Induction of the antioxidant stress proteins heme oxygenase-1 and MSP23 by stress agents and oxidised LDL in cultured vascular smooth muscle cells

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Abstract Enhanced expression of the antioxidant stress proteins heme oxygenase-1 (HO-1) and macrophage stress protein (MSP23) by oxidative stress agents and oxidatively modified low density lipoproteins (LDL) was investigated in cultured porcine aortic smooth muscle cells. Treatment of smooth muscle cells with glucose oxidase, $CdCl_2$ or diethylmaleate resulted in a time-dependent (6–48 h) induction of HO-1 and MSP23 expression. Exposure of cells to 100 μg protein/ml highly oxidised LDL increased the expression of HO-1 and MSP23 within 24 h, and the induction was dependent on the degree of LDL oxidation. The induction of HO-1 and MSP23 may thus play an important cytoprotective role against oxidative stress in atherogenesis.

Key words: Smooth muscle cell; Heme oxygenase-1; MSP23; Oxidative stress protein; Oxidized low density lipoprotein; Atherosclerosis

1. Introduction

An imbalance between the production of reactive oxygen species (ROS) and cellular antioxidant defence mechanisms may result in oxidative stress, which has been implicated in the pathogenesis of several human diseases including atherosclerosis [1]. Oxidatively modified low density lipoproteins (LDL) modulate the function of vascular cells [2], such as smooth muscle cells (SMC), which in atherogenesis undergo migration and proliferation and are thus central in the pathology of occluding arterial lesions [3]. An investigation of the response of SMC to stress agents and oxidised LDL may provide further insights into mechanisms leading to cellular dysfunction in atherogenesis.

The induction of intracellular antioxidant proteins may act as a cytoprotective defence against oxidative stress. These 'stress' proteins include the heat shock proteins, and the most abundant subset hsp70 has been detected in atherosclerotic lesions [4] and is induced in human endothelial cells treated with oxidised LDL [5]. The microsomal 32 kDa protein heme oxygenase-1 (HO-1) is another stress-induced protein, which catalyses the rate-limiting step in the conversion of heme via biliverdin and carbon monoxide to bilirubin [6], an efficient free radical scavenger [7]. HO-1 is induced by ROS, sulfhydryl-reactive agents, heavy metals and depletion of glutathione [8–10]. In murine macrophages, a cytosolic 23 kDa stress protein (MSP23) is induced in response to oxidative and sulfhydryl-reactive agents [11] and oxidised LDL [12]. The amino acid sequence of MSP23 is homologous with that of a family of stress inducible antioxidant enzymes, including the C22 component of alkyl hydroperoxide reductase of *Salmonella typhimurium* and the thiol-specific antioxidant protein of *Saccharomyces cerevisiae* [13,14], which inhibits the inactivation of glutamine synthetase by a thiol/Fe^{3+}/O_2 mixed function oxidation system [15]. This family of antioxidant proteins has recently been termed peroxiredoxins, as they seem to also have peroxidase activity [16].

This study has investigated for the first time the expression of both HO-1 and MSP23 in cultured porcine vascular SMC treated with the sulfhydryl-reactive agents cadmium chloride and diethylmaleate (DEM), glucose oxidase (GO) or oxidatively modified low density lipoproteins.

2. Materials and methods

2.1. Cell culture

Porcine aortic medial explants were cultured in DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine, 30 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin in an incubator at 37°C with a 5% CO_2/95% air atmosphere. Smooth muscle cells (SMC) grew out from the explants within 2–3 weeks and were subcultured into 75 cm^2 culture flasks when confluent. Cells were confirmed as SMC by their typical 'hill and valley' morphology and positive immunofluorescence staining with a monoclonal antibody against α-actin. SMC between passages 3 and 6 were used in all experiments.

2.2. Treatment of smooth muscle cells with stress agents

SMC were seeded into 24-well culture plates at a density of 10^5 cells/ml 3 days prior to incubation with stress agents. Cells were cultured in the presence or absence of the stress agents for 6–48 h at 37°C in a 5% CO_2 atmosphere. The final concentrations of stress agents were as follows: 100 μM DEM, 2.5–10 μM $CdCl_2$, 5 mU/ml GO and native, mildly oxidised or highly oxidised LDL (100 μg protein/ml). Exposure to the stress agents was terminated by gently washing the cells twice with ice-cold Dulbecco's phosphate buffered saline. Lactate dehydrogenase (LDH) release from porcine SMC was determined to assess the viability of cells after the stress treatments. LDH activity was assayed in the culture media by using a diagnostic reagent based on the oxidation of lactate to pyruvate.

