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SHORT COMMUNICATIONS

Oxidative damage to lipids and α_1-antiproteinase by phenylbutazone in the presence of haem proteins: protection by ascorbic acid

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Abstract—Phenylbutazone is an anti-inflammatory drug with numerous side-effects that restrict its clinical use. In the presence of myoglobin, or of haemoglobin plus H_2O_2, phenylbutazone accelerates the peroxidation of lipids (arachidonic acid and rat liver microsomes) and causes the inactivation of α-antiproteinase, so that this protein can no longer inhibit elastase. We propose that haem proteins oxidize phenylbutazone into a damaging free radical. Ascorbic acid inhibits these pro-oxidant actions of phenylbutazone.

Non-steroidal anti-inflammatory drugs (NSAIDs*), mainly developed as inhibitors of cyclooxygenase [1], are still frequently used as analgesics, and in the treatment of inflammatory joint disease [1–3]. Phenylbutazone was one of the first NSAIDs to be introduced, in the 1950s. Although phenylbutazone is effective in diminishing the symptoms of rheumatoid arthritis, it can produce serious side-effects [3]. Its therapeutic profile is unusual in that it is especially effective against ankylosing spondylitis, but its side-effects mean that it is not an agent of choice in the treatment of rheumatoid arthritis or osteoarthritis [3]. Despite this, phenylbutazone is still widely sold "over the counter" in many countries.

The unusual therapeutic profile of phenylbutazone suggests that it may have actions additional to cyclo-oxygenase inhibition. Oxygen-derived species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and possibly hydroxyl radical ($^{\cdot}OH$) are generated at sites of inflammation and tissue injury and may aggravate the damage taking place (reviewed in Refs 4 and 5). This is especially true in inflamed rheumatoid joints and in injured brain, where traumatic microbleeding and release of intracellular iron from injured cells can raise the iron content of the injured area [5–7]. Hydrogen peroxide produced at a site of injury (e.g. by activated phagocytic cells) might interact with haemoglobin to produce oxidants that stimulate lipid peroxidation [8–10]. In addition, excess H_2O_2 can release iron ions from the haem ring in a form that can stimulate lipid peroxidation and other free radical reactions [9, 11].

Myoglobin also reacts with H_2O_2 to form oxidants that stimulate lipid peroxidation [10], and excess H_2O_2 again releases iron ions from the haem [12]. These reactions of myoglobin may contribute to free radical damage during myocardial reperfusion injury [13]. In addition, muscle injury as a result of violent exercise or during the "crush syndrome" can release myoglobin into the circulation, where it could interact with any H_2O_2 available to cause oxidative damage [13–15].

In the present paper, we have studied the influence of phenylbutazone upon oxidative damage caused by myoglobin/H_2O_2 and haemoglobin/H_2O_2 systems. Damage both to lipids and to α_1-antiproteinase was studied. The latter is the most important inhibitor of serine proteinases (especially elastase) in human body fluids, yet it is highly susceptible to oxidative inactivation, thus accelerating proteolytic damage at sites of injury [16].

* Abbreviations: NSAID, non-steroidal anti-inflammatory drug; TBA, thiobarbituric acid; SOD, superoxide dismutase.

Materials and Methods

Reagents. All reagents, including α_1-antiproteinase (type A9024), catalase (type C100), erythrocyte copper–zinc superoxide dismutase (SOD) and phenylbutazone, were from the Sigma Chemical Co. (Poole, U.K.). Oxy- and met-haemoglobin were prepared and purified by gel filtration and concentrations determined as in Refs 9 and 12. Units of catalase are μmol H_2O_2 decomposed/min under the reaction conditions described in the Sigma catalogue. Units of SOD are as defined by the cytochrome *c* assay [17]. Rat liver microsomes were obtained by differential pelleting and washed as described in Ref. 18.

