

Remifentanil Protects Human Keratinocyte Through Autophagic Expression

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Background: 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. Remifentanil can prevent the inflammatory response and can suppress inducible nitric oxide synthase expression in a septic mouse model. However, the effects of remifentanil on human keratinocyte and autophagy have yet to be fully elucidated during hypoxia-reoxygenation. Here we investigated whether remifentanil confers protective effect against hypoxia-reoxygenation in human keratinocyte and, if so, whether autophagy mediates this effect.

Methods: The human keratinocytes were cultured under 1% oxygen tension. The cells were gassed with 94% N₂, and 5% CO₂ and incubated for 24 h at 37°C. To determine whether the administration of affects human keratinocytes hypoxia-reoxygenation injury, cells were then exposed to various concentrations of remifentanil (0.01, 0.1, 0.5 and 1 ng/ml) for 2 h. After remifentanil treatment, to simulate reoxygenation and recovery, the cells were reoxygenated for 12 h at 37°C. Control group did not receive remifentanil treatment. Normoxia group did not receive hypoxia and remifentanil treatment for 36 h. 3-MA group was treated 3-methyladenine (3-MA) for 1h before remifentanil treatment. Cell viability was measured using a quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells. Cells were stained with fluorescence and analyzed with Western blot analysis to find out any relations with activation of autophagy.

Results: Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in RPT group HaCaT cells. Similarly, AO staining, red fluorescent spots appeared in RPT group HaCaT cells, while the Normoxia, control and 3-MA groups showed mainly green cytoplasmic fluorescence. We here examined activation of autophagy related protein under H/R-induced cells by Western blotting analysis. Atg5, Beclin-1, LC3-II (microtubule-associated protein 1 light chain 3 form II) and p62 was elevated in RPT group cells. But they were decreased when autophagy was suppressed by 3-MA (Fig. 5).

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

Key Words: Autophagy; Hypoxia; Keratinocyte; Remifentanil

INTRODUCTION

Wound healing is important not only for the skin repair but also for its beneficial effect to systemic physiological defenses. Reepithelialization initiated during the early stages of healing is important. This process includes the proliferation, migration, and differentiation of keratinocytes from the wound margins [1,2]. Skin tissue ischemia-reperfusion can occur during various stressful environments such as surgery and trauma. Pressure ulcer,

diabetic ulcer, and varicose ulcer also can give rise to Hypoxia-reoxygenation injury of keratinocytes [3–5]. Autophagy is a self-eating process that is important for balancing sources of energy at critical times in development and in response stress. Autophagy also plays a protective role in removing clearing damaged intra-

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cellular organelles and aggregated proteins as well as eliminating intracellular pathogens [6–8]. 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. Remifentanil can prevent the inflammatory response and can suppress inducible nitric oxide synthase expression in a septic mouse model [9]. After cardiopulmonary bypass for coronary artery surgery, remifentanil can also inhibit the release of biomarkers of myocardial damage [10]. However, the effects of remifentanil on human keratinocyte and autophagy have yet to be fully elucidated during hypoxia–reoxygenation. Here we investigated whether remifentanil confers protective effect against hypoxia–reoxygenation in human keratinocyte and, if so, whether autophagy mediates this effect.

MATERIALS AND METHODS

1. 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% CO₂. Media were changed every 3 days.

2. Hypoxia/reoxygenation of cultured human keratinocytes and drug treatment

The 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% N₂, and 5% CO₂ (Anaerobic System PROOX model 110; BioSpherix, USA) and incubated for 24 h at 37°C. To determine whether the administration of remifentanil (Ultiva; GlaxoSmithKline Pharmaceuticals, Uxbridge, UK) affects human keratinocytes hypoxia–reoxygenation injury, cells were then exposed to various concentrations of remifentanil (0.01, 0.1, 0.5 and 1 ng/ml) for 2 h. After remifentanil treatment, to simulate reoxygenation and recovery, the cells were reoxygenated for 12 h at 37°C. Control group did not receive remifentanil treatment. Normoxia group did not receive hypoxia and remifentanil treatment for 36 h. 3–MA group was treated 3–methyladenine(3–MA) for 1h before remifentanil treatment (Fig. 1).

