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Protective Effects of Verapamil against H_2O_2-Induced Apoptosis in Human Lens Epithelial Cells

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Abstract

Verapamil is used in the treatment of hypertension, angina pectoris, and atrial fibrillation. Recently, several studies have demonstrated that verapamil increased the optic nerve head blood flow and improved the retrobulbar circulation. All these show that verapamil is potentially useful for ophthalmic treatment. Thus, the aim of this study is to investigate whether verapamil could protect human lens epithelial cell (HLEC) from oxidative stress induced by H_2O_2 and the cellular mechanism underlying this protective function. The viability of HLEC was determined by the MTT assay and apoptotic cell death was analyzed by Hoechst 33258 staining. Moreover, Caspase-3 expression was detected by immunocytochemistry and flow cytometry analysis. We also detected Caspase-3 mRNA expression by reverse-transcription-polymerase chain reaction and the GSH content in cell culture. The results showed that oxidative stress produced significant cell apoptotic death and it was reduced by previous treatment with the verapamil. Verapamil was effective in reducing HLEC death mainly through reducing the expression level of apoptosis-related proteins, caspase-3, and increasing glutathione content. Therefore, it was suggested that verapamil was effective in reducing HLEC apoptosis induced by H_2O_2.

Key Words: Apoptosis, Caspase-3, Human lens epithelial cell, Verapamil

INTRODUCTION

Cataracts are the most frequent cause of treatable blindness worldwide (Foster, 1999). The effective medicines are in various stages of development, therefore no drugs are actually available to treat people with cataract. Oxidative stress in the lenses is the most common damaging factor for the development of cataract. The epithelial layer of the lens is the main target of the oxidative insult and any external insult may affect its antioxidant status (Spector, 1995; Ottonello *et al*., 2000). Apoptosis of lens epithelial cells, which is an important, intricate and finely orchestrated mechanism that maintains the homeostasis of viable cells (Thompson, 1995), is suggested to be a cause of cataract formation (Li *et al*., 1995). In the aqueous fluid and lenses of some cataract patients, the level of H_2O_2 is markedly raised (Spector and Garner, 1981) and there is clear evidence that H_2O_2 may be involved in the genesis of human nuclear cataract (Truscott and Augusteyn, 1977). The concentrations of the H_2O_2 in normal human lenses and aqueous humor are approximately 20 to 30 μM. However, one-third cataract patients had elevated H_2O_2 levels in lenses ranging from 2- to 7-fold higher than the normal range, and 30-fold greater than normal in aqueous humor (Bhuyan *et al*., 1986; Spector, 1995). Using *in vitro* organ culture studies, H_2O_2, a non-radical member of the active oxygen family, can easily penetrate lipid membranes and has been proved to be toxic to the lens (Fukui, 1976). At the molecular level, H_2O_2 generates hydroxyl radicals which could particularly damage to DNA, resulting in mutagenesis and leading to cataract formation (Imlay and Linn, 1988). In addition, hydroxyl radicals can also damage to both the cell membrane and cytosol regions as they cause a decrease in the levels of antioxidants (Spector *et al*., 1985; Richer and Rose, 1998). The human lens epithelial cell line, SRA 01/04, has been established by using the immortalizing gene of SV40 and is derived from a single cell with uniform characteristics. The SRA 01/04 cell line appears

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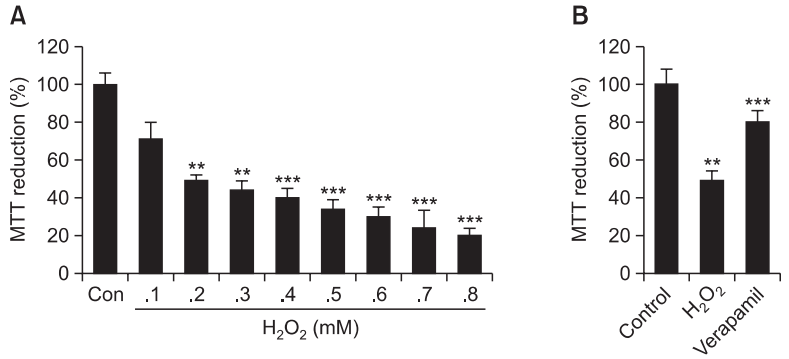

Fig. 1. Protective effects of verapamil on H_2O_2-induced damage on cultured HLEC. (A) H_2O_2-induced cytotoxicity inHLEC. (B) Effects of verapamil on H_2O_2-induced damage on cultured HLEC. Each value represents the mean ± S.E.M. obtained from four culture wells per experiment, determined in three independent experiments. ***p*<0.05 in comparison with control; ****p*<0.01 in comparison with cells exposed to H_2O_2 alone.

