Korean J Physiol Pharmacol
Vol 5: 381–388, October, 2001

KR 31378, a Potent Antioxidant, Inhibits Apoptotic Death of A7r5 Cells

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This work describes the pharmacological inhibition by KR 31378 and its acetyl metabolite, KR 31612, of the apoptotic cell death induced by H_2O_2 in the A7r5 cells. Exposure of A7r5 cells to H_2O_2 (0.5 mM) induced a concentration-dependent cytotoxicity in association with oligonucleosomal DNA fragmentation. H_2O_2-induced cell death was potently suppressed by KR 31378, KR 31612, α-tocopherol or trolox. Additionally, the apoptotic death of A7r5 cells (DNA ladders on electrophoresis) was also strongly suppressed by KR 31378 and KR 31612, but to a less degree by α-tocopherol and trolox. As a mechanistic study, incubation with H_2O_2 markedly showed a decreased Bcl-2 level and, in contrast, increased Bax protein and cytochrome C release, which were significantly and concentration-dependently reversed by KR 31378 and KR 31612 as well as by α-tocopherol and trolox. KR 31378 and α-tocopherol significantly reduced lipid peroxidation in accordance with reduced intracellular ROS and peroxyl radical. These results suggest that KR 31378 has a therapeutic potential against the apoptotic injury via mediation of anti-oxidative stress.

Key Words: KR 31378, α-Tocopherol, Oxidative stress, Apoptosis, Reactive oxygen species, Bcl-2, Bax, Cytochrome C

INTRODUCTION

Oxidative stress is a normally occurring and ubiquitous condition in which the generation of free radicals and reactive oxygen species (ROS) exceeds endogenous antioxidant defense and repair mechanisms (Kerr et al, 1996). Because free radicals and ROS are highly reactive, they have the potential to irreversibly damage lipids (via lipid peroxidation), proteins, and DNA in biological system. Excessive oxidative stress has been implicated in a number of the diseases, such as inflammation, ischemia-reperfusion injury, rheumatoid arthritis, cancer, and atherosclerosis (Halliwell & Gutteridge, 1992).

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Apoptosis, a morphological type of cell death, is an essential process controlling tissue homeostasis in multicellular organisms by which damaged or neoplastic cells are continually eliminated (Liu et al, 1996). A number of Bcl-2 family members have been identified in mammals: Bcl-2 and Bcl-X_L serve to inhibit apoptosis, whereas Bax, Bak, and Bad promote apoptosis (Reed, 1996; Kroemer, 1997). Recent evidence has shown that Bcl-2 blocks the release of cytochrome c from the mitochondria, an event that is necessary for caspase-3 activation (Kluck et al, 1997; Yang et al, 1997). Alternatively, the Bcl-2-related protein Bcl-X_L was also shown to block apoptosis via interaction with cytochrome c by binding to it and inhibiting its availability in the cytosol (Kharbanda et al, 1997). Thus, a number of studies have postulated that cell survival is associated with their ability to maintain homeostatic level of Bcl-2.

Recently, KR 31378 [(2S, 3S, 4R)-N"-cyano-N-

(6-amino-3, 4-dihydro-3-hydroxy-2-methyl-2-dimethoxy methyl-2*H*-benzopyran-4-yl)-N'-benzylguanidine] and KR 31612, its acetyl metabolite, were synthesized by the Korea Research Institute of Chemical Technology. Our studies have recently shown that KR 31378 strongly suppressed lipid peroxidation in association with electron paramagnetic resonance signals of superoxide anion and hydroxyl radicals, as did α-tocopherol.

Thus, it was aimed to examine the mechanism by which KR 31378 could suppress the DNA fragmentation and consequent cell death in A7r5s in comparison with KR 31612, its acetylated metabolite, α-tocopherol and trolox. Thus, we evaluated the ability of KR 31378 to scavenge peroxyl radicals and to reduce the intracellular ROS in A7r5s and to modulate the apoptotic proteins, Bcl-2 and Bax proteins, and cytochrome C release from mitochondria in H_2O_2-mediated apoptosis. α-Tocopherol and Trolox were used as reference agents.

