Biochem. J. (1981) **199**, 259–261
Printed in Great Britain

Inhibition of lipid peroxidation by the iron-binding protein lactoferrin

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(*Received 12 May 1981/Accepted 29 June 1981*)

Lactoferrin containing physiological amounts of iron is an inhibitor of lipid peroxidation induced by iron(III) salts and ascorbic acid. It might therefore help to protect neutrophils, inflammatory foci and secretions from metal-ion-dependent oxidative damage.

The iron-binding protein lactoferrin shares many common physical and chemical properties with transferrin. For example, both proteins bind two molecules of Fe(III) ion per molecule of protein with high affinity. Lactoferrin is, however, immunologically distinct from transferrin, and it occurs at both intracellular and extracellular locations, at which it has been suggested to act as an antibacterial agent because it binds iron essential to the growth of bacteria (Spitznagel *et al.*, 1972; Breton-Gorius *et al.*, 1980).

When neutrophils phagocytose bacteria, particles or immune complexes they show a rapid 'burst' of oxygen uptake associated with the production of oxygen-derived species such as superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) (Babior, 1978). In the presence of traces of iron salts, $O_2^{-\cdot}$ and H_2O_2 react together to form the hydroxyl radical $OH^{\cdot}$, which can attack and damage almost all biological molecules and induce lipid peroxidation (Halliwell *et al.*, 1980). It has been suggested that the $OH^{\cdot}$ that is generated when neutrophils engulf particles (Green *et al.*, 1979) is derived from the interaction of $O_2^{-\cdot}$ and H_2O_2 catalysed by iron-saturated lactoferrin (Ambruso & Johnston, 1981), which would make this protein an important component of the bacteria-killing mechanism. Release of lactoferrin from neutrophils into the surrounding medium would thus be expected to give rise to $OH^{\cdot}$-dependent damage and potentiate the inflammatory response and lipid peroxidation (Gutteridge *et al.*, 1979; Wong *et al.*, 1981).

However, in neutrophils (Bullen & Armstrong, 1979), as in milk (Fransson & Lönnerdal, 1980), lactoferrin has only a low iron content, well below saturation. In the present paper we show that partially iron-saturated lactoferrin has antioxidant properties, in that it can inhibit lipid peroxidation initiated by iron salts. Fe(II) ions, or Fe(III) ions in the presence of a biological reducing agent such as ascorbate, are powerful inducers of lipid peroxidation. Both free iron ions and ascorbate are readily available *in vivo* (Fong *et al.*, 1976; Wong *et al.*, 1981).

Materials and methods

Preparation of lactoferrin

Human breast milk samples, collected daily from several mothers at a nearby maternity hospital, were kept frozen at −20°C until needed. Lactoferrin was isolated on a heparin–Sepharose column by the method of Blackberg & Hernell (1980). After extensive dialysis against 20mM-NH_4HCO_3, the protein was freeze-dried and stored desiccated at 4°C. Polyacrylamide-gel electrophoresis at pH 8.6 gave a single band, which had a slightly slower mobility than transferrin. The isolated lactoferrin gave single precipitin lines with lactoferrin antibody (raised in rabbits by using commercially obtained lactoferrin). This antibody did not give precipitin lines with transferrin or whole serum. The iron saturation of the isolated protein was 0–4%, as determined by the method of Masson & Heremans (1968).

Other proteins

Human apotransferrin was obtained from Behring, Hoechst Pharmaceuticals (Hounslow, Middx., U.K.), and albumin (bovine RIA grade) was from Sigma Chemical Co. (Poole, Dorset, U.K.).

Iron saturation of proteins

Iron was added as $Fe(NH_4)_2(SO_4)_2$ to albumin, transferrin and lactoferrin (approx. 10mg of protein/ml in 0.2M-Tris/HCl/10mM-$NaHCO_3$ buffer,

0306-3275/81/100259-03$01.50/1

pH 7.3) to give the required iron saturation (20 or 100%) (Workman *et al.*, 1975). For these proteins 100% iron saturation was taken to be 1.4 μg of iron/mg of protein.

Lipid peroxidation

Bovine brain liposomes were prepared and their peroxidation, induced by 8 μM-$FeNH_4(SO_4)_2$ and 38 μM-ascorbate, was measured (by the thiobarbituric acid method) as described by Gutteridge (1977). Desferrioxamine methanesulphate (Desferal) was from CIBA (Horsham, West Sussex, U.K.). Each reaction mixture contained 2.5 mg of phospholipid liposomes suspended in 0.15 M-NaCl prepared in Chelex-treated water, pH 7.4.

Results

Addition of Fe(III) ions and ascorbate at physiological concentrations induced lipid peroxidation of bovine brain phospholipid liposomes, as followed by the thiobarbituric acid method. In agreement with previous results, peroxidation was strongly inhibited by the antioxidant propyl gallate and by the Fe(III)-ion chelator desferrioxamine (Gutteridge, 1977; Gutteridge *et al.*, 1979) (Table 1).

Apolactoferrin [containing no bound Fe(III)] was a powerful inhibitor of lipid peroxidation. This can be attributed to its iron-binding capacity, since iron-saturated lactoferrin had no inhibitory effect. Apoprotein 20% saturated with iron, which is approximately the physiological degree of saturation (Bullen & Armstrong, 1979; Fransson & Lönnerdal, 1980), was also inhibitory. Transferrin had similar effects to lactoferrin on lipid peroxidation, but albumin carried through the same procedures used to saturate these proteins with iron had no inhibitory effect. Inhibition is thus a specific property of these iron-binding proteins.

Table 1. *Effect of proteins on lipid peroxidation induced by ascorbate plus Fe(III) ions*

The results of a typical experiment are presented, but they were highly reproducible. The concentrations stated are the final concentrations in the reaction mixture. 'Iron-loaded' albumin was obtained by carrying bovine serum albumin through the procedures used to load lactoferrin with iron.

Addition to reaction mixture	Rate of peroxidation [thiobarbituric acid reaction (A_{532})] after 2 h incubation
None	0.50
Propyl gallate (76 μM)	0.02
Desferrioxamine (154 μM)	0.07
Lactoferrin (690 μg/ml)	
Apoprotein	0.08
20% saturated with iron	0.08
100% saturated with iron	0.52
Transferrin (770 μg/ml)	
Apoprotein	0.08
20% saturated with iron	0.10
100% saturated with iron	0.54
Albumin (770 μg/ml)	
Not iron-treated	0.54
20% saturated with iron	0.56
100% saturated with iron	0.58

Discussion

Both lactoferrin and transferrin at physiological degrees of iron saturation are inhibitors of iron-dependent lipid peroxidation. This is due to their iron-binding capacity, since saturation of the proteins with iron abolished their inhibitory effect, and, indeed, might have caused them to have a pro-oxidant effect (Table 1), although the effects were small and might have been due to traces of 'free' iron, loosely bound to non-specific sites on the protein, that had not been removed during preparation. Secretion of lactoferrin from neutrophils in large amounts during phagocytosis might therefore be a mechanism by which surrounding tissues are protected against damage. This may be particularly important during inflammatory joint disease, since synovial fluid from patients with such disease contains 'free' iron at micromolar concentrations and swarms with phagocytic cells (Wong *et al.*, 1981; Gutteridge *et al.*, 1981). Lactoferrin in milk might play a role in preventing peroxidation of the milk lipids and the resulting rancidity.

We thank the Wellcome Trust, the Cancer Research Campaign and the Medical Research Council for financial support.

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