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Original Article

Adenovirus-mediated overexpression of catalase attenuates oxLDL-induced apoptosis in human aortic endothelial cells via AP-1 and C-Jun N-terminal kinase/extracellular signal-regulated kinase mitogen-activated protein kinase pathways

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Abstract

In a variety of vascular disorders, endothelial cells (ECs) are exposed to high levels of reactive oxygen species (ROS) generated intercellularly. Recently, several anti-oxidants, including catalase, have been suggested to be cytoprotective against the development of atherosclerosis. The object of this study was to investigate whether adenovirus-mediated gene transfer of catalase in ECs can attenuate ROS production and cell apoptosis under oxidized low density lipoprotein (oxLDL) stimulation. Adenovirus-mediated gene transfer of human catalase gene (Ad-Cat) resulted in a high level of catalase overexpression in human arterial EC (HAEC), which manifested a time-dependent increase in cell viability under the exposure of oxLDL and decreased oxLDL-induced apoptosis. Phosphorylation studies of ERK1/2, JNK, and p38, three subgroups of mitogen activator protein kinase demonstrated that catalase overexpression suppressed JNK phosphorylation and increased ERK1/2 phosphorylation. NF-κB and AP-1 were induced after the exposure of HAECs to oxLDL. While catalase overexpression was found to inactivate AP-1, it had no effect on NF-κB activity. These results provide the evidence that overexpression of catalase in ECs attenuates ROS production and cell apoptosis under oxLDL stimulation. The protective effect is mediated through the downregulation of JNK and the upregulation of ERK1/2 phosphorylation as well as AP-1 inactivation. This observation supports the feasibility of catalase gene transfer to human endothelium to protect against oxidant injury.

Keywords: Catalase; Endothelial cells; OxLDL; Atherosclerosis; Apoptosis

1. Introduction

Disruption of the delicate balance between pro-oxidants and anti-oxidants has been implicated in the pathophysiology of cardiovascular disorders [1]. A plethora of information suggests that oxidants, such as hydrogen peroxides (H_2O_2) and lipid peroxide, induce a number of proatherogenic changes in vascular cells that are present in the atherosclerotic artery [2]. Most of these effects could be prevented by the addition of extracellular anti-oxidants, suggesting further propagation of oxidation [3]. Studies based on anti-oxidant gene expression to examine the effects of oxidants on cells also corroborate the premise that intracellular oxidative stress might play an important role in the transduction of oxidative stress from external sources to intracellular sites [4]. Previously, Shingu et al. [5] showed that endothelial cells (ECs) and smooth muscle cells (SMCs) have very low levels

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of catalase activity and, therefore, are more susceptible to damage by H_2O_2. In contrast, transient overexpression of catalase in human umbilical vein ECs has been found to confer protection against H_2O_2-mediated oxidative stress [6]. Moreover, overexpression of human catalase gene has been shown to decrease oxidized lipid-induced cytotoxicity in vascular SMCs [7]. These results suggest that catalase is a key enzyme in the protection of cells against oxidative injury. Several studies have attempted to overexpress the catalase enzyme in various cell types and have examined its effect in preventing oxidation-related damage [6,7]. However, the regulatory mechanism of catalase protein on human ECs exposed to oxidative stress still remains unclear.

Low density lipoprotein (LDL) is among the major risk factors and predictors for the development of atherosclerosis. Manifested as an increase in free radicals, oxidized LDL (oxLDL) perturbs endothelial functions via several routes, including a decrease in NO production, an increase in plasminogen activator inhibitor-1, recruitment of monocytes, and through the promotion of cell apoptosis [8,9]. It is thought that oxLDL-induced signal transmission requires the binding to specific cellular receptors to activate and stimulate a wide spectrum of host responsive systems [10]. This requires the activation of multiple signaling molecules in transduction pathways, for example protein-tyrosine kinase (PTK), oxLDL receptor-associated serine/threonine kinase, Ras, Raf-1, IκB kinase, MEK, mitogen-activated protein kinases (MAPKs), etc., [11–13]. These molecules may converge or diverge and often have "cross-talk" properties, which result in a complicated signaling network that exert mutual influence. Subsequently, the signals further transduce to downstream pathways and activate numerous transcriptional factors, including AP-1, NF-κB, and ATF-2 [14], which trigger a large amount of genes encoded for inflammatory mediators and cytokines [10]. These elicited that cytokines are believed to be responsible for cell proliferation, differentiation, immunoregulation, and cytotoxicity [11]. Currently, the effects of oxLDL and catalase overexpression on the signaling pathways and the events involved in the transduction of programmed death signal into the nucleus of human arterial ECs (HAECs) have not been well-defined.

In this study, adenovirus-mediated gene transfer was used to overexpress catalase in HAECs to obviate concerns regarding the purity of the enzyme and preparation or fluctuations in catalase protein delivery. Our results demonstrate that HAECs enriched in catalase are resistant to the damaging effects of oxLDL. The AP-1 and JNK/ERK MAPK pathway is the major regulatory mechanism leading to the protective effects of catalase overexpression.

2. Methods

2.1. Culture of HAECs

HAECs were obtained as cryopreserved tertiary cultures from Cascade Biologics (OR, USA) and were grown in EC growth medium (medium 200, Cascade Biologics) supplemented with 2% FBS, 1 μg/ml of hydrocortisone, 10 ng/ml of human epidermal growth factor, 3 ng/ml of human fibroblast growth factor, 10 μg/ml of heparin, 100 U/ml of penicillin, 100 pg/ml of streptomycin, and 1.25 μg/ml of Fungizone. The cells were used between passages 3 and 8. The purity of the cultures was verified by staining with monoclonal antibody against human von-Willebrand factor (vWF) [15].

2.2. Preparation and oxidation of LDL

Human LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of fasting plasma samples from healthy adult males and extensively dialyzed under nitrogen for 24 h at 4 °C against phosphate-buffered saline (PBS, 5 mmol/l phosphate buffer and 125 mmol/l NaCl, pH 7.4). This native LDL (nLDL) was oxidized by dialysis for 24 h at 37 °C against 10 μmol/l $CuSO_4$ in PBS, as previously described [16]. The extent of oxidation was monitored by measuring thiobarbituric acid-reactive substance (TBARS) and agarose gel electrophoresis. The levels of LDL oxidation were 7.2 ± 0.1 nmol/l for oxLDL and 1.1 ± 0.1 nmol/l for nLDL of TBARS per milligram protein, respectively. The cholesterol content of nLDL and oxLDL was determined using a cholesterol enzymatic kit (Merck, Darmstadt, Germany).

