Free Rad. Res., Vol. 28, pp. 611–621
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Cellular Thiol Production and Oxidation of Low-Density Lipoprotein

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Accepted by Prof. V. Darley-Usmar

(Received 1 October 1997; In revised form 5 January 1998)

Compelling evidence suggests that low-density lipoprotein (LDL) is oxidized by cells within the arterial intima and that, once oxidized, it is profoundly atherogenic. The precise mechanism(s) by which cells promote the oxidation of LDL *in vivo* are not known; *in vitro*, however, oxidation of LDL can be enhanced by a number of differing mechanisms, including reaction with free and protein-bound metal ions, thiols, reactive oxygen species, lipoxygenase, myeloperoxidase and peroxynitrite. This review is concerned with the mechanisms by which cells enhance the oxidation of LDL in the presence of transition metals; in particular, the regulation, pro- and anti-oxidant consequences, and mechanism of action of cellular thiol production are examined, and contrasted with thiol-independent oxidation of LDL in the presence of transition metals.

Keywords: Oxidized low-density lipoprotein, thiol, transition metal, atherosclerosis

INTRODUCTION

The hypothesis that oxidized low-density lipoprotein (OxLDL) is responsible for the deposition of lipid within macrophages in early atherosclerotic lesions was first propounded over ten years ago.[1] The concept was immediately appealing. While the increased atherosclerotic risk associated with elevated serum concentrations of low-density lipoprotein (LDL) was established,[2] it was clear that native LDL did not cause macrophage foam cell formation.[3] Oxidation of LDL confers recognition by macrophage scavenger receptors, leading to uncontrolled uptake of this particle, lipid deposition, and foam cell formation *in vitro*.[3,4] The presence of LDL oxidation products in atherosclerotic tissues[5–7] implies this process also occurs *in vivo*. It has since become increasingly clear that OxLDL exerts a diverse array of pro-atherogenic effects, including induction of colony-stimulating factors,[8] monocyte chemotactic protein-1[9] and proinflammatory cytokines,[10] increasing monocyte adhesion to endothelial cells.[11]

However, despite extensive research effort, the mechanism of LDL oxidation in the artery wall remains controversial.[12,13] That the parameters of LDL oxidation *in vitro*, are clearly defined is

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due largely to the work of Hermann Esterbauer and his colleagues during the late 1980s and early 1990s.[14–19] I first met Hermann in 1990, at a Lipid Group/Society for Free Radical Research Joint Colloquium of the Biochemical Society, held in Aberdeen. As a lipid biochemist, newly recruited to the field of free radical biology, I was intrigued by the concept of LDL oxidation resistance and its modulation by lipid-soluble antioxidants, which Hermann presented at this meeting.[19] However, we soon became much more familiar with the published work from Esterbauers' laboratory, in our attempts to apply the same kind of rigour to our cellular studies of LDL oxidation. In particular, the absolute dependence of these experiments upon the presence of transition metals meant much could be inferred from Esterbauers' classic studies of LDL oxidation by copper ions,[14–19] and our aims refined to an investigation of the mechanisms by which cells facilitate the oxidation of LDL by transition metals.

An understanding of the biochemical mechanisms underlying the formation of OxLDL *in vivo* could indicate important potential targets for drug intervention.[20] It seems unlikely, given the high concentrations of plasma antioxidants, that LDL oxidation occurs in the circulation; indeed, levels of lipid hydroperoxides found in circulating lipoproteins are extremely low, and levels of plasma lipophilic antioxidants are not reduced in atherosclerosis.[21,22] It was suggested, therefore, that LDL oxidation might occur within the intimal tissue of the artery wall, where antioxidants could become depleted by, and the LDL exposed to, oxidant stress exerted by cells therein.[20] Recent evidence, however, suggests human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of α-tocopherol and ascorbate, indicating the atherosclerotic artery is not, overall, an antioxidant-deficient tissue.[7] But local depletion of antioxidants mediated, for example, by clusters of macrophages within the vessel wall, could result in heterogeneous arterial antioxidant defences. The composition, and in particular the transition metal content, of such putative cellular microenvironments,[20] are critical factors when attempting to assess the relevance of cellular mechanisms of LDL oxidation derived from studies performed *in vitro*.

