

Induced expression of manganese superoxide dismutase by non-toxic concentrations of oxidized low-density lipoprotein (oxLDL) protects against oxLDL-mediated cytotoxicity

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Oxidized low-density lipoprotein (oxLDL) affects macrophages and plays a critical role in the development of atherosclerosis. In the present paper, we demonstrate that high concentrations of oxLDL provoked apoptosis of human Mono-Mac-6 cells, which was blocked by diphenylene-iodonium (DPI), an inhibitor of flavin-containing enzymes, such as NADPH oxidase, suggesting the involvement of reactive oxygen species (ROS). Importantly, pre-treatment of cells with low concentrations of oxLDL prevented apoptosis in response to high concentrations of oxLDL by up-regulating manganese superoxide dismutase (MnSOD). DPI prevented expression of MnSOD by oxLDL, whereas inhibitors of cytochrome P450 (methoxalen) or xanthine oxidase (allopurinol)

did not, thus pointing to a role of NADPH-oxidase-derived ROS in oxLDL-induced MnSOD expression. Transfection of cells with MnSOD antisense, but not scrambled antisense, oligonucleotides significantly attenuated oxLDL-mediated MnSOD expression and hindered cytoprotective effects of non-toxic oxLDL concentrations. Our findings suggest that up-regulation of MnSOD by low concentrations of oxLDL is critical for protection towards oxLDL-mediated cytotoxicity.

Key words: apoptosis, lipoprotein, macrophage, manganese superoxide dismutase (MnSOD), p53, reactive oxygen species (ROS).

INTRODUCTION

Atherosclerosis, a disease of the arterial wall, is characterized by cholesterol accumulation and culminates in potentially life-threatening conditions, such as heart attack, stroke and angina. Unfortunately, complete pathogenesis of the disease is poorly understood. Recent evidence suggest that atherosclerosis is a chronic inflammatory disease of the blood-vessel wall [1–3]. An early event, the development of arterial damage and fatty deposits, is the uptake of oxidatively modified low-density lipoprotein (oxLDL) by macrophages, their retainment in the arterial wall and formation of lipid-laden foam cells. Therefore, subendothelial macrophages are a major cellular component of atherosclerotic lesions and macrophage-derived foam cells in developing atherosclerotic lesions may originate from recruitment of circulating monocytes or from migration of resident tissue macrophages [4]. OxLDL plays a key role in the pathogenesis of atherosclerosis [5,6] and accumulation of oxLDL in the arterial wall is a characteristic feature of disease progression [1].

Apoptosis, a form of genetically programmed cell death, plays an important role in regulating cellularity of the arterial wall. Apoptosis of resident macrophages is recognized within early atherosclerotic lesions, based on morphological studies [7]. It has been shown that activation of the cellular suicide pathway of macrophages may be crucial to the development of atherosclerosis [8,9]. Although the significance of apoptosis in atherosclerosis is not fully understood, it has been proposed that apoptotic cell death contributes to plaque instability, rupture and thrombus formation [10]. It is appreciated that oxLDL can induce apoptosis in macrophages [11], and this has been implicated in the pathogen-

esis of atherosclerosis. It is known that activation of macrophages by oxLDL elicits an oxidative burst, which is prevented by diphenylene-iodonium (DPI), thus pointing to a role of the NADPH oxidase in producing reactive oxygen species (ROS) [12].

Superoxide and other ROS have been implicated in the initiation and progression of several pathophysiological conditions. At the same time, increasing evidence suggests that ROS have an important role in signal transduction [13,14]. Several intracellular sources contribute to the production of ROS, such as cyclo-oxygenases, cytochrome P450, nitric oxide synthase, lipoxygenases, mitochondrial respiration, NADPH oxidase and xanthine oxidase. Manganese superoxide dismutase (MnSOD) is a mitochondrial enzyme that catalyses the dismutation of superoxide anions to hydrogen peroxide and O₂. MnSOD is a primary antioxidant defence system against superoxide radicals in addition to preventing subsequent formation of potentially toxic free radical by-products [15]. Several studies have shown that increased expression of MnSOD protects against oxidative stress [16,17]. Indeed, MnSOD is up-regulated by a variety of pro-inflammatory mediators, such as TNF- α (tumour necrosis factor- α), LPS (lipopolysaccharide), IL-1 β (interleukin-1 β) and IFN- γ (interferon- γ) [18,19]. Considerable evidence suggests that ROS may play an important role in MnSOD induction [20]. However, signalling pathways that are responsible for MnSOD expression are far from being elucidated fully. Incubation of human macrophages with oxLDL resulted in mRNA expression of MnSOD, and MnSOD activity was significantly increased in atherosclerotic intima compared with the media of rabbit aorta [21]. On the other side, little is known about the signal transduction pathway involved in MnSOD induction by oxLDL.