2.3. Preparation of recombinant adenoviruses

A human catalase cDNA containing the entire coding sequence was subcloned into the adenovirus shuttle plasmid vector, pAd-PGK, which contains a promoter of the human phosphoglycerate kinase and a polyadenylation signal of bovine growth hormone. A recombinant adenovirus (Ad-Cat) was generated by homologous recombination and amplified in human embryonic kidney 293 cells as previously described [17]. Viruses were purified by CsCl density gradient centrifugation, and virus titer determination was as previously described [17]. Purified virus was stored in 10 mmol/l Tris–HCl (pH 8.0), 2 mmol/l $MgCl_2$, and 4% (vol/vol) sucrose at –80 °C until used for the experiments. For infection, ~3×10^5 cells were seeded on a 10-cm^2 Petri dish and treated with selected adenovirus at the indicated multiplicity of infection (MOI, or plaque forming unit per cell) for 48 h. Culture medium was changed for incubation longer than 48 h.

2.4. Western blot analysis

Western blot analysis was performed as described previously [18]. Briefly, the cell lysate (20 μg) was subjected to 12% SDS-PAGE electrophoresis and transferred onto PVDF membranes. Membranes were incubated with rabbit polyclonal antibody against catalase (1:1000, OXIS, OR, USA) then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:3000, Sigma, MO, USA), the signal

was detected using Chemiluminescence Reagent Plus (NEN, MA, USA). The intensity of each band was quantified using a densitometer. Anti-α-tubulin antibody (1:1000, Oncogen, CA, USA) was used to quantify as an internal control.

In other experiments, the protein was subjected to western blotting with antibodies (1:1000) directed against mouse anti-human procaspase-3, mouse anti-human caspase-9, mouse anti-human Bcl-2 (Neomarkers, CA, USA); rat anti-human Bax (PharMingen, CA, USA); rabbit anti-human phospho-JNK, mouse anti-human phopho-ERK1/2, rabbit anti-human phospho p38, rabbit anti-human total JNK, rabbit anti-human total ERK1/2, and goat anti-human cytochrome *c* (1:500, Cell Signaling, MA, USA).

2.5. Immunocytochemical localization of catalase

HAECs with or without adenoviral infection were washed with PBS and fixed with 4% paraformaldehyde in PBS at RT for 15 min. The samples were treated with PBS containing 1% Triton X-100 for 5 min, and then blocked with PBS containing 5% BSA at RT for 1 h. Rabbit anti-human catalase antibody (1:400, R&D, MN, USA) was added and incubated for 1 h. After rinsing with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:400, Vector, CA, USA) at 37 °C for 1 h. Following a wash with PBS, the cells were mounted using Vectashield mounting medium (Vector) and examined with a fluorescence microscope. For negative controls, the primary antibodies were omitted, and only negligible immunofluorescence was observed.

2.6. Catalase activity assay

Catalase activity was quantified as described previously [19]. Briefly, 100 μl of sonicated cell extracts (100 μg) were mixed with 2.9 ml of 19 mmol/l H_2O_2 in 50 mmol/l potassium phosphate buffer (pH 7.0). The time required for the OD 240 nm of the reaction mixture to change 0.05 was monitored as a measure of catalase activity. One unit of catalase activity is equal to 17 s. Thus, activity units of the samples were expressed as units per milligram of cell lysate protein.

2.7. Detection of H_2O_2 and superoxide production

The effect of catalase overexpression on H_2O_2 and superoxide (O_2^-) production in HAECs was determined by a fluorometric assay using DCFH-DA and DHE as the probe, respectively [20]. Confluent HAECs (10^4 cells/well) in 48-well plates were infected with Ad-Cat at 25 MOI for 48 h. Cells were washed with HBSS, and incubate in HBSS containing 20 μmol/l DCFH-DA or 10 μM DHE at 37 °C for 45 min. The fluorescence intensity (relative fluorescence units) was detected at 485/530 nm and 485/590 nm for excitation/emission for DCFH corresponding to H_2O_2 and ethidium to O_2^-, respectively, using a fluorescence microplate reader. To determine the effect of oxLDL on H_2O_2 and O_2^- generation, fluorescence intensity was also measured immediately after oxLDL (40 μg/ml) added at the predetermined intervals for 30 min.

2.8. Caspase activity assay

Cells were seeded in 96-well plates at 2×10^5/100 μl/well and cultured at 37 °C for 48 h. After treatments, cells were gently washed twice with PBS, 25 μl of 1% Triton X-100 (Sigma) was added to each well and the cells were subjected to four cycles of freezing and thawing. An equal amount of cellular protein of each group was added to a reaction mixture containing 50 μmol/l of the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Promega, WI, USA), followed by the incubation at 30 °C for 60 min. Free 7-amino-4-methyl coumarin (AMC) liberated upon enzymatic cleavage was detected by cytofluor 2300 with excitation/emission of 360/460 nm, respectively.

2.9. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was measured by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [15]. Cells infected with Ad-Cat at various MOI for 48 h were incubated in different concentrations of oxLDL for 24 h. MTT was added to each well and the metabolized MTT was determined by the absorbance at 530 nm, with 690 nm as reference. The cells incubated with control medium were considered 100% viable.

2.10. Propidium iodide staining and TdT-mediated dUTP nick end-labeling assay

Treated and control HAECs were stained with 5 μg/ml propidium iodide (Sigma) at 4 °C for 30 min and examined under fluorescence microscopy. Moreover, cells fixed with 1% paraformaldehyde and the 3′ ends of DNA were labeled with TdT-mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer's recommendation (Boehringer Mannheim, Germany) to detect apoptotic cells. Fluorescein-dUTP-labeled cells were observed under fluorescence microscope.

2.11. Cellular DNA fragmentation

DNA fragmentation of cells was evaluated by labeling the DNA strand breaks with 5-bromo-2′-deoxy-uridine (BrdU) using TdT. These sites were identified by staining with monoclonal antibody, BrdU monoclonal antibody, labeled with peroxidase following the protocol of the manufacturer (Cellular DNA fragmentation ELISA, Boehringer Mannheim). The amount of BrdU-labeled DNA released into the cytoplasm of apoptotic cells was quantified by ELISA [21].

2.12. cDNA microarray analysis

Total RNA of HAECs was isolated using Trizol reagent (Invitrogen, CA, USA) from HAECs under three conditions,

namely untreated cells, cells in the presence of oxLDL, and catalase-transfected cells in the presence of oxLDL. Aliquots of 10 μg RNA were used to analyze gene expression of apoptosis by Human Apoptosis Q Series arrays (Super Array Inc., Bethesda, MD, USA). Relative amounts of mRNA transcripts were quantified using the molecular Imager System and Molecular Analyst Imaging software (Super Array Inc.). The relative amount of a given gene transcript was estimated by comparing its signal intensity with the signal derived from GAPDH and β-actin.

2.13. Electrophoretic mobility shift assay

Preparation of nuclear extracts and conditions for electrophoretic mobility shift assay (EMSA) reactions were described previously [15]. The 22-mer synthetic double-stranded oligonucleotides used as NF-κB and AP-1 probes in the gel shift assay were (5′-AGTTGAGGGGACTTTCCCAGGC-3′; 3′-TCAACTCCCCTGAAAGGGTCCG-5′) and (5′-ATTC GATCGGGGCGGGGCGAG-C-3′; 3′-TAAGCTAGCCC CG CCCCGCTCG-5′), respectively.

2.14. Statistical analysis

Values are expressed as the mean ± S.E.M. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a P value <0.05 being considered significant.