CELLULAR OXIDATION OF LOW-DENSITY LIPOPROTEIN *IN VITRO*

Although the oxidative mechanism(s) responsible for cellular oxidation of LDL *in vivo* are not clearly established, several candidate oxidizing species, involving both transition metal-dependent and -independent reactions, have been identified.[23–27] Specific markers of oxidation elicited by lipoxygenase,[23] myeloperoxidase[24,25] and peroxynitrite[26,27] have been identified in human atherosclerotic lesions, and imply that these species contribute to LDL oxidation *in vivo*; their roles in cellular oxidation of LDL *in vitro* have been the subject of recent excellent reviews.[20,28]

Further, these enzymatic mechanisms are not the predominant mechanism(s) by which cells oxidize LDL *in vitro*; cells which do not possess lipoxygenase, myeloperoxidase, or nitric oxide synthase activities can efficiently oxidize LDL.[29,30] Indeed, virtually all of the cell types associated with atherosclerotic lesions, including endothelial cells,[31] smooth muscle cells,[32] lymphocytes,[33] platelets[34] and monocyte/macrophages,[35] and some which are not, such as HepG2 hepatoma cells,[36] can oxidize LDL *in vitro*. Probably the most important factor, and the one generating criticism of cellular oxidation studies,[12,13,20,28] is that in order to elicit the gross oxidative and structural changes conferring macrophage scavenger receptor recognition, nearly all of these systems have an absolute requirement for trace amounts of transition metals to be present in the cell culture medium. This is usually supplied by use of serum-free Hams F10 medium,[31–35] or by use of Hanks' buffer supplemented with transition metals.[37,38] Human monocytes, stimulated with opsonized zymosan, are reported to oxidize LDL in the

absence of transition metals;[39] however, recent data suggest this oxidation is due to the contaminating iron ions in the zymosan preparations.[40] 'Minimally' or mildly oxidized LDL (mmLDL), which is recognized by the native LDL receptor, can be formed in the absence of exogenously supplied transition metals, and in the presence of serum;[9,41] however, the mechanisms by which co-cultures of endothelial and smooth muscle cells generate mmLDL are not understood. This review focuses on the mechanisms by which arterial cells can enhance the oxidation of LDL by transition metals, and, in particular, the evidence that cellular production of free thiols accelerates the oxidation of LDL.

CELLULAR THIOL PRODUCTION AND LDL OXIDATION

Thiols, including protein-bound thiols, undergo auto-oxidation in the presence of transition metals and oxygen, generating oxygen- and sulphur-centred radicals.[42–51] Two initial observations, made over a decade ago, suggested a role for sulphur-containing molecules in cellular oxidation of LDL. Millimolar concentrations of reduced glutathione, and other compounds with free -SH groups, promote the oxidation of LDL in the absence of cells in Hams F10 medium.[52] Further, oxidation of LDL by arterial smooth muscle cells required the presence of both L-cystine and free transition metals.[32] More recently, Sparrow and Olszewski[53] demonstrated the dependency of LDL oxidation by rabbit endothelial cells and mouse peritoneal macrophages, on cellular production of free thiols (predominantly L-cysteine) in Hams F10 culture medium. Thiol production, and LDL oxidation, were inhibited by glutamate, which blocks L-cystine uptake by the x_c^- transporter.[53]

Stimulated primary human monocytes,[54] and human (THP-1) macrophages[29,55] oxidize LDL by a mechanism which is largely dependent on L-cystine, and requires a threshold level of thiol production.[55] Accumulation of intracellular ceroid, an insoluble fluorescent pigment found within lipid-laden macrophage foam cells, is also dependent upon L-cystine.[56] Intriguingly, non-arterial cells, such as HepG2 hepatoma cells,[36] enhance LDL oxidation by a thiol-dependent mechanism, suggesting any cell type which actively recycles L-cystine to free thiol will accelerate LDL oxidation in the presence of transition metals. In contrast, cells which do not exhibit 'thiol recycling', such as rat A10 smooth muscle cells,[54] oxidize LDL at a much slower rate. It is noteworthy that cellular LDL oxidation is not completely inhibited in the absence of thiol recycling, or following the removal of L-cystine from the culture medium[29,53–55] arguing that thiol-independent pathways exist by which cells enhance the effects of transition metals[37,38] (see below). Significantly, cellular thiol production is not sufficient to initiate oxidation of freshly isolated LDL (<9 h), but does enhance the oxidation of older preparations,[57] implying endogenous 'seeding' lipid peroxides are required for thiols to facilitate LDL oxidation.

