Superoxide dismutase and catalase inhibit oxidized low-density lipoprotein-induced human aortic smooth muscle cell proliferation: Role of cell-cycle regulation, mitogen-activated protein kinases, and transcription factors

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Received 26 August 2005; received in revised form 13 February 2006; accepted 20 February 2006
Available online 5 April 2006

Abstract

Several antioxidant enzymes, including copper, zinc-superoxide dismutase (Cu, Zn-SOD) and catalase, have been suggested to be protective against the proliferation of vascular smooth muscle cells exposed to oxidative stress. In the present study, we investigated effects of Cu, Zn-SOD and/or catalase on oxLDL-induced proliferation of, and intracellular signaling in, human aortic smooth muscle cells (HASMCs). HASMCs were transfected with adenovirus carrying the human Cu, Zn-SOD gene and/or the human catalase gene. This resulted in a high level of Cu, Zn-SOD and/or catalase overexpression and decreased oxLDL-induced proliferation. Cu, Zn-SOD and/or catalase also arrested cell cycle progression, which was associated with decreased expression of cyclin D1, cyclin E, CDK2, and CDK4 and upregulation of p21Cip1 and p27Kip1. Phosphorylation studies on ERK1/2, JNK, and p38, three major subgroups of mitogen activator protein kinases, demonstrated that Cu, Zn-SOD and/or catalase overexpression suppressed ERK1/2 and JNK phosphorylation. Gel-mobility shift analysis showed that oxLDL caused an increase in the DNA binding activity of activator protein-1 (AP-1) and nuclear factor κB (NF-κB), which was inhibited by Cu, Zn-SOD and/or catalase overexpression. These results provide the first evidence that overexpression of Cu, Zn-SOD and/or catalase in HASMCs ameliorates the cell proliferation caused by oxLDL stimulation and that this inhibitory effect is mediated via downregulation of ERK1/2 and JNK phosphorylation and AP-1 and NF-κB inactivation. These observations support the feasibility of the increase of Cu, Zn-SOD and/or catalase expression in human smooth muscle cells as a means of protection against oxidant injury.

Keywords: Antioxidant enzymes; Smooth muscle cells; oxLDL; Cell cycle; Proliferation

1. Introduction

Proliferation of vascular smooth muscle cells (VSMCs) is a prominent feature in the pathophysiology of hypertension and atherosclerosis and in restenosis after angioplasty and stent placement [1]. Recently, it was reported that reactive oxygen species, such as superoxide anions (O2•–) and
hydrogen peroxide (H$_2$O$_2$), can stimulate proliferation of VSMCs [2]. Most of these effects can be prevented by addition of extracellular antioxidants [3]. The results of a study based on antioxidant enzyme gene expression to examine the effects of oxidants on cells corroborated 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 of antioxidant enzyme activity and therefore are more susceptible to damage by H$_2$O$_2$. In addition, transient overexpression of catalase has been found to confer protection against H$_2$O$_2$-mediated oxidative stress in human umbilical vein endothelial cells and against oxLDL-induced apoptosis in human aortic endothelial cells [6,7]. Moreover, overexpression of the human catalase gene has been shown to decrease oxidized lipid-induced proliferation of VSMCs [8]. These results suggest that antioxidant enzymes protect cells against oxidative injury. Several studies have overexpressed antioxidant enzymes in various cell types and examined their effects in preventing oxidant-induced abnormality [7–10]. However, the effects and the mechanism of action of antioxidant enzymes on the proliferation of human arterial smooth muscle cells (HASMCs) exposed to oxidative stress still remain unclear.