3. Results

3.1. Overexpression of catalase reduces H_2O_2 production in HAECs

HAECs were infected with Ad-Cat at various MOI for 48 h, and their catalase expression detected by western blot analysis. As shown in Fig. 1A, high levels of catalase were expressed in a dose-dependent manner in Ad-Cat-infected cells but not in cells infected with Ad-GFP (green fluorescence protein) or control virus, Ad-PGK. Moreover, cellular catalase activity was increased about 18-fold with Ad-Cat infection compared to cells infected with Ad-PGK or untransfected cells (Fig. 1B). In catalase-transfected cells, the catalase expression was strongly located throughout the cytoplasm by immunofluorescent staining. In contrast, in untransfected cells the immunofluorescence was weakly present (Fig. 1C). These results suggest that infection of Ad-Cat greatly increased the amount of functional catalase protein within HAECs.

To determine whether high level of catalase affects the production of O_2^- and H_2O_2 in oxLDL-treated HAECs, cells infected with Ad-Cat and Ad-PGK at 25 MOI for 48 h were incubated with 40 μg/ml of oxLDL. Compatible amounts of O_2^- were generated among catalase overexpression and control cells with (Fig. 2A) or without oxLDL treatment

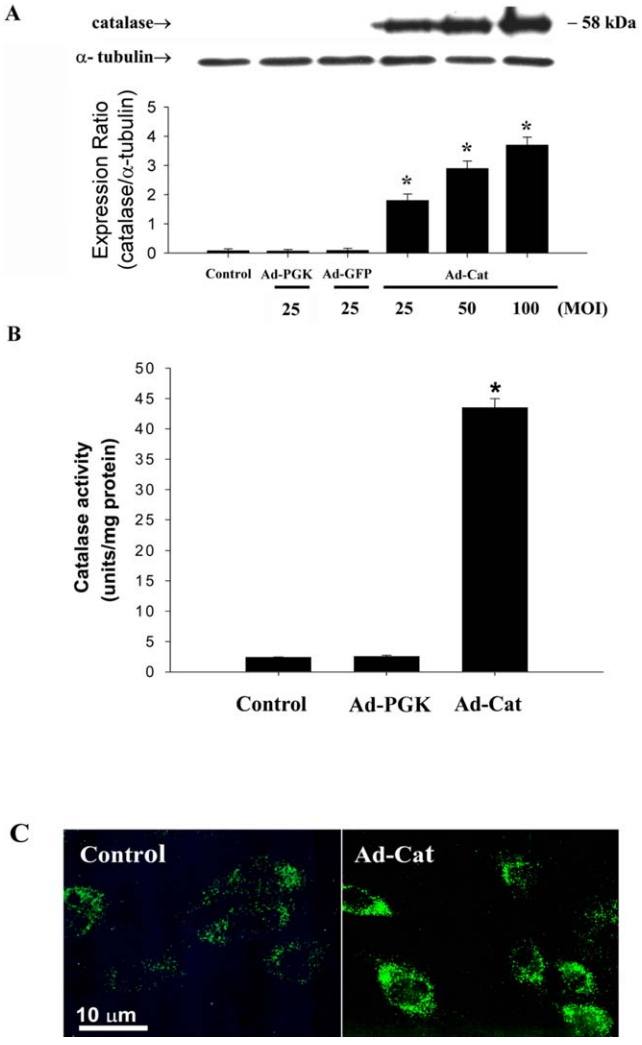

Fig. 1. Catalase expression and activity of Ad-Cat-infected HAECs. (A) Cells without infection (control), or infected with Ad-Cat at various MOI and control virus (Ad-PGK) were lysed at 48-h post infection and analyzed by western blot for catalase expression using catalase antibody. Densitometric analysis was conducted with PhotoCap software to semiquantify western blot data. Three independently experiments gave similar results. The summarized data (mean ± S.E.M.) from three separate experiments is shown in the bar graph. * $P < 0.05$ compared with control, Ad-PGK infected, and Ad-GFP infected. (B) Catalase activities of control, Ad-PGK-, or Ad-Cat (25 MOI)-infected cells measured by H_2O_2 disappearance at OD 240 nm for 3 min ($n = 3$). (C) Cells were fixed and immunostained with anti-catalase antibody at 24-h post Ad-Cat (25 MOI) infection for fluorescence microscopy.

(Fig. 2B), suggesting that overexpression of catalase does not significantly alter the O_2^- production in HAECs. Nonetheless, H_2O_2 level in cells was significantly reduced with catalase overexpression in untreated cells (Fig. 2C) and oxLDL-treated cells (Fig. 2D). The H_2O_2 levels were significantly increased by oxLDL treatment compared to control cells in a time-dependent manner (Fig. 2D).

3.2. Catalase overexpression increase cell viability

To investigate whether overexpression of catalase protect cells from the damage of oxLDL-, Ad-Cat-, and Ad-PGK-

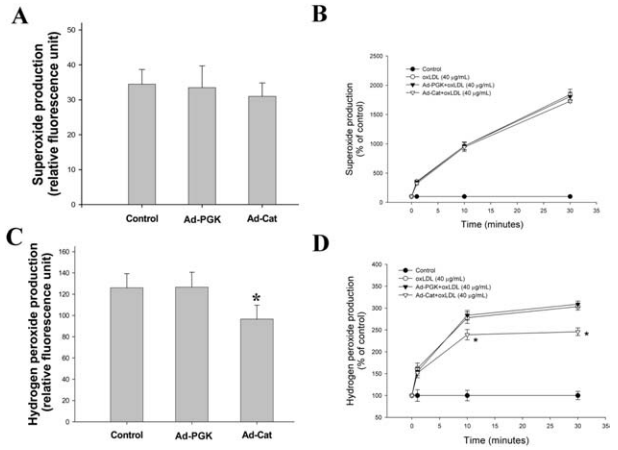

Fig. 2. Effect of Ad-Cat infection on O_2^- and H_2O_2 production in HAECs. HAECs were infected with Ad-Cat at 25 MOI for 48 h, DHE and DCFH-DA were then added for O_2^- and H_2O_2 detection, respectively. After incubation for 45 min at 37 °C, ethidium (A) and DCFH (C) fluorescence were measured. (B,D) Amount of O_2^- (B) and H_2O_2 (D) production in oxLDL-stimulated HAECs, where cells were infected with control virus, Ad-PGK, or with Ad-Cat at 25 MOI for 48 h, followed with stimulation of 40 μg/ml of oxLDL. Fluorescence readings were taken at 1, 10, and 30 min. Control, cells without treatment. Data represent mean ± S.E.M. of three independent experiments (with separate ECs isolates) performed in triplicate. * $P < 0.05$ compared with the cells without Ad-Cat infection (Fig. 2C) or compared with the oxLDL-treated cells without Ad-Cat infection (Fig. 2D).