REGULATION OF CELLULAR THIOL PRODUCTION AND LDL OXIDATION

Release of Free Thiols

Inflammatory conditions, associated with the pathogenesis of atherosclerosis, can induce thiol recycling by macrophages. Thus, release of acid-soluble free thiols from macrophages is stimulated by tumour necrosis factor (TNF) and by lipopolysaccharide (LPS);[58,59] our own data suggest induction of thiol release by human (THP-1) monocytes can be induced or inhibited, by protein kinase C (PKC) agonists and inhibitors, respectively.[60] Supra-physiological concentrations of insulin and glucose may also enhance thiol production by human monocytes.[61]

L-Cystine Uptake

Induction of thiol release by arterial cells requires increased uptake of L-cystine, usually mediated

by the x_c^- - transporter.[62] Transport of L-cystine is the requisite for glutathione synthesis, and is induced by oxidized LDL,[63] oxidative stress,[64] sodium arsenite,[65] by TNF-α and LPS,[66] and by activation of PKC.[60] Thus, uptake of L-cystine and release of free thiols show concerted responses to identical stimuli.[58–60,66] In general, cellular responses to oxidative stress include increased synthesis of cellular antioxidants, like glutathione;[67] release of free thiols may also be an extracellular defence mechanism, providing protection against immune oxidative injury.[58,59,68] In particular, release of free thiols can supply lymphocytes, which cannot transport L-cystine, with L-cysteine for glutathione synthesis.[58,59,68] Thus, it is only in the presence of transition metals that thiol recycling causes paradoxically damaging effects, leading to increased oxidative damage and LDL oxidation.

Thiol-Dependent LDL Oxidation

Thiol recycling and cellular LDL oxidation show similar regulatory control. TNF-α enhances LDL oxidation by macrophages and endothelial cells;[69] similarly, pathological concentrations of insulin, insulin-like growth factor-1 (IGF-1) and glucose enhance the oxidation of LDL by human monocytes.[61] Stimulation of PKC is required for oxidation of LDL by human monocytes, via a mechanism which is partially superoxide-independent.[70] However, perhaps the most convincing demonstration that thiol recycling, and LDL oxidation, can both be induced by oxidative stress exerted *in vivo*, comes from a study using human umbilical vein endothelial cells (HUVECs) isolated from smokers. In the presence of free iron, these cells oxidize LDL, via a mechanism which is strongly thiol-dependent, to a much greater degree than those isolated from non-smokers.[71] Importantly, HUVECs isolated from smokers had higher intracellular levels of glutathione than those isolated from non-smokers, highlighting the fact that this pathway is central to the supply of cellular antioxidants.[71]

The effects of cellular thiols on LDL oxidation may be further enhanced by the microenvironment created by the cells themselves. Activated macrophages, found clustered within atherosclerotic lesions,[72,73] can acidify their extracellular space by extrusion of H^+[74] or lactic acid.[75] Thiol-enhancement of LDL oxidation, in the presence of iron ions, is accelerated at acidic pH,[76] possibly by increasing the solubility of Fe^{3+} or the efficiency of LDL modification[77] (reviewed in Ref. [78]).

THIOL-INDEPENDENT REDUCTION OF TRANSITION METALS BY CELLS

Human monocyte-derived macrophages,[54] macrophages[29,37,38,53,55] and rat A10 smooth muscle cells[54] all exhibit a small, but significant, amount of LDL oxidation which requires the presence of transition metals, but is not thiol-dependent. Recent evidence shows macrophages can directly reduce both iron and copper ions in the absence of L-cystine, and enhance LDL oxidation.[37,38] A proportion of cell-mediated reduction of transition metals is due to direct trans-plasma membrane electron transport (TPMET),[38] which can be regulated by hormones, cytokines and growth factors.[79] The relative contributions of thiol-dependent and -independent pathways to LDL oxidation in Hams F10 medium can only be inferred by studies in the presence or absence of L-cystine;[29,32,53,54] it is likely that the rate of LDL oxidation will be determined by the availability of redox-active metals, a process augmented by cellular production of thiols[20] (see below).

