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PROMOTION OF OXIDATIVE DAMAGE TO ARACHIDONIC ACID AND α_1-ANTIPROTEINASE BY ANTI-INFLAMMATORY DRUGS IN THE PRESENCE OF THE HAEM PROTEINS MYOGLOBIN AND CYTOCHROME C

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Abstract—A mixture of myoglobin and hydrogen peroxide (H_2O_2) causes peroxidation of arachidonic acid. This peroxidation is greatly accelerated by adding phenylbutazone, which is effective even in the absence of H_2O_2. A wide range of other drugs was examined for their ability to exert similar pro-oxidant effects. We found that meclofenamic acid and flufenamic acid stimulated myoglobin-dependent lipid peroxidation, but only in the presence of H_2O_2. Ascorbic acid inhibited peroxidation both in the presence and in the absence of these drugs. Phenylbutazone, meclofenamic acid and flufenamic acid could also cause damage to proteins (as measured by inactivation of α_1-antiproteinase) in the presence of myoglobin and H_2O_2. The mitochondrial protein cytochrome c can also stimulate lipid peroxidation in the presence of H_2O_2. Phenylbutazone and meclofenamic acid, but not flufenamic acid, enhanced the peroxidation, which was again inhibited by ascorbic acid. However, only phenylbutazone caused inactivation of α_1-antiproteinase in the presence of cytochrome c and H_2O_2. Since respiring mitochondria generate superoxide radicals and H_2O_2, catalysis of lipid peroxidation and of the formation of drug-derived radicals by cytochrome c could be a mechanism contributing to mitochondrial damage by drugs.

Key words: myoglobin; free radical; flufenamic acid; meclofenamic acid; lipid peroxidation; cytochrome c

It is widely thought that several drugs used in the treatment of human disease exert some or all of their side-effects by being converted into damaging free radicals [1–3]. Drug-derived radicals might be produced by direct reaction of drugs with oxygen-derived free radicals generated *in vivo* [3, 4]. In addition, they could arise because many drugs can be oxidized into radicals by haem-containing proteins, such as myeloperoxidase [1, 6], prostaglandin synthetase [2], myoglobin [7, 8] or haemoglobin [5]. Myeloperoxidase is released from activated neutrophils at sites of inflammation, and could lead to formation of drug-derived radicals in extracellular fluids [1]. Myoglobin and haemoglobin can be liberated at sites of tissue injury [8, 9]; for example, bleeding is frequently seen in the inflamed rheumatoid joint [10]. Anti-inflammatory drugs that have been shown to be capable of generating damaging radicals include penicillamine [4], phenylbutazone [2, 5], indomethacin [11] and certain metabolites of sulphasalazine [12].

In the present paper, we have screened a wide range of drugs for the ability to promote free radical damage, using myoglobin–H_2O_2† as a model oxidizing system [5, 7, 13]. Free radical damage was assessed by measuring lipid peroxidation (using arachidonic acid as a substrate) and protein damage, using as a model system the inactivation of α_1-antiproteinase, an important inhibitor of certain serine protease enzymes in human body fluids [4, 14]. α_1-Antiproteinase often undergoes oxidative inactivation at sites of inflammation, such as the rheumatoid joint [14]. We show additionally that the mitochondrial protein, cytochrome c, can promote oxidative damage to lipids and proteins in the presence of certain drugs; this is a potential mechanism by which drugs can cause mitochondrial damage *in vivo*. Cytochrome c is also easily released from damaged mitochondria and so could exert pro-oxidant effects at other sites.

MATERIALS AND METHODS

Reagents. All reagents, including horse-heart cytochrome c, microperoxidase 11, porcine pancreatic elastase, α_1-antiproteinase (type A9024), myoglobin (horse heart) and drugs (except hydroxychloroquine) were from the Sigma Chemical Co. (Poole, Dorset, U.K.). Hydroxychloroquine was from Sterling-Winthrop (Guildford, Surrey, U.K.). In some experiments, cytochrome c was purified by chromatography on Sephadex G25 to remove contaminating peptide fragments.

