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Original Article

The Effect of Propofol on Hypoxic damaged-HaCaT Cells

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Background: 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 intracellular organelles and aggregated proteins as well as eliminating intracellular pathogens. The purpose of the present study was to examine the protective effect of propofol against hypoxic damage using keratinocytes.
Methods: Human keratinocytes (HaCaT cells) were obtained from the American Type Culture Collection. Propofol which were made by dissolving them in DMSO were kept frozen at -4℃ until use. The stock was diluted to their concentration with DMEM when needed. Prior to propofol treatment cells were grown to about 80% confluence and then exposed to propofol at different concentrations (0, 25, 50, 75, 100 μM) for 2 h pretreatment. Cell viability was measured using a quantitative colorimetric assay with thiazolyl blue tetrazolium bromide (MTT assay), and fluorescence microscopy and western blot analysis were used for evaluation of autophagy processes.
Results: The viability of propofol-treated HaCaT cells was increased in a dose-dependent manner. Propofol did not show any significant toxic effect on the HaCaT cells. The autophagy inhibitor, 3-methyladenine, reduced cell viability of hypoxia-injured HaCat cells. Fluorescence microscopy and western blot analysis showed propofol induce autophagy pathway signals.
Conclusions: Propofol enhanced viability of hypoxia-injured HaCaT cells and we suggest propofol has cellular protective effects by autophagy signal pathway activation.

Key Words: Autophagy; Hacat cell; Hypoxic damage; Propofol

INTRODUCTION

Tissue hypoxia is a feature common to many skin diseases including cirrhosis and cancer. It also occurs as a consequence of hemodynamic shock and surgery. Chronic ischemia, a pathology associated with peripheral vascular disease and diabetes, is a significant cause of skin ulceration [1,2]. The decreased quantity of tissue oxygen levels triggers metabolic and ionic disorders, reduces the levels of intracellular redox buffers, and causes expression of specific genes including immediate-early stress genes in keratinocytes [3]. 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 intracellular organelles and aggregated proteins as well as eliminating intracellular pathogens [4-6]. While keratinocyte apoptosis and necrosis have been shown to occur during hypoxia, the role of keratinocyte autophagy remains controversial. However, now widely recognized that autophagy is required for protein and organelle turnover and is typically a homeostatic cellular response to starvation. The role of autophagy in skin disease has been comprehensively presented in recent study [7,8]. Propofol (2, 6-diisopropylphenol) is

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a widely used agent for the induction and maintenance of anesthesia during surgical procedures. Propofol is also used as a sedative for ICU patients. Propofol has a structural similarity to the endogenous antioxidant vitamin E and exhibits antioxidant activities [9]. Therefore, it shows the protective effect on the hydrogen peroxide induced apoptosis in cardiac cells [10] and myocardial ischemia and reperfusion injury in rats [11]. However, the effects of propofol on human keratinocyte and autophagy have yet to be fully elucidated during hypoxia induced cell death. The purpose of the present study was to examine the protective effect of propofol against hypoxic damage using keratinocytes.

MATERIALS AND METHODS

1. Reagents

Propofol (2, 6-diisopropylphenol) diluted with dimethyl sulfoxide (DMSO), The following reagents were obtained commercially: 3-[4,5-dimethylthiazol -2-yl]2,5-diphenyl tetrazolium bromide (MTT), acridine orange, monodansylcadaverine (MDC), 3-methyladenine (3-MA, class III PI3K inhibitor) was obtained from Calbiochem (La Jolla, CA, 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. 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℃ in a humidified atmosphere with 5% CO2. Media were changed every 3 days.

3. Treatment of propofol

Propofol which were made by dissolving them in DMSO were kept frozen at -4℃ until use. The stock was diluted to their concentration with DMEM when needed. Prior to propofol treatment cells were grown to about 80% confluence and then exposed to propofol at different concentrations (0, 25, 50, 75, 100 μM) for 2 h pretreatment. Cells grown in medium containing an equivalent amount of DMSO without propofol served as control. The experimental protocol was shown in Fig. 1.