3. MTT assay

Cell viability was measured using a quantitative colori–

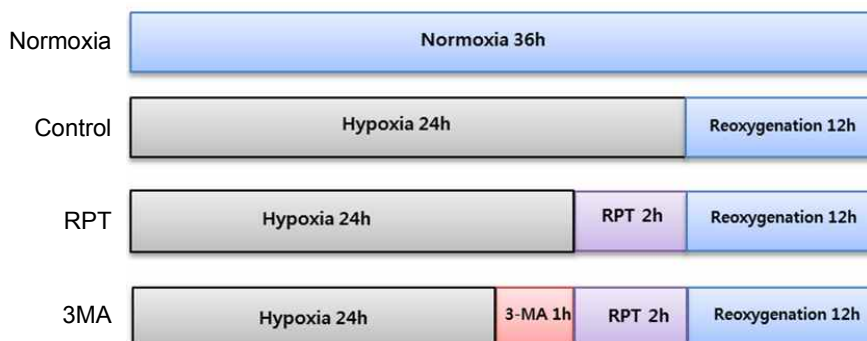


Fig. 1. The experiment protocols. In vitro experiments are shown. Normoxia = normoxia group, Control = no remifentanil treatment group, RPT = remifentanil posttreatment group, 3MA = Both 3–MA and remifentanil treatment group. *P < 0.05 as compared with control group.

metric 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 μ l MTT (final concentration 0.5 mg/mL) for 1.5 h at 37°C. The reaction was terminated by addition of 200 μ l DMSO. Cell viability was measured by an ELISA reader (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength.

4. Fluorescence microscopy (with 1 ng/ml remifen-tanil treatment)

Cells were grown on coverslips and treated with HaCaT cells. After 24 h, cells were stained with 0.05 mM MDC, a selective fluorescent marker for autophagic vacuoles, at 37°C 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 green fluorescence in the cytoplasm and nucleus. Cells were stained with 1 μ g/mL acridine orange for 15 min and washed with PBS. AVOs formation was obtained under a confocal microscope LSM 700 (Carl Zeiss, Germany).

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

Cells (2×10^6) were washed twice in ice-cold PBS, resuspended in 200 μ l ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 μ l/ml aprotinin and 2 μ l/ml leupeptin] and incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 20 μ g 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 (1:3,000), Beclin-1 (1:1,000) from Abcam, p62 (1:1,000), Atg5 (1:500), from Santa Cruz.

6. Statistical analysis

All experiments were repeated five 5 times. Multiple groups were compared using one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. The data were expressed as the mean \pm standard deviation (SD). Values of $P < 0.05$ were considered significant (SPSS 13.0 Software, SPSS Inc., Chicago, IL, USA).

RESULT

1. Effect of remifentanil treatment on cell proliferation

The proliferation assay was performed 36 h after treatment with remifentanil. The cell proliferation rate (%) was calculated and compared with the control group. Control group had lower cell proliferation than normoxia group. However, the cell proliferation rate was higher in the 0.01, 0.1, 0.5 and 1 ng/ml groups compared to the control group ($P < 0.05$) (Fig. 2A). In 3-MA group, similar to the results, the cell proliferation rate was lower than remifentanil group ($P < 0.05$) (Fig. 2B). Among all of the concentrations, 1 ng/ml remifenatnil represented the highest cell viability. Based on this result, all subsequent experiments were performed with 1 ng/ml remifenatnil.

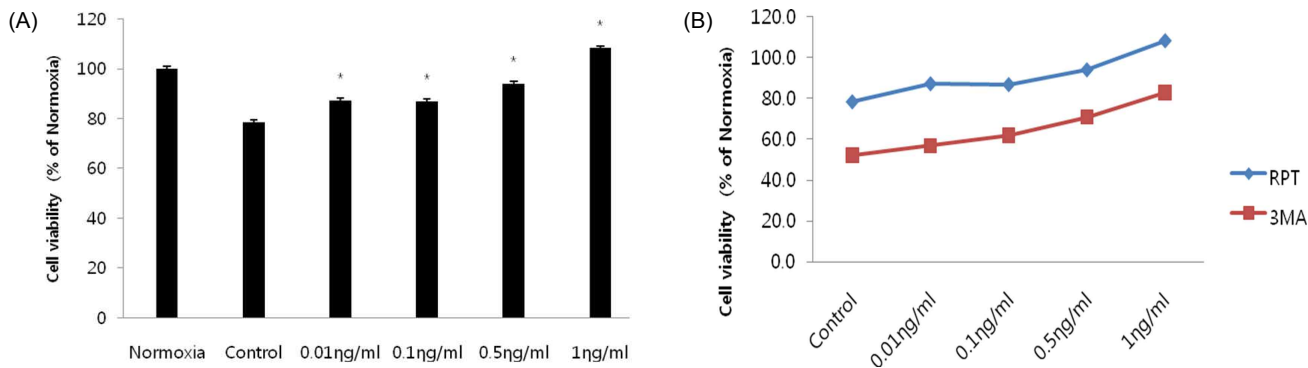


Fig. 2. Effect of remifentanil on cell viability in human keratinocytes by MTT assay. (A) Effect of remifentanil on HaCaT cells under H/R condition by MTT assay. (B) Cell viability comparison between RPT and 3MA groups.