Dissolution of drugs. Acetyl salicylate, chloroquine, diclofenac, hydroxychloroquine, meclofenamic acid, mercaptopurine, naproxen and quinine

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† Abbreviations: H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; TBA, thiobarbituric acid.

were water soluble at the concentrations given. Other drugs (flufenamic acid, indomethacin, paracetamol, D-penicillamine, phenylbutazone and piroxicam) were dissolved in a minimum volume of 1% (w/v) Na_2CO_3, the pH was then adjusted to 7.4 with HCl and the solution made up to volume. Some drugs (3′-azido-3′-deoxythymidine, azothioprine, methotrexate, D-penicillamine disulphide, prednisolone, quinacrine, sulphasalazine and tolmetin) were only soluble in ethanol. Control experiments showed that ethanol had no effect on the assays themselves at the concentrations stated [5].

Assays of lipid peroxidation. Reaction mixtures contained (unless otherwise stated) the following reagents in a final volume of 1 mL: 25 mM NaH_2PO_4–Na_2HPO_4 buffer, pH 7.4, 0.5 mM arachidonic acid (dissolved in dimethylsulphoxide), 50 μM myoglobin, the drug under investigation, and 0.5 mM H_2O_2. They were incubated at 37° for 10 min. Peroxidation was measured by the TBA test in the presence of butylated hydroxytoluene (0.2% w/v) to suppress any peroxidation during the test itself [15]. The final concentration of dimethylsulphoxide was 0.3 M. Although dimethylsulphoxide is a powerful scavenger of hydroxyl radicals, these radicals do not contribute to arachidonic acid peroxidation in this system and control experiments showed no interference with the assay.

Assays of α_1-antiproteinase and elastase. These were carried out at 37° essentially as described in Ref. [5]. α_1-Antiproteinase, 1.1 μM, (0.06 mg/mL) was preincubated in PBS, pH 7.4 for 15 min at room temperature with combinations of the following reagents: myoglobin (50 μM) or cytochrome c (50 μM), H_2O_2 (500 μM), ascorbic acid (1 mM) and meclofenamic or flufenamic acids (200 μM) in a final volume of 0.975 mL. Porcine pancreatic elastase (0.025 mL of 1.2 mg/mL) was then added and the residual elastase activity measured after a further 15 min incubation by making up the volume to 2.9 mL with additional PBS and initiating the reaction with 0.1 ml of *N*-succ-ala-ala-ala-*p*-nitroanilide elastase substrate (12 mg/ml).

Spectral determinations were performed at 20° using a Shimadzu UV2101 recording spectrophotometer with a Peltier temperature control attachment. Addition of H_2O_2 to myoglobin generates the myoglobin iron (IV) ferryl-species which is stable for at least 30 min in the absence of added drugs. To ascertain the effects of the drugs on the ferryl-myoglobin spectrum, myoglobin in 25 mM phosphate buffer, pH 7.4, was mixed in a cuvette with H_2O_2 to give 50 μM final concentrations of both substances. Meclofenamic and flufenamic acids were then added to 200 μM final concentrations and the spectrum recorded immediately after mixing and after a 10 min interval.

RESULTS

Effects of drugs on myoglobin-dependent lipid peroxidation

A mixture of myoglobin and H_2O_2 causes peroxidation of arachidonic acid, and this peroxidation is accelerated in the presence of the anti-inflammatory drug phenylbutazone. Phenylbutazone also accelerates peroxidation in the absence of added H_2O_2 (Ref. [5] and Table 1). A wide range of anti-inflammatory and other drugs in therapeutic use (listed in Table 2) was screened using this peroxidation system, at concentrations in the range 50 μM–1 mM. Most had no effect on peroxidation at the concentrations tested and some inhibited peroxidation (Table 2). However, a significant stimulation of lipid peroxidation was observed with flufenamic acid and meclofenamic acid (Table 1). Figure 1 shows the concentration dependence of these effects. Stimulation of myoglobin–H_2O_2-dependent peroxidation by these drugs was maximal at a concentration of around 200 μM, and decreased at higher concentrations (Fig. 1). Peroxidation enhanced by the fenamic acids required the presence of H_2O_2, unlike the case of phenylbutazone (Table 1). Control experiments showed that none of these drugs interfered with the assay used to measure peroxidation, nor did the drugs themselves generate a chromogen in the assay.

As previously observed with phenylbutazone [5], ascorbic acid inhibited the peroxidation (Table 1). Figure 2 shows that addition of either fenamic acid to the myoglobin–H_2O_2 system caused a loss of the myoglobin (IV) (ferryl-myoglobin) spectrum, confirming that these drugs are interacting with H_2O_2-activated myoglobin.