METHODS

Chemicals

KR 31378 were generously donated by the Korea Research Institute of Chemical Technology (Daejon, Korea) and dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution. α-Tocopherol (Sigma-Aldrich Co., Seoul, Korea) was dissolved in ethanol and trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, Alexis Biochemicals, USA) in DMSO. β-Phycoerythrin (β-PE) (Sigma-Aldrich Co., St. Loues, MO) and 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (Wako Pure Chemical Co., Osaka, Japan) were dissolved in 75 mM phosphate buffer (pH 7.0). Hydrogen peroxide (Junsei Chemical Co., Japan) was dissolved in 10 mM phosphate buffer (pH 7.4) before use. Human LDL (Sigma-Aldrich Co., St. Louse, MO) was dissolved in distilled water at a concentration of 1 mg/ml. 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide was from Sigma-Aldrich Co. (St. Loues, MO).

Cell cultures

A7r5 cells (ATCC CRL-1444, smooth muscle cell line derived from thoracic aorta of BDIX rat) were cultured in Dulbecco's modified Eagle's medium (DM EM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were subcultured after trypsinization with a solution of 0.05% trypsin-0.02% EDTA (Gibco BRL, Grand Island, NY, USA). Cells were grown to confluence at 37°C in 5% CO_2 on culture dishes.

Determination of cell viability

According to a mitochondrial tetrazolium assay (MTT), cells were seeded 1×10^5 cells/well in 96-well tissue culture plates. The confluent cells received DM EM medium with 1% FBS plus drugs from 5 h prior to stimulation with H_2O_2, and then were exposed to H_2O_2 for 18 h. After incubation, 20 μl/well of an MTT solution (5 mg/ml PBS) was added and incubated for 2 h. The plates were shaken for 20 min and the OD measured at 570~630 nm using ELISA (Bio-Tek instruments Inc., Winooski, VT, USA).

DNA fragmentation assays

After incubation in the absence and presence of the drugs for 5 h, cells ($1\sim5\times10^6$) were exposed to H_2O_2 (1 mM) for 18 h. At harvest, trypsinized cells were pelleted by centrifugation. Oligonucleosomal fragmentation of genomic DNA was determined as previously described (Wyllie, 1980). Cells were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for 1~3 h at 55°C, followed by addition of RNase A to 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol).

Equivalent amounts of DNA (15~20 μg) were loaded into wells of a 1.6% agarose gel and electrophoresed in 0.5×TAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Gel pictures were taken by UV transillumination with a Polaroid camera. Bands were quantified by Molecular Analyst Software using Bio-Rads Image Analysis System (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analyses

For determination of Bcl-2 and Bax protein levels, cells were grown in 100-mm tissue culture dishes and treated with the indicated compounds. After washing,

the cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0); 150 mM NaCl; 0.02% sodium azide; 100 μg/ml phenylmethylsulflonyl fluoride; 1 μg/ml aprotinin and 1% Triton X-100. Following centrifugation at 12,000 rpm, 50 μg of total protein of each sample was loaded into a 12% SDS-PAGE gel, and transferred to nitrocellulose membrane (Amersham Pharmacia Biotechlogy, Piscataway, NJ, USA). The blocked membranes were then incubated with the antibody of Bcl-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Mitochondrial cytochrome c was prepared following procedures. After washing cells (12×10^6) once with ice-cold PBS, cell pellets were resuspended in buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM surcose. The cells were homogenized and then centrifuged twice at 750 g for 10 min at 4°C. The harvested supernatants were centrifuged at 10,000 g for 10 min at 4°C, and the resulting mitochondrial pellets were dissolved in 1XSDS sample buffer. Western blots were preformed as described above with the antibody of cytochrome C (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The immunoreactive bands were visualized using chemiluminescent reagent of Supersignal® West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL). The signals of the bands were quantified using Calibrated Imaging Densitometer (GS-710, Bio-Rad Laboratories, Hercules, CA, USA). The protein concentration of the lysate was determined using Bio-Rad DC assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Assay of intracellular ROS

Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2',7'-dichlorofluororescine diacetate (DCFH-DA) into DCFH.