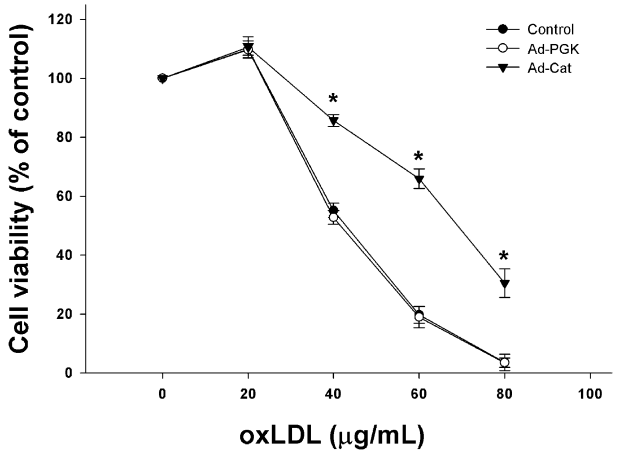

Fig. 3. Effect of catalase on cell viability of oxLDL-treated HAECs as measured by MTT assay. Cells were treated with oxLDL of 40 μg/ml with or without Ad-Cat infection. The results are as expressed in percentage of viable cell in untransfected cells grown in media containing 10% FCS. Data are mean ± S.E.M. ($n = 3$). * $P < 0.05$ compared with control and Ad-PGK infected.

infected HAECs were incubated with oxLDL at various concentrations (20–80 μg/ml) for 24 h followed by MTT assay. As illustrated in Fig. 3, low concentration (20 μg/ml) of oxLDL increased cell viability, while drastic cell death was observed with oxLDL concentration higher than 40 μg/ml. At higher levels of oxLDL concentration, catalase significantly increased the cell viability so that less than 20%, and only 2% of cells were survived when treated with 60 and 80 μg/ml of oxLDL, respectively, whereas, cell viability were increased to 65% and 29% with Ad-Cat infection, respectively. These data suggest that catalase overexpression prevents ECs cytotoxicity caused by oxLDL. Moreover, infection of Ad-Cat and Ad-PGK did not alter the cell viability without oxLDL treatment (data not shown).

3.3. Effects of catalase overexpression on cell apoptosis

To determine whether overexpression of catalase inhibit the apoptotic effect induced by oxLDL, HAECs, infected with Ad-Cat, Ad-PGK at 25 MOI for 48 h, were treated with 40 μg/ml oxLDL for 24 h, followed with propidium iodide staining. Confocal microscopic examination of cells showed nuclear fragmentation and condensation in a majority of cells treated with oxLDL but not in untreated or Ad-Cat-infected cells (Fig. 4A), suggesting that Ad-Cat infection inhibited oxLDL-induced apoptosis. Moreover, using TUNEL assay,

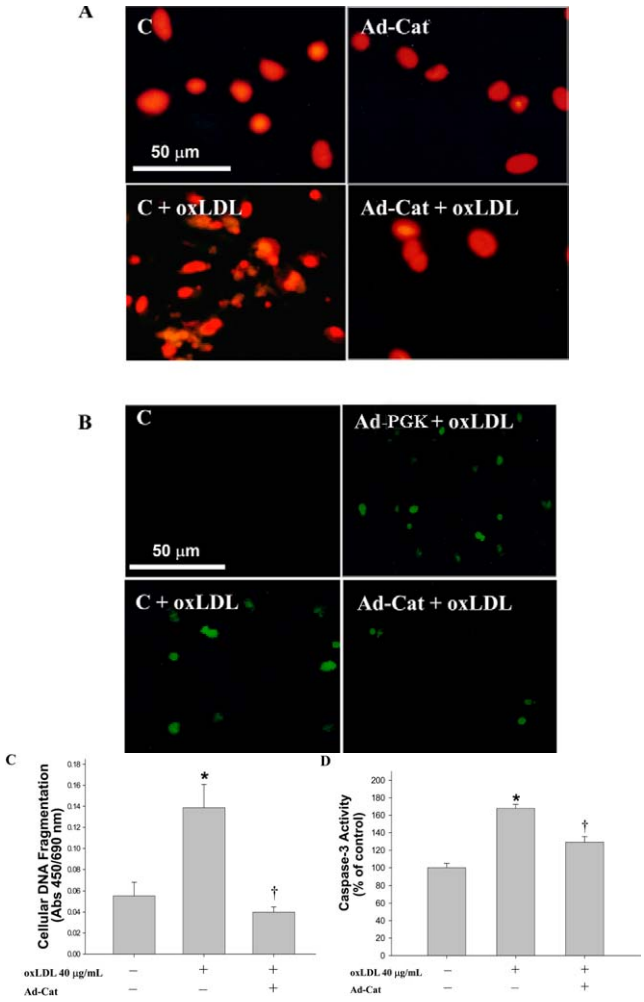

Fig. 4. Effects of catalase overexpression on oxLDL-induced HAEC apoptosis. HAECs were infected without control, or with Ad-Cat at 25 MOI for 48 h, followed by oxLDL treatment. (A) Propidium iodide staining of cell nucleus. (B) TUNEL staining. (C) Quantification of cellular DNA fragmentation of oxLDL-and Ad-Cat-treated cells. DNA fragmentation in supernatants of cell culture and lysate was measured by ELISA. (D) Treated HAECs were analyzed for caspase-3 activity using the fluorogenic substrate Ac-DEVD-AMC. * $P < 0.05$ compared with control, † $P < 0.05$ compared with oxLDL-treated cells.

more fluorescence-positive cells were detected in control and Ad-PGK-infected cells treated with oxLDL than in Ad-Cat-infected and untreated cells (Fig. 4B), suggesting that catalase overexpression suppressed oxLDL-induced DNA fragmentation. The degree of BrdU labeling showed that oxLDL significantly increased the amount of DNA fragmentation in the control and Ad-PGK-infected cells while its level exhibited marked suppression in Ad-Cat-infected cells (Fig. 4C). While the cells treated with oxLDL exhibited substantial DNA fragmentation as evidenced by DNA laddering, little or no DNA fragmentation was detected in non-infected control or Ad-Cat-infected cells treated with oxLDL (data not shown).

Caspase-3 activity, an executioner of apoptosis [22], was determined by measuring the proteolytic cleavage of a fluorogenic substrate, Ac-DEVD-AMC. As shown in Fig. 4D, caspase-3 activity was significantly increased in cells treated with oxLDL for 6 h. In contrast, the activation of caspase-3 by oxLDL was suppressed in cells infected with Ad-Cat (Fig. 4D).

3.4. Gene expression profiling by cDNA microarray analysis

To investigate the mechanism underlying anti-apoptotic effects of catalase in oxLDL-treated HAECs, a cDNA microarray with apoptotic-related gene was used to study their gene regulation profile. The results showed that oxLDL treatment caused many genes to upregulate significantly, such as caspase-9, caspase-3 and Bax, and others to downregulate, such as Bcl-2 (Table 1). Moreover, infection of Ad-Cat counteracted the downregulation of Bcl-2 by oxLDL while it had no effect on the gene expression of caspase-9 and Bax. Some of these genes regulated patterns were confirmed by western blot analysis which showed that the treatment of oxLDL suppressed the protein levels of procaspase-3 and Bcl-2, and increased the levels of caspase-9, cytochrome *c* and Bax (Fig. 5). The downregulation of procaspase-3 and Bcl-2 by oxLDL was upregulated by further treatment of Ad-Cat, suggesting that the anti-apoptotic effect of catalase may be mediated through the upregulation of Bcl-2 and the downregulation of procaspase-3.