Assays of lipid peroxidation. Reaction mixtures (1 mL final volume) contained (unless otherwise stated) 25 mM NaH_2PO_4/Na_2HPO_4 buffer pH 7.4, 0.4 mM arachidonic acid or 0.25 mg/mL of rat liver microsomal protein, 50 μM (as haem) myoglobin or haemoglobin, and 0.5 mM H_2O_2. They were incubated at 37° for 10 min (arachidonic acid) or 20 min (microsomes). Phenylbutazone was added to give the final concentrations stated. Peroxidation was measured by the thiobarbituric acid (TBA) test in the presence of butylated hydroxytoluene (to suppress any peroxidation during the test itself) as described in Ref. 18.

Assays of α_1-antiproteinase and elastase were carried out as in Ref. 19.

Results

Stimulation of myoglobin-dependent oxidative lipid damage by phenylbutazone. As expected [8, 10], a mixture of myoglobin and H_2O_2 stimulated peroxidation of arachidonic acid, as measured by the TBA test. The amount of peroxidation varied somewhat depending on the batch of commercial arachidonic acid used, but the effects of phenylbutazone and antioxidants were always consistent. Phenylbutazone stimulated the peroxidation of arachidonic acid by myoglobin/H_2O_2 to an extent increasing with phenylbutazone concentration up to 0.5 mM (e.g. see Table 1). Even when H_2O_2 was omitted, phenylbutazone caused arachidonic acid peroxidation in the presence of myoglobin (Table 1), the effect again being maximal at 0.5 mM phenylbutazone. Peroxidation in all three systems was inhibited by ascorbic acid, the iron ion chelator desferrioxamine and, to a lesser extent, by cysteine (Table 1). These molecules may be preferentially oxidized by activated haem proteins [20–22]. Mixing myoglobin with H_2O_2 caused formation of a ferryl myoglobin [12] spectrum (Fig. 1), which was stable for at least 20 min. Adding phenylbutazone caused a rapid loss of this spectrum.

Peroxidation of arachidonic acid in the presence of myoglobin and phenylbutazone (no H_2O_2) was not significantly inhibited by addition of catalase (500 U/mL), SOD (400 U/mL) or bovine serum albumin (0.5 mg/mL).

Table 1. Stimulation by phenylbutazone (PB) of arachidonic acid (AA) or microsomal (MIC) peroxidation in the presence of myoglobin (MB)

Reaction mixture	Amount of peroxidation A_{532}
AA/MB (blank)	0.00
AA/MB/H_2O_2	0.18
AA/MB/H_2O_2/cysteine (5 mM)	0.12
AA/MB/H_2O_2/desferrioxamine (500 μM)	0.07
AA/MB/H_2O_2/ascorbate (500 μM)	0.04
AA/PB	0.00
MB/PB	0.00
AA/MB/PB	0.62
AA/MB/PB/cysteine	0.13
AA/MB/PB/desferrioxamine	0.08
AA/MB/PB/ascorbate	0.12
AA/MB/PB/H_2O_2	0.54
AA/MB/PB/H_2O_2/cysteine	0.23
AA/MB/PB/H_2O_2/desferrioxamine	0.22
AA/MB/PB/H_2O_2/ascorbate	0.29
MIC/MB (blank)	0.00
MIC/PB	0.00
MB/PB	0.00
MIC/MB/PB	0.20
MIC/MB/PB/catalase (500 U/mL)	0.18
MIC/MB/PB/SOD (400 U/mL)	0.22
MIC/MB/PB/ascorbate (500 μM)	0.00
MIC/MB/H_2O_2	0.08
MIC/MB/H_2O_2/PB	0.13
MIC/MB/H_2O_2/PB/ascorbate (500 μM)	0.07

Experimental conditions are described in Materials and Methods. A PB concentration of 0.5 mM was used. Concentrations quoted are the final concentrations in the reaction mixture. PB alone (tested up to 1 mM) gave no chromogen in the TBA test, nor did it have any effect if added at the end of the reaction with the TBA reagents.

