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Induction of Antioxidant Stress Proteins in Vascular Endothelial and Smooth Muscle Cells: Protective Action of Vitamin C Against Atherogenic Lipoproteins

RICHARD C.M. SIOW[a], HIDEYO SATO[b], DAVID S. LEAKE[c], TETSURO ISHII[b], SHIRO BANNAI[b] and GIOVANNI E. MANN[a,*]

[a]*Centre for Cardiovascular Biology and Medicine, GKT School of Biomedical Sciences, King's College London, Campden Hill Road, London W8 7AH, UK;* [b]*Department of Biochemistry, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan;* [c]*Cell and Molecular Biology Research Division, School of Animal and Microbial Sciences, University of Reading, UK*

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Elevated levels of lipid peroxidation and increased formation of reactive oxygen species within the vascular wall in atherosclerosis can overwhelm cellular antioxidant defence mechanisms. Accumulating evidence implicates oxidatively modified low density lipoproteins (LDL) in vascular dysfunction in atherosclerosis and oxidized LDL have been localized with in atherosclerotic lesions. We here report that human oxidatively modified LDL induce expression of 'antioxidant-like' stress proteins in vascular cells, involving increases in the activity of L-cystine transport, glutathione synthesis, heme oxygenase-1 and the murine stress protein MSP23. Moreover, treatment of human arterial smooth muscle cells with the dietary antioxidant vitamin C markedly attenuates adaptive increases in endogenous antioxidant gene expression and affords protection against smooth muscle cell apoptosis induced by moderately oxidized LDL. As vascular cell death is a key feature of atherosclerotic lesions and may contribute to the plaque 'necrotic' core, cap rupture and thrombosis, our findings suggest that the cytoprotective actions of vitamin C could limit plaque instability in advanced atherosclerosis.

Keywords: Antioxidant stress proteins, vascular smooth muscle, vascular endothelial cells, vitamin C, low density lipoproteins, MSP23, heme oxygenase-1, apoptosis

INTRODUCTION

Atherogenesis is associated with elevated levels of lipid peroxidation and increased formation of reactive oxygen species within the vascular wall, which could overwhelm cellular antioxidant defence mechanisms.[1] Native low density lipoprotein (LDL) initially accumulates in the subendothelial space of arteries where it becomes progressively oxidatively modified. Oxidized

* Corresponding author. Tel.: 44-171-333-4450. Fax: 44-171-333-4450. E-mail: giovanni.mann@kcl.ac.uk.

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LDL are now widely accepted as major factors contributing to the initiation and progression of atherosclerosis and have also been localized within atherosclerotic lesions.[2,3] The oxidant insult mediated by oxidized LDL at the vessel wall results in vascular dysfunction,[4] and induction of a multitude of responses in vascular endothelial and smooth muscle cells (reviewed in [5,6]), including compensatory antioxidant defence mechanisms.[7,8]

Dietary antioxidants attenuate the cytotoxicity of oxidatively modified LDL[9,10] and restore endothelial function in patients with coronary artery disease, diabetes and hypercholesterolemia.[11–13] Moreover, low plasma vitamin C concentration has been implicated as an independent predictor of unstable coronary artery disease,[14] and thus antioxidant vitamins continue to be advocated for both the treatment and prevention of atherosclerosis.[15,16] This review summarizes our findings and other reports that induction of antioxidant-like stress proteins in vascular endothelial and smooth muscle cells in atherosclerosis provides adaptive mechanisms to counteract oxidative stress induced by atherogenic lipoproteins. Moreover, we review our recent findings that oxidatively modified LDL induce the L-cystine-glutathione pathway and the stress proteins heme oxygenase-1 (HO-1) and murine stress protein (MSP23) in vascular smooth muscle cells. The cytoprotective actions of vitamin C spare glutathione (GSH) in vascular cells exposed to oxidatively modified LDL, attenuating adaptive responses in antioxidant gene expression and protecting against apoptotic cell death.