Effect of drugs on myoglobin–H_2O_2-dependent inactivation of α_1-antiproteinase

The myoglobin–H_2O_2 system can cause oxidative damage not only to lipids, but also to proteins [5]. Since protein damage is an important consequence of oxidative stress *in vivo*, we examined the effect of drugs on this process, using α_1-antiproteinase as a model system. The activity of α_1-antiproteinase was measured by its ability to inhibit the serine protease elastase. Neither flufenamic acid nor meclofenamic acid had any significant effect on the activity of elastase itself (Table 3, column 1). Flufenamic acid slightly decreased the ability of α_1-antiproteinase to inhibit elastase (Table 3, column 2, line 6). However, mixtures of either drug with myoglobin and H_2O_2 caused much greater decreases in the elastase inhibitory capacity of α_1-antiproteinase, seen as rises in the elastase activity measured. Damage to α_1-antiproteinase could be prevented by adding 1 mM ascorbic acid (Table 3, column 3).

Cytochrome c-dependent lipid peroxidation

Several drugs in therapeutic use produce mitochondrial damage, so it was of interest to see whether the abundant mitochondrial haem protein cytochrome c could catalyse the formation of damaging drug-derived species. Of the drugs tested, only phenylbutazone induced lipid peroxidation in the presence of cytochrome c (Table 4). Cytochrome c plus H_2O_2 induced some peroxidation, as expected [16–18]. Addition of phenylbutazone or (to a lesser extent) meclofenamic acid, but not flufenamic acid, stimulated the peroxidation caused by cytochrome c (Table 4). Purification of the commercial cytochrome c by gel filtration on a Sephadex G25 column to remove any contaminating haem peptides did not affect its ability to stimulate lipid peroxidation.

Table 1. Stimulation of myoglobin–H_2O_2-dependent peroxidation of arachidonic acid by certain anti-inflammatory drugs

Addition to assay	Extent of peroxidation (A_{532})
Arachidonic acid–myoglobin	0.174 ± 0.022
plus phenylbutazone	0.693 ± 0.082*
plus meclofenamic acid	0.174 ± 0.025
plus flufenamic acid	0.172 ± 0.019
Arachidonic acid–myoglobin–H_2O_2	0.419 ± 0.015
plus phenylbutazone	0.856 ± 0.012*
plus meclofenamic acid	0.684 ± 0.018*
plus flufenamic acid	0.667 ± 0.014*
Arachidonic acid–myoglobin–H_2O_2–ascorbic acid	0.195 ± 0.009
plus phenylbutazone	0.167 ± 0.002
plus meclofenamic acid	0.132 ± 0.006
plus flufenamic acid	0.133 ± 0.005

Peroxidation was measured by the TBA test and expressed as absorbance at 532 nm (mean ± SD, N = 5 separate experiments). Drugs were added to give the following final concentrations: phenylbutazone 500 μM, meclofenamic and flufenamic acids 200 μM. Where indicated, ascorbic acid was added to a final concentration of 1 mM.

* Significant stimulations ($P < 0.01$ using one-way ANOVA).

Table 2. Effects of anti-inflammatory and other drugs on myoglobin-dependent lipid peroxidation

Drugs which had no significant effect on lipid peroxidation:
- Acetylsalicylate
- 3'-Azido-3'-deoxythymidine (AZT)
- Azothioprine
- Chloroquine
- D-Penicillamine disulphide
- Hydroxychloroquine
- Indomethacin
- Methotrexate
- Naproxen
- Piroxicam
- Prednisolone
- Quinine
- Tolmetin

Drugs which inhibited lipid peroxidation:
- Diclofenac (34)
- Mercaptopurine (32)
- Paracetamol (55)
- D-Penicillamine (30)
- Quinacrine (58)
- Sulphasalazine (39)

Assays were performed as described in Table 1 with each assay containing arachidonic acid, myoglobin, H_2O_2 and drug. Concentrations of 50 μM–1 mM were tested for each drug. For drugs found to inhibit the peroxidation, the mean percentage inhibition produced by 1 mM drug is given in parentheses. Drugs are listed in alphabetical order.

As a further control, commercial microperoxidase did not cause lipid peroxidation in the presence of H_2O_2. Cytochrome c-dependent peroxidation, with or without drugs present, was inhibited by ascorbic acid (Table 4).

Cytochrome c could also promote protein damage, in that α_1-antiproteinase was inactivated by mixtures of cytochrome c, H_2O_2 and phenylbutazone (Table 5). Again, ascorbic acid inhibited the damage. As observed previously [5], phenylbutazone itself inhibits elastase somewhat (Table 5, column 1). By contrast, α_1-antiproteinase was not markedly affected by mixtures of cytochrome c and H_2O_2 with flufenamic or meclofenamic acids (Table 5).