3.5. Involvement of ERK1/2 and JNK in catalase-transfected oxLDL-stimulated HAECs

Previous studies have shown that oxLDL can activate MAPKs in the signaling pathway leading to apoptosis [14,23]. To examine whether MAPKs were regulated through phosphorylation, cell lysates were subjected to western blot analysis. As shown in Fig. 6, phosphorylation levels of JNK1/2 and p38 were significantly increased at 5 min following oxLDL addition, whereas phosphorylated of ERK1/2 was mildly increased. Interestingly, catalase transfection completely abolished JNK phosphorylation induced by oxLDL, while it elevated the ERK1/2 phosphorylation (Fig. 6). These results suggest that catalase inhibits JNK activation while it increases ERK1/2 activity. Besides, phosphorylation of p38 did not appear to respond to catalase transfection.

Table 1
Expression changes of apoptosis-related genes analyzed by cDNA microarray

Gene name	Description	Ratio		
		oxLDL/control	oxLDL + Ad-Cat/control	oxLDL + Ad-Cat/oxLDL
p53	Tumor protein p53 (Li-Fraumeni syndrome)	15.8	1.9	0.1
DFFA	DNA fragment factor-45	7.3	2.3	0.3
Caspase-8 (FLICE)	Apoptosis-related cysteine protease	7.1	2.4	0.3
Bik	BCL2-interacting killer	5.0	3.3	0.7
Caspase-9 (Mch6)	Caspase-9, apoptosis-related cysteine protease	4.7	4.2	0.9
DFF40 (CAD)	DNA fragmentation factor, 40 kD, beta subunit	4.7	1.8	0.4
Bak	BCL2-antagonist/killer 1	3.1	2.4	0.8
TRAIL	TNF (ligand) superfamily, member 10	2.8	2.1	0.8
CIDE-A	Cell death inducing DFFA-like effector A	2.8	1.4	0.5
TRAF1	TNF receptor-associated factor 1	2.7	0.6	0.2
Caspase-3 (cpp32)	Caspase-3, apoptosis-related cysteine protease	2.6	1.3	0.5
HRK	Harakiri, BCL2-interacting protein (contains only BH3 domain)	2.6	1.6	0.6
Caspase-1 (ICE)	Caspase 1, apoptosis-related cysteine protease	2.6	2.1	0.8
mdm2	Mouse double minute 2, human homolog of; p53-binding protein	2.2	1.4	0.6
bax	BCL2-associated X protein	2.2	2.6	1.2
bar	Apoptosis regulator	2.0	1.6	0.8
Caspase-10 (mch4)	Caspase-10, apoptosis-related cysteine protease	2.0	2.3	1.2
IAP-1	Inhibitor of apoptosis protein 1	2.0	2.8	1.4
bcl-2	B-cell CLL/lymphoma 2	0.7	2.5	3.6

* Control: HAECs without any treatment. oxLDL: HAECs treated with 40 μg/ml of oxLDL for 2 h. oxLDL + Ad-Cat: HAECs infected with Ad-Cat at 25 MOI for 48 h, and then treated with 40 μg/ml of oxLDL for 2 h. TNF: tumor necrosis factor.

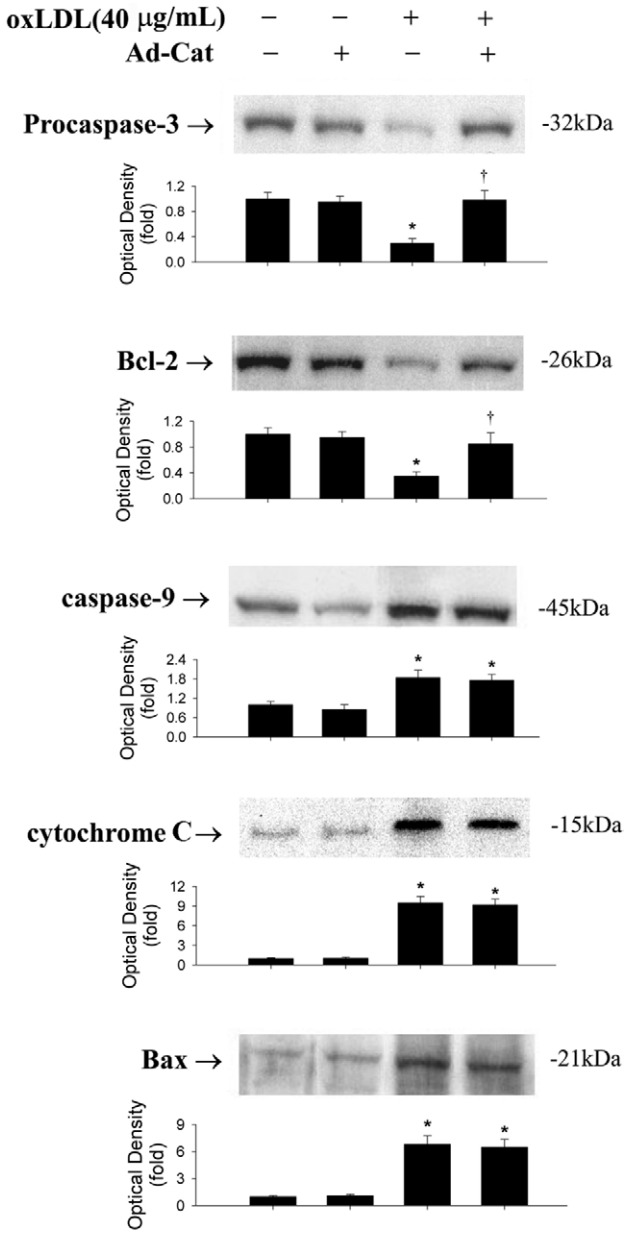

Fig. 5. Western blot analysis of apoptotic-related proteins in HAECs. Cells were not infected or infected with Ad-Cat of 25 MOI for 48 h. Cells were subsequently stimulated with or without 40 μg/ml oxLDL for 12 h at 37 °C. Equal amounts of protein were resolved by SDS-PAGE followed by western blot analysis with antibodies against each specific protein. The summarized data from three separate experiments is shown in the chart. * $P < 0.05$ compared with control, † $P < 0.05$ compared with oxLDL-treated cells.