LDL-INDUCED CYSTINE–GLUTATHIONE PATHWAY

Synthesis of GSH is dependent on availability of intracellular L-cysteine, and cellular transport of L-cystine is often rate-limiting for GSH synthesis.[17] L-Cystine is incorporated into cells via the Na^{+}-independent anionic amino acid transporter system x_c^-, previously characterized in human umbilical vein endothelial cells, in which exposure to oxidative stress results in a rapid decrease in intracellular GSH followed by adaptive increases in GSH biosynthesis and L-cystine transport.[18] Within cells, L-cystine is rapidly reduced to the sulfhydryl form L-cysteine, which is used for GSH and protein synthesis, whereas extracellularly L-cysteine is autoxidized to the disulfide L-cystine. In order to maintain intracellular L-cysteine levels, there is a continuous cycling of L-glutamate efflux and L-cystine influx via system x_c^-, driven not only by the cellular metabolism of L-cysteine but also by the extracellular redox state.[19] GSH is also involved in the recycling of the dietary antioxidants vitamin E, vitamin C and β-carotene to their reduced forms in order to maintain the reducing milieu within cells.[20,21]

Oxidatively modified LDL induces oxidative stress in vascular cells,[3,8] and our recent studies in human cultured umbilical artery smooth muscle cells revealed that LDL induced adaptive increases in intracellular GSH and L-cystine transport were dependent on the degree of LDL oxidative modification (Figure 1A). Exposure of smooth muscle cells to mildly and highly oxidized LDL for 24 h, but not native LDL, significantly increased initial rates of L-cystine transport. When smooth muscle cells were pretreated with vitamin C (100 µM for 24 h), the medium removed and cells then challenged with fresh medium containing oxidatively modified LDL, adaptive increases in L-cystine transport were markedly attenuated. As shown in Figure 1B, pretreatment of smooth muscle cells with vitamin C also markedly attenuated LDL-induced adaptive increases in intracellular GSH levels, with protection detected after 3 h pretreatment and at concentrations between 20 and 50 µM (Figure 1C,D). Moreover, when compared with α-tocopherol (100 µM for 24 h, see Ref. [8]), vitamin C was far more effective in attenuating the adaptive responses to highly oxidized LDL. Interestingly, even basal levels of L-cystine transport activity were decreased following pretreatment of cells with vitamin C

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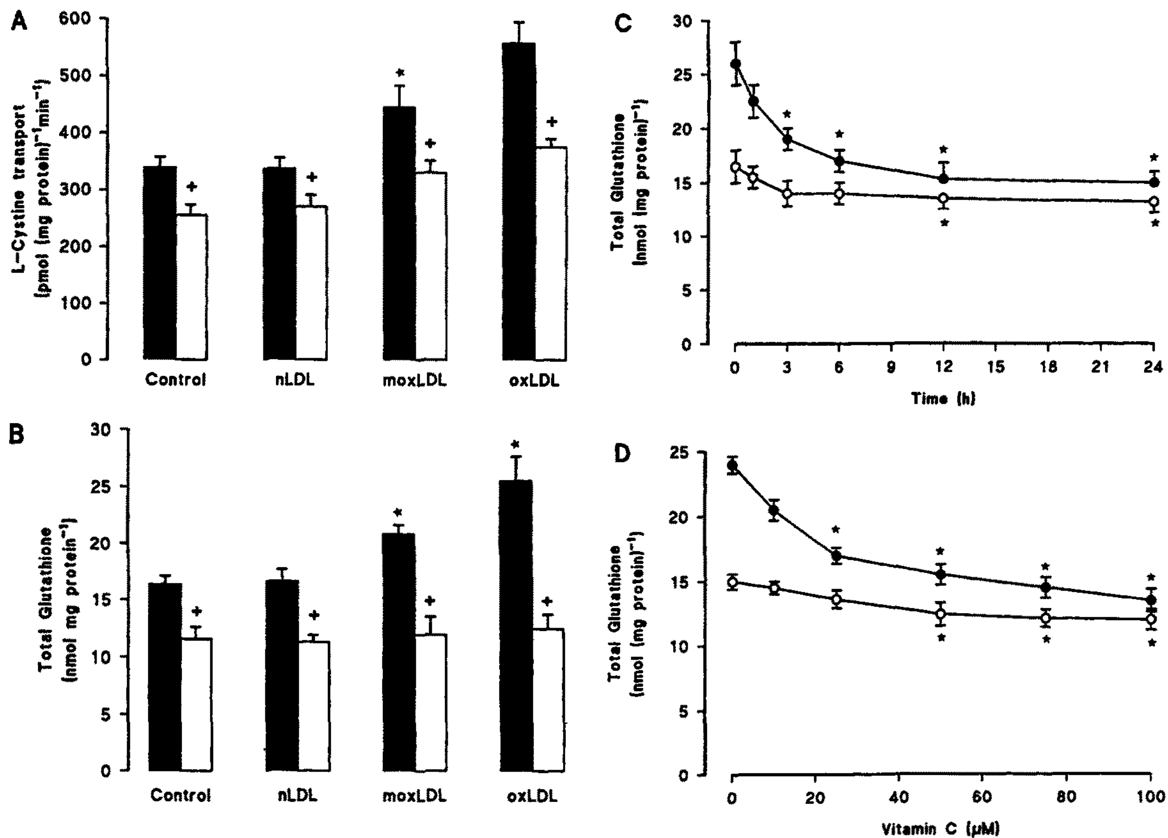