Abbreviations used: AS-ODN, antisense oligonucleotide; DAPI, 4,6-diamidino-2-phenylindole; DOTAP, dioleoyltrimethylammonium propane; DPI, diphenylene-iodonium; LPS, lipopolysaccharide; MM6, Mono-Mac-6; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; L-NAME, N^G-nitro-L-arginine methyl ester; nLDL, native low-density lipoprotein; ODN, oligonucleotide; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; TNF- α , tumour necrosis factor- α .

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In the present study, we examined protective mechanisms evoked by low oxLDL concentrations on subsequent oxLDL-induced apoptosis. Non-toxic concentrations of oxLDL induced MnSOD expression in human macrophages, which in turn protected towards oxLDL-mediated apoptosis and blocked accumulation of the tumour suppressor p53. We conclude that ROS are critical for oxLDL in inducing MnSOD.

EXPERIMENTAL

Materials

A p53 polyclonal antibody was purchased from Santa Cruz Biotechnology. Rabbit antiserum against MnSOD was a gift from Dr Kohtaro Asayama (Department of Pediatrics, Yamanashi Medical University, Yamanashi, Japan). A secondary antibody, horseradish-peroxidase-conjugated donkey anti-rabbit Ig, was obtained from Amersham Biosciences. The Cellular DNA fragmentation ELISA kit and DOTAP (dioleoyltrimethylammonium propane) liposomal transfection reagent were provided by Roche. Synthetic phosphorothioate MnSOD antisense oligonucleotides (AS-ODNs), sense oligonucleotides (ODNs) or scrambled AS-ODNs were purchased from MWG-Biotech (Ebersberg, Germany). All other reagents were obtained from Sigma Chemical Co.

Cell culture

The human monocytic cell line Mono-Mac-6 (MM6) was grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 1% (w/v) non-essential amino acids, 1 mM sodium pyruvate, 9 µg/ml bovine insulin and antibiotics in a humidified 5% CO₂ atmosphere at 37 °C. When the cells were 80–90% confluent, they were exposed to native LDL (nLDL), oxLDL and/or inhibitors (pre-incubated for 2 h) at the doses and times indicated.

Oxidation of nLDL

nLDL (1 mg/ml) was oxidized by exposure to 3 µM CuSO₄ in PBS at room temperature (21–23 °C) for 28–30 h. Oxidation was terminated by adding EDTA (pH 8.0) to a final concentration of 200 µM, followed by dialysis against PBS/100 µM EDTA (pH 8.0) at 4 °C. The degree of nLDL oxidation was quantified by an increased relative mobility on agarose gels (Lipidophor® AII In 12 kit; Technoclone, Heidelberg, Germany), indicating an enhanced negative charge of oxidized lipoprotein [12]. The relative mobility of oxLDL on agarose gels as an index for lipoprotein oxidation was 2.0–2.5 compared with that of nLDL. Protein content of oxLDL and nLDL was measured by the Lowry method [21a]. Low-density lipoprotein concentrations are given as µg of protein per ml solution.

Cell viability assays

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] test

The MTT test is widely used for determination of cell viability and cytotoxicity of oxLDL [22,23]. Cells were seeded at a density of 1×10^4 cells/well in 96-well, flat-bottom microtitre plates and incubated with nLDL/oxLDL in 0.2 ml of culture medium for the times indicated. Following incubations, 10 µl of a MTT solution (5 mg/ml in PBS) was added and incubations were continued for 4 h. Supernatants were removed followed by the addition of

150 µl of a solution containing 4% (v/v) HCl and 96% (v/v) propan-2-ol. The A_{550} of each well was determined with an automated plate reader. Survival was calculated relative to the staining value of an untreated control (100%).

Trypan Blue staining

To measure cell viability, an aliquot of cells was incubated in 0.1% (w/v) Trypan Blue for 3 min, and cells were viewed under a light microscope. Dead cells are permeable to Trypan Blue, whereas viable cells exclude the dye. Four separate fields in the light microscope were used to assess Trypan Blue staining. By counting 100 cells, the percentage of viable cells was calculated.

Mitochondrial membrane potential

Following individual incubations, cells were loaded with 40 nM of the fluorochrome 3,3'-dihexyloxycarbocyanide iodide (DiOC₆) for 10 min. The dye accumulates in mitochondria that contain an intact membrane potential. Mitochondrial membrane potential was measured on a FACSCalibur flow cytometer (Becton Dickinson), using CellQuestPro software. At least 10 000 cells were accumulated for analysis. Results are given as a percentage of total cells that show a high mitochondrial membrane potential.

DNA fragmentation

Cellular DNA fragmentation was measured using a commercially available cellular DNA fragmentation ELISA kit (Roche), according to the manufacturer's instruction. DNA fragmentation was expressed as an increase of the A_{450} .