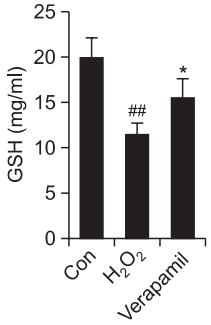

Fig. 2. Effects of verapamil on the activities of glutathione in H_2O_2-induced HLEC. Data are mean ± S.D. (n=4) obtained from three independent experiments. $^{\#\#}p$<0.01 in comparison with control, **p*<0.05 compared with H_2O_2-treated cultures alone.

to be an excellent model system for investigating the cellular functions of the human lens epithelium under the oxidative damage induced by H_2O_2 in this study.

Verapamil belongs to the dihydropyridine family of a potent calcium channel blocker and is used in the treatment of hypertension, angina pectoris, and atrial fibrillation. The primary mechanism of verapamil is via L-type calcium channel blockade, which prevents excessive calcium influx into cardiomyocytes as well as smooth muscle cells of the coronary vasculature. *In vitro*, verapamil impaired the toxicity including morphological change, cell viability and apoptosis caused by rubratoxin B (Nagashima and Goto *et al*, 2000). For ophthalmic treatment, several studies have demonstrated that verapamil increased the optic nerve head blood flow measured by laser Doppler flowmetry (Netland *et al*., 1996) or improved the retrobulbar circulation (Netland *et al*., 1995). In recent years, there has been an upsurge of interest in unraveling the roles of Ca^{2+} in the pathophysiology of cataract. It is believed that an understanding of the mechanisms, which mediate pathological Ca^{2+} overload as occurs in the process of cataract formation and disturbances in calcium homeostasis, is associated with various forms of cataract.

All these show that verapamil as a calcium channel blocker is potentially useful for ophthalmic treatment. Here, we investigated the protective role of verapamil in human lens epithelial cells (HLEC) and the cellular mechanism underlying this protective function. Our results indicate that verapamil in human lens epithelial cell cells protects against H_2O_2-induced cell death, and this protection involves the inhibition of caspase-3 activation.

MATERIALS AND METHODS

Cell culture and treatment

The human lens epithelial cell line were cultured in Dulbeccos modied Eagle's medium (DMEM; Gibco,) with 10% fetal bovine serum (FBS; Gibco). Cells were grown in a 25 cm^2 culture dish (Corning) in a 5% CO_2 environment at 37°C. After the cells 80% confluent, they were passaged.

The cells were cultured with different concentrations of H_2O_2 (0: control group, 0.1- 0.8 mM) for 24 h. When the effects of verapamil on cells were studied, verapamil (25 μg/ml) was added for 30min prior to H_2O_2 treatment. 30 min later, 0.2 mM H_2O_2 was added and incubated for 24 h in growth media.

Hoechst staining

Apoptotic cell death was analyzed by Hoechst 33258 (Sigma) staining. Cells were fixed with 4% paraformaldehyde for 1h at 37°C and then rinsed twice in PBS. After this cells were incubated with 2 mg/ml Hoechst 33258 for 30 min at 37°C and cells were visualized under fluorescence microscope.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 1 h at 37°C. After rinsed with PBS, they were blocked with 10% goat serum. The cells were incubated with rabbit caspase3 (1:200; Sigma) for 2 h at 37°C. The cells were rinsed three times in PBS. Then they were incubated with the corresponding secondary antibodies, Cy3-conjugated anti-rabbit IgG (1:100; Sigma). DAPI (Invitrogen) was used for counterstaining. The cells were photographed with a fluorescence microscope (VANOX-S; Olympus, Melville, NY, USA).

Reverse-transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells following the protocol of an RNeasy mini kit. cDNA was synthesized by using a PrimeScript RT reagent kit (Takara, Tokyo, Japan). PCR was carried out with the following program: 94°C, 1 min; 94°C, 30 s, 52°C, 30s, 72°C, 45 s, 30 cycles; 72°C, 5 min. The PCR products were electrophoresed on 1% agarose gels and detected by ultraviolet excitation.