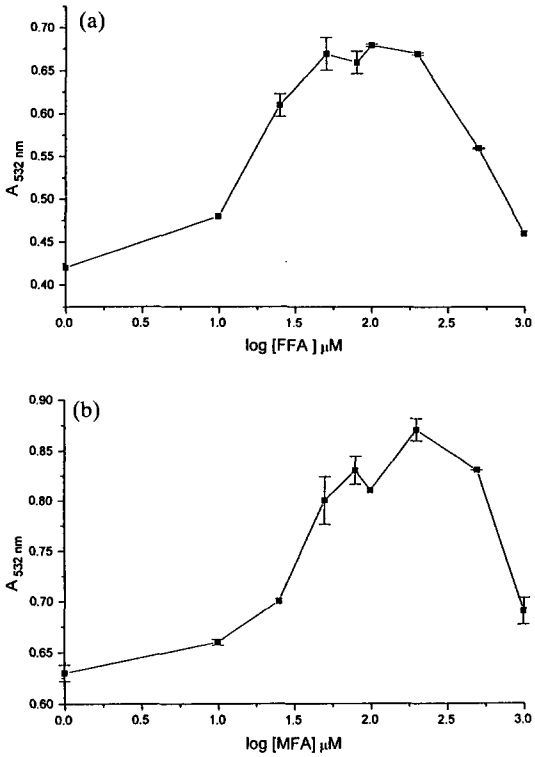

Fig. 1. Concentration dependence of the stimulation of myoglobin–H_2O_2-dependent arachidonic acid peroxidation by fenamic acids. Drugs were included in the reaction mixtures at the final concentrations given. Each point represents the mean ± SD (five experiments). (a) Flufenamic acid; (b) meclofenamic acid.

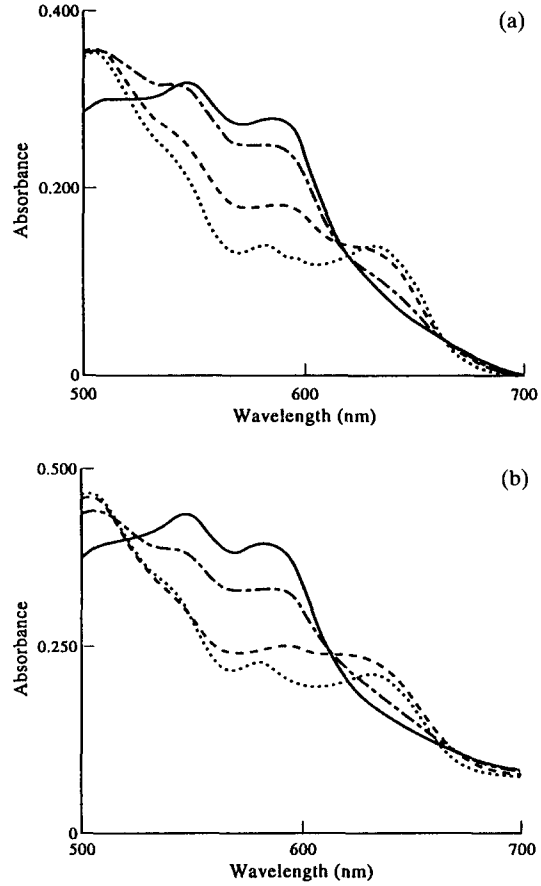

Fig. 2. Spectra of (···) myoglobin (50 μM) in 25 mM phosphate buffer, pH 7.4; (—) myoglobin plus H_2O_2 (0.5 mM); myoglobin plus H_2O_2 to which (a) meclofenamic acid (200 μM), and (b) flufenamic acid (200 μM) had been added and the spectrum recorded immediately after mixing (---) and after 10 min (---). Addition of H_2O_2 to myoglobin generates the ferryl-myoglobin [iron (IV)] species, which was stable over the time course of the experiment. However, addition of fenamic acids caused its disappearance, suggesting that myoglobin iron (IV) is reduced and the drugs are oxidized.

