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Vascular Non-protein Thiols: Prooxidants or Antioxidants in Atherogenesis?

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Abstract-cell-mediated lipoprotein oxidation may be due to generation of non-protein thiols (NP-SH) from cystine with formation of oxidizing species. However, NP-SH, especially GSH, may also exert antioxidant effects *in vitro* and *in vivo*. To further investigate whether vascular NP-SH could be prooxidants or antioxidants in atherosclerosis, we have correlated the aortic content of NP-SH with that of lipoperoxides in 10 rabbits fed on a fat-enriched atherogenic diet for 9 weeks. As compared to 7 control rabbits, aortic NP-SH and lipoperoxides were significantly increased in the fat-fed animals. The levels of NP-SH were strongly and inversely correlated with those of lipid peroxidation in the atherosclerotic aorta (r_S −0.92, $P < 0.0001$ for thiobarbituric acid reactive substances, and r_S −0.80, $P < 0.01$ for fluorescent damage products of lipid peroxidation). A similar trend was evident also in the control rabbits (r_S −0.60 for both indices of lipid peroxidation). Thus, the present data suggest that vascular NP-SH exert significant antioxidant–antilipoperoxidative effects *in vivo* especially in fat diet-related atherogenic conditions.

Keywords: Atherosclerosis, non-protein thiols, lipoprotein, lipid peroxidation, oxidative stress

INTRODUCTION

A plethora of evidence indicates that vascular oxidative stress and lipoprotein oxidation represent focal aspects of atherosclerosis.[1–3] Various mechanisms, either metal ion-dependent or -independent, have been proposed to be potentially implicated in lipoprotein oxidation.[1–3] In recent years, the problem of thiol-driven lipoprotein oxidation has emerged. This oxidation is mediated by cells typically present in atherosclerotic lesions, such as endothelial and smooth muscle cells and macrophages, and it seems related to cellular generation of non-protein thiols (NP-SH) from cystine, with formation of reactive oxygen species in the presence of redox-active metals and/or of oxidizing thiyl radicals.[4–6] Similar prooxidant mechanisms could also be involved for reduced glutathione (GSH),[7] which represents the bulk of cell NP-SH.[8] However,

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GSH has both direct and indirect antioxidant properties, these latter being related to the activity of key antioxidant enzymes, such as glutathione peroxidase.[8] Moreover, antioxidant effects of cysteine might also be operative, given its role as both radical scavenger and physiological precursor of glutathione biosynthesis in the so-called gamma-glutamyl cycle.[8] Thus, the problem of possible prooxidant or antioxidant effects of NP-SH in atherogenesis remains open, also considering that the aforementioned evidences of thiol-dependent oxidative damage arise essentially from *in vitro* studies.

To add further insight into this problem, we have investigated the relationship between the aortic levels of NP-SH and those of lipid peroxidation in an *in vivo* model of fat diet-related atherosclerosis in the rabbit. The results indicate that NP-SH may exert significant antioxidant–antilipoperoxidative effects in atherogenesis.

MATERIALS AND METHODS

Ten male New Zealand white rabbits (body weight 1.9–2.2 kg) received an atherogenic diet consisting of 89.5% standard rabbit pellets, 0.5% cholesterol, 5% lard and 5% peanut oil for 9 weeks.[9,10] The detailed composition of this diet has previously been reported.[9] In the same study we have shown that such a fat diet supplementation in the rabbit results in significant morphological changes of the aortic intimal surface, which becomes covered by sudanophilia (i.e., atherogenic lipid infiltration) for about 60% of its extension, whereas no sudanophilia is detectable in normal animals.[9]

Blood samples were obtained by ear bleeds before initiation of the assigned diet and just before sacrifice.[9,10] Plasma total cholesterol and triglycerides were determined using enzymatic commercial procedures (Boehringer Biochemia Robin, Milano, Italy).[9,10]

Reagents were from Sigma Aldrich s.r.l., Milano, Italy.