Enclosing the reaction tubes in aluminium foil to exclude light also failed to affect phenylbutazone-stimulated peroxidation in the presence of myoglobin (with or without H_2O_2), but the chain-breaking antioxidants Trolox C and butylated hydroxytoluene inhibited peroxidation, as expected (data not shown).

Phenylbutazone also stimulated peroxidation of a different lipid substrate, rat liver microsomes, in the presence of myoglobin. Table 1 (lower half) shows representative data. Again, catalase or SOD did not inhibit peroxidation, but ascorbate did.

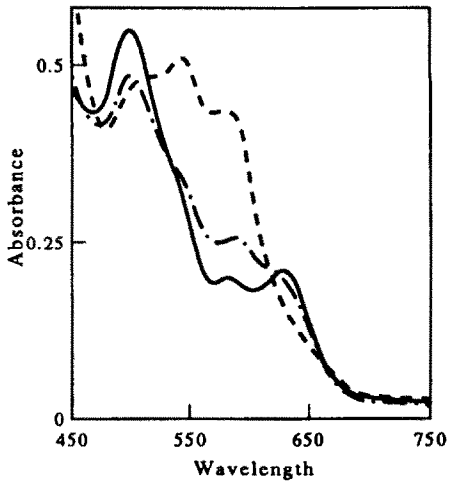

Fig. 1. Spectra of (——) myoglobin (50 μM) in 25 mM phosphate buffer pH 7.4; (– – –) myoglobin plus H_2O_2 (0.5 mM); and (—·—) myoglobin plus H_2O_2 to which phenylbutazone (0.5 mM) was added and the spectrum recorded immediately after mixing.

Action of phenylbutazone on haemoglobin-dependent peroxidation. Both methaemoglobin and oxyhaemoglobin can stimulate lipid peroxidation weakly in the presence of H_2O_2 [8]. Phenylbutazone again stimulated peroxidation of arachidonic acid in this system. In contrast to the results obtained with myoglobin, phenylbutazone gave much less, if any, peroxidation in the absence of H_2O_2. Table 2 shows representative data. SOD (400 U/mL) did not inhibit peroxidation in the presence of haemoglobin, H_2O_2 and phenylbutazone.

Inactivation of α_1-antiproteinase in the presence of haem proteins and phenylbutazone. The ability of α_1-antiproteinase to inhibit elastase was not significantly affected by including any of the following in the reaction mixture at the concentrations used: myoglobin, haemoglobin, H_2O_2, mixtures of haem proteins with H_2O_2, or phenylbutazone (Table 3, column B). The activity of elastase was also unaffected by these reagents, except that high concentrations of phenylbutazone inhibited (1 mM by 50–60%: Table 3, column A). Higher levels of myoglobin than those used here inhibited elastase activity to some extent, perhaps by acting as a competitive substrate for the

Table 2. Haemoglobin-dependent peroxidation of arachidonic acid (AA): stimulation by phenylbutazone (PB)

Reaction mixture	Amount of peroxidation (A_{532}) in presence of oxyhaemoglobin	in presence of methaemoglobin
AA	0.00	0.00
AA/PB (0.1 mM)	0.01	0.00
(0.2 mM)	0.00	0.03
(0.5 mM)	0.10	0.04
(1.0 mM)	0.15	0.07
AA/H_2O_2	0.04	0.03
AA/H_2O_2/PB (0.2 mM)	0.19	0.17
(0.5 mM)	0.32	0.29
(1.0 mM)	0.55	0.38

Experimental conditions were as described in Materials and Methods. Compounds were added to give the final concentrations stated.