2.3. Western blot analysis

HO-1 and MSP23 protein expression were determined by im-

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Abbreviations: HO-1, heme oxygenase-1; MSP23, macrophage stress protein; SMC, smooth muscle cells; ROS, reactive oxygen species; nLDL, native low density lipoprotein; moxLDL, mildly oxidised LDL; oxLDL, highly oxidised LDL; DEM, diethylmaleate; GO, glucose oxidase.

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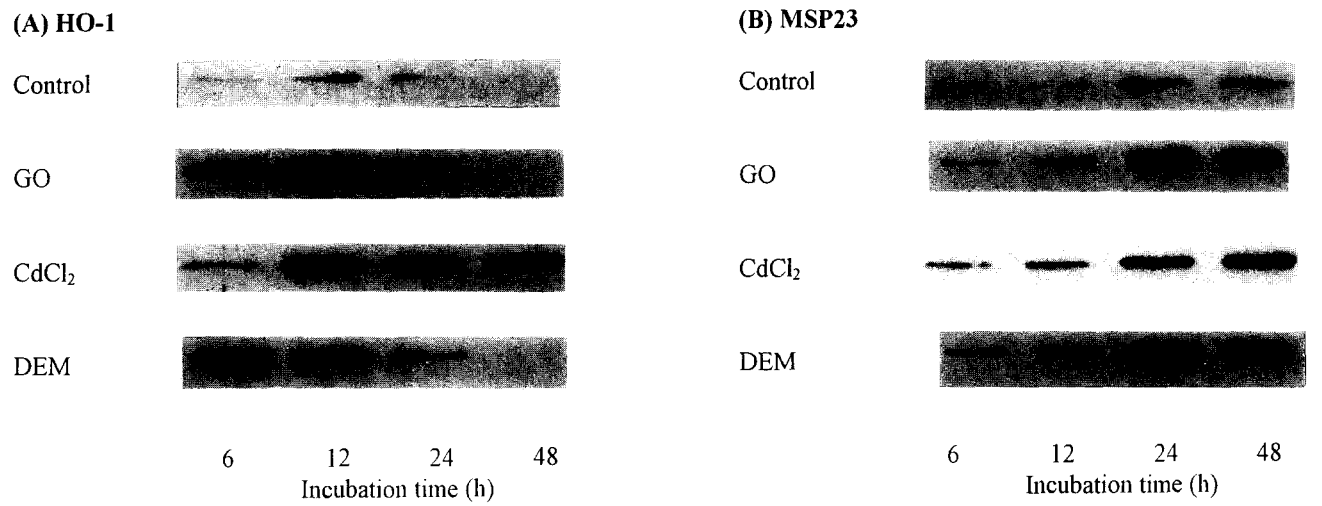

Fig. 1. Time-dependent enhancement of HO-1 (A) and MSP23 (B) expression in porcine aortic smooth muscle cells exposed to stress agents. Cells were incubated for 6–48 h in the absence (control) or presence of 5 mU/ml glucose oxidase (GO), 10 μM $CdCl_2$ or 100 μM diethylmaleate (DEM). HO-1 and MSP23 expression were determined by Western blot analysis. Data are representative of experiments in 3 different cell cultures.

munoblotting. SMC proteins were solubilised in buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM Tris-HCl, pH 6.8) and boiled for 5 min. Sample total protein content was determined using the BCA protein assay reagents, and an equal protein concentration from each sample was then boiled in a mixture of 1% 2-mercaptoethanol and 0.05% bromophenol blue for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis using a 13% acrylamide resolving gel [17]. Separated proteins were then transferred to a polyvinylidine difluoride membrane and probed with either a polyclonal rabbit anti-rat heme oxygenase antibody [9] or a polyclonal rabbit anti-mouse MSP23 antibody [13]. A horseradish peroxidase conjugated goat anti-rabbit secondary antibody was used in conjunction with enhanced chemiluminescence to visualise the HO-1 or MSP23 bands on autoradiography film.