The aortas were excised from the aortic valve to the iliac bifurcation and placed in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, plus 1.0 mM EDTA (buffer A); after removing the fat, perivascular connective tissue and adventitia, they were homogenized in Chelex-100 treated and argon-flushed ice-cold 100 mM potassium phosphate buffer, pH 7.4, plus 0.5 mM EDTA (buffer B). After centrifugation at 400× *g* for 10 min, the relative supernatant was used for the assay of NP-SH and lipid peroxidation.

NP-SH, of which GSH represents the main biochemical pool, were measured spectrophotometrically at 412 nm, after homogenate treatment with 4% sulphosalicylic acid to precipitate proteins and reaction of the relative supernatant with 0.8 mM 5,5′dithiobis-2-nitrobenzoic acid (Ellman's reagent) in 0.2 M potassium phosphate buffer, pH 8.5, plus 1.0 mM EDTA.[9–11] Values were calculated as nmoles NP-SH/g tissue, using a molar extinction coefficient of 13,600.[9–11] Recovery of GSH added to buffer B before aortic tissue homogenization was total.

To improve specificity, lipid peroxidation was investigated through two different indices, namely thiobarbituric acid reactive substances (TBARS) and fluorescent damage products of lipid peroxidation (FDPL). TBARS were measured spectrophotometrically according to the method of Ohkawa *et al.*[12] as previously reported.[9,10] The pink chromogen was extracted with n-butanol plus pyridine (15 : 1, v/v) and read at 532 nm against an appropriate blank. Values were calculated as nmol TBARS/g tissue, using a molar extinction coefficient of 154,000. It should be noted that this method can detect, besides malondialdehyde, monofunctional aldehydes, such as alkenals and alkadienals, which represent the bulk of aldehydic by-products of lipid peroxidation in tissue systems.[13,14]

FDPL, which are sensitive indicators of lipid peroxidation *in vivo* and tend to remain at oxidant burden sites,[15,16] were assessed after homogenate lipid extraction with chloroform/methanol (2 : 1, v/v) and water addition to separate aqueous

and chloroform phases.[11,15] The organic layer was dried under a stream of argon, resuspended in chloroform and subjected to spectrofluorometric study at 360 nm excitation and 430 nm emission. Results are given as units of relative fluoresence (URF)/g tissue.

Results were calculated as means ± SEM, and analyzed by Wilcoxon's test and Mann–Whitney *U* test, where appropriate;[17] correlations were studied by Spearman rank correlation coefficient (r_S) calculation,[17] with a *P* value less than 0.05 being considered as statistically significant.

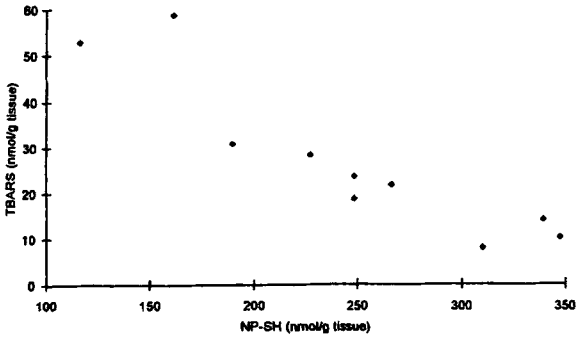

FIGURE 1 Scatterplot showing the relationship between the levels of NP-SH and those of TBARS in the aortic tissue of fat-fed rabbits; r_S −0.92, $P < 0.0001$.

RESULTS AND DISCUSSION

After 9 weeks of a hyperlipidic diet, plasma cholesterol and triglycerides values rose significantly from 49 ± 13 and 57 ± 14.5 mg/dl to 1090 ± 140 and 595 ± 97 mg/dl, respectively ($P < 0.05$).