The intensity of fluorescence reflects enhanced oxidative stress. To measure the intracellular ROS, A7r5 were preincubated for 30 min in the absence and the presence of either KR31378 ($10^{-7}\sim10^{-5}$ M) or α-tocopherol ($10^{-7}\sim10^{-5}$ M). Thereafter, cells were stimulated without and with H_2O_2 (10^{-5} M for 20 min), and then incubated in the dark for 2 h at 37°C in 50 mM phosphate buffer (pH 7.4) containing 5 μM DCFH-DA. This agent is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the fluorescent polar derivative, DCFH and thereby trapped within the cells. The quantity of DCFH fluorescence in the supernatant was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm by using Fluorescence Plate Reader (Bio-Tek instruments, Inc., Winooski, VT, USA). Results were expressed as % control fluorescence intensity.

Peroxyl radical absorbing capacity (PRAC)

The assay for peroxyl radical scavenging is based on production of peroxyl radicals by AAPH (3 mM) with subsequent oxidation of the reporter protein, β-PE (16.7 nM), in a volume of 2 ml with 75 mM phosphate buffer (pH 7.0) in 24 well plates. After adding AAPH, loss of fluorescence was measured every 5 min at the emission of 590 nm and excitation of 485 nm using Fluorescence Plate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Trolox (1 μM) was used as a reference for PRAC assay. The fluorescence just prior to addition of the AAPH was estimated as the 100% value for that sample. The PRAC values were calculated as follows: PRAC=[area of compound − area of blank]/[area of 1 μM trolox − area of blank], where 1 PRAC unit is the value of 1 μM of trolox.

Measurement of lipid peroxidation

Confluent A7r5 cells from 24-well plates were washed with phosphate-buffered solutions. DMEM with antibiotics and 10% fetal bovine serum were added in a total volume of 0.5 ml/well. Cells were preincubated without and with either KR 31378 (10^{-6}, 10^{-5}, 10^{-4} M) or α-tocopherol (10^{-6}, 10^{-5}, 10^{-4} M) at 37°C for 30 min. Then, A7r5 alone or A7r5 plus 0.1 μM H_2O_2 were exposed to native LDL (100 μg/ml) for 24 h. Oxidation was stopped at 4°C by addition of EDTA (200 μM). Aliquots were assayed for malondialdehyde (MDA).

Statistical analysis

The results are expressed as means ± SEM. Statistical differences between groups were determined by paired or unpaired Student's t-test or analysis of variance. $P<0.05$ was considered to be significant.

RESULTS

Effect on cell viability

H_2O_2 (0.1 ~ 1 mM) exerted a concentration-dependent cytotoxic effect on A7r5 cells as determined by MTT assay in the serum-free medium (Fig. 1, Inset).

After incubation in the medium containing 0.5 mM H_2O_2 for 18 h, the cell viability was reduced to 71.7%. Cell death was concentration-dependently reduced upon incubation with KR 31378, KR 31612, α-tocopherol and trolox ($10^{-7} \sim 10^{-5}$ M, each), respectively (Fig. 1).

Antiapoptotic effect

Exposure of A7r5 cells to 0.5 mM H_2O_2 induced oligonucleosomal DNA fragmentation in a time-dependent and concentration-dependent manner, and the maximum values were observed after 18 h incubation (data not shown). At 18 h after exposure to 0.5 mM H_2O_2, cells showed morphological characteristics of apoptosis, including cell shrinkage and chromatin concensation relative to control cells (data not shown). Treatment with KR 31378 strongly suppressed the H_2O_2 (0.5 mM)-induced DNA laddering ($P<0.001$), whereas KR 31 612, α-tocopherol and trolox showed an inhibition but to a less degree than KR 31378 (Fig. 2).