Vascular lesions form during several pathological processes involving the accumulation of oxidized low-density lipoprotein (oxLDL) in the vascular wall [1]. OxLDL exerts various biological effects, such as chemotaxis, cell proliferation, or cytotoxicity, on SMCs that are potentially involved in the development of atherosclerosis [11]. It is thought that the effects of oxLDL on SMC proliferation requires its binding to specific cellular receptors to activate and stimulate a wide spectrum of host responsive systems [12]. 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, and mitogen-activated protein kinases (MAPKs) [12]. These molecules may have converging or diverging effects and often show “cross-talk” properties, which results in a complicated signaling network in which they can mutually affect each other’s action. Subsequently, the signals are transduced to downstream pathways and activate numerous transcriptional factors, including AP-1, NF-κB, and ATF-2, triggering the expression of many genes and ultimately leading to biological changes, such as cell proliferation [13]. Currently, the effects of oxLDL and antioxidant enzyme overexpression on cell cycle-related proteins and the signaling pathways associated with proliferation of HASMCs are poorly understood. In this study, adenovirus-mediated gene transfer was used to overexpress Cu, Zn-SOD and/or catalase in HASMCs in order to avoid concerns regarding enzyme purity or fluctuations in Cu, Zn-SOD or catalase protein delivery. Human SODs consist of three isozymes, namely cytosolic or copper-zinc SOD (Cu, Zn-SOD), manganese SOD (Mn-SOD) localized in mitochondria, and extracellular form of Cu, Zn-SOD (EC-SOD) [14]. Cu, Zn-SOD is the predominant isoform of SOD and has been proposed to play a key role in atherosclerosis [9]. The purpose of this research is to investigate whether the effects of Cu, Zn-SOD and/or catalase overexpression on cell cycle-related proteins, MAPKs, and transcriptional factors pathways are associated with proliferation of oxLDL-treated HASMCs. Our results show that HASMCs overexpressing Cu, Zn-SOD and/or catalase are resistant to the proliferative effects of oxLDL. A number of cell cycle regulatory proteins, such as cyclin D1, cyclin E, CDK2, CDK4, p21$^{Cip1}$, and p27$^{Kip1}$, were identified as downstream targets in the growth-inhibition activity of antioxidant enzymes. The JNK/ERK MAPKs and AP-1/NF-κB pathway is the major signal pathway leading to the antiproliferative effects of antioxidant enzyme overexpression.

2. Materials and methods

2.1. HASMC cultures

HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR, USA), were grown in culture flasks in smooth muscle cell growth medium (M231,Cascade Biologics Inc.) supplemented with 5% smooth muscle growth supplement (SMGS), penicillin (100 units/mL), streptomycin (100 pg/mL), and Fungizone (1.25 μg/mL) at 37°C in a humidified 5% CO$_2$ atmosphere. The growth medium was changed every other day until confluence. Cells were used between passages 3 and 8. All cells were synchronized in serum-free media for 24 h prior to experimentation.

2.2. LDL preparation and oxidation

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 for 24 h at 4°C against phosphate-buffered saline (PBS; 5 mM phosphate buffer, 125 mM NaCl, pH 7.4). This native LDL was oxidized by dialysis for 24 h at 37°C against 10 μM CuSO$_4$ in PBS according to a published protocol [7,15], then the oxidized LDL (oxLDL) was dialyzed for 24 h at 4°C against PBS containing 0.3 mM EDTA. The extent of oxidation was monitored by measuring thiobarbituric acid-reactive substances (TBARS) and by agarose gel electrophoresis. The level of LDL oxidation was $8.8\pm1.6$ nM of TBARS per milligram of protein in the present study. The protein content of oxLDL was determined using the Bio-Rad protein assay (Bio-Rad, München, Germany).

2.3. Preparation of recombinant adenoviruses

A human Cu, Zn-SOD or catalase cDNA containing the entire coding sequence was subcloned into the adenovirus shuttle plasmid vector, pAdPGK, which contains a promoter
of human phosphoglycerate kinase and a polyadenylation signal of bovine growth hormone. A recombinant adenovirus was generated by homologous recombination and amplified in human embryonic kidney 293 cells for large-scale viral production; viral titers were determined as described previously [7]. Purified virus was stored in 10 mM Tris–HCl, pH 7.4, 1 mM MgCl₂, and 10% (v/v) glycerol at −80 °C until used for experiments. For infection, ∼3 × 10⁵ cells were seeded on a 100 mm Petri dish and treated for 48 h with the selected adenovirus(es) at the indicated multiplicity of infection (MOI), then the culture medium was replaced with fresh medium.

2.4. MTT assay of cell viability

As an index of cell viability, mitochondrial dehydrogenate activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described by Lin et al. [16]. The absorbance after oxLDL treatment, normalized to that for cells incubated in control medium, was used as a measure of cell viability, control cells being considered 100% viable.