FIGURE 1 Activation of the L-cystine – glutathione pathway in human vascular smooth muscle cells exposed to oxidatively modified low density lipoproteins. (A) initial rates of L-cystine transport (50 µM, 2 min) were measured in human umbilical artery smooth muscle cells (HUASMC) incubated in the absence (filled bars) or presence of vitamin C (100 µM for 24 h, open bars) and then challenged for 24 h with 100 µg protein ml^{-1} native (nLDL), mildly oxidized (moxLDL) or highly oxidized (oxLDL) LDL. (B) total intracellular GSH was determined in parallel experiments using cells treated in the absence (filled bars) or presence (open bars) of vitamin C. (C) Total intracellular GSH was determined in HUASMC pretreated for the specified times in the absence or presence of 100 µM vitamin C prior to incubation for a further 24 h in the absence (○) or presence (●) of 100 µg protein ml^{-1} oxLDL. (D) Concentration-dependent effects of vitamin C (0–100 µM for 24 h) on total intracellular GSH levels in HUASMC treated in the absence (○) or presence (●) of 100 µg protein ml^{-1} oxLDL. Values denote means ± SE ($n = 3$–5 different cell cultures).), $^{*}P < 0.05$ relative to values in the absence of vitamin C, $^{+}P < 0.05$ relative to control values in the absence of vitamin C (see Siow *et al.*[7]).

(Figure 1A), suggesting that under *in vitro* culture conditions cells may already have been exposed to a degree oxidative stress. Our observation that vitamin C reduced L-cystine transport and intracellular GSH levels in untreated human umbilical artery smooth muscle cells is consistent with its ability to spare GSH in human red blood cells and rat astrocytes.[22,23] Our findings provided the first evidence that physiologically relevant concentrations of vitamin C spare endogenous adaptive antioxidant responses in smooth muscle cells exposed to atherogenic lipoproteins. In our studies, the degree of LDL Cu^{2+}-oxidation was carefully defined based on measurements of their electrophoretic mobilities and lipid hydroperoxide content.[24]

Intracellular levels of vitamin C in human cultured umbilical vein endothelial cells are low, but increase rapidly following addition of physiological concentrations of vitamin C to the culture medium and protect cells against the cytotoxic actions of H_2O_2.[25] Auto-oxidation of

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ascorbic acid to dehydroascorbate provides a substrate which is more readily taken up by cells via glucose transporters.[26] Although free radicals within oxidized LDL may be quenched by vitamin C,[27] and hence spare intracellular GSH, our study in human umbilical artery smooth muscle cells was designed to minimize quenching of free radicals within oxidized LDL during incubation of cells with the lipoprotein. Smooth muscle cells were pretreated with vitamin C (3–24 h in the absence of LDL) and then exposed to oxidatively modified LDL for another 24 h in the absence of vitamin C, and hence it is seems more likely that attenuation of adaptive increases in the activity of the L-cystine-glutathione pathway were due to intracellular antioxidant actions of vitamin C.

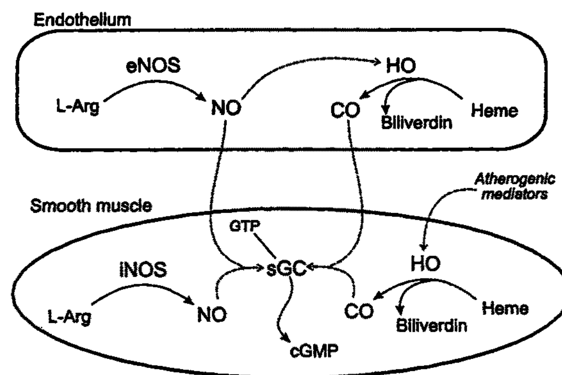

FIGURE 2 Schematic model of the heme oxygenase signalling pathway in vascular endothelial and smooth muscle cells. Heme oxygenases (HO) metabolize heme to generate the antioxidant biliverdin and CO, which like NO, stimulates sGC resulting in increased intracellular cGMP levels and vascular relaxation of smooth muscle cells. (see Siow *et al.*[8]).