As compared to 7 control rabbits, the levels of aortic NP-SH were increased in the fat-fed animals (245 ± 23.8 vs 141.6 ± 8.3 nmol/g tissue, $P = 0.01$), as were those of both TBARS and FDPL (27 ± 5.4 vs 11.5 ± 1.05 nmol TBARS/g tissue, $P < 0.025$, and 206.5 ± 14.5 vs 125 ± 11.8 URF/g tissue, $P < 0.01$). There was a strong inverse correlation between the levels of NP-SH and those of lipoperoxides detected in the aortic tissue of fat-fed rabbits (r_S −0.92, $P < 0.0001$ for TBARS, Figure 1; r_S −0.80, $P < 0.01$ for FDPL). A similar trend was evident also in the control rabbits, although the level of statistical significance was not reached probably as a consequence of the relatively small number of animals studied (r_S −0.60 for both TBARS and FDPL). These data therefore suggest that vascular NP-SH exert a significant antilipoperoxidative activity *in vivo* especially in fat diet-related atherogenic conditions.

It could be speculated that the more the lipid in the arterial wall the less thiol will be, but the more lipoperoxide. In this regard, however, we have previously shown that both GSH and lipoperoxides are significantly increased in the rabbit aorta after only 10 days supplementation of the same fat-enriched diet used in the present study, in spite of the absolute absence of aortic lipid infiltration;[9] similar results have recently been reported with other fat-enriched atherogenic diets.[18] Moreover, an inverse relationship between the levels of NP-SH and those of lipoperoxides was also present in the control rabbits, which lack aortic lipid infiltration.[9] These data indicate that vascular levels of NP-SH and lipoperoxides are not simply related to the degree of vascular lipid infiltration.

After initial evidence of a possible prooxidant role of cell NP-SH in lipoprotein oxidation, Santanam and Parthasarathy[19] have recently shown that cellular cysteine generation does not contribute to the initiation of lipoprotein oxidation itself, and that both cysteine and GSH can effectively act as powerful antioxidants against metal-dependent and -independent lipoprotein peroxidation. These authors have suggested that thiol depletion may adversely affect the redox status of the cells and favor oxidative atherogenic processes, whereas increased extra- and intracellular thiol levels could exert relevant antioxidant–antiatherogenic effects. Our results obtained in an *in vivo* model of fat diet-induced atherosclerosis in the rabbit are apparently in line with such a point of view, indicating a significant

antilipoperoxidative activity of NP-SH in the vascular tissue. GSH represents a crucial antioxidant *in vivo*, since it favors vitamin E regeneration[20] and the activity of key enzymes, such as glutathione peroxidase and transferase, able to remove peroxides and aldehydic by-products of lipid peroxidation involved in atherogenic processes.[1–3,8] Moreover, GSH has direct antioxidant effects related to scavenging of oxidant species and complexation-inactivation of catalytically-active transition metals, especially copper.[8,21,22] Cysteine may also behave as a radical scavenger and it can augment the GSH pool by acting as a physiological precursor of cell GSH biosynthesis.[8,19]

We and other authors,[9,18,23] have shown increased GSH levels and glutathione peroxidase hyperactivity, associated with lipoperoxide burden, in the atherosclerotic aorta of fat-fed rabbits. Whether such increased GSH levels could reflect lipoperoxide-related *de novo* biosynthesis as suggested by Darley-Usmar *et al.*[24] for macrophages remains to be elucidated. In spite of vascular antioxidant activation, aortic atherosclerotic lesions and lipid peroxidation become nonetheless apparent in the hyperlipidemic animals, possibly reflecting an inadequate antioxidant capacity of GSH and the GSH redox-cycle to cope with a fat diet-related oxidant burden at vascular level. Indeed, the glutathione redox-cycle plays a pivotal role in biomolecule antioxidant protection especially in vascular parietal cells.[25–29] In such a context, it is noteworthy that lipoprotein oxidation seems to represent essentially a local event occurring in the arterial wall environment,[1–3] and it may be so modulated by the endogenous antioxidant defences. These aspects and our experimental data suggest that administration of GSH biochemical precursors without inhibitory effects on GSH biosynthesis, such as gamma-glutamyl cysteine,[8] could potentially be helpful at further enhancing GSH-related antioxidant capability of the arterial wall against oxidative atherogenic injury. This issue deserves further specific investigations.

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