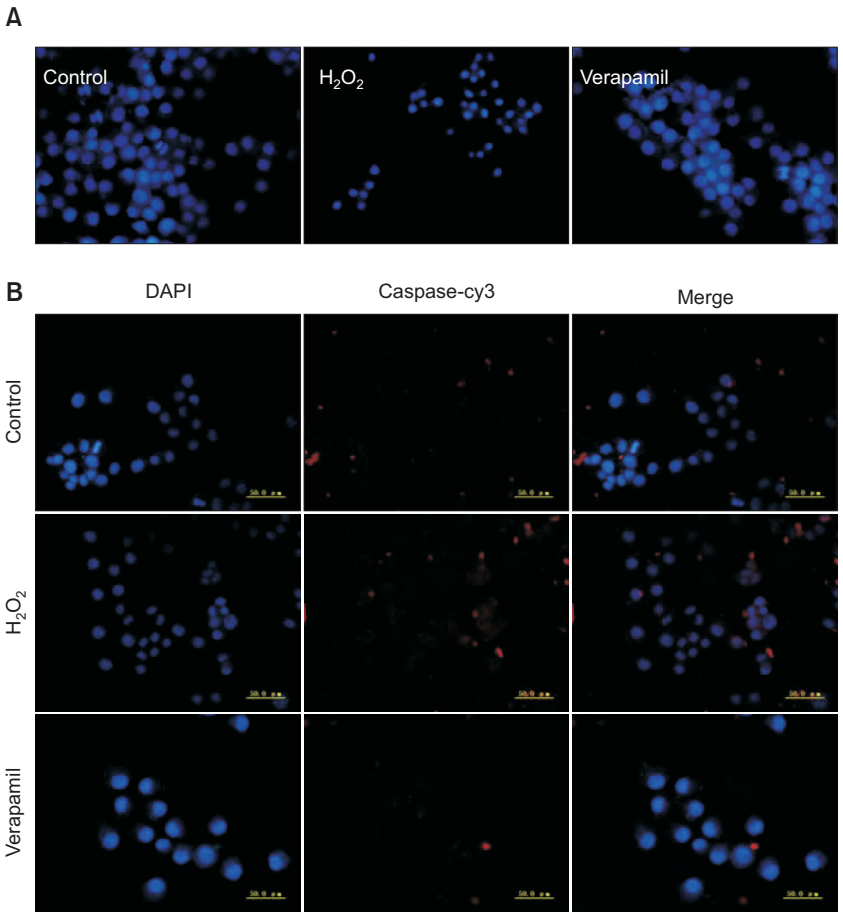

Fig. 3. (A) HLEC were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. (A) Control cells; (B) cells treated with H_2O_2 alone; (C) cells treated with H_2O_2 and verapamil. The figures are representative for three different experiments. (B) Effects of verapamil on Caspase-3 expression. The Caspase-3 expression was examined by immunocytochemistry in HLEC treated with H_2O_2. DAPI was used for counterstaining. The bar is 50 μm in the figures.

Flow cytometry analysis of apoptotic cells

Human Lens epithelial cells were used for caspase-3 detection. The first appropriate antibody (1:100; Sigma) was added for 60 min at room temperature. The cells were then washed twice with PBS, treated with the anti-rabbit Cy3 conjugated antibody (1:200; Sigma) and incubated in darkness. Samples were allowed to incubate in the dark for 30 min at room temperature, then washed and filtered through a 200 mesh filter before being resuspended in 500 μl of PBS, ready to be analyzed by flow cytometry.

Assays for glutathione (GSH)

The oxidative injury inflicted to the cells was assessed by the amount of (GSH) activity released into the media by using commercially available kits (Jiancheng Bioengineering, China). All procedures completely complied with the manufacture's instructions.

Statistical analysis

Data are expressed as means±SD with $p<0.05$ considered significant. Statistical significance was assayed by one-way analysis of variance (ANOVA) followed by Scheffe' test using Origin software.

RESULTS

Verapamil alleviated H_2O_2-induced apoptosis of HLEC

The viability of the HLEC which induced by the different concentrations of H_2O_2 (0: control group, 0.1- 0.8 mM) for 24 h was detected by MTT assay. Cell viability was significantly reduced in a H_2O_2-concentration dependent manner (Fig.1A). Consequently, 0.2 mM H_2O_2 (48.8±1.1% of control cell viability) was chosen for subsequent experiments. The Cell viability was higher in the cells which were pretreatment with verapamil (viability of 79.3 ± 3.3% for verapamil concentrations of 25 μg/ml) than the cells which treated with H_2O_2 alone (Fig. 1B). Thus, it was possibly concluded that verapamil was effective for the protection of HLEC.

Assays for glutathione (GSH)

H_2O_2 resulted in a marked decrement in the content of GSH in HLEC and verapamil can increase the activity of GSH after H_2O_2-induced .The content of GSH in Control group, H_2O_2 group and verapamil group are 19.8, 11.4, 15.4 (G/L) (Fig. 2).