4. MTT assay

Cell viability assay Cell viability was measured using a quantitative colorimetric assay with thiazolyl blue tetrazolium bromide (MTT, AMResco), showing the mitochondrial activity of living cells. HaCaT cells (3 × 104) were seed in 96-well plates. After drug treatment as indicated, cells were incubated with 100 μl MTT (final concentration 0.5 mg/mL) for 1.5 h at 37℃. The reaction was terminated by addition of 100 μl DMSO. Cell viability was measured by an ELISA reader (Tecan, Männedorf, Switzerland) at 620 nm excitatory emission wavelength.

5. Fluorescence microscopy

Cells were grown on coverslips and treated with HaCaT

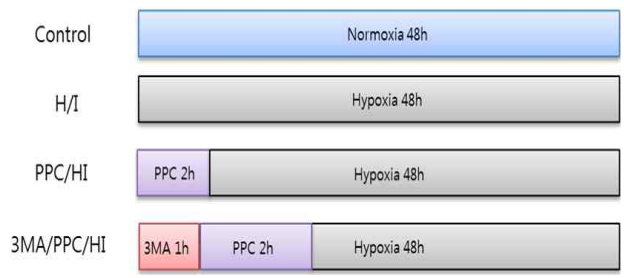

Fig. 1. The experiment protocols. In vitro experiment are shown. Control = Normoxia ; H/I = Hypoxia Injury; PPC = Propofol Preconditioning; 3-MA = 3-Methyladenine with treatment.

cells. 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 μg/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

Cells (2 × 106) were washed twice in ice-cold PBS, resuspended in 200 μl ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/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 μg of proteins were resoved 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).

RESULT

1. Propofol improved the cell viability of hypoxic damage HaCaT cells

The effect of propofol on HaCaT cells was investigated

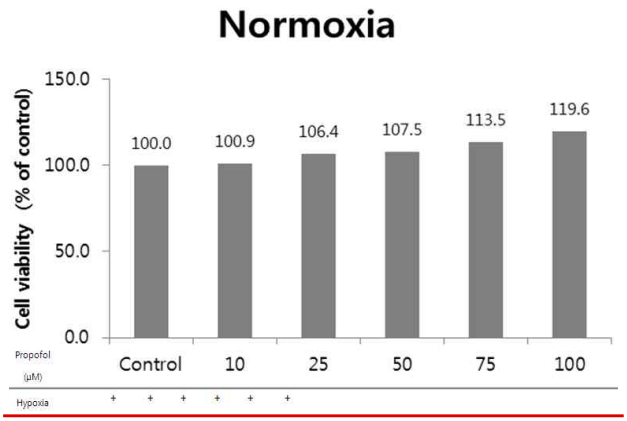

Fig. 2. Effect of propofol preconditioning on cell viability. The normal HaCaT cells were treated with different concentrations (0-100 μM) of propofol. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay, as previously described H/I, Hypoxia/Injury. Values are represented as the percentage of viable cells; cells exposed to normoxia were considered as 100% viable.

