ELSEVIER

FEBS Letters 342 (1994) 49–52

FEBS 13834

FEBS LETTERS

Peroxynitrite releases copper from caeruloplasmin: implications for atherosclerosis

Jason A. Swain[a], Victor Darley-Usmar[b], John M.C. Gutteridge[a,*]

[a]*Unit of Critical Care, Department of Anaesthesia and Intensive Care, Royal Brompton Hospital National Heart & Lung Institute, Sydney Street, London, SW3 6NP, UK*

[b]*Department of Biochemical Sciences, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, UK*

Received 2 February 1994; revised version received 17 February 1994

Abstract

Peroxynitrite may be formed in the vasculature by the reaction of superoxide with nitric oxide. When the blue copper-containing protein, caeruloplasmin, is incubated with peroxynitrite, copper is released, and ferroxidase activity and the blue colouration are lost. When plasma from normal subjects is incubated with peroxynitrite, the oxidant reacts with numerous plasma constituents but is still able to release copper from caeruloplasmin. As the ferroxidase activity of caeruloplasmin is lost in plasma in the presence of peroxynitrite, a second ferroxidase activity associated with peroxidised lipids, and not inhibited by azide, is formed.

Key words: Peroxynitrite; Caeruloplasmin; Reactive plasma copper; Atherosclerosis; Ferroxidase activity

1. Introduction

Oxidation of low-density lipoproteins (LDL) is thought to occur in the artery wall and results in a modified form of LDL recognised by the macrophage scavenger receptor [1–4]. Uptake of LDL modified in this way is not down-regulated and results in the formation of lipid-laden foam cells frequently associated with early atherosclerotic lesions [5].

The lipid moiety of LDL contains polyunsaturated fatty acids which are normally protected by strategically placed antioxidants. These may, however, be overwhelmed, leading to lipid peroxidation and oxidative modification of LDL protein [4].

Copper salts are extremely effective pro-oxidants in vitro for oxidatively modifying LDL [6]. They readily decompose lipid peroxides to generate aldehydic intermediates [4,6], and it is these aldehydes which are believed to play a major role in vivo in altering LDL protein structure leading to scavenger receptor recognition [7].

Plasma does not normally contain chelatable forms of copper able to participate in lipid peroxidation reactions [8]. Storage or mishandling of plasma, however, can cause such copper to be released from caeruloplasmin which stimulates peroxidation of plasma lipids to form a copper–lipoprotein complex with ferroxidase activity [8,9].

We show here that peroxynitrite is extremely effective in vitro at releasing copper from caeruloplasmin, even when the protein is in its normal plasma environment, and suggest that similar mechanisms may occur in vivo.

2. Materials and methods

1,10-phenanthroline, calf thymus DNA, apotransferrin and caeruloplasmin (human) were obtained from the Sigma Chemical Co., Poole, Dorset. All other reagents were of the highest purity available from Fisons Scientific Equipment, Loughborough, Leics., UK. Caeruloplasmin radial immunodiffusion plates were obtained from Behring-Hoechst, Middx., UK.

2.1. Caeruloplasmin

Caeruloplasmin obtained as a solution containing 50 mg/ml was further purified by passing it through a column (5 × 0.5 cm) of Sephadex G10, and eluting with 0.15 M NaCl, pH 7.4. Protein concentration was determined by radial immunodiffusion, and application of the molar extinction coefficient for caeruloplasmin (10^{-4} $M^{-1} \cdot cm^{-1}$) at $A_{610\ nm}$. Commercially obtained caeruloplasmin treated as above still contained trace amounts of phenanthroline-chelatable copper (see Table 1).

2.2. Peroxynitrite synthesis

Based on the method previously described [10], equal volumes of 2 M $NaNO_2$ and 2.5 M H_2O_2 in 2.9 M HNO_3 were mixed by syringe on ice. The mixture (12 ml) was stabilized by the addition of 6.6 ml 4.2 M NaOH. Unreacted H_2O_2 was removed by passing the solution over MnO_2. The resulting peroxynitrite was stored frozen at −20°C, and only the top yellow layer retained for use. The concentration of peroxynitrite was determined by applying a molar extinction coefficient of 1,670 $M^{-1} \cdot cm^{-1}$ at $A_{302\ nm}$ against an appropriate blank prepared from peroxynitrite in 0.1 M potassium phosphate buffer, pH 7.4. The latter procedure was also used for the preparation of peroxynitrite decomposition products.

* Corresponding author.