Staining of nuclei with DAPI (4,6-diamidino-2-phenylindole)

To analyse changes in nuclear morphology, cells were collected by centrifugation, washed with PBS, resuspended in PBS and mounted on glass slides. After fixation with 3% (w/v) paraformaldehyde, cells were stained for 20 min at room temperature in PBS containing 1 µg/ml DAPI and were examined with a Zeiss fluorescent microscope. Apoptosis was characterized by chromatin condensation. Non-apoptotic or control cells exhibited diffuse chromatin staining. Quantification of nuclear condensation was performed by blinded counting of 300 cells for each sample.

Western blot analysis

Protein lysates were prepared from cells and the protein amount was quantified by the method of Bradford [23a] with a Bio-Rad Protein Assay Kit. Protein lysate (60 µg) was separated by SDS/PAGE, blotted on to nitrocellulose and then blocked with 5% (w/v) skimmed milk powder in TTBS buffer [100 mM Tris, pH 7.5, 0.9% (w/v) NaCl and 0.1% (v/v) Tween 20] for 1 h at room temperature. The membrane was then incubated with rabbit antiserum against MnSOD (1/10 000) or with rabbit polyclonal antibody against p53 (1/1000) in TTBS buffer with 5% (w/v) skimmed milk powder at 4 °C overnight. After washing, membranes were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (1/2000) for 1 h at room temperature. Proteins were detected by ECL® (enhanced chemiluminescence; Sigma). Where indicated, blots were stripped and reprobed with a polyclonal antibody against actin to confirm equal protein loading.

Transient transfection

Synthetic phosphorothioate MnSOD-specific AS-ODN corresponding to the initiation site of MnSOD translation (22-mer, CACGCCGCCGACACAACATTG) and sense ODN (control) at the same site (22-mer, CAATGTTGTGTCGGGCGGCGTG) were used [24]. Scrambled AS-ODN (22-mer, AGCCCCGC-CCGAGTAGGATCTG) was used as an additional control. Cells (3×10^6 cells) were treated with MnSOD AS-ODN, control ODN or scrambled AS-ODN for 8 h, using DOTAP for transfection, according to the manufacturer's instructions. The efficiency of ODN treatment was controlled by MnSOD Western blotting.

Statistical analysis

Each experiment was performed at least three times and representative images are shown. Data are presented as means \pm S.D., unless stated otherwise. The unpaired Student's *t* test was used to evaluate the significance of differences between groups, accepting $P < 0.05$ as the level of significance.

RESULTS

OxLDL-mediated cytotoxicity in MM6 cells

To examine the cytotoxic effects of oxLDL, MM6 cells were incubated with increasing concentrations of oxLDL or nLDL for the times indicated and cell viability was determined by the MTT test. As shown in Figures 1(A) and 1(B), oxLDL-mediated cytotoxicity increased over time and the most effective concentration turned out to be 200–400 $\mu\text{g/ml}$ oxLDL.

OxLDL at concentrations of $< 50 \mu\text{g/ml}$ was not toxic for MM6 cells. Importantly, oxLDL, but not nLDL, caused cell death. It is known that oxLDL-induced cell death is mediated by ROS. We have demonstrated previously that the NADPH oxidase inhibitor DPI blocked oxLDL-induced ROS production in macrophages [12]. In supporting these findings, we observed that DPI markedly attenuated oxLDL-mediated cytotoxicity (Figure 1B).

The impact of oxLDL on cell viability was confirmed by the Trypan Blue exclusion method. As shown in Table 1, treatment of MM6 cells with 200 $\mu\text{g/ml}$ oxLDL significantly decreased cell viability from 98% in controls, i.e. untreated cells, to 32%.

At 200 $\mu\text{g/ml}$ nLDL and low concentrations of oxLDL (50 $\mu\text{g/ml}$), cell viability was not diminished. In addition, cell viability was then followed by determination of mitochondrial membrane potential. Results shown in Table 1 are compatible with data obtained by the Trypan Blue exclusion method.

OxLDL-induced apoptosis in MM6 cells

Treatment of MM6 cells with oxLDL, but not nLDL, caused apoptotic cell death (Figures 2A and 2B).

Incubations with 200 $\mu\text{g/ml}$ oxLDL induced DNA fragmentation, whereas nLDL did not (Figure 2A). As shown by DAPI staining, treatment with oxLDL, but not nLDL, induced chromatin condensation and fragmentation of the nucleus (Figure 2B). Counting apoptotic cells that exhibited a distinct apoptotic morphology revealed that $64 \pm 4\%$ of cells enter programmed cell death. The percentage of apoptosis under basal conditions (non-treated cells) did not exceed 3%. To determine the role of ROS in oxLDL-induced apoptosis, cells were treated with oxLDL in the absence or presence of DPI. Evidently, looking at DNA fragmentation or chromatin condensation, oxLDL-induced apoptosis was markedly attenuated in the presence of DPI. In this case, the overall rate of apoptosis decreased to $27 \pm 2\%$.