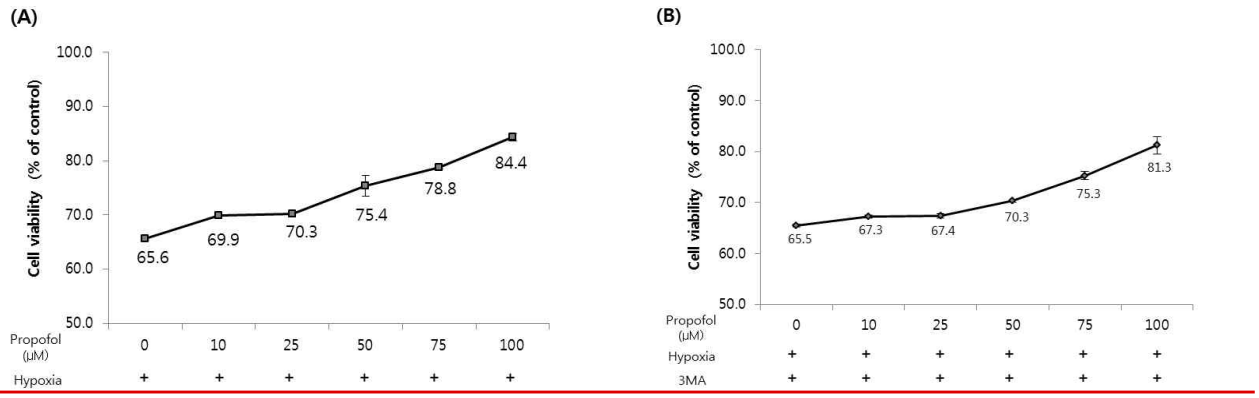

Fig. 3. Effect of propofol preconditioning on Hypoxia induced cell viability. The cells were pre-conditioned with increasing concentrations of propofol (0-100 μM) 48 h of hypoxia condition. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylterazolium bromide (MTT) assay, as previously described.

over a wide concentration range. In particular, the hypoxia injury dependent programed cell death was suppressed by propofol preconditioning. Our results showed that the viability of propofol-treated HaCaT cells were increased in a dose-dependent manner. Propofol did not show any significant toxic effect on the HaCaT cells (Fig. 2). We next investigated the effect of various concentrations of propofol (0, 25, 50, 75, 100 μM) on the hypoxia-induced cells and discovered that propofol significantly protected the HaCaT cells from hypoxia-induced cell cytotoxicity (Fig. 3A). The role of autophagy in the H/R damage against of HaCaT cells was further confirmed by the autophagy inhibitor 3-MA, an inhibitor of class III phosphoinositide 3-kinase (PI3K). As shown in (Fig. 3B), 3-MA (5 mM) 1 h before H/R (reoxygenation 12h after hypoxia for 24 h) significantly reduced cell viability of propofol-treated HaCaT cells. This decrease in the cell viability was accompanied by an increase in cell death, indicating that the inhibition of autophagy by 3-MA sensitized HaCaT cells to H/R damage.

2. Propofol treatment leads to induction of autophagy in HaCaT cells

Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in propofol-treated HaCaT cells (Fig. 4). Similarly, AO staining, red fluorescent spots appeared on propofol -treated HaCaT cells, while the nomal control group, and the cells co-treated with 5 mmol/L 3-MA and hypoxic-injured group showed mainly green cytoplasmic fluorescence (Fig. 5). We here examined activation of autophagy related protein under hypoxia-induced cells by Western blotting analysis. The recruitment of LC3 to the membrane occurs via an Atg5-dependent mechanism, and thus Atg5 is essential for autophagosome formation vivo [12]. Atg5 and Beclin-1, LC3-II (microtubule-associated protein 1 light chain 3 form II), p62 was elevated in PPC/HI group cells but Hypoxia-induced group significantly reduced in HaCaT cells (Fig. 6). The Atg5 were increased when autophagy was induced by propofol, and

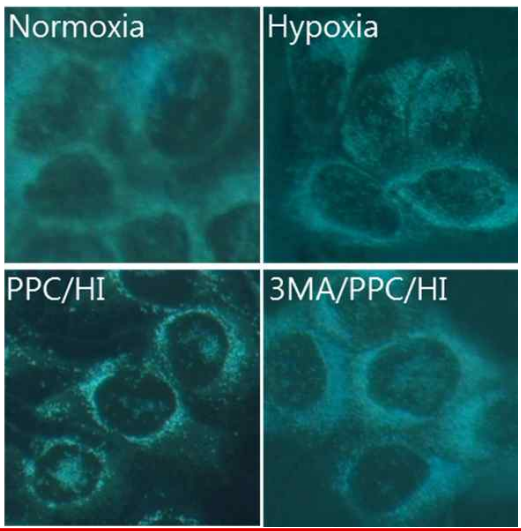

Fig. 4. Fluorescence microscopic (× 400) analysis of autophagosome in the H/I injured HaCaT cells. Hypoxia caused accumulation of autophagosomes containing partially digested cytoplasmic contents compared to the control group. The PPC during H/R dramatically increased formation of autophagosomes and the autophagy pathway inhibitor 3-MA blocked formation of autophagosomes by PPC. H/I, Hypoxia/Injury; PPC, propofol precondition-ing; 3-MA, 3-Methyladenine.