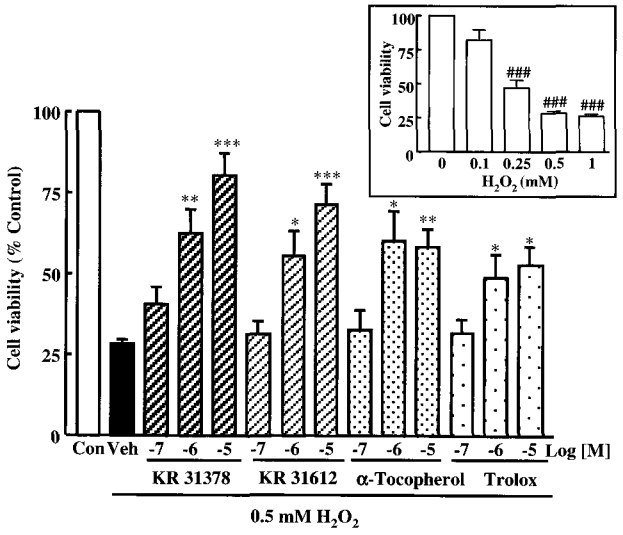

Fig. 1. Effect of KR 31378, KR 31612, α-tocopherol and trolox on A7r5 cells death induced by H_2O_2. A7r5 cells (1×10^4 cells/well) were incubated with 0.5 mM H_2O_2 for 18 h in the medium with 1% FBS, and cell viability was determined by MTT assay. The drugs were added 5 h before H_2O_2 addition. Inset: Concentration-dependent increases in H_2O_2 (0.1 ~ 1 mM)-induced cell death. Values are means ± SEM of three different preparations with quadruplicate experiments. ###: $P<0.001$ vs. zero H_2O_2. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ vs. Vehicle.

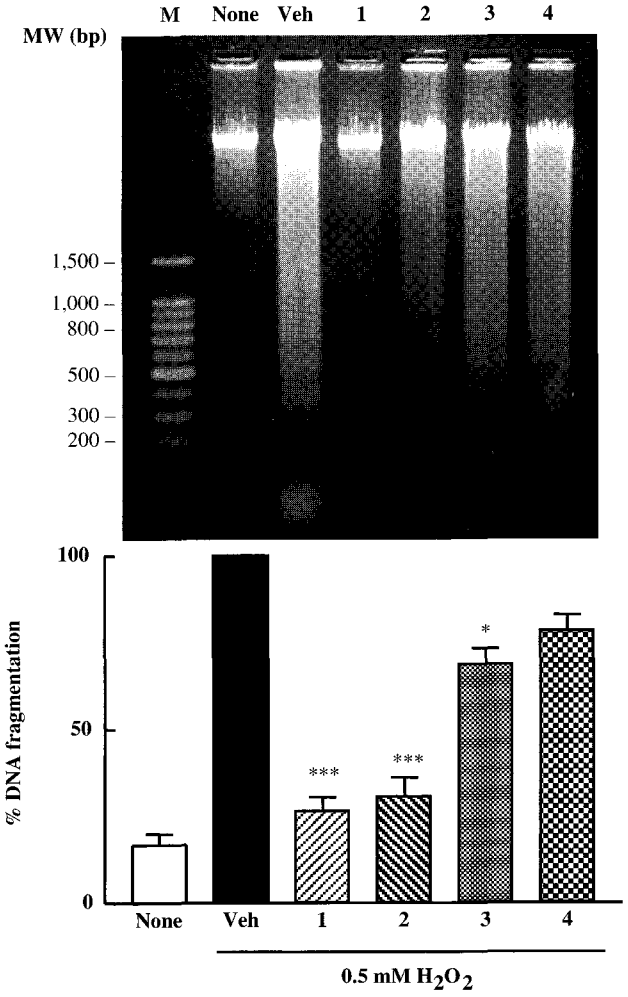

Fig. 2. Representative graphs showing DNA laddering after exposure to 0.5 mM H_2O_2 under pretreatment with vehicle (Veh), 10^{-5} M KR 31378 (lane 1), 10^{-5} M KR 31612 (lane 2), 10^{-5} M α-tocopherol (lane 3) and 10^{-5} M trolox (lane 4). None represents absence of H_2O_2. Preincubation of A7r5s with drugs for 5 h prior to and during H_2O_2 exposure significantly suppressed DNA laddering compared to H_2O_2 alone (Veh). M represents the 100 bp DNA Ladder markers. Lower panel shows the results of densitometric analysis representing means ± SEM from three experiments. *: $P<0.05$; ***: $P<0.001$ vs. Vehicle.

Western blot analyses

Fig. 3 is showing the expression of Bcl-2 and Bax protein. Bax was significantly elevated with increasing concentrations of H_2O_2 from 0.1 to 1 mM, whereas Bcl-2 level was concentration-dependently decreased (data now shown).