SSDI 0014-5793(94)00219-L

2.3. Ferroxidase activities

Total ferroxidase (Fox) activity of plasma was measured kinetically at 30°C as the oxidation of a ferrous salt to the ferric state and its loading onto apotransferrin to form the pink diferric transferrin absorbing at 460 nm [11]. Ferroxidase 1 activity was that part of the total activity inhibited by 1 mM azide. Activity is expressed as IU/ml.

2.4. Measurement of chelatable copper

Phenanthroline-chelatable copper was determined as previously described [8] and quantitated with reference to standards of cupric chloride by measuring the amount of malondialdehyde released from DNA by the phenanthroline–copper complex in the presence of the reducing agent, mercaptoethanol. Controls were introduced to assess the amount of TBA-reactive material arising in incubated plasma that did not come from the specific DNA reaction with copper–phenanthroline.

2.5. Incubation of native caeruloplasmin and plasma with peroxynitrite

Caeruloplasmin (3.6 μmol/l) was incubated for 90 min at 37°C with 1.5 mmol/l peroxynitrite in 0.1 M potassium phosphate buffer, pH 7.4, and compared with a control incubated with decomposed peroxynitrite. Plasma (0.375 ml in the incubation mixture volume of 1 ml) was similarly incubated for 90 min at 37°C with peroxynitrite.

2.6. Total plasma thiobarbituric acid reactivity

200 μl of plasma sample incubated with peroxynitrite was added to 0.5 ml of 1% (w/v) thiobarbituric acid in 0.05 M NaOH and 0.5 ml 28% (w/v) trichloroacetic acid. The mixture was heated at 100°C for 10 min. When cool, 3 ml of butan-1-ol was added, and the tube contents vortex-mixed before centrifugation at 3,000 rpm for 6 min. TBA reactivity in the upper organic layer was measured spectrofluorimetrically against malondialdehyde (MDA) standards prepared from 1,1,3,3-tetramethoxypropane similarly treated.

3. Results

Incubation of native caeruloplasmin with increasing concentrations of peroxynitrite caused a progressive release of chelatable copper, with a concentration of 1.5 mmol/l peroxynitrite releasing all the copper (25.1 μM) present in caeruloplasmin (Fig. 1 and Table 1). Coinciding with copper release from caeruloplasmin was loss of ferroxidase 1 activity, and loss of antibody recognition in a radial immunodiffusion assay (Table 1). Spectral changes over this time-course showed a loss of absorbance at 610 nm and an increase at 460 nm (Fig. 2). These changes were associated with copper release from caeruloplasmin and loss of ferroxidase activity.

Freshly taken plasma (heparinised) from three normal subjects was pooled for analysis. In agreement with previous studies [12] the plasma had a caeruloplasmin concentration of 2.28 ± 0.27 μmol/l, a ferroxidase activity of 0.780 ± 0.075 IU/ml and no phenanthroline-chelatable copper present. Incubation of plasma (diluted 1 in 2.7 in the reaction) with peroxynitrite (1.5 mmol/l) at 37°C for 90 min caused substantial release of phenanthroline-chelatable copper (Table 2) compared with controls. Simultaneously, ferroxidase 2 activity (copper-oxidised lipoprotein) increased (Table 2). Evidence consistent with oxidation of plasma lipids during incubation with peroxynitrite, was provided by the large increase in thiobarbituric acid-reactive substances compared to the controls (Table 2).

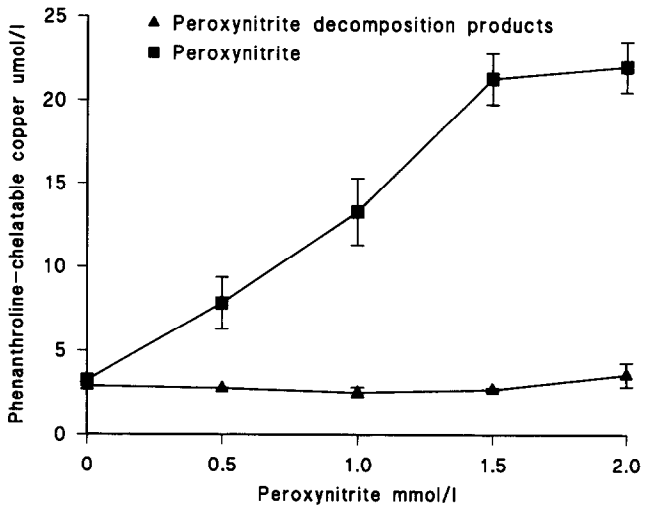

Fig. 1. Phenanthroline-chelatable copper (μmol/l) released from caeruloplasmin following incubation at 37°C for 90 min with increasing (mmol/l) concentrations of peroxynitrite.