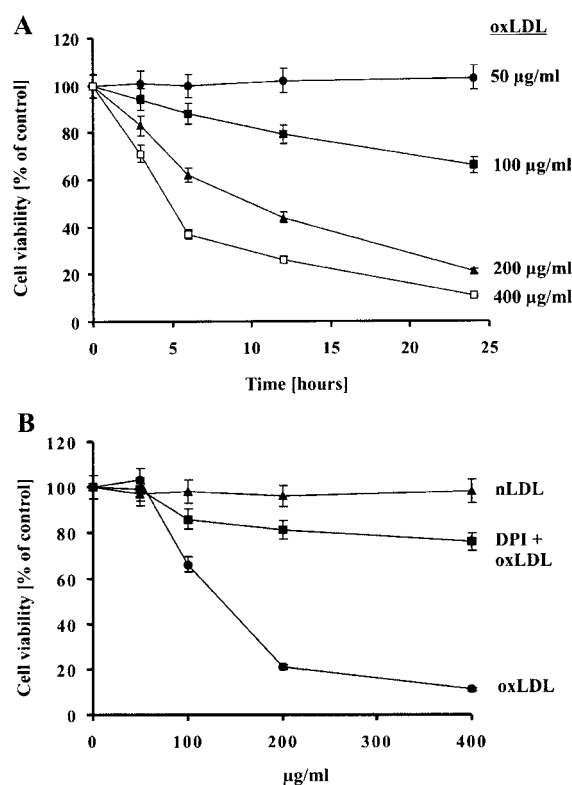


Figure 1 Cell viability of MM6 cells in response to oxLDL

MM6 cells were seeded in 96-well plates at a density of 10^4 cells/well. Cultures received serial dilutions of oxLDL and incubations were continued for the times indicated. (A) Time-dependent (0–24 h; 50–400 $\mu\text{g/ml}$ oxLDL) and (B) concentration-dependent (0–400 $\mu\text{g/ml}$ oxLDL and nLDL; 24 h) effects of oxLDL. Cells were incubated in the absence or presence of 30 μM DPI and oxLDL-induced cytotoxicity was measured. Mitochondrial dehydrogenase activity (MTT assay) was assayed as an index of cell viability. Results are means \pm S.D. ($n = 4$).

Table 1 Effect of non-toxic oxLDL pre-stimulation on oxLDL-induced cell death

MM6 cells were cultured in six-well plates and were exposed to the indicated concentrations of nLDL or oxLDL for 24 h. In some experiments, cells were pre-exposed for 16 h to 50 $\mu\text{g/ml}$ oxLDL, followed by the addition of 200 $\mu\text{g/ml}$ oxLDL for 24 h. The percentage of viable cells (Trypan Blue staining) and the percentage of total cells with high mitochondrial membrane potential were calculated as outlined in the Experimental section. Results are means \pm S.D. of three independent experiments.

Treatment	% of viable cells (Trypan Blue staining)	% of total cells with high mitochondrial membrane potential
Control (untreated)	98.1 \pm 7.3	98.5 \pm 5.9
nLDL (200 $\mu\text{g/ml}$)	96.5 \pm 8.7	98.3 \pm 6.1
oxLDL (50 $\mu\text{g/ml}$)	97.3 \pm 10.4	98.3 \pm 5.7
oxLDL (200 $\mu\text{g/ml}$)	32.8 \pm 5.1	53.1 \pm 3.4
oxLDL (50 $\mu\text{g/ml}$) + oxLDL (200 $\mu\text{g/ml}$)	86.6 \pm 7.5	94.2 \pm 8.3

Apoptosis with pre-treatment of non-toxic concentrations of oxLDL

Little attention has been attributed so far to the exposure of macrophages with non-toxic concentrations of oxLDL, as may occur in the early stages of atherosclerotic development. We were interested whether pre-treatment with non-toxic concentrations of oxLDL modulates oxLDL-induced apoptosis in MM6 cells. We pre-exposed macrophages for 16 h to 50 $\mu\text{g/ml}$ oxLDL,