MECHANISM OF LDL OXIDATION IN HAMS F10 MEDIUM: THIOLS AND IRON IONS

Thiols (RSH) may enhance the oxidation of lipids (LH) within LDL, in the presence of free iron (the

predominant metal in Hams F10 medium) by a number of differing mechanisms. These include generation of thiyl radicals,[42,43] production of superoxide, hydrogen peroxide, or hydroxyl radicals,[44–49] and reduction of transition metal ions[50,51] (reactions (1)–(10)). Differing thiol species may also exert effects by differing mechanisms.[80] The following reactions can occur following thiol ionisation: $RSH \leftrightarrow RS^- + H^+$ (pK_a 8.4).

$$RS^- + Fe^{3+} \rightarrow RS^\bullet + Fe^{2+} \quad (1)$$

$$RS^\bullet + LH \rightarrow RSH + L^\bullet \quad (2)$$

$$RS^- + RS^\bullet \rightarrow RSSR^{\bullet-} \quad (3)$$

$$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^- \quad (4)$$

$$O_2^- + Fe^{3+} \leftrightarrow O_2 + Fe^{2+} \quad (5)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (6)^*$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^- \quad (7)$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^\bullet + OH^- \quad (8)$$

$$LO^\bullet + LH \rightarrow L^\bullet + LOH \quad (9)$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad (10)$$

*(Reaction markedly accelerated in the presence of superoxide dismutase.)

Thiyl Radicals

Formation of thiyl radicals (1) can initiate lipid peroxidation[42,43] by abstraction of hydrogen from polyunsaturated fatty acids (2), or by formation of sulphonyl radicals which can form adducts with double bonds.[42] The reactivity of thiyl radicals towards unsaturated fatty acids is increased by an increased distance between the reactive S• and the ionic groups of the attacking molecule, decreased numbers of ionic functions, and increased lipophilicity of the attacking thiyl radical.[43] However, these properties of thiyl radicals contrast directly with the demonstrated abilities of cysteine and cysteinyl derivatives to catalyze oxidation of LDL in Hams F10 medium: cysteine > homocysteine > cysteinylglycine > glutathione > N-acetylcysteine.[54] This lack of correlation with thiyl radical reactivity implies initiation of lipid peroxidation by these species is probably not the major mechanism by which cells facilitate the oxidation of LDL; an argument confirmed and extended by Santanam and Parthasarathy[57] who demonstrated cellular generation of L-cysteine was not sufficient to initiate LDL oxidation in freshly isolated LDL.

Superoxide

Thiol autoxidation results in increased production of oxidizing species (4–7), such as superoxide,[44,45] which can promote the oxidation of LDL in the presence of transition metals,[81] or redox cycle Fe^{3+} to Fe^{2+} (5). Production of superoxide by smooth muscle cells is L-cystine dependent,[32] associating superoxide production and thiol-dependent LDL oxidation. However, thiol-enhanced LDL oxidation is, at best, only partially dependent upon superoxide radicals. Superoxide dismutase (SOD) could only partially inhibit the oxidation of LDL by cysteine[54,80] and homocysteine[54] in the presence of iron[54] or copper[80] and was ineffective against glutathione-enhanced oxidation of LDL.[54,80] While Jessup *et al.*[82] have argued SOD may be an inappropriate test for the involvement of superoxide radicals in cell-mediated oxidation, due to its metal-chelating properties and to the development of a pro-oxidant activity by heat inactivation, it is difficult to argue that a lack of effect due to SOD does not imply a lack of superoxide involvement *per se* in thiol-enhanced LDL oxidation.