INDUCTION OF THE STRESS PROTEIN HEME OXYGENASE BY OXIDIZED LDL

Increased expression of the stress response protein HO-1 in human atherosclerotic lesions[28] and vascular endothelial and smooth muscle cells exposed to oxidized LDL[29,30] may serve a multi-factorial role, via metabolism of heme to the antioxidant biliverdin and the vasodilator carbon monoxide (CO).[31,32] These adaptive responses may contribute to the maintenance of vascular tone and patency in atherosclerotic vessels, compensating partially for diminished nitric oxide (NO) generation and activity[4,33] in atherogenesis. Recent evidence indicates that there is a diminished GSH-related enzymatic antioxidant shield within human atherosclerotic lesions,[34] and thus induction of HO-1 could provide an alternative antioxidant defence mechanism. The scheme in Figure 2 summarizes the potential role that the heme oxygenase-CO. (CO) and L-arginine-NO signalling pathways may play in modulating endothelial and smooth muscle cell function in atherogenesis. NO and CO stimulate soluble guanylyl cyclase (sGC) resulting in elevated intracellular cGMP levels, which in vascular smooth muscle leads to relaxation.[35] Although recent studies have implicated CO as a mediator of vascular relaxation,[36,37] it is worth noting that not all studies report similar findings[38] and that studies *in vitro* reveal that CO is an order of magnitude less effective than NO in activating sGC.[35] Atherogenic mediators are known to decrease the expression and activity of endothelial NO synthase (eNOS) while inducing HO-1 and inducible NO synthase (iNOS) in smooth muscle cells.[8] Under conditions of diminished NO, induction of HO-1 in endothelial and smooth muscle cells may generate biliverdin to counteract the oxidative stress induced by atherogenic lipoproteins whilst elevated cGMP levels induced by CO could modulate vascular smooth muscle tone.

The role of HO-1 induction in cellular antioxidant defences in atherogenesis may be of greater importance than its involvement in vascular relaxation mediated by CO. The clinical complications arising from atherosclerosis are directly related to elevated free radical generation and oxidation of lipids in the sub-endothelial space.[1–5] Induction of HO-1 by atherogenic and inflammatory mediators such as oxidized LDL and cytokines could ameliorate the insult to cells

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by restoring the balance of antioxidants and pro-oxidants in the vascular wall. In the context of this review, it is of interest to note that there is increasing evidence that low serum concentrations of bilirubin have been implicated as an independent risk factor for coronary artery disease.[39] Protection against atherosclerosis afforded by bilirubin may be due to its ability to protect LDL from oxidative modification by a variety of pro-oxidants and to potentiate actions of vitamin C and E.[40,41]

We have previously demonstrated that mildly and highly oxidized LDL, but not native LDL, induce a time- (6–48 h) and concentration- (10–100 μg protein ml^{-1}) dependent expression of HO-1 in cultured vascular smooth muscle cells.[30] In similar studies in rat aortic smooth muscle cells, we confirmed that mildly oxidized and highly oxidized LDL, but not native LDL, increased mRNA levels for HO-1 (Figure 3A). Ishikawa *et al.*[29] have recently reported that induction of HO-1 mRNA expression and activity in co-cultures of human aortic endothelial and smooth muscle cells by mildly oxidized LDL, oxidized lipid metabolites or hemin inhibits monocyte chemotaxis (see Figure 3B), an early event in atherogenesis.[2] These authors attributed their findings to the generation of bilirubin and biliverdin by endothelial cells, since monocyte chemotaxis was attenuated by pretreatment with