Table 3. Inactivation of α_1-antiproteinase (α_1-AP) by phenylbutazone (PB)/haem/H_2O_2 mixtures

Addition to reaction mixture	% of elastase activity Column A α_1-AP absent	Column B α_1-AP present
—	100	0
HB	88	6
H_2O_2	100	0
PB 0.05 mM	87	0
PB 0.1 mM	84	6
PB 0.5 mM	51	3
PB 1.0 mM	43	3
HB/H_2O_2	100	3
HB/PB (0.5 mM)	55	22
H_2O_2/PB (0.5 mM)	83	5
HB/H_2O_2/PB (0.05 mM)	86	13
HB/H_2O_2/PB (0.1 mM)	81	20
HB/H_2O_2/PB (0.5 mM)	81	78
HB/H_2O_2/PB (1.0 mM)	72	71
—	100	1
MB	100	5
H_2O_2	100	0
MB/H_2O_2	100	7
MB/PB (1.0 mM)	66	34
MB/H_2O_2/PB (0.05 mM)	100	47
MB/H_2O_2/PB (0.1 mM)	100	78
MB/H_2O_2/PB (0.1 mM)/ascorbate (1 mM)	100	12
MB/H_2O_2/PB (0.5 mM)	89	87
MB/H_2O_2/PB (0.5 mM)/ascorbate (1 mM)	100	29
MB/H_2O_2/PB (1.0 mM)	55	54

α_1-AP (0.06 mg/mL) was preincubated in phosphate-buffered saline pH 7.4 [19] for 15 min at room temperature with a combination of the following reagents, where indicated: myoglobin (MB)/haemoglobin (HB) (50 μM, as haem), H_2O_2 (500 μM) and PB. Porcine pancreatic elastase (30 μg/mL elastase, final concentration) was then added and the residual elastase activity measured after a further 15 min.

enzyme (elastase can attack a wide range of proteins). Mixtures of haem proteins with H_2O_2 and phenylbutazone at pH 7.4 did not affect elastase activity more than could be accounted for by the effect of phenylbutazone alone. However, such mixtures did cause loss of the ability of α_1-antiproteinase to inhibit elastase. Table 3, column B shows some representative data. Ascorbic acid, which itself has no effect on elastase or α_1-antiproteinase, prevented the loss of α_1-antiproteinase activity.

Discussion

There is considerable current interest in the possibility that therapeutic agents are metabolized to toxic free radicals. Oxidation of drugs to free radicals could be catalysed by myeloperoxidase [23, 24], gastric peroxidase [25], prostaglandin synthetase [26] or by reaction of drugs with oxygen radicals generated at sites of inflammation [27]. The present paper shows that phenylbutazone accelerates oxidative damage to lipids by myoglobin ($\pm H_2O_2$) and by haemoglobin in the presence of H_2O_2. Haem protein/H_2O_2/phenylbutazone mixtures can also inactivate α_1-antiproteinase, preventing it from inhibiting elastase. The simplest explanation of our results is that haem proteins oxidize phenylbutazone into a free radical that is capable of initiating lipid peroxidation and inactivating α_1-antiproteinase. This would explain the loss of ferryl myoglobin on addition of phenylbutazone (Fig. 1)—the drug is oxidized by the ferryl protein into a damaging radical. Phenylbutazone-stimulated lipid peroxidation and inactivation of α_1-antiproteinase could be deleterious at sites of chronic inflammation and tissue injury, where haem proteins can become available to catalyse such reactions [4–9, 13–15].

One unexplained aspect of our work is that mixtures of myoglobin and phenylbutazone in the absence of H_2O_2 caused lipid peroxidation and α_1-antiproteinase inactivation. Neither catalase nor SOD inhibited, so that O_2^- and H_2O_2 do not appear to be required.

Our results may be related to the side-effects that phenylbutazone can cause when administered to patients with rheumatoid arthritis (who produce an excess of oxygen radicals [4–6] and may have "available" haem proteins at sites of inflammation [6]). It is important to consider free radicals as potential mediators of the side-effects of a wide range of drugs and food additives [27, 28]. Ascorbic acid decreased the pro-oxidant effects of phenylbutazone, perhaps by reducing the phenylbutazone radical into an inactive form and/or by reacting preferentially with the haem ferryl species. Rheumatoid patients often show sub-normal concentrations of ascorbic acid in their body fluids [29], which may make them more prone to side-effects due to drug derived radicals.

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