Superoxide dismutation generates hydrogen peroxide (6) and, via the Fenton reaction (7), hydroxyl radicals, both of which could enhance LDL oxidation. Indeed, it is possible that the lack of effect due to SOD was caused by the alternate generation of these highly reactive species. However, both cell- and thiol-enhancement of LDL oxidation are unaffected by the addition of catalase, or the hydroxyl radical scavenger, mannitol;[31,83] further, inhibition of cellular

catalase, by aminotriazole, does not increase oxidation of LDL by macrophages.[54]

Reduction of Transition Metals

The half-life of thiol autoxidation, and the susceptibility of a range of cysteinyl derivatives to iron-catalysed oxidation, correlate strongly with the extent of LDL oxidation in Hams F10 medium.[54] These findings, together with the comparative lack of firm evidence for involvement of initiating thiyl or superoxide radicals, lead to the conclusion that the primary mechanism by which thiols propagate the oxidation of LDL is by reduction of Fe^{3+} to Fe^{2+}(1).[54] The reaction of lipid hydroperoxides with Fe^{2+} is much faster ($k_2 \sim 1.5 \times 10^3 M^{-1} s^{-1}$) than their reaction with Fe^{3+}, thereby accelerating the decomposition of lipid peroxides within the LDL particle, and generating alkoxyl and peroxyl radicals which propagate lipid peroxidation (8–10). The mechanism by which LDL oxidation is facilitated by cellular thiols appears, therefore, to rely upon redox cycling of free iron, the presence of 'seeding' levels of lipid peroxides, and is essentially propagative in nature.

THIOLS AND COPPER IONS

Copper and iron are known to oxidatively modify LDL by differing mechanisms: iron-dependent, in contrast to copper dependent, oxidation of LDL, requires the presence of a physiological reductant such as enzymatically generated superoxide or a free thiol.[84,85] These differing requirements are due to the fact that LDL can mediate the reduction of copper, but not iron ions,[85] and are reflected in the greater resistance of LDL core lipids to iron-oxidation in the absence of a suitable reductant.[86] Studies performed with thiols, such as cysteine, homocysteine and glutathione, in the presence of iron or copper ions, have yielded apparently paradoxical pro- and anti-oxidant roles for thiol compounds.[54,80] Thus, homocysteine[80,87] and cysteine[57,76,87] are reported to inhibit[57,76,87] or promote[80] copper oxidation of LDL. In contrast, studies performed using iron,[76] or Hams F10 culture medium[29–36,40,54,76] indicate thiols promote, or do not affect[57] LDL oxidation. Most, if not all,[57,80] of these conflicts were resolved by a recent study indicating thiol compounds enhance the oxidation of LDL by iron, but effectively inhibit copper oxidation of LDL.[88] Copper-dependent oxidation of LDL was effectively inhibited by glutathione > homocysteine > cysteine, while iron-dependent LDL oxidation stimulated by cysteine > homocysteine > glutathione.[88] Interestingly, while cystine and homocystine disulphides did not stimulate or inhibit LDL oxidation, glutathione disulphide (GSSG) and methionine, the S-methylated derivative of homocysteine, effectively inhibited LDL oxidation by copper ions.[88] While free thiols can act as free radical scavengers,[89] the mechanism of GSSG-mediated inhibition is unknown and could be due to a non-thiol specific chelation of either Cu^{2+} or Cu^{+}.[88] It can be predicted, therefore, that cellular thiol production will accelerate iron-oxidation of LDL, but exert repressive effects in the presence of copper. In contrast, direct reduction of transition metals by macrophages (see above) will enhance both copper-, and iron-, dependent LDL oxidation;[37,38] thiol-dependent and -independent pathways of cell-mediated LDL oxidation may, therefore, exert mutually antagonistic effects in the presence of copper ions.

RELEVANCE OF THIOL RECYCLING *IN VIVO*

Cellular oxidation of LDL can be enhanced by *thiol-dependent*[29,32,53,54] and *-independent* mechanisms[37,38] (Figure 1), which can be independently regulated, and are critically dependent upon the presence of iron[29,32,53,54] and/or copper.[37,38] But do atherosclerotic lesions contain free iron or copper? Catalytically active free iron and copper ions are normally not found in the plasma or interstitial fluid, but