Immunocytochemistry

The changes of nuclear morphology were assessed by

Hoechst 33258 staining after H_2O_2-treatment. The control HLEC nuclei had a regular and oval shape (Fig. 3A). However, when the cell was exposed to H_2O_2 for 24 h, nuclear condensation and fragmentation were appeared. Verapamil treatment rescued the H_2O_2 induced nuclear morphological change.

To investigate that verapamil improved HLEC survival by decreasing apoptosis, the level of active caspase-3 was analyzed. The caspase-3 which plays a critical role in apoptosis was assessed by immunocytochemistry staining (Fig. 3B). After HLEC were treated with 0.2 mM H_2O_2 for 24 h, caspase-3 is implicated. While caspase-3 activity was significantly decreased in verapamil-treatd cells compared to H_2O_2-induced.

The effect of verapamil on caspase-3 in HLEC-induced by H_2O_2

As shown in Fig. 4, mRNA expression of caspase-3 was significantly upregulated when treated with H_2O_2 compared with control group. While in the verapamil group, Caspase-3 mRNA expression was significantly lower than in the H_2O_2 group.

The caspase-3 positive cell of each group of HLEC was tested by flow cytometry. In the presence of H_2O_2, the Caspase-3 expression increased to 20.08% while that of control group was 2.06%. In the verapamil group, the caspase-3 positive cell was declined to 2.44% (Fig. 5A). The data also showed that caspase-3 fluorescence intensity of H_2O_2 group was higher than that of control group nearly to 4.6-fold, and verapamil effectively decreased the caspase-3 fluorescence intensity nearly to 1.4-fold of control group' s (Fig. 5A).

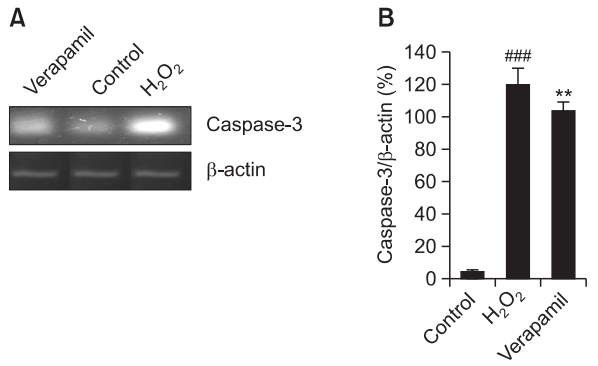

Fig. 4. Effects of verapamil on H_2O_2-induced mRNA expression of Caspase-3 in HLEC. DNA strips show expression levels of Caspase-3 on agarose gel electrophoresis. ###p<0.01 versus control, **p<0.01 versus H_2O_2.

DISCUSSION

This study describes the effect of verapamil on the oxidative damage in cultured Human lens epithelial cells induced by H_2O_2. It has been reported that verapamil treatment significantly attenuated oxidative damage, levels of pro-inflammatory mediators in quinolinic acid treated animals (Kalonia *et al*., 2011). Verapamil decreased simvastatin-induced apoptosis rate and also prevented simvastatin-induced DNA laddering (Cheng *et al*., 2003). These results and those of the present study suggest that verapamil maybe has a cytoprotective effect.

Cataract is the leading cause of blindness and visual impairment in the world (Resnikoff S *et al*., 2004). Cataract development has a strong relationship with increasing age in both humans and animals. There is considerable evidence to support the concept that oxidative stress and the generation of reactive oxygen species (ROS) can accelerate cataract development through damage to lens epithelial cells (Spector *et al*, 1985, Spector, 1995; Wakamatsu *et al*., 2008). Excessive generation of reactive oxygen species (ROS) from either the