2.5. Detection of O₂•− and H₂O₂ production

The effect of SOD and/or catalase overexpression on O₂•− and H₂O₂ production in HASMCs was determined by a fluorometric assay using dihydroethidium (DHE, Sigma) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) as the probe, respectively [8, 17]. Confluent HASMCs (10⁴ cells/well) in 48-well plates were transfected with AdSOD, AdCat, or AdCO at 50 MOI for 48 h. Cells were washed with HBSS, and incubated in HBSS containing 100 μM DHE or 20 μM DCFH-DA at 37 °C for 45 min. The fluorescence intensity (relative fluorescence units) was detected at 485 nm/590 nm and 485 nm/530 nm for excitation/emission for corresponding ethidium to O₂•− and H₂O₂, respectively, using a Fluorescence Microplate Reader. To determine the effect of oxLDL on O₂•− and H₂O₂ generation, fluorescence intensity was also measured immediately after oxLDL (20 μg protein/mL) addition at the different intervals for 60 min.

2.6. BrdU incorporation in HASMCs

HASMCs were cultured in 96-well plates at a concentration of 10⁴ cells/well. To monitor DNA synthesis, BrdU labeling was performed using a kit from Roche (5-bromo-2′-deoxy-uridine labeling and detection kit III, Roche, Basel, Switzerland). The labeling reagent was added to the medium at a final concentration of 100 μM and incubation was continued for 3 h. The culture medium was removed and each well filled with 100 μL of fixative solution for 30 min at −20 °C, then the fixative was removed and each well washed 3 times with 100 μL of Hank’s balanced salt solution (HBSS) before 100 μL of anti-BrdU-POD Fab fragments was added to each well for 30 min at 37 °C. The anti-BrdU-POD solution was removed and each well rinsed three times with 100 μL of HBSS, then 100 μL of peroxidase substrate (tetramethyl benzidine, TMB) was added to each well for 30 min at room temperature. The absorption of the samples was then measured at 450 nm/690 nm by spectrophotometry (Dynatech MR5000). All results are expressed as a percentage of the control value.

2.7. Cell cycle analysis

Analysis of the DNA content and the movement of the cells through the mitotic cycle were performed by flow cytometry 24 h after cell stimulation. HASMCs were harvested, fixed in 70% ethanol, and stored in a −20 °C freezer for 30 min, then were washed twice with ice-cold PBS and incubated with 10 μL of RNAase (20 μg/mL, Sigma, MO, USA) and 50 μL of propidium iodide (20 μg/mL, Sigma). Cell cycle phase analysis was performed by flow cytometry using a FACScan (Becton Dickinson, NJ, USA), and the percentage of cells in different phases of the cell cycle analyzed using ModFitLT software (BD).

2.8. Western blot analysis

Western blot analyses for Cu, Zn-SOD and catalase were performed as described previously [16]. Briefly, samples of cell lysate (20 μg of protein) were subjected to 12% SDS-PAGE and transferred to PVDF membranes, which were then treated with 3% nonfat milk in 0.1 M phosphate buffer for 1 h at room temperature to block nonspecific binding of antibody. Membranes were incubated with rabbit polyclonal antibody against catalase or Cu/Zn SOD (1:1000, Oxis Research, OR, USA), then with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:3000, Sigma), bound antibodies being detected using Chemiluminescence Reagent Plus (NEN, MA, USA). The intensity of each band was quantified using a densitometer. Anti β-actin antibodies (1:1000, Oncogen, CA, USA) were used to quantify used β-actin as an internal control. In other studies, antibodies employed include rabbit against human phospho-JNK, rabbit against human phospho-p38, rabbit against human total JNK, rabbit against human total ERK1/2, mouse against human phospho-ERK1/2, goat against human total p38 (1:1000, Cell Signaling, MA, USA), rabbit anti-human cyclin D1 (Santa Cruz, CA, USA), mouse anti-human cyclin E (Upstate, MA, USA), rabbit anti-human CDK2 (Santa Cruz), mouse anti-human CDK4 (BD), rabbit anti-human p21cip1 (Santa Cruz), or rabbit anti-human p27kip1 (Santa Cruz).

2.9. Electrophoretic mobility shift assay (EMSA)

The preparation of nuclear extracts and the conditions for EMSA reactions have been described previously [7]. Nuclear extracts (5 μg of protein) were incubated with [γ-³²P]ATP-labeled oligonucleotide probes and resolved on a 6% native
polyacrylamide gel, which was dehydrated, then exposed to X-ray film for 3 h. The 22-mer synthetic double-stranded oligonucleotides used as the NF-κB and AP-1 probes in the gel shift assay were, respectively (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; 3'-TCA ACT CCC CTG AAA GGG TCC G-5') and (5'-ATT CGA TCG GGG CGG GGC GAG C-3'; 3'-TAA GCT AGC CCC GCC CCG CTG G-5').