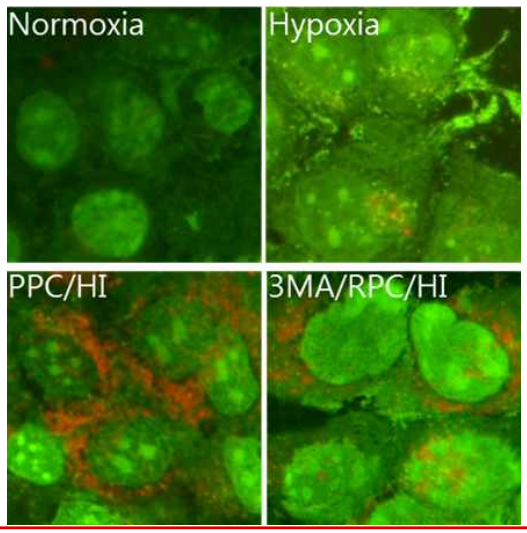

Fig. 5. Fluorescence microscopic (× 400) analysis of autophagosome in the hypoxia injured HaCaT cells. Stained with acridine orange the green shows where the dye has stained the nucleus and the red is where the cell is starting to 'digest' parts of itself in small capsules called autophagosomes.

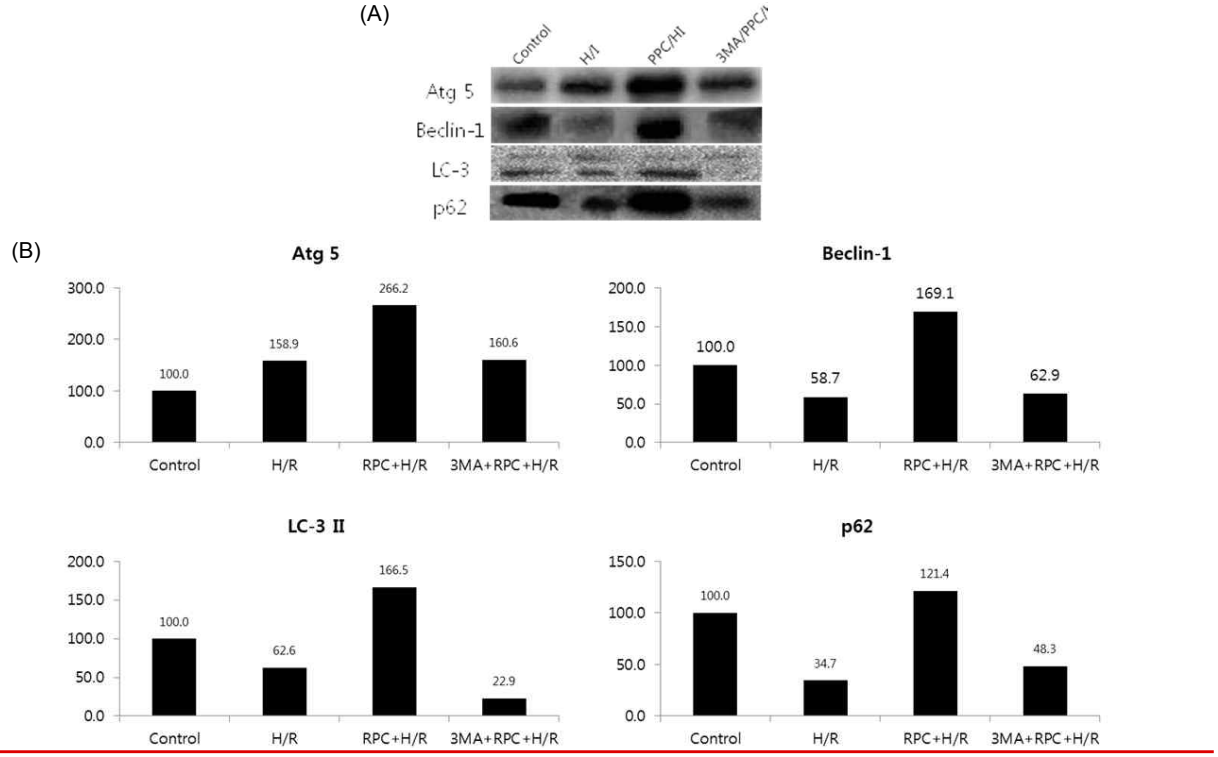

Fig. 6. (A) Western blot analysis. Expression of Atg5, LC3-II, Beclin-1 and p62 in Hypoxia-induced HaCaT cells preconditioned with propofol and 3-MA. (B) Quantification of Atg5, LC3-II, Beclin-1 and p62. Each band shown above was quantified by the densitometric scan. H/R, Hypoxia injury; PPC, propofol preconditioning; 3-MA, 3-Methyladenine.

they were decreased when autophagy was suppressed by 3-MA.

DISCUSSION

The aim of the present study was to examine the protective effect of propofol on hypoxia-induced human keratinocytes and to investigate possible mechanisms of protection. For the first time, we provide in vitro evidence of potential therapeutic value of propofol in hypoxia-induced skin via inhibiting cell death activate autophagy pathway. Results showed that pretreatment with propofol reduced hypoxia stress and subsequent apoptosis in HaCaT cells subjected to hypoxia injury. Autophagy is widely known as an important process in cell physiology, for both cell survival and death. While autophagy is an important mechanism by which the cell rids itself of potentially harmful constituents and helps maintain normal cellular functioning and homeostasis, its precise role during skin hypoxia injury and disease where keratinocytes are involved remains uncertain. A consensus is now emerging that autophagy does not precede cell death but may be a physiologically protective mechanism which favors cell survival [13]. The diverse role of autophagy in liver diseases has been recently reviewed [7,14,15]. To our knowledge