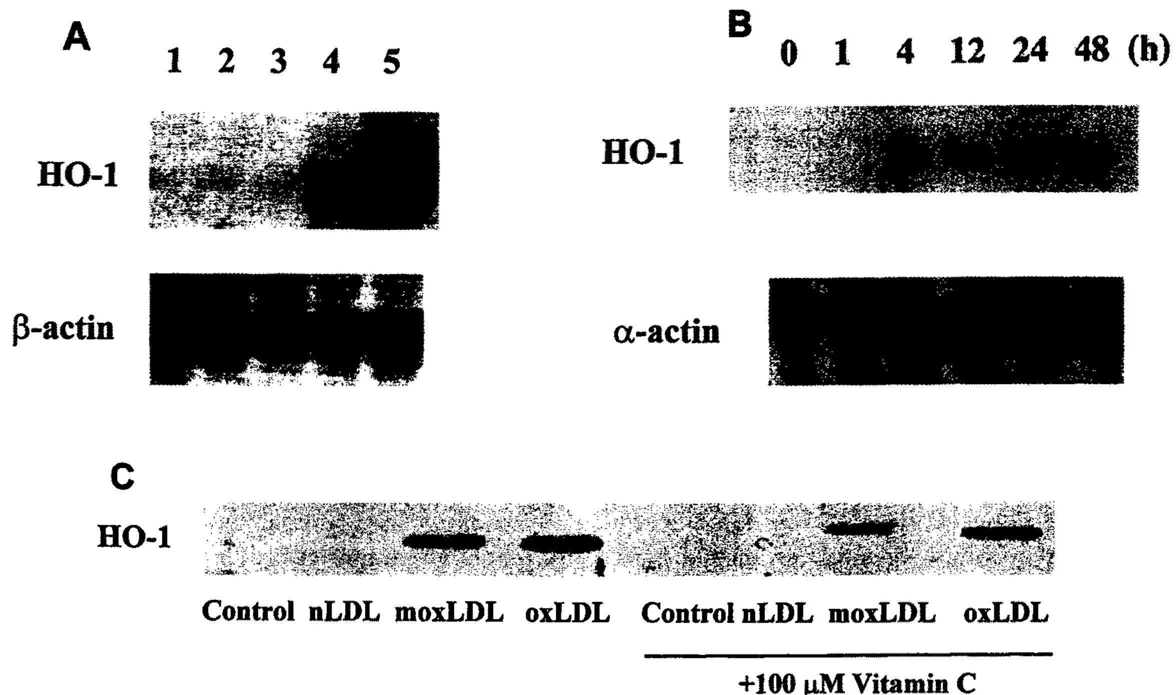

FIGURE 3 Induction of HO-1 mRNA in vascular smooth muscle cells and cytoprotective actions of vitamin C. (A) Northern blot of HO-1 mRNA expression in rat aortic smooth muscle cells cultured *in vitro*. Cells were incubated for 8 h in the absence (lane 1) or presence of 100 (μg protein) ml^{-1} native (nLDL, lane 2), mildly oxidized (moxLDL, lane 3), or highly oxidized (OxLDL, lane 4) LDL or with 10 μM $CdCl_2$ (lane 5). (B) time course of HO-1 mRNA induction in co-cultures of human aortic endothelial and smooth muscle cells by mildly oxidized LDL (taken from Ishikawa *et al.*[29]). Co-cultured cells were exposed for 0–48 h to 350 μg ml^{-1} co-culture modified LDL, prepared by prior incubation of native LDL in other co-cultures for 16 h. HO-1 and α-actin mRNA levels were determined by Northern blot analysis. (C) human umbilical artery smooth muscle cells were treated for 24 h with 100 (μg protein) ml^{-1} native (nLDL), mildly oxidized (moxLDL) or highly oxidized (oxLDL) in the absence or presence of vitamin C. Cells were pretreated for 24 h with vitamin C (100 μM), the medium removed and cells challenged with 100 (μg protein) ml^{-1} oxidatively modified LDL for a further 24 h. Expression of HO-1 protein was determined by Western blot analysis (taken from Siow *et al.*[42]).

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these products of heme metabolism and reversed by inhibition of HO activity. Our recent studies in human cultured umbilical artery smooth muscle cells have established that highly oxidized LDL induces HO-1 expression to a greater degree than mildly oxidized LDL (Figure 3C) and that pretreatment of cells with physiological concentrations of vitamin C (10–100 μM) attenuates the adaptive increase in HO-1 expression induced by highly oxidized LDL.[42] Taken together with our recent report that vitamin C attenuates the adaptive increases in GSH levels in human smooth muscle cells treated with oxidized LDL,[7] these findings provide convincing evidence that antioxidant supplementation with vitamin C may be anti-atherogenic. As endothelium-derived NO synthesis and the sensitivity of target smooth muscle cells may be decreased in atherosclerosis, induction of the heme oxygenase-CO pathway could provide an important adaptive mechanism for moderating the severity of ischaemia, haemorrhage, thrombosis and atherosclerosis.