2.10. Statistical analysis

All data were expressed as mean ± S.E.M.. The difference in mean values among different groups was analyzed by one-way ANOVA and subsequent post hoc Dunnett test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Overexpression of Cu, Zn-SOD and/or catalase reduces the proliferation of oxLDL-treated HASMCs

HASMCs were incubated for 24 h with 10–100 μg protein/mL of oxLDL, then an MTT assay was performed. Low concentrations (10 or 20 μg protein/mL) of oxLDL increased cell viability (125.1 ± 4.1% and 138.2 ± 3.1%, $n = 3$, $P<0.05$, respectively, compared to control cells), while marked cell death was seen at oxLDL concentrations higher than 60 μg protein/mL (57.0 ± 6.4% for 60 μg protein/mL of oxLDL compared to control cells, $n = 3$, $P<0.05$). This result is similar to that in a previous report [18] showing that oxLDL has a dual effect on the cell cycle in endothelial cells, inducing proliferation at low concentrations and apoptosis at higher concentrations. HASMCs incubated with 20 μg protein/mL of oxLDL showed a significant 23% increase in BrdU uptake over a 24 h period compared to controls (Fig. 1). To determine whether overexpression of Cu, Zn-SOD and/or catalase affected oxLDL-induced cell proliferation, cells were transfected with AdSOD or AdCat, both together (AdCO), or the control AdPGK. Transfection with AdSOD or AdCat greatly increased the amount and activity of Cu, Zn-SOD or catalase in HASMCs detected by immunofluorescent staining, Western blot, and enzyme activity assay (data not shown). The expression of Cu, Zn-SOD and catalase in HASMCs cotransfected with the Cu, Zn-SOD and catalase genes (50 MOI of each) was similar to that in cells transfected with only Cu, Zn-SOD or catalase (data not shown). As shown in Fig. 1, the oxLDL-induced proliferation of HASMCs was almost completely suppressed following transfection with AdSOD or AdCat, the level of BrdU incorporation being similar to that in control cells (97 ± 1% and 95 ± 1%, respectively). In addition, transfection with both AdSOD and AdCat gave a more significant reduction in BrdU incorporation (91 ± 1%). Infection with AdSOD, AdCat, AdCO, or AdPGK in the absence of oxLDL treatment did not alter cell viability (data not shown) or cell proliferation (Fig. 1).

3.2. Effects of Cu, Zn-SOD and/or catalase overexpression on $O_2^{•⁻}$ and $H_2O_2$ production in oxLDL-treated HASMCs

To determine whether SOD and/or catalase overexpression affected $O_2^{•⁻}$ and $H_2O_2$ production in oxLDL-treated HASMCs, cells were transfected for 48 h with AdSOD, AdCat, AdCO, or the control vector, AdPGK, then incubated with or without 20 μg protein/mL of oxLDL for 24 h at 37°C. BrdU uptake was then measured as described in Section 2. The results are as expressed as a percentage of that in non-transfected untreated controls. The data are the mean ± S.E.M. ($n = 3$). $P<0.05$ compared to untreated non-transfected controls, $P<0.05$ compared to oxLDL-treated non-transfected or AdPGK-transfected cells, $P<0.05$ compared to oxLDL-treated AdSOD-transfected cells, $P<0.05$ compared to oxLDL-treated AdCat-transfected cells.

3.3. Overexpression of Cu, Zn-SOD and/or catalase arrests cell cycle progression in oxLDL-treated HASMCs

Flow cytometric analysis was then used to determine whether the antioxidant enzyme-induced cell growth inhibition was due to arrest at a specific point of the cell cycle. As shown in Table 1, flow cytometric analysis of the DNA
Fig. 2. Effect of AdSOD and/or AdCat infection on O$_2^•−$ and H$_2$O$_2$ production in HASMCs with or without oxLDL stimulation. HASMCs were infected with AdSOD and/or AdCat at 50 MOI for 48 h. DHE and DCFH-DA were then added for O$_2^•−$ and H$_2$O$_2$ detection, respectively. After incubation for 45 min at 37 °C, ethidium (A) and DCFH (C) fluorescence were measured. O$_2^•−$ (B) and H$_2$O$_2$ (D) production in oxLDL stimulated HASMCs infected with control virus, AdPGK, AdSOD, AdCat, or AdCO at 50 MOI for 48 h were measured at the intervals of 3, 10, 20, 30, and 60 min. Control, cells without treatment. Data represents mean ± S.E.M. of three independent experiments performed in triplicate. *P < 0.05 compared with the cells without Ad infection (A and C) or compared with the oxLDL-treated cells without Ad infection (B and D).