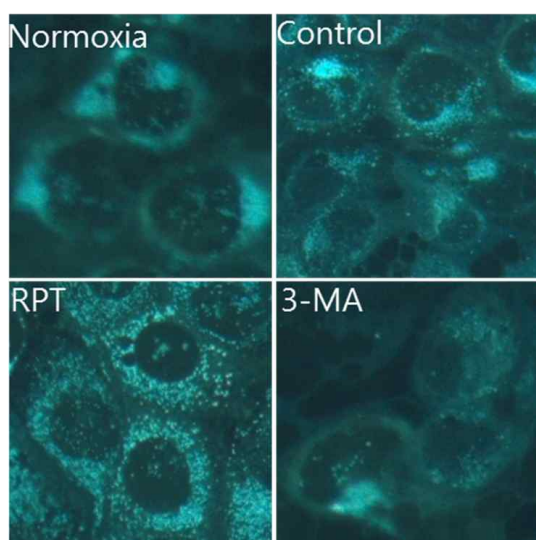


Fig. 3. MDC staining of cytoplasmic vacuoles induced by remifentanil treated in human keratinocytes.

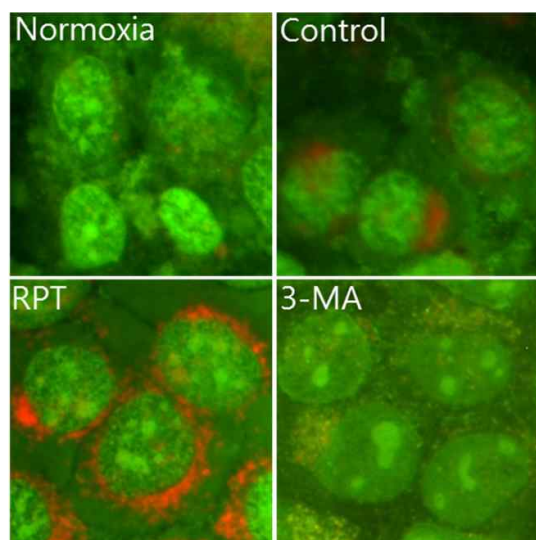


Fig. 4. AO staining of autophagosome formation induced by remifentanil treated in human keratinocytes.

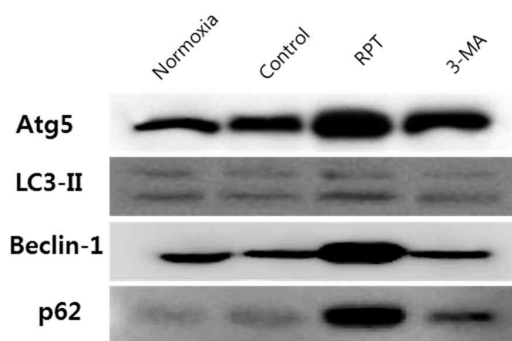


Fig. 5. Effects of remifentanil on autophagy markers in human keratinocytes.

2. Effect of remifentanil treatment on autophagy activation

Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in RPT group HaCaT cells (Fig. 3). Similarly, AO staining, red fluorescent spots appeared in RPT group HaCaT cells, while the Normoxia, control and 3-MA groups showed mainly green cytoplasmic fluorescence (Fig. 4). We here examined activation of autophagy related protein under H/R-induced cells by Western blotting analysis. Atg5, Beclin-1, LC3-II (microtubule-associated protein 1 light chain 3 form II) and p62 was elevated in RPT group cells. But they were decreased when autophagy was suppressed by 3-MA (Fig. 5).

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 treatment increased the proliferation of human keratinocytes with hypoxia–reoxygenation injury (Fig. 2A). But the autophagy pathway inhibitor 3–MA blocked the protective effect of remifentanil against H/R injury (Fig. 2B). In autophagic specific staining MDC and AO, RPT group induced more autophagic expression than normoxia, control group and 3–MA groups. Atg5 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]. Beclin–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]. 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 treatment 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. In summary, the present study shows that remifentanil treatment increases the human keratinocytes proliferation rate and stimulates the expression of autophagy under hypoxia–reoxxygenation 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 may have a beneficial effect in the recovery of wound from hypoxia–reoxxygenation injury.

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