3.6. Effects of catalase overexpression on NF-κB and AP-1 activity in oxLDL-treated HAECs

NF-κB or AP-1 activation has been implicated in the oxLDL-induced expression of inflammatory cytokines [24,25]. Gel-shift assays were performed to determine the effect of catalase overexpression to the activation of NF-κB and AP-1 in oxLDL-treated HAECs. As shown in Fig. 7, low levels of basal NF-κB and AP-1-binding activity were detected in control cells, while treatment of oxLDL (40 μg/ml for 30 min) significantly increased the binding activity of

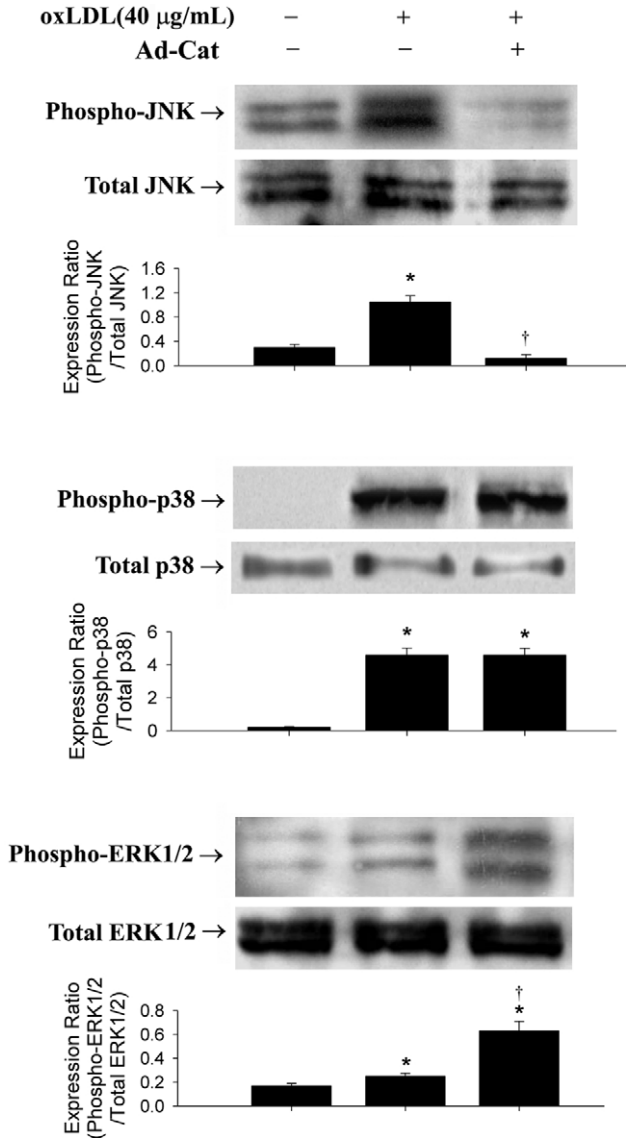

Fig. 6. Effects of catalase overexpression on various MAPKs phosphorylation in oxLDL-stimulated HAECs analyzed by western blot. Control cells or Ad-Cat cells infected with 25 MOI for 48 h were stimulated with 40 μg/ml oxLDL for 5 min at 37 °C. Equal amounts of protein were subjected to immunoblotting with specific antibodies. The summarized data from three separate experiments are shown in the chart. * $P < 0.05$ compared with control, † $P < 0.05$ compared with oxLDL-treated cells.

NF-kB and AP-1. These binding activities were blocked by a 100-fold excess of unlabeled NF-kB and AP-1 targeted oligonucleotides (data not shown). When HAECs were pre-infected with Ad-Cat, the AP-1 binding was reduced by 40% compared with oxLDL treatment only. However, the NF-κB-binding activity was not changed with catalase overexpression.

4. Discussion

We have shown that catalase overexpression can increase cell viability and reduce apoptosis in oxLDL-treated HAECs. Our data demonstrate that the protective effects of

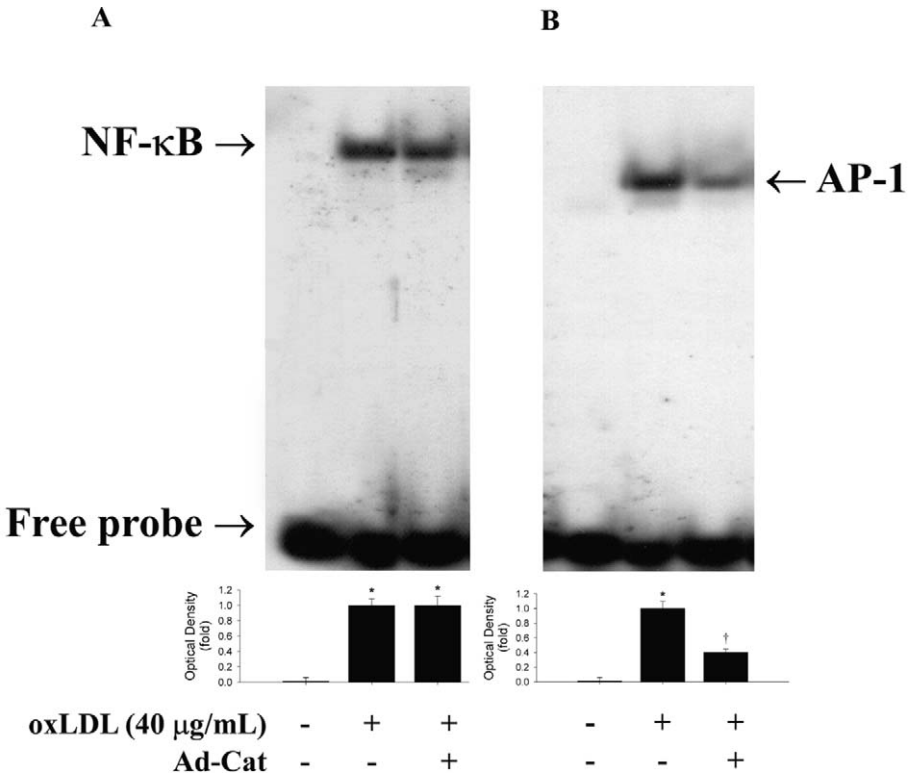

Fig. 7. Effects of catalase overexpression on NF-κB and AP-1 activation in oxLDL-stimulated HAECs by EMSA. Control cells or Ad-Cat-infected cells (25 MOI for 48 h) were stimulated with 40 μg/ml oxLDL for 30 min at 37 °C. Nuclear extracts were prepared and tested for DNA-binding activity of NF-κB and AP-1. A representative result from three separate experiments was shown. The summarized data from three separate experiments are shown in the chart. * $P < 0.05$ compared with control, † $P < 0.05$ compared with oxLDL-treated cells.

catalase overexpression may be mediated via the regulation of JNK and ERK1/2 phosphorylation, as well as AP-1 activity, without altering p38 and NF-κB activity.

LDL is considered to be the main atherogenic class of lipoproteins. An elevated LDL level is one of the most important risk factors for atherosclerosis and cardiovascular morbidity [2,3]. Continuous interaction between plasma LDL and arterial endothelium seems to be the major factor pertinent to the development of atherosclerosis [26]. Overexpressing Cu/Zn SOD or catalase in transgenic mice protects LDL against vascular cell-mediated oxidation and reduces oxLDL-induced apoptosis in SMCs [27]. These data suggest that alteration of the anti-oxidant status in the arterial wall may change the pathogenesis of atherosclerosis. An increase in the activity of anti-oxidant enzymes may reduce the development of atherosclerosis. The present study demonstrates that increase of endogenous catalase in HAECs reduces oxLDL-mediated cell damage. These data add to the reports of other investigators showing that overexpression of catalase through gene therapy is able to protect ECs against oxidative injury.