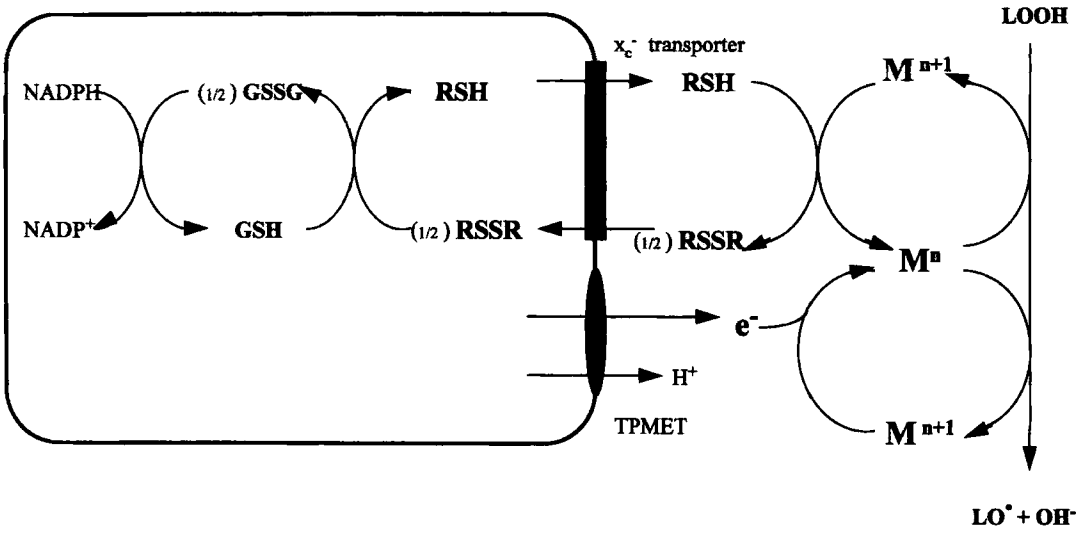

FIGURE 1 Proposed pathways by which cells can reduce transition metals, and accelerate LDL oxidation. TPMET, trans-plasma membrane electron transport; RSH, free thiol; RSSR, thiol disulphide; LOOH, lipid hydroperoxide; M^n, reduced transition metal (e.g. Fe^{2+}, Cu^+); Mn^{+1}, oxidized transition metal (e.g. Fe^{3+}, Cu^{2+}); GSH, intracellular glutathione (reduced); GSSG, intracellular glutathione (oxidized); NADP+/NADPH, nicotinamide adenine dinucleotide phosphate (oxidized/reduced, forms respectively).

bound to proteins such as transferrin,[90] albumin[91] and caeruloplasmin,[92] and are unlikely to be present in normal arterial tissue. Epidemiological studies examining the relationship between iron stores and risk for atherosclerosis have proved inconclusive;[93–95] similarly, premature atherosclerosis is not a prominent feature of iron- or copper-overload in haemachromatosis[96] or Wilson's disease,[97] respectively.

However, metal ions may become available locally, under pathological conditions such as the cellular necrosis occurring in advanced atherosclerotic lesions. Gruel extracted from human aortic atherosclerotic lesions can contain significant amounts of copper (0–28 μM) or iron ions (0–7 μM), stimulate lipid peroxidation, generate hydroxyl radicals,[98] and catalyze oxidation of LDL by macrophages.[99] Catalytically active iron and copper are also released from mechanically disrupted early lesions and from normal arterial walls.[100] However, human atherosclerotic material does not contain elevated levels of *o*-tyrosine, a specific marker of copper-mediated oxidative damage, arguing against a role for free metal ions as catalysts of LDL oxidation in the artery wall.[101] Alternatively, protein-bound metal ions may be involved in enhancing cellular oxidation of LDL;[102–104] recent evidence indicates intact caeruloplasmin can exert pro-oxidant effects,[102] and enhance the oxidation of LDL by monocytes, endothelial and smooth muscle cells.[103,104] Regulation of free iron levels in cells may also play a role in the formation of oxidized lipids in atherosclerotic tissue: atherosclerosis-susceptible strains of mice, fed a high cholesterol diet, had increased levels of intracellular iron relative to resistant strains.[105]

In conclusion, the pro- or anti-oxidant consequences of cellular thiol production appear inextricably linked with the putative catalytic action of free or protein-bound transition metals within the arterial intima. Lack of information on the composition, and transition metal content, of cellular microenvironments within the vessel wall hinder our assessment of the physiological relevance of this cellular mechanism; direct evidence is needed to demonstrate the transition-metal dependent link between induction of thiol recycling by inflammatory mediators, and lipoprotein oxidation *in vivo*.

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