DISCUSSION

The present study has established three things. First, only a few of the drugs tested could accelerate lipid peroxidation under our reaction conditions. Second, in the presence of H_2O_2 and haem proteins, meclofenamic acid, flufenamic acid and phenylbutazone are oxidized (presumably to free radicals) that can accelerate lipid peroxidation and protein damage. Third, mixtures of cytochrome c and H_2O_2, already known to stimulate lipid peroxidation [16–18] and oxidize electron donors [19], can interact with phenylbutazone to cause oxidative damage in the same manner. As observed with myoglobin [5], cytochrome c–phenylbutazone mixtures can accelerate lipid peroxidation even in the absence of added H_2O_2.

Phenylbutazone was the most damaging drug examined, although the concentration required to give the maximum damage (500 μM) is greater than that required for the fenamic acids (200 μM). Damage to arachidonic acid is still, however, observed at lower concentrations. All three drugs are non-steroidal anti-inflammatory drugs and microbleeding (which releases haemoglobin) often accompanies both acute and chronic inflammation. Myoglobin has been used in these experiments to replace haemoglobin as it is much more stable and the interactions of phenylbutazone with ferryl-haemoglobin and ferryl-myoglobin are very similar [5]. Production of H_2O_2 by activated neutrophils at sites of inflammation provides a source of H_2O_2 which can combine with the haemoglobin to generate ferryl species which may interact with the drugs. Although activated haem proteins are not such powerful oxidants as the hydroxyl radical, they are much more enduring, being stable for at least the 30 min over which these experiments were performed and possibly much longer. It is thus conceivable that damage to lipids and proteins may be induced *in vivo* even in the presence of low concentrations of the drugs. Phenylbutazone is damaging in the presence of haem proteins without H_2O_2 addition, presumably because sufficient peroxide is generated

Table 3. Inactivation of α_1-antiproteinase by drug–myoglobin–H_2O_2 mixtures

Addition to reaction mixture	Elastase activity (ΔA/min × 10^3)		
	Activity of elastase alone	Activity of elastase when α_1-antiproteinase present	Activity of elastase when α_1-antiproteinase and ascorbic acid present
None	558 ± 37	45 ± 14	32 ± 15
Meclofenamic acid	518 ± 27	49 ± 57	48 ± 28
Myoglobin–H_2O_2	614 ± 88	36 ± 17	16 ± 15
Myoglobin–meclofenamic acid	524 ± 24	62 ± 52	42 ± 24
Myoglobin–meclofenamic acid–H_2O_2	566 ± 42	334 ± 62*	37 ± 27
Flufenamic acid	538 ± 32	126 ± 12	137 ± 33
Myoglobin–H_2O_2	559 ± 38	32 ± 29	15 ± 21
Myoglobin–flufenamic acid	497 ± 16	126 ± 29	102 ± 34
Myoglobin–flufenamic acid–H_2O_2	547 ± 26	273 ± 33*	172 ± 55

α_1-Antiproteinase (0.06 mg/mL) was preincubated in PBS, pH 7.4 [5], for 15 min at room temperature with a combination of the following reagents, where indicated: myoglobin (50 μM), H_2O_2 (500 μM), ascorbic acid (1 mM) and meclofenamic or flufenamic acid (200 μM). Ascorbic acid has no effect on elastase or α_1-antiproteinase. Porcine pancreatic elastase (30 μg/mL, 1.2 μM, final concentration) was then added and the residual elastase activity measured after a further 15 min. Activities shown are the mean ± SD of six individual determinations.

* Significant rise in elastase activity (decrease in α_1-antiproteinase activity), $P < 0.01$ by one-way ANOVA.

Table 4. Stimulation of cytochrome c–H_2O_2-dependent peroxidation of arachidonic acid by anti-inflammatory drugs

Addition to assay		Extent of peroxidation (A_{532})
Arachidonic acid–cytochrome c		0.170 ± 0.014
plus phenylbutazone	(0.2 mM)	0.186 ± 0.017
	(1.0 mM)	0.820 ± 0.051*
plus meclofenamic acid	(0.2 mM)	0.157 ± 0.039
	(1.0 mM)	0.181 ± 0.017
plus flufenamic acid	(0.2 mM)	0.108 ± 0.011
	(1.0 mM)	0.185 ± 0.044
Arachidonic acid–cytochrome c–H_2O_2		0.415 ± 0.019
plus phenylbutazone	(0.2 mM)	0.972 ± 0.026*
	(1.0 mM)	1.196 ± 0.041*
plus meclofenamic acid	(0.2 mM)	0.613 ± 0.079*
	(1.0 mM)	0.650 ± 0.055*
plus flufenamic acid	(0.2 mM)	0.342 ± 0.025
	(1.0 mM)	0.319 ± 0.028
Arachidonic acid–cytochrome c–ascorbic acid–H_2O_2		0.206 ± 0.013
plus phenylbutazone	(1.0 mM)	0.503 ± 0.035
plus meclofenamic acid	(1.0 mM)	0.249 ± 0.017

Experimental conditions are as described for Table 1 with cytochrome c replacing myoglobin. Concentrations quoted are the final concentrations in the reaction mixtures, which contained 50 μM cytochrome c. The data shown are mean ± SD (N = 5). Where indicated, ascorbic acid was added to a final concentration of 1 mM.