Bcl-2 protein was present at relatively high level in control samples (27.1±4.2 relative density) and the protein level was markedly decreased to 0.07±0.01 relative density under application of 0.5 mM H_2O_2, which was markedly recovered by pretreatment with 10^{-5} M KR 31378 (18.1±2.4 relative density), 10^{-5} KR 31612 (12.7±1.0 relative density) and in less degree by 10^{-5} α-tocopherol, but little by trolox (10^{-5} M) (Fig. 3A). In contrast, Bax protein was little identified in the control sample, but it was markedly increased by 21.8±3.0 relative density under 0.5 mM H_2O_2. KR 31378 and KR 31612 (10^{-5} M, each) markedly inhibited Bax protein expression up to 1.1 ±0.5 and 2.4±0.7 relative densities, respectively. α-Tocopherol and trolox showed inhibitory effects, but comparatively to a low degree (Fig. 3B).

In addition, the cytochrome C release level was markedly increased in the presence of 0.5 mM H_2O_2 to 47.5±5.6 relative density, which was strongly suppressed by KR 31378 (10^{-5} M). KR 31612, α-tocopherol and trolox (10^{-5} M, each) also significantly decreased the cytochrome C release (Fig. 4).

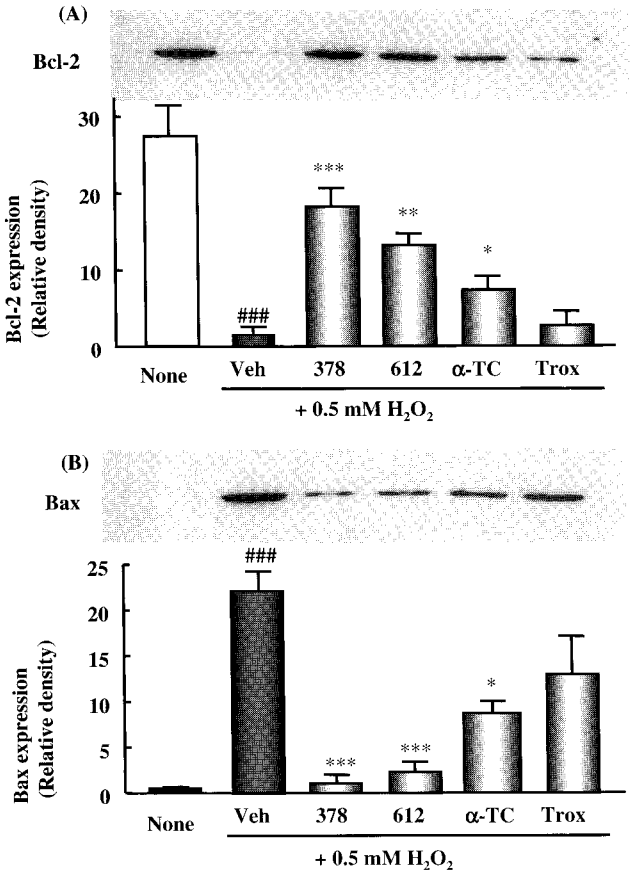

Fig. 3. Representative Western blots of A7r5 cell homogenates for Bcl-2 and Bax protein, and their corresponding densitometric analyses. (A) Enhancing effect of KR 31378 and other three compounds on H_2O_2 (0.5 mM)-induced suppression of Bcl-2 protein level. (B) Inhibitory effect of KR 31378 and other three compounds on H_2O_2 (0.5 mM)-induced increased Bax protein level. Values are means±SEM from three different preparations. ###: $P<0.001$ vs. None, *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ vs. Vehicle.

Scavenging of intracellular ROS and peroxyl radical

The intracellular ROS concentration was determined by measuring the intensity of fluorescence. Incubation of DCFH-loaded cells in the medium containing H_2O_2 (10^{-7}~10^{-5} M) for 2 h showed a concentration-dependent increase in fluorescence intensity, and the intensity was 317.8±7.38% by H_2O_2 at 10^{-5} M. KR 31378 and KR 31612 (10^{-7}, 10^{-6} and 10^{-5} M, each) significantly suppressed the increased