content in HASMCs showed that oxLDL treatment alone caused a significant increase in the percentage of cells in S phase and G2/M phase (from 3.6 ± 0.4% to 11.4 ± 1.0% and from 5.0 ± 0.9% to 10.3 ± 0.6%, respectively) and a significant decrease in the G0/G1 populations (from 91.3 ± 1.2% to 78.3 ± 0.7%) relative to control cultures. In oxLDL-treated AdSOD-transfected cells, the percentage of cells in S phase was significantly reduced to 5.6 ± 0.2% compared to the 11.4 ± 1.0% and 10.1 ± 0.3% seen, respectively, in oxLDL-treated control cells or oxLDL-treated AdPGK-transfected cells, while the G2/M population was significantly reduced to 8.2 ± 0.3% from 10.3 ± 0.6% or 10.4 ± 1.1%; this was accompanied by a significant accumulation of cells in G0/G1 phase. Similar results were obtained after AdCat or AdCO transfection. These data show that Cu, Zn-SOD and/or catalase inhibits DNA synthesis in oxLDL-treated HASMCs via inhibition of the cell transition from the G0/G1 to the S phase of the cell cycle.

3.4. Cu, Zn-SOD- and/or catalase overexpression-induced cell-cycle arrest is associated with decreased protein expression of G1 cyclins and CDKs and with upregulation of p21$^{Ckip1}$ and p27$^{Kip1}$ in oxLDL-treated HASMCs

Progression through the cell cycle from G0/G1 phase requires the sequential activation of cyclin D1/CDK4 and cyclin E/CDK2 activity [19]. Since the above data showed that Cu, Zn-SOD and/or catalase expression induces cell-cycle arrest at G0/G1, we therefore investigated the mechanism involved. To achieve this, we transfected cells for 48 h with AdCat, or AdCO before oxLDL treatment, then analyzed the expression of different components of the cyclin-dependent complexes that are important in regulating cell cycle progression. As shown in Fig.3A–D, oxLDL alone caused a marked increase in expression of cyclin D1, CDK4, cyclin E, and CDK2, while the expression of all
four was significantly reduced in oxLDL-treated cells overexpressing Cu, Zn-SOD and/or catalase. We also assessed the effect of Cu, Zn-SOD and/or catalase on expression of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, two cyclin-dependent kinase inhibitors (CDKIs) which are known to regulate the entry of the cells into S phase [20], and found that expression of one or both enzymes resulted in a significant induction of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> compared to oxLDL treatment alone (Fig. 3E and F).

3.5. Involvement of ERK1/2 and JNK in the effects of Cu, Zn-SOD and/or catalase on oxLDL-stimulated HASMCs

Since cell proliferation during exposure to various stimuli is linked to activation of MAPKs [21], we therefore determined whether oxLDL-stimulated cell proliferation in HASMCs was associated with MAPK activation. As shown in Fig. 4, phosphorylation of ERK, p38, and JNK was significantly increased 5 min after addition of 20 µg protein/mL of oxLDL. Interestingly, transfection with Cu, Zn-SOD and/or catalase completely abolished the oxLDL-induced phosphorylation of ERK and JNK, but had no effect on p38 phosphorylation. To determine whether the anti-proliferative effect of the antioxidant enzymes was mediated through the inactivation of the MAPK pathway, the effect of antioxidant enzymes on DNA synthesis was examined after treatment of HASMCs for 1 h with 30 µM PD98059 (an ERK inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor). As shown in Fig. 5, PD98059 or SP600125 significantly inhibited oxLDL-induced BrdU incorporation by control cells, while SB203580 did not. PD98059 had a significantly greater effect on cells transfected with AdSOD, AdCat, or AdCO than on cells treated with the control vector, AdPGK. Moreover, AdCat and AdCO also displayed an additive effect on BrdU incorporation when compared with SB203580 treatment alone. In contrast, AdSOD, AdCat, or AdCO transfection had no additional effect to SP600125 alone on oxLDL-induced proliferation.