2.4. Preparation of LDL

LDL (density 1.019–1.063 g/ml) was isolated from normal human blood by ultracentrifugation in the presence of EDTA, followed by dialysis against (mM): NaCl 154, NaH_2PO_4 16.7, Na_2HPO_4 21.1, EDTA 0.1, pH 7.4, as described previously [18]. Mildly oxidised LDL was formed by incubating LDL at 100 μg protein/ml at 37°C in (mM): NaCl 137, KCl 2.68, NaH_2PO_4 8.1, KH_2PO_4 1.47, pH 7.4 with $CuSO_4$ (net concentration 5 μM above the EDTA present). The absorbance at 234 nm was monitored to measure the conjugated dienes produced [19] until the absorbance had increased by 0.2. 1 mM EDTA was then added to stop the oxidation. Highly oxidised LDL was formed by incubating the LDL in the above way but for 24 h before adding 1 mM EDTA. The density of the oxidised LDL was then raised to 1.2 g/ml with KBr (in the presence of Chelex-100) and they were concentrated by ultracentrifugation and dialysed against several changes of (mM): NaCl 154, NaH_2PO_4 16.7, Na_2HPO_4 21.1, EDTA 1, pH 7.4. They were then sterilised by membrane filtration (0.2 μm) and their protein [20] and lipid hydroperoxide [21] contents and mobility in agarose gels determined. The lipid hydroperoxides in the native, mildly oxidised and highly oxidised LDL of the LDLs used were 40, 64 and 80 nmol/mg protein respectively and the relative electrophoretic mobilities of moxLDL and oxLDL (compared to nLDL) were 1.3 and 4.6 respectively.

2.5. Materials

Dulbecco's modified Eagles medium (DMEM), lactate dehydrogenase assay reagent, cadmium chloride and diethylmaleate were purchased from Sigma Chemical Co., Poole, UK. Glucose oxidase was purchased from Calbiochem, Nottingham, UK. Horseradish peroxidase conjugated goat anti-rabbit immunoglobuin and BCA protein assay reagents were purchased from Pierce, Chester, UK. Polyvinylidine difluoride membrane (Immobilon-P) was purchased from Millipore, Watford, UK. Enhanced chemiluminesence Western blotting detection reagents and Hyperfilm-MP autoradiography film were purchased from Amersham International, Amersham, UK. All chemicals used were of analytical grade.

3. Results

Fig. 1 shows the time-dependent enhanced expression of HO-1 and MSP23 in SMC exposed to GO, DEM or $CdCl_2$. The basal expression of HO-1 in SMC was low over the 48 h incubation period. Expression was enhanced in SMC after 6–12 h exposure to 5 mU/ml GO or 100 μM DEM and then decreased to basal levels by 48 h (Fig. 1A). 10 μM $CdCl_2$ did not enhance the expression of HO-1 after 6 h but expression was maximal by 12 h and remained elevated for 48 h. The basal expression of MSP23 was low over the 48 h incubation period. SMC exposed to 5 mU/ml GO, 100 μM DEM and 10 μM $CdCl_2$ showed a marked time-dependent enhancement of MSP23 expression, with maximal levels being observed at 48 h (Fig. 1B).

Maximal expression of HO-1 and MSP23 in SMC treated with 100 μM $CdCl_2$ occurred after 24 h and 48 h respectively (Fig. 1). As shown in Fig. 2, $CdCl_2$ (2.5–10 μM) resulted in a concentration-dependent enhancement of HO-1 and MSP23 expression.

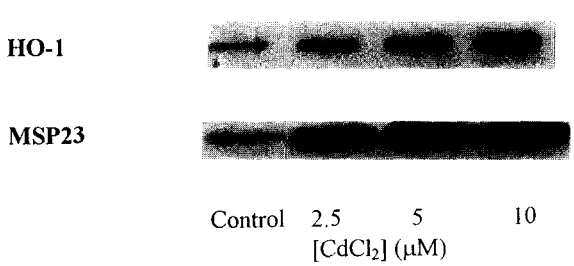

Fig. 2. Concentration-dependent enhancement of HO-1 and MSP23 expression in porcine aortic smooth muscle cells determined by Western blot analysis. Cells were incubated in the absence (control) or presence of increasing concentrations of $CdCl_2$ (2.5–10 μM) for either 24 h (HO-1) or 48 h (MSP23). Data are representative of experiments in 3 different cell cultures.

As shown in Fig. 3, these stress proteins were also induced in a time-dependent manner following exposure to 100 μg protein/ml highly oxidised LDL, a dose shown to produce a maximal response in macrophages [12]. Although the expression of either stress protein was not enhanced within 6 h, the levels were increased maximally after 24 h. Within 48 h the expression of MSP23 had decreased, whereas HO-1 levels were still elevated. When SMC were incubated with native LDL, mildly oxidised LDL or highly oxidised LDL (100 μg protein/ml) for 48 h, there was a greater level of HO-1 and MSP23 induction with increased LDL oxidation (Fig. 4).

Release of lactate dehydrogenase into the culture medium of cells exposed for 48 h to 5 mU/ml GO, 100 μM DEM, 10 μM $CdCl_2$, 100 μg protein/ml nLDL, moxLDL and oxLDL was within 10% of that measured in the medium from control cells, indicating that the various stress regimes were not cytotoxic to porcine vascular smooth muscle cells (data not shown).