4. Discussion

Copper salts are particularly effective in modifying isolated low density lipoproteins for recognition by the

Table 1
The effect of peroxynitrite (1.5 mmol/l) on native caeruloplasmin

	Caeruloplasmin (3.6 μmol/l)	Caeruloplasmin (3.6 μmol/l) + decomposition products of peroxynitrite	Caeruloplasmin (3.6 μmol/l) + peroxynitrite
Phenanthroline-chelatable copper (μmol/l)	3.6 ± 0.2	3.7 ± 0.7	26.5 ± 1.2*
Total ferroxidase activity (IU/ml)	0.718 ± 0.029	0.697 ± 0.027	0.030 ± 0.011*
Ferroxidase 1 (IU/ml)	0.679 ± 0.036	0.659 ± 0.032	0.001 ± 0.016*
Ferroxidase 2 (IU/ml)	0.038 ± 0.008	0.038 ± 0.006	0.029 ± 0.006
Immunoreactive caeruloplasmin (μmol/l)	3.5 ± 0.1	3.5 ± 0.1	0.0 ± 0.0*

Values shown are concentrations in the reaction and are the means ± SD, $n = 3$, Student's t-test, $^*P < 0.001$. All samples were incubated at 37°C for 90 min.

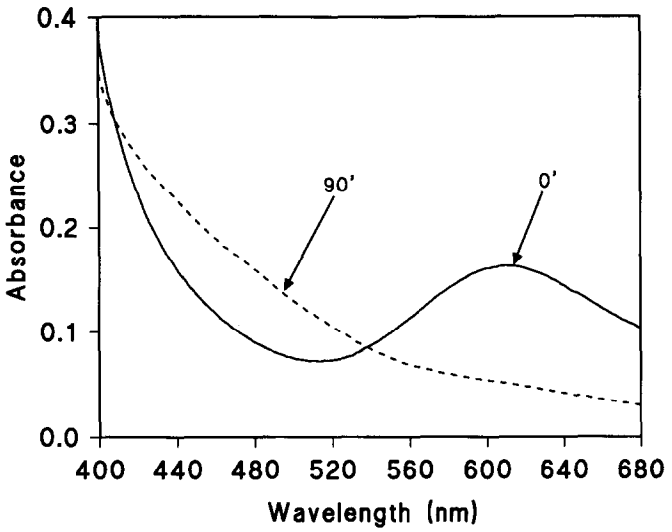

Fig. 2. Spectral changes (400–680 nm) in caeruloplasmin (5 μmol/l) incubated with peroxynitrite (1.5 mmol/l) from 0–90 min.

acetyl, or scavenger, receptor of macrophages [6]. This may in part be explained by the ability of copper to decompose lipid peroxides to form aldehydes [13], and perhaps direct interaction with the protein. Low molecular mass copper complexes, however, with redox active properties towards organic and inorganic oxygen radical reactions, are not present in freshly taken plasma or serum from normal healthy individuals [8]. Such copper, chelatable to 1,10-phenanthroline, does arise, however, when plasma is stored or mishandled [9], and can degrade DNA when redox cycled in the phenanthroline assay [8]. Almost all the copper present in plasma taken from normal healthy subjects is structurally part of the protein, caeruloplasmin, which contains some 6–7 copper ions per molecule. This copper is essential to the ferroxidase activity (oxidation of ferrous ions to the ferric state) of caeruloplasmin and, when released, causes oxidation of plasma lipoproteins and the appearance of a second ferroxidase (Fox 2) [9,14,15] with an activity not inhibited by azide [16]. When native caeruloplasmin is incubated with peroxynitrite, substantial amounts of copper are released from the protein that can be chelated by 1,10-phenanthroline and redox-cycled to damage DNA. As expected, copper loss caused a decrease in ferroxidase activity, and a loss of caeruloplasmin's characteristic blue colour ($A_{610\ nm}$). Since lipoproteins were not present, ferroxidase 2 activity was not increased. Incubation of freshly taken normal pooled plasma with peroxynitrite also caused release of phenanthroline-chelatable copper, representing 24% of the total copper present, in spite of the fact that peroxynitrite reacts with many other plasma constituents. The copper released in plasma caused the oxidation of plasma lipoproteins, increasing the amount of ferroxidase 2 activity as well as TBA-reactive material, although the latter is a non-specific measure of lipid peroxidation.

Our studies show that peroxynitrite, formed by the reaction of superoxide and nitric oxide, has the potential to release chelatable redox active copper from the major plasma copper-containing protein caeruloplasmin. Peroxynitrite may contribute to the pathogenesis of atherosclerosis by modifying LDL directly, as well as shown here by releasing copper from caeruloplasmin, which would then promote lipid peroxidation [17–19]. Whether peroxynitrite is formed in the vasculature is still unclear, although indirect evidence supports the hypothesis that superoxide and nitric oxide are formed simultaneously in atherosclerotic lesions [20,21]. This study supports the idea that caeruloplasmin is a likely source of the redox-active copper recently detected in atherosclerotic post-mortem material [22], and this may have arisen as a consequence of direct damage by peroxynitrite.