INDUCTION OF MSP23 IN VASCULAR CELLS BY OXIDIZED LDL

Previous studies in murine macrophages identified a cytosolic 23 kDa stress protein (MSP23) which is induced in response to oxidative, sulfhydryl-reactive agents and oxidized LDL.[43,44] 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 cervisiae*, which inhibits the inactivation of glutamine synthethase by a thiol/Fe^{3+}/O_2 mixed function oxidation.[45–47] This family of antioxidant proteins has been named peroxiredoxins as they appear to have peroxidase activity. We recently reported the first evidence that expression of MSP23 is enhanced in vascular smooth muscle cells exposed to electrophiles (diethylmaleate), heavy metals ($CaCl_2$) and oxidized LDL.[30] MSP23 was only induced maximally after 24–48 h incubation with the stress agents, whereas in the same study HO-1 was already induced within 6–12 h. These findings suggested that HO-1 may be important in initial cellular antioxidant responses to oxidative stress, which then trigger the subsequent induction of other antioxidant systems such as MSP23. More recently, we have examined expression of MSP23 in vascular cells cultured from different blood vessels. MSP23 expression in freshly isolated human umbilical artery smooth muscle was negligible, whereas basal levels were detectable by Western blot analyses in extracts of human cultured umbilical artery smooth muscle cells, umbilical vein endothelial cells and rat cultured aortic smooth muscle cells. As in the case of L-cystine transport (see [7] and Figure 1A), it seems probable that the culture conditions (95% air, 5% CO_2) expose cells to enhanced oxidative stress, perhaps accounting for the basal expression of MSP23 in vascular endothelial and smooth muscle cells. This conclusion is consistent with findings in murine peritoneal macrophages, where MSP23 was not expressed in freshly isolated macrophages but was gradually detected following culture of cells in an oxygen-rich incubator.[43,45] Nevertheless, basal expression of these stress proteins has not precluded us from characterizing time- and concentration-dependent effects of oxidatively modified LDL on antioxidant gene expression in vascular endothelial and smooth muscle cells.

VITAMIN C PROTECTS AGAINST LDL-INDUCED SMOOTH MUSCLE APOPTOSIS

Oxidized LDL induces vascular cell death by apoptosis,[48,49] and the presence of apoptotic cells in atherosclerotic and restenotic lesions[50,51] has clinical implications for atherogenesis. Vascular cell apoptosis contributes to the accumulation of necrotic debris within the lesion core and

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instability of the fibrous plaque cap leading to its rupture and thrombosis.[51] The extent to which LDL becomes oxidized within atherosclerotic lesions has not been well defined and levels of oxidation may differ within lesions. As the toxicity of oxidized LDL appears to increase with the degree of oxidative modification,[52,53] this may contribute to the impaired vascular reactivity in patients with coronary heart disease.[4]

We have recently reported cytoprotective effects of vitamin C against apoptosis of human smooth muscle cells induced by oxidatively modified LDL *in vitro*.[54] Moderately oxidized LDL, with the peak level of lipid hydroperoxides, but not highly oxidized LDL, caused smooth muscle cell apoptosis within 6 h. Moreover, moderately oxidized LDL (300 μg protein ml^{-1}, 24 h), caused an increase in cellular DNA fragmentation, whereas native, mildly or highly oxidized LDL had negligible effects on DNA fragmentation (Figure 4A). Pretreatment of cells with vitamin C (100 μM for 24 h) attenuated DNA fragmentation induced by moderately oxidized LDL (Figure 4A), with protection observed at vitamin C concentrations above 50 μM. Binding of annexin V, a marker of early apoptosis, was observed in cells treated for 6 or 24 h with moderately oxidized LDL (300 μg protein ml^{-1}), whereas no binding was detected in smooth muscle cells exposed to native, mildly or highly oxidized LDL (see [54]). Smooth muscle cells treated with moderately oxidized LDL for 24 h, but not for 6 h, were permeable to propidium iodide, suggesting that cells progressed to a late stage of apoptosis after prolonged exposure to moderately oxidized LDL. Pretreatment of smooth muscle cells with vitamin C abolished the early (6 h) annexin V binding and attenuated propidium iodide staining in cells exposed to moderately oxidized LDL for 24 h, suggesting that sparing intracellular GSH protected cells against apoptosis (see [54]).