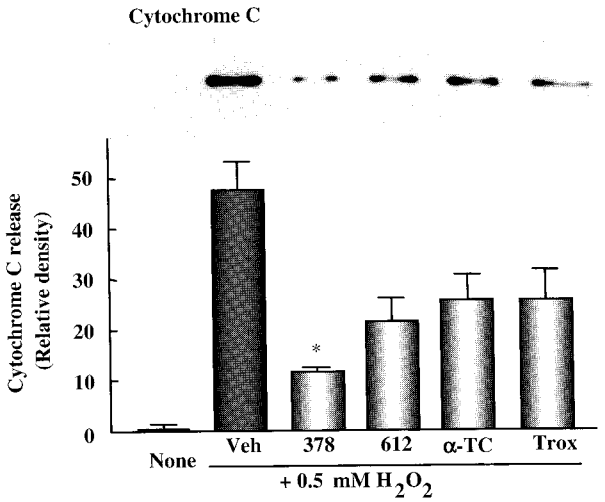

Fig. 4. Representative Western blots of A7r5 cell homogenates for cytochrome C, and their corresponding densitometric analyses. Effects of KR 31378 and other two compounds on H_2O_2 (0.5 mM)-induced increase in cytochrome C release into cytosol. Values are means±SEM from three different preparations. *: $P<0.05$ vs. Vehicle.

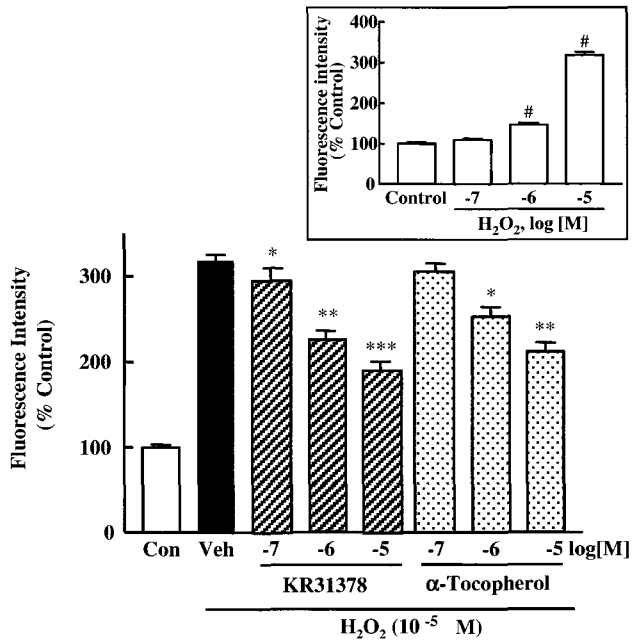

Fig. 5. Inhibitory effects of KR 31378 and α-tocopherol on the increased intracellular ROS stimulated by H_2O_2 (10^{-5} M) in A7r5 cells. Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2',7'-dichloro-fluororescine diacetate (DCFH-DA) into DCFH. KR 31378 and α-tocopherol were added 5 h prior to and during exposure to H_2O_2 (10^{-5} M). Inset: H_2O_2-concentration-dependent changes in fluorescence intensity indicative of intracellular ROS. Values are means±SEM from three different preparations with quadruplicate experiments. #: $P<0.05$ vs. Control; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ vs. Vehicle.