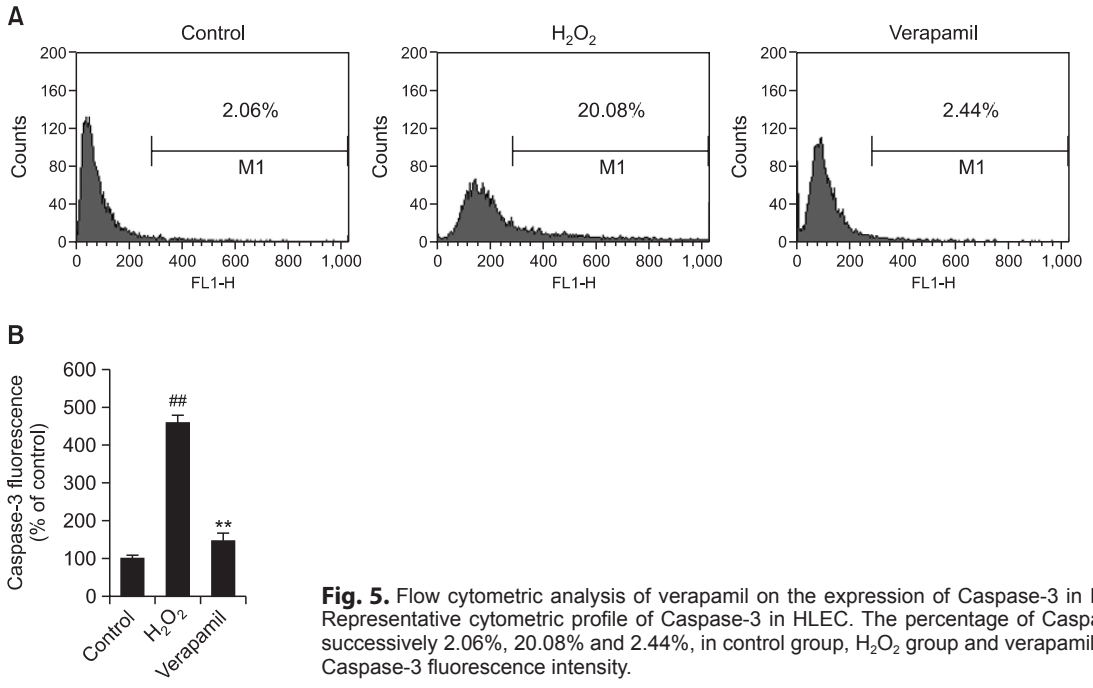

Fig. 5. Flow cytometric analysis of verapamil on the expression of Caspase-3 in HLEC. (A) Representative cytometric profile of Caspase-3 in HLEC. The percentage of Caspase-3 was successively 2.06%, 20.08% and 2.44%, in control group, H_2O_2 group and verapamil group (B) Caspase-3 fluorescence intensity.

environment or from mitochondria of the lens epithelial cells can damage cellular macromolecules such as proteins, DNA, and lipids, leading to opacication of the lens and compromising lens transparency. In this present study hydrogen peroxide also causes a decrease in viability of HLEC by MTT detection. Cells stained with fluorescent DNA binding dye, Hoechst 33258, displayed typical morphological features of apoptosis with condensed nuclei and verapamil treatment significantly reduced the apoptotic cells.

Oxidative stress causes cell death when intracellular levels of metabolic and antioxidant enzymes (especially glutathione related enzymes) and substrates (glutathione) are exhausted (Naval *et al*., 2007). During oxidative stress, glutathione metabolism cycling in HLEC was significant. This process also has been reported in many other cell types and proposed as a mechanism of cellular self-defense by supporting the maintenance of a reduced thiol reduction potential (Keppler *et al*. 1999). Thus, in order to investigate the possible mechanism of verapamil protecting HLEC from oxidative stress induced by H_2O_2, glutathione was detected. The ultimate results indicated that pretreatment with verapamil caused a significant enhancement of the glutathione content.

Some previous studies have shown that apoptosis of lens epithelial cells (LECs) plays an important role in the development of several types of cataract (Li *et al*., 1995), such as hydrogen peroxide-induced cataract (Spector, 1995). While the mechanism of apoptosis is complex and involves a cascade of reactions, one of the key steps leading to apoptosis is the leakage of cytochrome C from the mitochondria and activation of caspase-3. The present study shows that caspase-3 expression increased when HLEC was treated with H_2O_2. However, pretreatment with verapamil decreased both the caspase-3 expression and apoptotic rate. These results suggest that verapamil plays a role as a negative regulator of apoptosis by inhibiting the autocatalytic maturation of caspase-3, a key mediator of apoptosis in mammalian cells. For the above reasons, we speculated that verapamil has a protective effect against H_2O_2-induced damage. Caspase-3 might be one of the main effector proteins in the effects of verapamil preventing H_2O_2-induced apoptosis.

In summary, the present study demonstrated that verapamil could protect HLEC from hydrogen peroxide-induced oxidative damage. Our results showed verapamil is effective in reducing cell death induced by H_2O_2 by decreasing caspase-3 expression. This protection of verapamil may be not only due to its ability to inhibit cell death induced by H_2O_2, but also its possibility to improve glutathione mechanism cycling via maintaining the glutathione content. Our data suggest that verapamil may be a useful compound to prevent acute damage during oxidative stress.

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