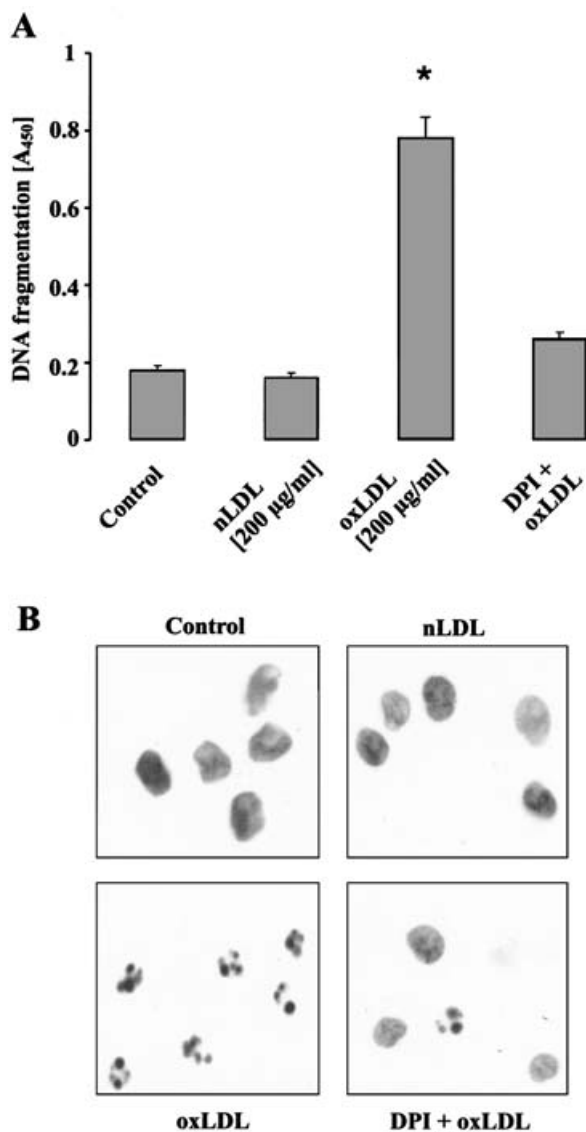


Figure 2 Effect of oxLDL on apoptosis

Cells were left untreated or were treated with 200 $\mu\text{g/ml}$ nLDL or oxLDL in the presence or absence of 30 μM DPI. (A) Cells were exposed to oxLDL or nLDL for 24 h and DNA fragmentation was determined by a cell-death-detection ELISA. Results are means \pm S.D. ($n = 4$). * $P < 0.05$ relative to controls. (B) Cells were incubated for 8 h with 200 $\mu\text{g/ml}$ nLDL or oxLDL, stained with DAPI, and chromatin condensation was examined with a Zeiss fluorescent microscope. Images are representative of four independent experiments.

followed by the addition of 200 $\mu\text{g/ml}$ oxLDL for the times indicated to induce apoptosis. When cells were treated with low doses of oxLDL (50 $\mu\text{g/ml}$) for times beyond 16 h, cells did not display apoptotic features. As shown in Figures 3A and 3B, pre-stimulation with 50 $\mu\text{g/ml}$ oxLDL prevented oxLDL-mediated apoptosis, when looking for DNA fragmentation or assaying for chromatin condensation. A similar protective effect on cell viability was noticed using the Trypan Blue exclusion assay (Table 1).

OxLDL-mediated expression of MnSOD

Considering that pre-treatment with non-toxic concentrations of oxLDL attenuated cell death we were interested to look into

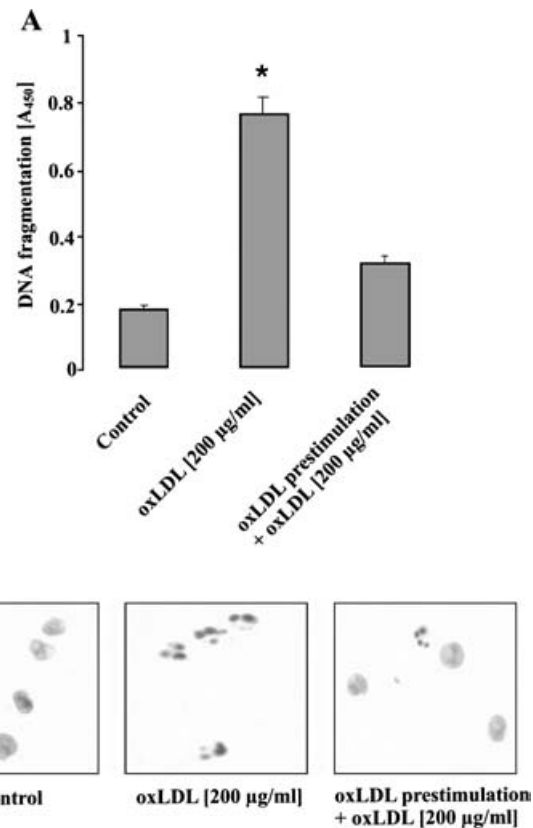


Figure 3 Non-toxic oxLDL pre-stimulation prevented oxLDL-induced apoptosis

MM6 cells were pre-treated with 50 $\mu\text{g/ml}$ oxLDL for 16 h, and were incubated further with 200 $\mu\text{g/ml}$ oxLDL. (A) Cells were exposed for 24 h to oxLDL (200 $\mu\text{g/ml}$) and DNA fragmentation was determined by ELISA. Results are the means \pm S.D. ($n = 4$). (B) Cells were incubated for 8 h with 200 $\mu\text{g/ml}$ oxLDL, stained with DAPI and examined for chromatin condensation.

a potential protective mechanism, such as MnSOD expression. As determined by Western blot analysis, MnSOD expression was significantly induced by non-toxic concentrations of oxLDL (50 $\mu\text{g/ml}$), with maximal responses seen at 8 and 16 h (Figure 4A). nLDL did not alter MnSOD protein expression. In order to determine the source of ROS that might be involved in MnSOD up-regulation, cells were treated for 2 h with different inhibitors of ROS production before the addition of 50 $\mu\text{g/ml}$ oxLDL for 16 h.