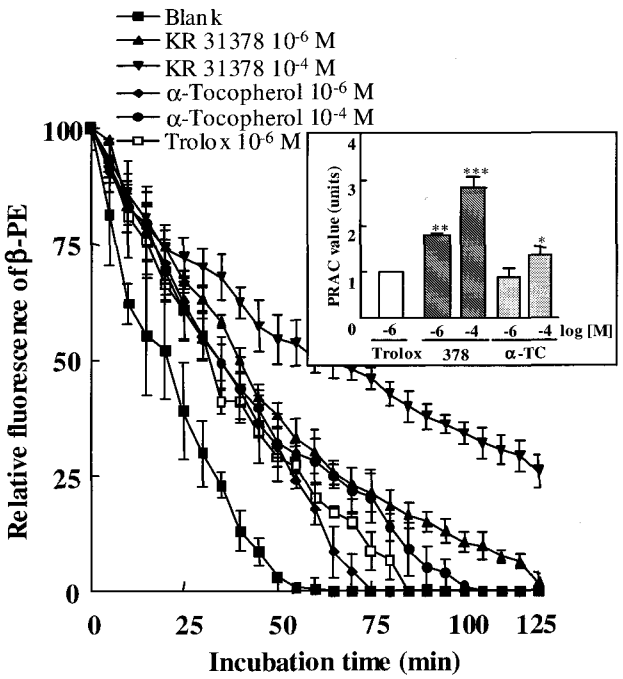

Fig. 6. Quenching of β-phycoerythrin (β-PE) with different concentrations of KR-31378 (378) and α-tocopherol (α-TC). The reaction mixture contains 1.67×10^{-8} M β-PE, 3×10^{-3} M AAPH with either 10^{-6} and 10^{-4} M KR 31378 or 10^{-6} and 10^{-4} M α-tocopherol and 10^{-6} M trolox. Inset: Peroxyl radical absorbing capacity (PRAC) values in unit. Each point represents mean± SEM of three measurements. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ vs. 10^{-6} M trolox.

fluorescence stimulated by H_2O_2 (10^{-5} M) in a concentration-dependent manner, as did α-tocopherol (Fig. 5).

Peroxyl radical absorbing ability of KR 31378 was identified by employing β-PE, and AAPH was used as a source of peroxyl radicals. In the presence of 10^{-6} and 10^{-4} M of KR 31378, the extinction curve showed a right shift, suggestive of its large scavenging effect (Fig. 6). The relative peroxyl radical absorbing capacity (in unit) calculated for KR 31378 (10^{-6} and 10^{-4} M) were 2.1±0.7 ($P<0.01$) and 3.4 ±0.3 ($P<0.001$) (Fig. 6 and Inset). The values of KR-31378 were significantly higher than those for α-tocopherol ($10^{-6}\sim10^{-4}$ M).

Antiperoxidative action

Upon incubation of the cells with LDL (100 μg/ml) and H_2O_2 (0.1 μM), MDA production was significantly increased, reaching a plateau at 24 h (25.2± 1.1 nmol MDA/mg protein, $P<0.01$). KR 31378 at 10^{-6}, 10^{-5} and 10^{-4} M significantly reduced the product of MDA to 15.2±1.1, 12.5±0.4 and 10.9± 0.6 nmol MDA/mg protein, respectively. The antiperoxidative effect of KR 31378 was similar to that of α-tocopherol (Fig. 7).

DISCUSSION

In the present study, 1) KR 31378 effectively protected A7r5 cells from H_2O_2-induced cell death in association with oligonucleosomal DNA fragmentation, 2) incubation with H_2O_2 markedly decreased the Bcl-2 protein and increased the Bax protein in association with cytochrome C release, which were

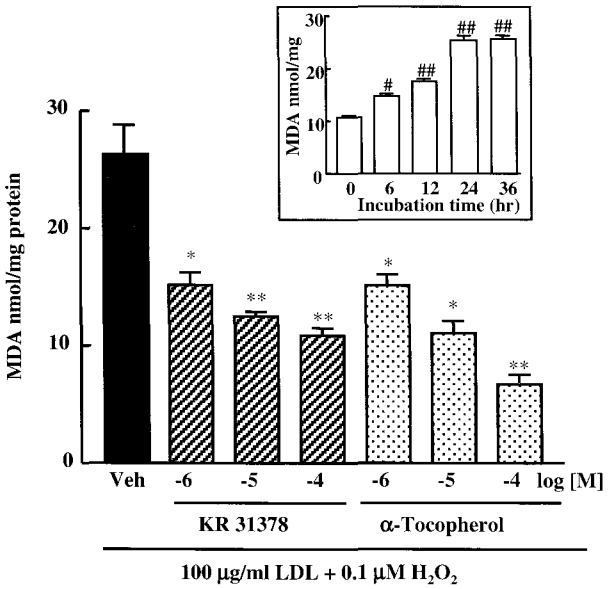

Fig. 7. Potent inhibitory effects of KR 31378 and α-tocopherol on TBARS formation (lipid peroxidation) of low density lipoprotein (LDL) in A7r5 cells plus 0.1 M H_2O_2 for 24 h. Inset: Time-course of H_2O_2-induced lipid peroxidation. Values are means ± SEM from three different preparations with triplicate experiments. #: $P<0.05$; ##: $P<0.01$ vs. zero hour, *: $P<0.05$; **: $P<0.01$ vs. Vehicle.

significantly reversed by KR 31378 and in less degree by KR 31612, α-tocopherol and trolox, and 3) KR 31378, KR 31612, α-tocopherol and trolox all significantly reduced the increased production of intracellular ROS and KR 31378 more effectively scavenged peroxyl radical than α-tocopherol. 4) KR 31 378 elicited a strong antiperoxidative effect.