![Fig. 3. Western blot analysis showing that overexpression of Cu, Zn-SOD and/or catalase alters the expression of cell cycle regulatory proteins in HASMCs. Cells were either not transfected or were transfected for 48 h with 50 MOI of AdSOD, AdCat, or AdCO, then were incubated with or without 20 µg protein/mL of oxLDL for 24 h at 37 °C. Equal amounts of cell lysate protein were subjected to SDS-PAGE followed by Western blot analysis using antibodies against cyclin D1 (A), CDK4 (B), cyclin E (C), CDK2 (D), p21<sup>Cip1</sup> (E), or p27<sup>Kip1</sup> (F) as described in Section 2. β-Actin was processed in parallel as an internal control for protein loading and the staining for the test protein band normalized to that of the β-actin band. The results are shown as the fold increase in expression relative to that in untreated nontransfected controls. The data are the mean ± S.E.M. (n = 3). * P < 0.05 compared to untreated controls, † P < 0.05 compared to oxLDL-treated cells.](image-url)
Table 1
Effect of overexpression of Cu, Zn-SOD and/or catalase on the cell cycle distribution

<table>
<thead>
<tr>
<th></th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.3 ± 1.2</td>
<td>3.6 ± 0.4</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>AdPGK</td>
<td>88.7 ± 0.6</td>
<td>5.8 ± 0.7</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>AdCat</td>
<td>88.7 ± 1.1</td>
<td>5.2 ± 0.5</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>AdSOD</td>
<td>90.6 ± 1.3</td>
<td>4.5 ± 0.7</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>AdCO</td>
<td>89.6 ± 0.5</td>
<td>4.6 ± 0.2</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>oxLDL</td>
<td>78.3 ± 0.7</td>
<td>11.4 ± 1.0</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td>AdPGK + oxLDL</td>
<td>79.5 ± 1.1</td>
<td>10.1 ± 0.3</td>
<td>10.4 ± 1.1</td>
</tr>
<tr>
<td>AdSOD + oxLDL</td>
<td>86.2 ± 0.3</td>
<td>5.6 ± 0.2</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>AdCat + oxLDL</td>
<td>86.8 ± 0.6</td>
<td>6.0 ± 0.2</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>AdCO + oxLDL</td>
<td>85.7 ± 1.6</td>
<td>7.2 ± 0.9</td>
<td>7.1 ± 0.8</td>
</tr>
</tbody>
</table>

Cells were not transfected or were transfected for 48 h with 50 MOI of AdSOD, AdCat, AdCO, or AdPGK, then were incubated with or without 20 μg protein/mL of oxLDL for 24 h at 37 °C. The cells were harvested using trypsin, fixed with ethanol, and treated with RNase and EDTA, then DNA was stained using propidium iodide and the percentage of cells in each phase was determined by FACS profile analyzer. The data are the mean ± S.E.M. (n = 3).

*P < 0.05 compared to the untreated non-transfected control.
†P < 0.05 compared to oxLDL-treated non-transfected or AdPGK-transfected cells.
3.6. Effect of Cu, Zn-SOD and/or catalase overexpression on NF-κB and AP-1 activity in oxLDL-treated HASMCs

Since transcriptional regulation involving NF-κB or AP-1 activation has been implicated in the proliferation of HASMCs [22], EMSA was performed to determine whether oxLDL induced NF-κB or AP-1 activation. As shown in Fig. 6A, low levels of basal NF-κB binding activity were detected in unstimulated control serum-starved cells, and treatment with oxLDL (20 μg protein/mL for 15 min) resulted in a 3.3-fold increase in NF-κB binding activity; this was specific for NF-κB, as it was undetectable in the presence of a 100-fold excess of unlabeled NF-κB oligonucleotide (data not shown). Fig. 6B shows that serum-starved HASMCs contained low levels of active AP-1, which were increased 2.6-fold by oxLDL stimulation. When the effect of Cu, Zn-SOD and/or catalase on the activation of NF-κB and AP-1 in oxLDL-treated HASMCs was examined in HASMCs transfected with AdSOD, AdCat, or AdCO and stimulated with 20 μg protein/mL oxLDL for 15 min, the NF-κB binding activity was reduced to 43%, 23%, and 20% of the levels in control cells treated with oxLDL and the AP-1 binding activity to 60%, 31%, and 18%.