Our results suggest that oxLDL immediately induced H_2O_2 production. Previous studies have shown that a direct or indirect stimulation by H_2O_2 activated various cellular pathways, including calcium release, tyrosine kinases, MAPKs and NF-κB, as well as a subsequent increase in some gene products, such as Fas, macrophage colony-stimulating factor, and intercellular adhesion molecule-1 [28–30]. SMCs stably transfected with human catalase are not only resistant to the H_2O_2-induced cytotoxicity [7] but also inhibit the angiotensin I-induced H_2O_2 production and hypertrophy through the inhibition of phosphorylation of p38MAPK [31]. These findings suggest that catalase overexpression protects SMCs against the oxidative stress of H_2O_2, whereas the protection mechanism of catalase for the ECs remains unknown. To study the protection mechanism of catalase, ECs and SMCs were incubated with oxLDL in the presence or absence of catalase [32]. However, limitations have been identified with the usage of exogenous catalase in that the contaminated impurities may non-specifically alter oxLDL. Furthermore, the negative charge of catalase may be repelled from the cell surface, thus preventing it from destroying H_2O_2 near the cell. In using adenovirus-mediated gene transfer, a considerably high level of catalase was detected, suggesting an effective gene transfer. We have demonstrated that the overexpressed catalase in HAECs reduced the oxLDL-induced H_2O_2 elevation and cytotoxicity but not the O_2^- production (Fig. 2).

Phosphorylation status of MAPKs plays an important role in signal transduction of environmental and extracellular stimuli to cellular responses [33,34]. Recently, several studies have demonstrated that oxLDL activated several members of the MAPK family including ERK1/2 and JNK, and p38 in cultured ECs [23,35]. However, their activation to the

protection mechanism of catalase remains unclear. The ERK pathway plays a major role in regulating cell growth and differentiation, being highly induced in response to growth factors, cytokines, and phorbol esters [36,37]. It is also activated by some conditions of stress, particularly oxidant injury, and in such circumstances is believed to confer a survival advantage to cells [38,39]. Inhibition of ERK enhanced ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerated reperfusion injury in isolated perfused heart [40]. In contrast, the activation of JNK and p38 is most frequently associated with induction of apoptosis [41,42]. Disruption of JNK in mice contributes to the suppression of apoptotic responses [43]. Our results demonstrate for the first time that catalase overexpression activates ERK1/2 while counteracting the activation of JNK by oxLDL in HAECs. Thus, one of the mechanisms by which catalase overexpression reduces oxLDL-induced apoptosis involves the reduction of JNK and the increase of ERK1/2 activation.

The mechanism that accounts for the pro-apoptotic action of JNK remains to be elucidated. Recent studies suggest that JNK may contribute to the apoptotic response by regulating the intrinsic cell death pathway involving the mitochondria [44]. The Bcl-2 subfamily (anti-apoptosis) and the Bax subfamily (pro-apoptosis) are associated with the regulation of mitochondria-related apoptosis [45]. Yamamoto et al. [21] suggested that JNK pathway phosphorylates and inactivates Bcl-2. In this study, we found that catalase overexpression decreased the phosphorylation of JNK and increased the level of Bcl-2. It is possible that Bcl-2 expression mediates, in part, the effects of JNK on apoptosis. Moreover, we have demonstrated that oxLDL increases the levels of pro-apoptotic molecules, including Bax and caspase-9, while reducing the anti-apoptotic molecules, such as Bcl-2 and procaspase-3. This altered equilibrium between pro-apoptotic and anti-apoptotic molecules resulted in the occurrence of apoptosis. Our results showed that overexpression of catalase augmented the levels of procaspase-3 and Bcl-2, whereas it did not affect the expression of caspase-9, cytochrome *c*, and Bax. These data imply that Bcl-2 activation is involved in the protection effect of catalase overexpression against oxLDL-induced apoptosis. It may be consistent with the point that Bcl-2 inhibited apoptosis directly at caspase-3 [46]. In addition, the protection of catalase in oxLDL-induced apoptosis may also be mediated through ER pathway, in turn, through downregulating caspase-12 and then inactivating caspase-3, but not including caspase-9 and cytochrome *c* [47]. Further investigation is needed to define the detail regulation of the special apoptotic state more clearly.

Several transcriptional factors have been shown to be phosphorylated and subsequently activated by MAPKs. JNK phosphorylates members of the AP-1 transcription factors (c-Jun, JunB, and JunD) and the AP-1-related transcriptional factor, ATF2 [48], resulting in an increase in their transcription activities. Indeed, it has been established that JNK regulates AP-1 transcription activity in vivo and it is likely that increased AP-1 activity mediates, in part, the effects of the JNK signaling pathway [48]. It is thought that certain nuclear oncoproteins, such as c-Fos, c-Jun, (components of AP-1), as well as NF-κB, which contains transcriptional regulatory activity play important roles in apoptosis [49]. oxLDL appears to be a stimulator of NF-κB and AP-1 that are thought to play a predominant role in EC activation [50,51]. oxLDL provokes a sustained elevation of c-Jun expression, AP-1 DNA binding, and transactivation activity in ECs. Moreover, the LDL induction of AP-1 is preceded by a rapid activator for c-Jun [52]. In the present study, pretreatment with catalase overexpression suppressed the AP-1-binding activity augmented by oxLDL, whereas NF-κB activity was not changed. These findings raise the possibility that catalase overexpression may reduce cell apoptosis through the reduction of AP-1 activity.

In summary, we have shown that overexpression of catalase protects HAECs against oxLDL damage, implying that H_2O_2 are involved in oxLDL-induced apoptosis. The anti-apoptosis effect of catalase may be mediated through increase of Bcl-2, activation of ERK1/2, and inactivation of JNK and AP-1. These results suggest that anti-oxidant enzymes are potentially valuable models for studying the role of oxidative stress in atherosclerosis and catalase gene transfer has potential for vasoprotection against oxLDL damage.

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References

[1] Halliwell B. Oxidants and human disease: some new concepts. FASEB J 1987;1:358–64.

[2] Khan BV, Parthasarathy SS, Alexander RW, Medford RM. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J Clin Invest 1995;95:1262–70.

[3] Fuller CJ, Jialal I. Effects of antioxidants and fatty acids on low-density-lipoprotein oxidation. Am J Clin Nutr 1994;60:1010S–3S.

[4] Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radic Res 1996;25:57–74.

[5] Shingu M, Yoshioka K, Nobunaga M, Yoshida K. Human vascular smooth muscle cells and endothelial cells lack catalase activity and are susceptible to hydrogen peroxide. Inflammation 1985;9:309–20.

[6] Erzurum SC, Lemarchand P, Rosenfeld MA, Yoo JH, Crystal RG. Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA. Nucleic Acid Res 1993;21:1607–12.

