to determine the beneficial effect of remifentanil on human keratinocytes with hypoxia-reoxygenation injury and to investigate whether autophagy is associated with protective mechanism. We showed that remifentanil preconditioning increased the proliferation of human keratinocytes with

hypoxia-reoxygenation injury (Fig. 3A). And the autophagy pathway inhibitor, 3-MA, blocked the protective effect of remifentanil preconditioning against H/R injury (Fig. 3B).

In autophagic specific staining, MDC and AO stain, RPC/HR group induced more autophagic expression than control, H/R group and 3-MA groups. These findings mean that remifentanil preconditioning has cellular protective effect mediated by autophagy. Atg-5 can induce autophagy and also enhance the susceptibility of tumor cells to activate the intrinsic cell death pathway, for instance by ceramide or DNA-damaging agents [11]. Becline-1 regulates the kinase activity for the activation of mammalian Vps34 which is initial step of vesicle nucleation [12,13]. Lipid conjugation leads to the conversion of the soluble form of LC3 (named LC3-I) to the autophagic vesicle- associated form (LC3-II). LC3-II is used as a marker of autophagy because its lipidation and specific recruitment to autophagosomes provides a shift from diffuse to punctate staining of the protein and increases its electrophoretic mobility on gels compared with LC3-I [14]. The p62/SQSTM1 is a multifunctional adaptor protein that promotes turnover of polyubiquitinated protein aggregates through interaction with LC3 at the autophagosome [15,16].

In the western blot analysis, we showed that remifentanil treatment was found to increase expression of Atg5, Beclin-1, LC3-II and p62 proteins associated with autophagic expression. Depending on these results, we suggest remifentanil preconditioning stimulated human keratinocytes endogenous cellular protective effect against H/R injury through autophagy signal pathways activation.

The earlier studies have presented the effect of autophagy against H/R injury in other cells (cardiomyocyte, propofol post), but the effect of remifentanil on autophagy in human keratinocytes with hypoxia-reoxygenation injury has not been documented until this study. Of course, these protective effects were observed in human keratinocytes through autophagic activation, so in vivo and clinical trials would be needed to establish this therapeutic role for remifentanil.

We also point out another limitation of this study. The physiology of keratinocytes involves two important pathways of proliferation and differentiation for epidermis repair [17]. However, our results did not determine whether remifentanil treatment confers positive effect on differentiation in human keratinocytes. We acknowledge that additional studies using factors associated with differentiation of keratinocyte will be needed to determine the role of remifentanil for wound repair.

The functional relationship between apoptosis and autophagy is complex, in several scenarios; autophagy constitutes a stress adaptation that avoids cell, whereas in other cellular settings, autophagy constitutes an alternative pathway to cellular demise that is called autophagic cell death [18-21]. According to previous studies, autophagy plays two distinct roles during ischemia and reperfusion. In the ischemic phase, autophagy can be protective via AMPK activation and sequentially inhibition of mTOR signaling, but reperfusion after ischemia stimulates autophagic cell death through the different pathway. This autophagy process is beclin-1 dependent but AMPK independent [22].

Here we found that remifentanil preconditioning protected HaCaT human keratinocytes death due to H/R injury and this cellular protective effect is associated with autophagy activated by expression of Atg5, LC3-II, Beclin-1 signaling and accumulation of autophagic vacuoles. 3-MA, autophagy inhibitor, suppressed the induction of autophagy effectively and the protective effects on apoptosis. The authors examined caspase-3, Bcl-xl and Bax by western blot analysis. It's known that the caspase-3 and Bax are apoptotic proteins and Bcl-xl

is anti-apoptotic protein [23-25]. Our data showed that anti-apoptotic effect of remifentanil preconditioning mediated by mitochondria related pathway.

In summary, the present study shows that remifentanil preconditioning increases the human keratinocytes proliferation rate and stimulates the expression of autophagy under hypoxia-reoxygenation injury. No functional studies were performed to investigate the effects of remifentanil on the wound healing process.

Therefore, although the findings of this study are limited to an in vitro interpretation, we suggest that remifentanil preconditioning may have a beneficial effect in the recovery of wound from hypoxia-reoxygenation injury.

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