As shown in Figure 4(B), oxLDL-mediated MnSOD expression was neither affected by pre-treatment with the xanthine oxidase inhibitor allopurinol nor with the cytochrome P450 inhibitor methoxalen. However, MnSOD expression was significantly diminished by pre-treatment with DPI, an inhibitor of flavin-containing enzymes, such as NADPH oxidase. As DPI is known to inhibit nitric oxide synthase or the mitochondrial respiratory chain, we excluded these potential side effects by showing that neither the nitric oxide synthase inhibitor L-NAME (N^G -nitro-L-arginine methyl ester) nor the mitochondrial respiratory chain inhibitor rotenone blocked MnSOD expression by oxLDL (Figure 4C). We conclude that ROS, most likely derived from NADPH oxidase, participate in oxLDL-mediated MnSOD expression.

MnSOD AS-ODN impaired oxLDL-induced MnSOD expression

To connect oxLDL-mediated MnSOD expression and protection from apoptosis, we used MnSOD-specific AS-ODNs.

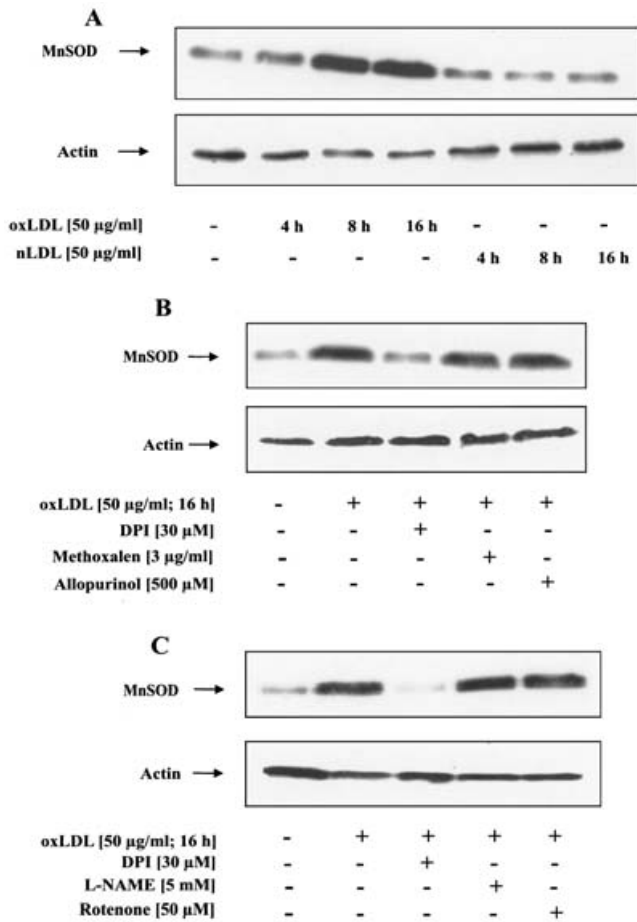


Figure 4 Effect of oxLDL on MnSOD protein expression

(A) Time course of MnSOD expression by oxLDL. MM6 cells were incubated with control medium or medium containing 50 µg/ml oxLDL or nLDL for the indicated times. (B) and (C) Cells were pre-treated with DPI (30 µM), methoxalen (3 µg/ml), allopurinol (500 µM), L-NAME (5 mM) or rotenone (50 µM) for 2 h before MM6 stimulation with 50 µg/ml oxLDL for 16 h. Expression of MnSOD in cell lysates was detected by Western blot analysis. Membranes were stripped and probed with an anti-actin antibody to control protein loading. Western blots are representative of at least three independent experiments.

Transfection efficiency was determined by Western blot analysis, looking at oxLDL-induced MnSOD protein levels under conditions of ODN treatment (Figures 5A and 5B). Western blots confirmed that oxLDL-mediated MnSOD expression was significantly decreased in cells that were transfected with MnSOD AS-ODN compared with cells transfected with either a control sense ODN or scrambled AS-ODN (Figure 5A). Using the experimental setting when MnSOD expression was attenuated by transfecting MnSOD AS-ODN, protection towards apoptosis as a result of oxLDL-pre-treatment was lost (Figure 5B).

These results suggest that protection towards oxLDL-induced apoptosis by non-toxic concentrations of oxLDL demands induction of MnSOD.