4. Discussion

The present study showed that overexpression of Cu, Zn-SOD and/or catalase attenuated the oxLDL-induced proliferation of HASMCs. Overexpression of Cu, Zn-SOD and/or catalase induced the arrest of cell cycle progression, down-regulation of expression of cyclin D1, cyclin E, CDK2, and CDK4, and upregulation of expression of the CDK inhibitors, p21<sup>Cip1</sup> and p27<sup>Kip1</sup>. Our data also demonstrated, for the first time, that the antiproliferative effect of overexpression of antioxidant enzymes was mediated via regulation of JNK and ERK1/2 phosphorylation and AP-1 and NF-κB activity, without altering p38 activity.

Elevated LDL levels is one of the most important risk factors for atherosclerosis and cardiovascular morbidity [1]. oxLDL deposition in vascular walls contributes to the proliferation and migration of VSMCs, the expression of adhesion molecules in endothelial cells, and impairment of endothelium-dependent vasorelaxation by the induction of oxidative stress. The overexpression of Cu, Zn-SOD or catalase in transgenic mice protects LDL against vascular cell-mediated oxidation and reduces oxLDL-induced apoptosis in SMCs [10]. Low concentrations of oxLDL (5–10 μg/mL) induce proliferation of human umbilical vein endothelial cells and this effect is blocked by co-addition of SOD and catalase [18]. These data suggest that alteration of the antioxidant status in the arterial wall may change the pathogenesis of atherosclerosis, and indicate that oxygen radicals are essential for oxLDL-induced cell proliferation and that an increase in the activity of antioxidant enzymes may therefore reduce
the development of atherosclerosis. Antioxidant enzymes have been shown to have protective actions on the cardiovascular system, including anti-inflammatory, anti-oxidative, and vasodilatory effects [23]. The present study demonstrated that an increase in endogenous Cu, Zn-SOD or catalase in HASMCs led to a reduction in oxLDL-mediated cell proliferation and that a greater effect was seen when the levels of both enzymes were increased. These effects may be due to Cu, Zn-SOD and catalase causing a reduction in levels of superoxide anions and hydrogen peroxide. The proliferation of HASMCs with AdSOD, AdCat, or AdCO was slightly reduced below 100% as compared to controls, because the controls may cause a small amount of proliferation resulted from the low production of ROS. These data add to the reports of other investigators showing that overexpression of Cu, Zn-SOD and/or catalase using gene therapy can protect SMCs against oxidative stimulation by inhibiting proliferation.

Our flow cytometric analysis data showed that the inhibition of HASMC proliferation by overexpression of Cu, Zn-SOD and/or catalase was mediated through G0/G1 cell-cycle arrest. This is consistent with the results of another study showing that increased glutathione peroxidase expression induces G0/G1 cell-cycle arrest in human breast cancer cells [24] and is closely correlated with decreased cell proliferation [25]. Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle regulatory proteins (including cyclins, CDKs, and CDKIs) provides an important mechanism for inhibition of growth [19,20]. Here, we showed that, in the presence of antioxidant enzymes, downregulation of cyclin D, cyclin E, CDK4, and CDK2 expression occurred in parallel with a decrease in DNA synthesis and cell growth inhibition and decreased entry into S phase. These observations suggest that the antiproliferative activity of antioxidant enzymes in HASMCs involves a multifaceted attack on multiple target molecules critically involved in growth inhibition.

The activity of the cyclin/CDK complex is affected by the action of specific CDKIs [20]. The CDKIs, p21Cip1 and p27Kip1, can bind tightly to, and inhibit the kinase activity of, several cyclin-CDK complexes, such as cyclin D-CDK4 and cyclin E-CDK2, and arrest cell growth at the G1 and G1/S boundary, and thus function in growth regulation [20]. Another possible explanation for the altered cyclin-CDK activity and G1 phase-arrested HASMCs after antioxidant enzyme treatment could therefore be the upregulation of p21Cip1 or p27Kip1 protein expression, and our data did indeed show significant upregulation of p21Cip1 and p27Kip1 during G1-phase arrest of oxLDL-treated HASMCs transfected with AdSOD, AdCat, or AdCO. Taken together, these data suggest that the G0/G1 arrest of HASMCs following antioxidant enzyme treatment is mainly due to the loss of CDK kinase components, including cyclin D, cyclin E, CDK4, and CDK2, and the inhibitory action of p21Cip1 and p27Kip1 on the CDK complexes. EUK-134, a SOD/catalase mimetic, attenuates serum-induced fetal pulmonary arterial SMC proliferation by an increase in p21 levels [26]. Although some studies have shown G0/G1 phase arrest due to antioxidant enzymes [24,26], to our knowledge, this is the first system-
atic study showing the involvement of each component of the CDKI-cyclin-CDK machinery during the cell-cycle arrest of VSMCs induced by antioxidant enzymes.