* Significant stimulations ($P < 0.05$ by one-way ANOVA).

during the course of aerobic incubation to sustain radical formation. The incidence of side-effects (possibly caused by drug-derived radicals) has led to a withdrawal of phenylbutazone from use in rheumatoid arthritis treatment in the U.K. Studies are currently underway to see whether increased oxidative damage can be measured in rheumatoid patients during treatment with fenamic acids.

Oxidative damage also occurs by interaction of phenylbutazone and meclofenamic acid with mixtures of cytochrome c and H_2O_2, presumably again by formation of a ferryl haem species. As observed with myoglobin, cytochrome c–phenylbutazone mixtures could accelerate lipid peroxidation even in the absence of H_2O_2. Respiring mitochondria generate both $O_2^{\cdot-}$ and H_2O_2 [20, 21]. Thus cytochrome c-dependent oxidation of drugs to damaging free radicals could be a mechanism of drug-dependent mitochondrial injury. In addition, damaged mitochondria at sites of tissue injury can

Table 5. Inactivation of α_1-antiproteinase by drug–cytochrome c–H_2O_2 mixtures

Addition to reaction mixture	Elastase activity (ΔA/min × 10^3)		
	Activity of elastase alone	Activity of elastase when α_1-antiproteinase present	Activity of elastase when α_1-antiproteinase and ascorbic acid present
None	531 ± 39	24 ± 13	28 ± 14
Phenylbutazone	254 ± 21	25 ± 22	29 ± 10
Cytochrome c–H_2O_2	528 ± 49	83 ± 61	42 ± 39
Cytochrome c–phenylbutazone	261 ± 25	21 ± 12	23 ± 19
Cytochrome c–phenylbutazone–H_2O_2	327 ± 26	283 ± 9*	13 ± 12
Flufenamic acid	440 ± 20	131 ± 32	90 ± 46
Cytochrome c–flufenamic acid	451 ± 28	104 ± 30	17 ± 18
Cytochrome c–flufenamic acid–H_2O_2	433 ± 24	180 ± 59	127 ± 30
Meclofenamic acid	518 ± 77	94 ± 32	66 ± 44
Cytochrome c–meclofenamic acid	522 ± 66	86 ± 57	97 ± 50
Cytochrome c–meclofenamic acid–H_2O_2	579 ± 14	104 ± 36	72 ± 20

Conditions were exactly as described in the legend to Table 3 except that cytochrome c (50 μM) replaced myoglobin. Phenylbutazone concentration was 0.5 mM.

* Significant rise in elastase activity (decrease in α_1-antiproteinase activity), $P < 0.01$ by one-way ANOVA.

leak cytochrome c and so could cause extra-mitochondrial oxidative damage.

Damage by these haem protein–H_2O_2 systems to α_1-antiproteinase was used as a model system for ability to cause protein damage in our experiments. It is also biologically relevant since α_1-antiproteinase has an exposed methionine group which is sensitive to oxidative damage and, as a major inhibitor of serine proteinases, has a protective effect on both structural and functional extracellular proteins. Damage to α_1-antiproteinase, apparently by free radicals, has been detected in the rheumatoid joint [14] and the generation of free radicals from anti-inflammatory drugs could exacerbate this.

Addition of ascorbate to the assays of lipid peroxidation and protein damage causes a decrease in the damage, possibly due to the ferryl species being reduced back to a lower oxidation state by the ascorbate in preference to oxidation by the drug. An additional explanation is that ascorbate reduces the drug-derived radical. The occurrence of side-effects of certain drugs may be due to the production of drug-derived radicals by haem proteins and H_2O_2. If these radicals can be inactivated by ascorbate, the possibility exists of co-administering the drug with ascorbate to prevent the formation of the damaging radicals. In this context, it is interesting that rheumatoid arthritis patients are often deficient in ascorbic acid and were particularly prone to the side-effects of phenylbutazone (discussed in Ref [5]).

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