Impact of oxLDL on p53 protein accumulation in MM6 cells

The tumour suppressor p53 is a key regulator for initiation and control of apoptotic cell death with the further notion that oxLDL-provoked p53 accumulation in human macrophages may transmit an apoptotic signal in response to oxLDL. As seen in Figure 6(A),

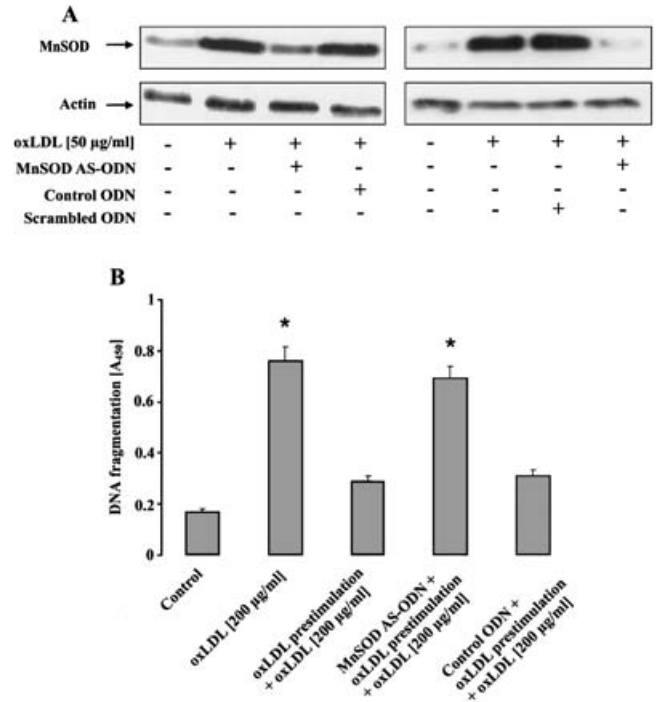


Figure 5 oxLDL-evoked protection is impaired by MnSOD AS-ODNs

(A) Following transfection with MnSOD-specific AS-ODN, sense ODN or scrambled AS-ODN, subsequent treatment of cells with 50 µg/ml oxLDL for 16 h, MnSOD protein expression was detected by Western blot analysis. Blots were stripped and reprobed for actin to confirm equal protein loading. (B) Cells were transfected with MnSOD AS-ODNs or sense ODNs and DNA fragmentation was measured after oxLDL pre-stimulation (50 µg/ml) for 16 h, followed by stimulation with oxLDL (200 µg/ml) for another 24 h. Results are means ± S.D. (n = 4). *P < 0.05 relative to control cells.

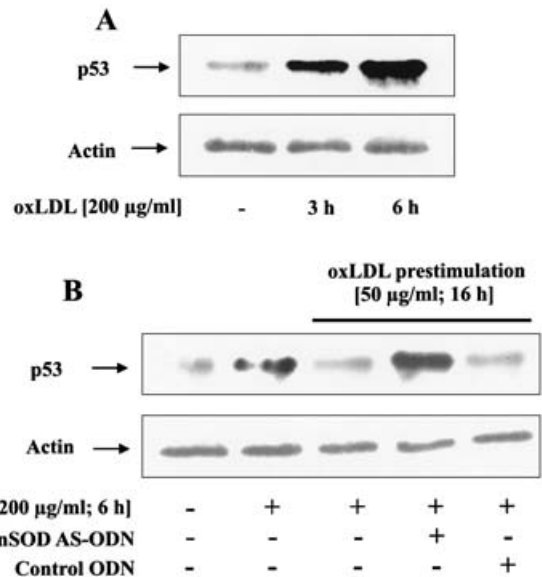


Figure 6 Accumulation of p53 under the impact of oxLDL

(A) MM6 cells were treated with 200 µg/ml oxLDL for the times indicated. Accumulation of p53 was followed by Western blot analysis. (B) Cells were transiently transfected with MnSOD AS-ODN or control ODN, pre-stimulated with 50 µg/ml oxLDL for 16 h, followed by stimulation with 200 µg/ml oxLDL for 6 h. Detection of p53 was performed by Western blot analysis. Expression of the housekeeping protein actin was followed to confirm equal protein loading. Results are representative of three independent experiments.

200 $\mu\text{g/ml}$ oxLDL induced p53 protein accumulation, as determined by Western blot analysis at 3 and 6 h.

Interestingly, p53 accumulation was significantly attenuated by pre-incubation of MM6 cells with 50 $\mu\text{g/ml}$ oxLDL for 16 h (Figure 6B). Transfection of MM6 cells with MnSOD AS-ODN, followed by pre-stimulation with non-toxic concentrations of oxLDL (50 $\mu\text{g/ml}$) before incubation with 200 $\mu\text{g/ml}$ oxLDL allowed the cells to fully regain p53 accumulation. We conclude that protection towards oxLDL-mediated apoptosis is correlated with an attenuated p53 response.