[7] Santanam N, Auge N, Zhou M, Keshava C, Parthasarathy S. Overexpression of human catalase gene decreases oxidized lipid-induced cytotoxicity in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 1999;19:1912–7.

[8] Choy JC, Granville DJ, Hunt DW, Mcmanus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. J Mol Cell Cardiol 2001;33:1673–90.

[9] Li D, Yang B, Mehta JL. Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas. Am J Physiol 1998;275:H568–76.

[10] Kita T, Kume N, Minami M, Hayashida K, Murayama T, Sano H, et al. Role of oxidized LDL in atherosclerosis. Ann NY Acad Sci 2001;947: 199–205.

[11] Irani K. Oxidant signaling in vascular cell growth, death, and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 2000;87: 179–83.

[12] Maziere C, Conte MA, Maziere JC. Activation of JAK2 by the oxidative stress generated with oxidized low-density lipoprotein. Free Radic Biol Med 2001;31:1334–40.

[13] Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. Circulation 2000;101:2889–95.

[14] Napoli C, Quehenberger O, De Nigris F, Abete P, Glass CK, Palinski W. Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. FASEB J 2000;14:1996–2007.

[15] Chen YH, Lin SJ, Chen JW, Ku HH, Chen YL. Magnolol attenuates VCAM-1 expression in vitro in TNF-alpha-treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. Brit J Pharmacol 2002;135:37–47.

[16] Lin SJ, Yen HT, Chen YH, Ku HH, Lin FY, Chen YL. Expression of interleukin-1beta and interleukin-1 receptor antagonist in oxLDL-treated human aortic smooth muscle cells and in the neointima of cholesterol-fed endothelia-denuded rabbits. J Cell Biochem 2003;88: 836–47.

[17] Shyue SK, Tsai MJ, Liou JY, Willerson JT, Wu KK. Selective augmentation of prostacyclin production by combined prostacyclin synthase and cyclooxygenase-1 gene transfer. Circulation 2001;103:2090–5.

[18] Chen YH, Lin SJ, Ku HH, Shiao MS, Lin FY, Chen JW, et al. Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF-alpha-treated human aortic endothelial cells. J Cell Biochem 2001;82:512–21.

[19] Luck H. Catalase. In: Bergmeyer H, editor. Methods of enzymatic analysis. New York: Academic Press; 1965.

[20] Brown MR, Miller Jr FJ, Li WG, Ellingson AN, Mozena JD, Chatterjee P, et al. Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells. Circ Res 1999;85:524–33.

[21] Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol 1999;19:8469–78.

[22] Abu-Qare AW, Abou-Donia MB. Biomarkers of apoptosis: release of cytochrome *c*, activation of caspase-3, induction of 8-hydroxy-2-deoxyguanosine, increased 3-nitrotyrosine, and alteration of p53 gene. J Toxicol Environ Health B Crit Rev 2001;4:313–32.

[23] Zhang X, Hu K, Li CY. Protection against oxidized low-density lipoprotein-induced vascular endothelial cell death by integrin-linked kinase. Circulation 2001;104:2762–6.

[24] Zhu Y, Liao HL, Lin JH, Verna L, Stemerman MB. Low-density lipoprotein augments interleukin-1-induced vascular adhesion molecule expression in human endothelial cells. Atherosclerosis 1999; 144:357–65.

[25] Xu XP, Meisel SR, Ong JM, Kaul S, Cercek B, Rajavashisth TB, et al. Oxidized low-density lipoprotein regulates matrix metalloproteinase-9 and its tissue inhibitor in human monocyte-derived macrophages. Circulation 1999;99:993–8.

[26] Diaz MN, Frei B, Vita JA, Keaney Jr JF. Antioxidants and atherosclerotic heart disease. New Engl J Med 1997;337:408–16.

[27] Guo Z, Van Remmen H, Yang H, Chen X, Mele J, Vijg J, et al. Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxidized LDL-induced apoptosis in mouse aortic cells. Arterioscler Thromb Vasc Biol 2001;21:1131–8.

[28] Favero TG, Zable AC, Abramson JJ. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. J Biol Chem 1995;270:25557–63.

[29] Sabri A, Byron KL, Samarel AM, Bell J, Lucchesi PA. Hydrogen peroxide activates mitogen-activated protein kinases and Na^{+}-H^{+} exchange in neonatal rat cardiac myocytes. Circ Res 1998;82:1053–62.

[30] Lo SK, Janakidevi K, Lai L, Malik AB. Hydrogen peroxide-induced increase in endothelial adhesiveness is dependent on ICAM-1 activation. Am J Physiol 1993;264:L406–12.

[31] Ushio-Fukai M, Alexander RW, Akers M, Griendling KK. p38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J Biol Chem 1998;273: 15022–9.

[32] Steinbrecher UP. Role of superoxide in endothelial-cell modification of low-density lipoproteins. Biochim Biophys Acta 1988;959:20–30.

[33] Hagemann C, Blank JL. The ups and downs of MEK kinase interactions. Cell Signal 2001;13:863–75.

[34] Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 2002;64:765–70.

[35] Go YM, Levonen AL, Moellering D, Ramachandran A, Patel RP, Jo H, et al. Endothelial NOS-dependent activation of c-Jun NH(2)-terminal kinase by oxidized low-density lipoprotein. Am J Physiol Heart Circ Physiol 2001;281:H2705–13.

[36] Johnson GL, Vaillancourt RR. Sequential protein kinase reactions controlling cell growth and differentiation. Curr Opin Cell Biol 1994; 6:230–8.

[37] He H, Wang X, Gorospe M, Holbrook NJ, Trush MA. Phorbol ester-induced mononuclear cell differentiation is blocked by the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059. Cell Growth Differ 1999;10:307–15.

[38] Stadheim TA, Kucera GL. c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for mitoxantrone- and anisomycin-induced apoptosis in HL-60 cells. Leuk Res 2002;26:55–65.

[39] Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, et al. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J Clin Invest 1997;100:1813–21.

[40] Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, et al. Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res 2000; 86:692–9.

[41] Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J Biol Chem 1996;271:31929–36.
[42] Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 1998;333:291–300.
[43] Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 1999;22:667–76.
[44] Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, et al. The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Mol Cell Biol 2002;22:4929–42.
[45] Gross A, Mcdonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Gene Dev 1999;13:1899–911.
[46] Coffey RN, Watson RW, Fitzpatrick JM. Signaling for the caspases: their role in prostate cell apoptosis. J Urol 2001;165:5–14.
[47] Guégan C, Przedborski S. Programmed cell death in amyotrophic lateral sclerosis. J Clin Invest 2003;111:153–61.
[48] Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239–52.
[49] Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–6.
[50] Draczynska-Lusiak B, Chen YM, Sun AY. Oxidized lipoproteins activate NF-kappaB binding activity and apoptosis in PC12 cells. Neuro Report 1998;9:527–32.
[51] Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. Arterioscler Thromb Vasc Biol 2000;20:1116–22.
[52] Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 1987;238:1386–92.