The phosphorylation status of MAPKs plays important roles during the proliferation and cell cycle transition triggered by various stimuli [21]. Recently, several studies have demonstrated that oxLDL activates a specific MAPK subgroup, i.e. ERK, in cultured smooth muscle cells and macrophages and that ERK activation is frequently associated with cell proliferation and differentiation [21]; however, the effect on other types of MAPKs (JNK and p38) needs to be elucidated. In addition, the effects of Cu, Zn-SOD and/or catalase expression on the phosphorylation of MAPKs in oxLDL-treated HASMCs associated with cell proliferation remains unclear. In the present study, we showed that oxLDL at a concentration of 20 μg protein/mL caused rapid and significant activation of the MAPKs members, ERK1/2, JNK, and p38 MAPK. Two of the commonly used blockers of the MAPK pathway that we tested (PD98059 and SP600125) reduced cell proliferation to control levels and negated the proliferative effects of oxLDL.

In contrast, it is unlikely that p38 phosphorylation is involved in oxLDL-induced proliferation, since SB203580, a specific p38 MAPK inhibitor, failed to inhibit the oxLDL-induced increase in BrdU incorporation under our experimental conditions. Our results also demonstrated that overexpression of Cu, Zn-SOD and/or catalase significantly decreased the oxLDL-induced phosphorylation of ERK1/2 and JNK in HASMCs and that the role of JNK appeared to be greater than that of ERK in inhibition of oxLDL-induced cell proliferation. Thus, it is reasonable that antioxidant enzyme treatment, which inhibits the oxLDL-induced phosphorylation of the ERK1/2 and JNK pathways, results in inhibition of cellular proliferation. One of the mechanisms by which Cu, Zn-SOD and/or catalase overexpression reduces oxLDL-induced cell proliferation therefore mainly involves reduction of JNK and ERK phosphorylation.

Two transcriptional factors, NF-κB and AP-1, have been shown to be phosphorylated and subsequently activated by MAPKs [27]. Activation of the redox-regulated transcriptional factors has been suggested to play a key role in this process, as they bind to the promoters of many genes involved in regulating cell growth and proliferation [13]. OxLDL appears to be a stimulator of NF-κB and AP-1, which are thought to play a predominant role in endothelial cell activation, resulting in atherogenesis. In our previous study [15], oxLDL caused significant induction of NF-κB and AP-1 DNA binding activity in HASMCs. In the present study, overexpression of Cu, Zn-SOD and/or catalase suppressed the oxLDL-induced increase in AP-1 and NF-κB activity. This is consistent with a report that active oxygen induces cell proliferation and NF-κB activation in liver and that catalase overexpression significantly inhibits this increase [28] and with another that MnSOD transgenic mice show reduced tumor promotion due to suppression of AP-1 activation [29]. MAPKs, such as ERK and JNK, play a central role in the regulation of AP-1 expression and activation [30]. Given the above findings, the present study raises the possibility that overexpression of Cu, Zn-SOD and catalase in HASMCs attenuates proliferation of oxLDL-stimulated cells and that this inhibitory effect is mediated via downregulation of ERK1/2 and JNK phosphorylation, followed by AP-1 and NF-κB inactivation.

In summary, we have shown that overexpression of Cu, Zn-SOD and/or catalase reduces oxLDL-stimulated HASMCs proliferation by several mechanisms, namely by arresting cell cycle progression, reducing levels of cyclin D1/CDK4 and cyclin E/CDK2 by upregulation of p21 and p27 expression, and inhibiting ERK and JNK phosphorylation. Furthermore, we have demonstrated that Cu, Zn-SOD and catalase potently inhibit AP-1 and NF-κB binding activities in oxLDL-treated HASMCs. These findings of the present study provide information on a mechanism potentially explaining the antiatherogenic and antiproliferative activities of antioxidant enzymes.

Acknowledgments

We thank Miss Hsiao-Jung Wang for technical assistance in manuscript preparation. This work was supported in part by grants from the National Science Council (NSC 94-2320-B002-048 and NSC 94-2314-B010-052) and the Program for Promoting University Academic Excellence (A92-B-FA09-2-4), Taiwan, Republic of China.

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