DISCUSSION

OxLDL is thought to play a major role in the pathogenesis of atherosclerosis [25]. Accumulation of oxLDL is a characteristic feature of atherosclerotic lesions and has a fundamental role in the progression of disease [1]. In the present paper, we show that pre-treatment of MM6 cells with low concentrations of oxLDL prevents apoptosis in response to high concentrations of oxLDL by up-regulating MnSOD. Apoptotic cell death within atherosclerotic lesions and its contribution to plaque rupture has been established, and it is demonstrated that macrophages are among the cells that undergo programmed cell death [26–28]. Apoptosis of macrophages may be beneficial for plaque stability, if apoptotic bodies are removed [26]. Indeed, studies have shown that oxLDL, but not nLDL, induces apoptosis in human macrophages [29,30]. Although molecular pathways controlling apoptosis in macrophages have not been precisely defined, it is known that ROS are critical for oxLDL-induced apoptosis. As shown in our previous study [12], the NADPH oxidase complex constitutes the main source of ROS in human macrophages under oxLDL treatment. We now extend this observation, demonstrating that oxLDL-mediated apoptosis is ROS-dependent. Thus incubation of MM6 cells with the NADPH oxidase inhibitor DPI attenuated apoptosis induced by oxLDL, as assessed by DNA fragmentation and chromatin staining. Although the production of oxidants and potentially pro-apoptotic signals are enhanced during the atherosclerotic process, recent findings also suggest that cytoprotective pathways are induced [31]. Unfortunately, molecular mechanisms that account for cytoprotection under the impact of oxLDL remain poorly defined. Recent results demonstrate that low doses of oxLDL protected cells against quinone-mediated oxidative stress by inducing glutathione synthesis [32]. Antioxidant response elements are needed in evoking glutathione synthesis by oxLDL and in protecting macrophages against oxidized-lipid-induced oxidative stress [33]. So far, little attention has been attributed to the exposure of macrophages to non-toxic concentrations of oxLDL, as may occur in the early stages of atherosclerotic development. In the present paper, we propose a role of MnSOD in protecting MM6 cells from oxLDL toxicity. We demonstrated that oxLDL-induced MnSOD up-regulation resulted in protection against oxLDL-mediated cytotoxicity. MnSOD expression by oxLDL has previously been described [21]. However, the authors suggested a pro-apoptotic role of MnSOD during oxLDL-induced apoptosis. In contrast, our study implies that MnSOD up-regulation by low doses of oxLDL protected against oxLDL-mediated cell death. Several lines of evidence suggested that ROS formation is critical for MnSOD up-regulation by inducers such as LPS and TNF- α [18–20]. We were therefore interested in whether or not ROS are involved in MnSOD expression by oxLDL. In order to determine the potential source of ROS that might be involved, we studied the effect of the NADPH oxidase inhibitor DPI. DPI, but not the xanthine oxidase inhibitor allopurinol or

the cytochrome P450 inhibitor methoxalen, significantly reduced oxLDL-elicited MnSOD expression. As DPI has been shown to inhibit nitric oxide synthase [34] and the mitochondrial respiratory chain [35], we excluded these potential side effects by showing that neither the nitric oxide synthase inhibitor L-NAME nor the mitochondrial respiratory chain inhibitor rotenone blocked oxLDL-induced MnSOD expression. In order to establish causation between oxLDL-mediated MnSOD expression and protection from apoptosis, MnSOD AS-ODNs were transfected into MM6 cells. We demonstrate that oxLDL-mediated MnSOD expression was significantly reduced by transfection of MnSOD AS-ODN, that in turn resulted in loss of protection towards oxLDL-induced apoptosis. p53 is a tumour-suppressor protein with established pro-apoptotic actions [36,37]. Often, cellular stress facilitates p53 accumulation by blocking p53 proteosomal degradation [36]. In atherosclerotic lesions, p53 co-localizes with apoptotic macrophages [38,39] and *in vivo* studies revealed that p53 deficiency is associated with increased atherosclerotic lesions [40,41]. In addition, oxLDL induces p53 accumulation in human macrophages [42]. A role of ROS has been suggested in facilitating p53 up-regulation by oxLDL, presumably by enhancing p53 protein synthesis [43]. Since p53 has been suggested as a pro-apoptotic transmitter in oxLDL toxicity, we were interested to follow p53 accumulation under the condition of oxLDL-elicited cytoprotection. We show that oxLDL markedly enhanced p53 accumulation and this effect was abrogated by pre-treatment with non-toxic oxLDL concentrations. Transfection of MnSOD AS-ODN, followed by pre-stimulation with non-toxic concentrations of oxLDL before incubation with 200 $\mu\text{g/ml}$ oxLDL, allowed cells to regain a complete p53 response. Our results demonstrate that pre-treatment of MM6 cells with non-toxic concentrations of oxLDL abrogated oxLDL-induced p53 accumulation via MnSOD up-regulation. We conclude that protection towards oxLDL-mediated apoptosis is mirrored by an attenuated p53 response.

Taken together, our results demonstrate that oxLDL not only induced cell death, as previously noted, but also protects from apoptosis via MnSOD up-regulation. Clarification of molecular mechanisms that regulate apoptosis may help to design new strategies for treatment of atherosclerosis and its major complications, the acute vascular syndrome.

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