In our results, H_2O_2-induced cytotoxicity as determined by MTT assay was concentration-dependently suppressed by incubation of A7r5 cells with 10^{-7}~10^{-5} M of KR 31378, KR 31612, α-tocopherol and trolox, respectively. In this experiment, treatment with KR 31378 strongly suppressed the H_2O_2 (0.5 mM)-induced DNA fragmentation, which showed signs of oligonucleosomal-sized fragmentation as evidenced by a ladder pattern indicating the occurrence of apoptotic cell death.

The family of Bcl-2 proteins has gained interest, because it has shown to enhance the cell survival by inhibiting apoptosis induced under various circumstances (White, 1996). Bax, homologues to Bcl-2, suppresses the ability of Bcl-2 to block apoptosis (Oltvai et al, 1993). We have shown that H_2O_2 induces a decrease in Bcl-2 protein expression, which has been demonstrated to be an antiapoptotic member of the Bcl-2 family by preventing cytochrome C release (Yang et al, 1997). Moreover, increased expression of Bax, a pro-apoptotic member of the same family by H_2O_2 was identified. Bax has been shown to facilitate cytochrome C release either by interacting with the permeability transition pore complex and/or by forming oligomers, which act as channels that trigger cytochrome C release from mitochondria, and activation of caspase cascade. Increasing number of reports has demonstrated that Bcl-2 and Bcl-XL block the mitochondrial permeability transition and cytochrome C release (Kluck et al, 1997; Yang et al, 1997). Our data showing that the apoptotic death of A7r5 cells was strongly inhibited by KR 31378 with significantly increased Bcl-2 protein expression are suggested to reflect that increased Bcl-2 might play an important role in inhibiting apoptotic cell death induced by application of H_2O_2. Otherwise, the results that H_2O_2-induced increased Bax expression was greatly suppressed by KR 31378 in association with inhibition of cytochrome C release suggest KR 31378 being a potential antiapoptotic agent.

Reactive free radicals generated from oxygen are potent mediators of tissue injury associated with many pathological conditions such as inflammatory and ischemic states. ROS such as the superoxide and hydroxyl radicals can act on membrane lipids or lipids in lipoprotein particles, and have been implicated in the development of atherosclerosis. In the present study, intracellular ROS was significantly and concentration-dependently increased in A7r5 cells by H_2O_2. The present results of suppression of ROS and peroxyl radicals by KR 31378 in association with scavenging of not only hydroxyl radical but also superoxide anion (data not shown) highlighted the capacity of KR 31378 to react with a wide spectrum of radicals.

The antioxidative effect of KR 31378 was further evident by the fact that increased production of thiobarbituric acid reactive substance in the LDL (by incubation with 0.1 M of H_2O_2 for 24 h) was significantly reduced by KR 31378 as well as by α-tocopherol (10^{-6}~10^{-4} M). α-Tocopherol is known as biologically and chemically the most active form of vitamin E and lipid peroxyl radical trapping and chain-breaking antioxidant. Interestingly, KR 31378 showed no pro-oxidant effect in contrast to α-tocopherol (Neužil et al, 1997).

Taken together, H_2O_2 potently induced apoptotic cell death in A7r5 cells in association with oligonucleosomal DNA fragmentation. KR 31378 and its acetyl metabolite, KR 31612, exert a strong inhibition of the apoptotic cell death by scavenging of intracellular ROS and peroxyl radical, and increasing Bcl-2 and inhibiting Bax protein and cytochrome C release. Further experiments are remained to elucidate how the expression of Bcl-2 and Bax proteins are modulated by the oxygen free radicals.

ACKNOWLEDGMENTS

This study was supported by the fund from Center for Bioactive Substances, Korea Research Institute of Chemical Technology, Daejon, Research Institute of Genetic Engineering, Pusan National University (KWH), Pusan and the Korea Science & Engineering Foundation (KWH), Korea.

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