Basal expression of the anti-apoptotic mediator Bcl-x_L was unaltered in human arterial smooth muscle cells treated with native, mildly or highly oxidized LDL (300 μg protein ml^{-1}, data not

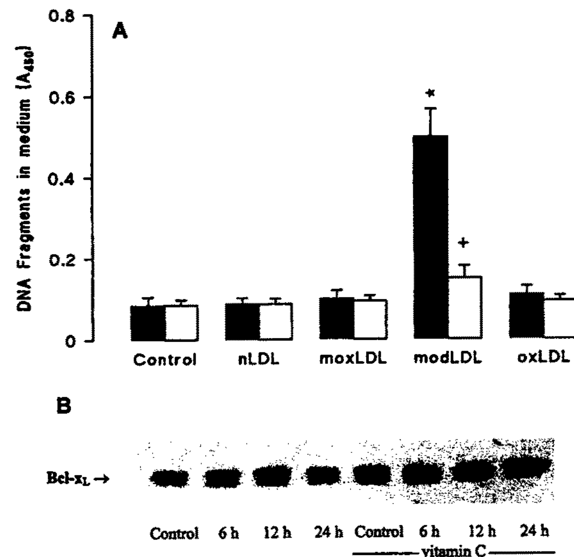

FIGURE 4 Vitamin protects human arterial smooth muscle cells against LDL-induced apoptosis. A, human umbilical artery smooth muscle cells were pretreated in the absence or presence of vitamin C (100 μM, 24 h), and then exposed to either native or moderately oxidized LDL for a further 24 h. Cellular DNA fragmentation determined by ELISA. Values denote means ± SE, $n = 3$–6 different cell cultures, * $P < 0.05$ relative to respective values in cells treated with native LDL, $^{+}P < 0.05$ relative to cells treated with moderately oxidized LDL in the absence of vitamin C. B, vitamin C maintains Bcl-x_L expression in human smooth muscle cells treated with oxidatively modified LDL. Cells were pretreated in the absence or presence vitamin C (100 μM, 24 h) prior to exposure to moderately oxidized LDL (modLDL, 300 μg protein ml^{-1}) for 6, 12 or 24 h or to culture medium alone (control). Bcl-x_L expression was determined by Western blot analysis (taken from Siow *et al.*[54]).

shown) and, as shown in Figure 4B, treatment with moderately oxidized LDL for 24 h significantly decreased Bcl-x_L expression. As with the L-cystine-glutathione pathway and heme oxygenase, pretreatment of human arterial smooth muscle cells *in vitro* with vitamin C (100 μM, 24 h) also prevented the decrease in Bcl-x_L expression induced by moderately oxidized LDL. We interpret these results as follows. It seems likely that in addition to its ability to scavenge free radicals, vitamin C may also modulate cellular apoptotic mediators such as CPP32, Bcl-2, and Bcl-x_L to protect cells against apoptosis. It has recently been shown that enhanced expression of Bcl-x_L protects against transplant atherosclerosis

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and endothelial cell activation,[55] suggesting that maintained or enhanced expression of anti-apoptotic genes by vitamin C supplementation may be protective in atherogenesis.

SUMMARY

The extent of LDL oxidation within atherosclerotic lesions remains a controversial issue, yet our findings suggest that protein modifications associated with highly oxidized LDL are not necessarily required for LDL-induced cytotoxicity. Although antioxidant dietary vitamins protect against vascular dysfunction, plaque instability and reduce the oxidative modification of LDL,[11,16] it is also possible that LDL modification may be arrested at a more atherogenic 'moderately oxidized' level by vitamin supplementation. Thus, moderately oxidized LDL could induce more smooth muscle cells within the lesion to undergo apoptosis than highly oxidized LDL. Prolonged dietary antioxidant supplementation could have different effects on advanced atherosclerotic lesions, since initially antioxidants would inhibit oxidative modification of LDL in the arterial wall and limit the formation of lesions. We have provided evidence that oxidized LDL induce endogenous antioxidant defence mechanisms in vascular smooth muscle cells which appear to be protective in atherogenesis. Although it is beyond the scope of this review to speculate whether antioxidants, such as vitamin C, may be pro-atherogenic through the arrest of LDL oxidation or inhibition of neointimal smooth muscle cell apoptosis, our findings support current consensus that antioxidant consumption reduces the risk of coronary heart disease.[15,16]

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