this is the first study to conclusively demonstrate autophagy in isolated primary human cells under conditions of hypoxic stress. However these studies failed to show a causal link between oxidative stress, induction of apoptosis and autophagy [16]. Recent studies have shown that the severity and duration of an ischemic insult determine whether autophagy is induced or not. Indeed these studies have shown that autophagy can delay the decision for a cell to die via apoptosis or necrosis [17]. However, crucially we show that inhibition of early autophagy with the class

III PI3K inhibitor, 3-MA, induces increased levels apoptosis during hypoxic condition. Importantly, inhibiting autophagy causes the lowering of mitochondrial membrane potential and leads to cell death. Specifically, keratinocytes would have been treated with propofol, showed MDC staining that was consistent throughout hypoxia. It can be seen that these keratinocytes are also resistant to cell death during hypoxia. Indeed, the reduction in cell death maybe in part due to the induction of autophagy. During hypoxic stress, autophagy appears to occur within the cells our data shows that this process is likely to be mediated by Beclin-1, LC3-II, Atg5 and p62. The antioxidant propofol decreased hypoxia induced oxidative stress. In our study, the viability of propofol in hypoxia induced HaCaT cells was first studied by MTT assay. The treatment with 25, 50, 75, 100, μM propofol in hypoxia induced HaCaT cells enhanced cell viability in a dose- dependent manner and 100 μM was the most effective dose. Our experiment is limited In vitro/In vivo experiments are required to gain a coherent understanding of mitochondria nexus under the more complex internal environment. Finally, our results suggest that propofol has cellular protective effects by autophagy signal pathway activation.

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