Acknowledgements: J.A.S. holds a British Heart Foundation PhD Studentship. J.M.C.G. thanks the British Lung Foundation, the British Oxygen Group and the British Heart Foundation for their financial support. We thank Professor Barry Halliwell for his careful reading of our manuscript.

Table 2
The effect of peroxynitrite (1.5 mM) on human plasma

	Plasma before incubation	Plasma after incubation	Plasma + decomposition products of peroxynitrite	Plasma + peroxynitrite
Phenanthroline-chelatable copper (μmol/l)	0	0.1 ± 0.0	0.2 ± 0.1	1.3 ± 0.4**
Total ferroxidase activity (IU/ml)	0.292 ± 0.028	0.267 ± 0.045	0.275 ± 0.022	0.198 ± 0.028***
Ferroxidase 1 (IU/ml)	0.270 ± 0.013	0.248 ± 0.029	0.262 ± 0.006	0.143 ± 0.039**
Ferroxidase 2 (IU/ml)	0.022 ± 0.014	0.019 ± 0.017	0.013 ± 0.017	0.055 ± 0.011
Thiobarbituric acid-reactive substances (TBARS) μmol/l)	0.54 ± 0.18	0.44 ± 0.20	0.41 ± 0.14	1.64 ± 0.34**

Values shown are reaction concentrations as means ± SD. Student's *t*-test, $n = 3$, *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$. All samples were incubated at 37°C for 90 min. TBARS are expressed as malondialdehyde equivalents.

References

[1] Brown, M.S. and Goldstein, J.L. (1983) Annu. Rev. Biochem. 52, 223–261.
[2] Witzum, J.L. and Steinberg, D. (1991) J. Clin. Invest. 88, 1785–1792.
[3] Yla-Hertuala, S. (1991) Ann. Med. 23, 561–567.
[4] Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) Free Rad. Biol. Med. 13, 341–390.
[5] Parthasarthy, S., Printz, D.J., Boyd, D., Joy, L. and Steinberg, D. (1986) Arteriosclerosis 6, 505–510.
[6] Esterbauer, H., Striegl, G., Puhl, H. and Rotheneder, M. (1989) Free Rad. Res. Commun. 6, 67–75.
[7] Haberland, M.E., Oleh, C.L. and Fogelman, A.M. (1984) J. Biol. Chem. 259, 11305–11311.
[8] Gutteridge, J.M.C. (1984) Biochem. J. 218, 983–985.
[9] Gutteridge, J.M.C., Winyard, P., Blake, D.R., Lunec, J., Brailsford, S. and Halliwell, B. (1985) Biochem. J. 230, 517–523.
[10] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Proc. Natl. Acad. Sci. USA. 87, 1620–1624.
[11] Johnson, D.A. Osaki, S and Frieden, E. (1967) Clin. Chem. 13, 142–150.
[12] Gutteridge, J.M.C. and Quinlan, G.J. (1993) Biochim. Biophys. Acta 1156, 144–150.
[13] Gutteridge, J.M.C. (1984) Mech. Ageing Dev. 25, 205–214.
[14] Topham, R.W. and Frieden, E. (1970) J. Biol. Chem. 245, 6698–6705.
[15] Sung, C.S.M. and Topham, R.W. (1973) Biochem. Biophys. Res. Commun. 53, 824–829.
[16] Williams, D.M., Christensen, D.D., Lee, G.R. and Cartwright, G.E. (1974) Biochim. Biophys. Acta 350, 129–134.
[17] Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T. and Moncada, S. (1992) Free Rad. Res. Commun. 17, 9–20.
[18] Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V.J., Darley-Usmar, V. and Moncada, S. (1993) FEBS Lett. 330, 181–185.
[19] Hogg, N., Darley-Usmar, V.M., Graham, A. and Moncada, S. (1993a) Biochem. Soc. Trans. 21, 358–362.
[20] Ohara, Y., Peterson, T.E. and Harrison, D.G. (1993) J. Clin. Invest. 91, 2541–2551.
[21] Mugge, A., Elwell, J.H., Peterson, T.E., Hofmeyer, T.G., Heistad, D.D. and Harrison, D.G. (1991) Circ. Res. 69, 1293–1300.
[22] Smith, C., Mitchinson, M.J., Aruoma, O. and Halliwell, B. (1992) Biochem. J. 286, 901–905.