4. Discussion

Our study provides the first evidence that expression of the antioxidant-like stress proteins HO-1 and MSP23 is enhanced in cultured vascular SMC exposed to oxidative stress agents and oxidatively modified LDL. These stress-induced proteins may play an important role in cellular antioxidant defence mechanisms, as antioxidant proteins like MSP23 are able to protect enzymatic activity from inactivation by ROS [15], and HO-1 generates bilirubin, a potent-free radical scavenger [7]. HO-1 was acutely induced in the SMC after 6–12 h exposure to GO, a hydrogen peroxide generator, the electrophilic agent DEM and the sulfhydryl-reactive heavy metal cadmium. In contrast, MSP23 was only maximally induced after 24–48 h incubation with these stress agents. HO-1 may therefore be important in the initial cellular antioxidant response to oxidative stress, which may then trigger the subsequent induction of other cellular antioxidant systems such as MSP23.

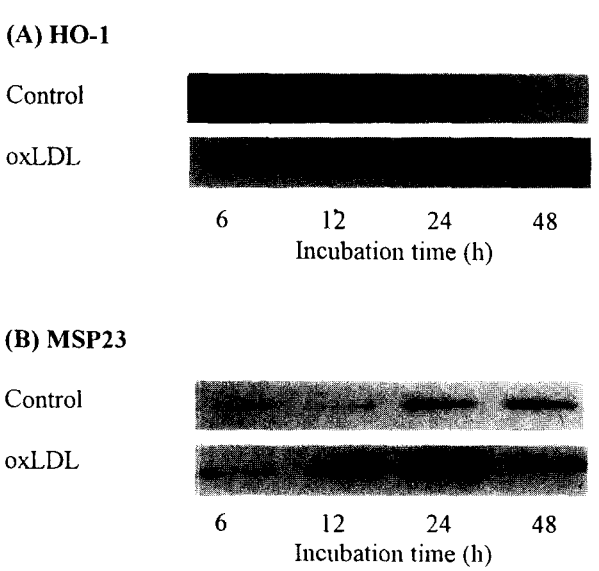

Fig. 3. Time-dependent enhancement of HO-1 (A) and MSP23 (B) expression in porcine aortic smooth muscle cells exposed to oxLDL determined by Western blot analysis. Cells were incubated for 6–48 h in the absence (control) or presence of 100 μg protein/ml oxLDL. Data are representative of experiments in 3 different cell cultures.

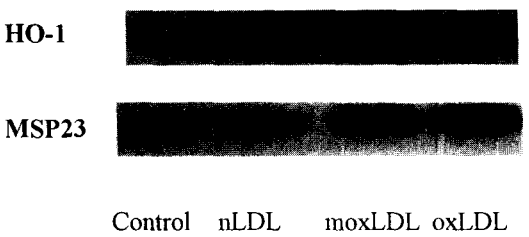

Fig. 4. Comparison of the effects of native, mildly oxidised and highly oxidised LDL on the expression of HO-1 and MSP23 by Western blot analysis. Cells were incubated for 48 h in the absence (control) or presence of 100 μg protein/ml native, mildly oxidised or highly oxidised LDL. Data are representative of experiments in 3 different cell cultures.

Oxidatively modified LDL has been implicated in the pathogenesis of atherosclerosis and may be a major source of free radical insult in the arterial wall [22]. HO-1 and MSP23 were only maximally induced in cultured SMC after 24–48 h incubation with oxLDL. In contrast to the early response of HO-1 to DEM, GO and cadmium, the delayed responses of HO-1 to oxLDL may be due to the different nature of oxidative stress presented by oxidatively modified LDLs, including binding to SMC LDL receptors [23], transfer of ROS to the plasma membrane or internalisation of the components of oxidised LDL, such as oxysterols and lipid hydroperoxides. Oxidatively modified LDL have also been shown to reduce endothelial cell nitric oxide synthesis which may lead to vasoconstriction [24,25]. The induction of HO-1 in vascular SMC by oxidatively modified LDL may also contribute to the maintenance of arterial patency in atherogenesis, as carbon monoxide generated by HO-1 activity has been shown to stimulate guanylate cyclase activity directly [26], which would elevate cGMP production in arterial SMC leading to vascular relaxation.

Our study demonstrates that vascular SMC induce protective responses to oxidative stress and oxidatively modified LDLs. Further understanding of the regulation of these responses and their sensitivity to dietary antioxidants could lead to alternative strategies for reducing atherogenesis.

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