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FREE RADICAL SCAVENGING AND INHIBITION OF LIPID PEROXIDATION BY β-BLOCKERS AND BY AGENTS THAT INTERFERE WITH CALCIUM METABOLISM

A PHYSIOLOGICALLY-SIGNIFICANT PROCESS?

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Abstract—It has been proposed that β-blockers and agents affecting Ca^{2+} metabolism might exert cardioprotective actions because of their ability to act as antioxidants *in vivo*. The feasibility of this proposal was tested by examining the reaction of a series of such compounds with various oxygen-derived species. None of the compounds tested was sufficiently reactive with superoxide radical, hydrogen peroxide or hypochlorous acid for scavenging of these species to be feasible *in vivo* at the drug concentrations present in patients given the usual therapeutic doses. All the drugs tested were powerful scavengers of hydroxyl radical except for flunarizine, which stimulated iron ion-dependent hydroxyl radical generation from hydrogen peroxide. However, none of the drugs significantly inhibited production of hydroxyl radicals in this system. Propranolol, verapamil and flunarizine had significant inhibitory effects on the peroxidation of rat liver microsomes in the presence of iron ions and ascorbic acid. All three compounds exerted weaker inhibitory effects on peroxidation of arachidonic acid caused by a mixture of myoglobin and H_2O_2: pindolol stimulated peroxidation in this system. It is concluded that the ability of β-blockers and "Ca^{2+}-blockers" to inhibit lipid peroxidation varies with the lipid substrate used and the mechanism by which peroxidation is induced. We conclude that suggestions that β-blockers and "Ca^{2+}-blockers" exert antioxidant effects *in vivo* are not well founded, although there is a possibility that verapamil and propranolol might have some inhibitory effects against peroxidation if they accumulate in membranes to a sufficiently-high concentration *in vivo*. We could not confirm the reported ability of propranolol to inhibit the enzyme xanthine oxidase.

Reperfusion of ischaemic myocardium is associated with distinctive pathological and physiological derangements which are collectively referred to as "reoxygenation injury". These derangements include arrhythmias, stunning and zones of infarction. Generation of reactive oxygen species has been suggested (with variable degrees of experimental evidence) to be involved in all these processes [1–5]. Thus it has been proposed that reoxygenation of ischaemic myocardium leads to generation of O_2^- and H_2O_2 within the tissue which can, in the presence of transition metal ions, become converted into highly-reactive hydroxyl radicals, ·OH [1]. Potential sources of transition metal ions to catalyse ·OH formation include the release of iron ions from damaged cells [6] and the ability of excess H_2O_2 to degrade myoglobin and release iron ions from the haem ring [7]. In addition, the initial reaction of myoglobin with H_2O_2 leads to formation of a powerful oxidant that can accelerate peroxidation of membrane lipids [8, 9] and it has been suggested that myoglobin–H_2O_2 interactions are an important component of reoxygenation injury [8]. The nature of this powerful oxidant is not absolutely clear; it is not ·OH [9, 10] but may be a tyrosine peroxyl radical [11]. Another reactive oxygen species that can damage the myocardium is hypochlorous acid (HOCl) generated by the enzyme myeloperoxidase from activated neutrophils infiltrating the reoxygenated tissue [4].

Calcium ions are often involved in the injury to cells that can be produced by oxidative stress [1, 12, 13] and calcium may also play a direct role in reoxygenation injury (reviewed in [4, 14]). Thus "calcium blockers" such as verapamil, nifedipine and diltiazem may improve myocardial function after reoxygenation [14]. However, pharmacological agents rarely exert biological effects by a single mechanism of action and it has been suggested that several agents acting to alter Ca^{2+} metabolism could themselves have antioxidant properties [15–18]. In addition propranolol, a β-blocker widely used in the management of cardiovascular disorders, has been reported to be an inhibitor of lipid peroxidation [19] and to decrease xanthine oxidase activity [20]. Xanthine oxidase is an enzyme that may be important in producing free radicals in ischaemic/reperfused heart [1]. Some other β-blockers have also been claimed to inhibit lipid peroxidation [21]. Such observations are also relevant to atherosclerosis, since both free radical reactions (reviewed in Ref. 22) and Ca^{2+} [23] are involved in development of

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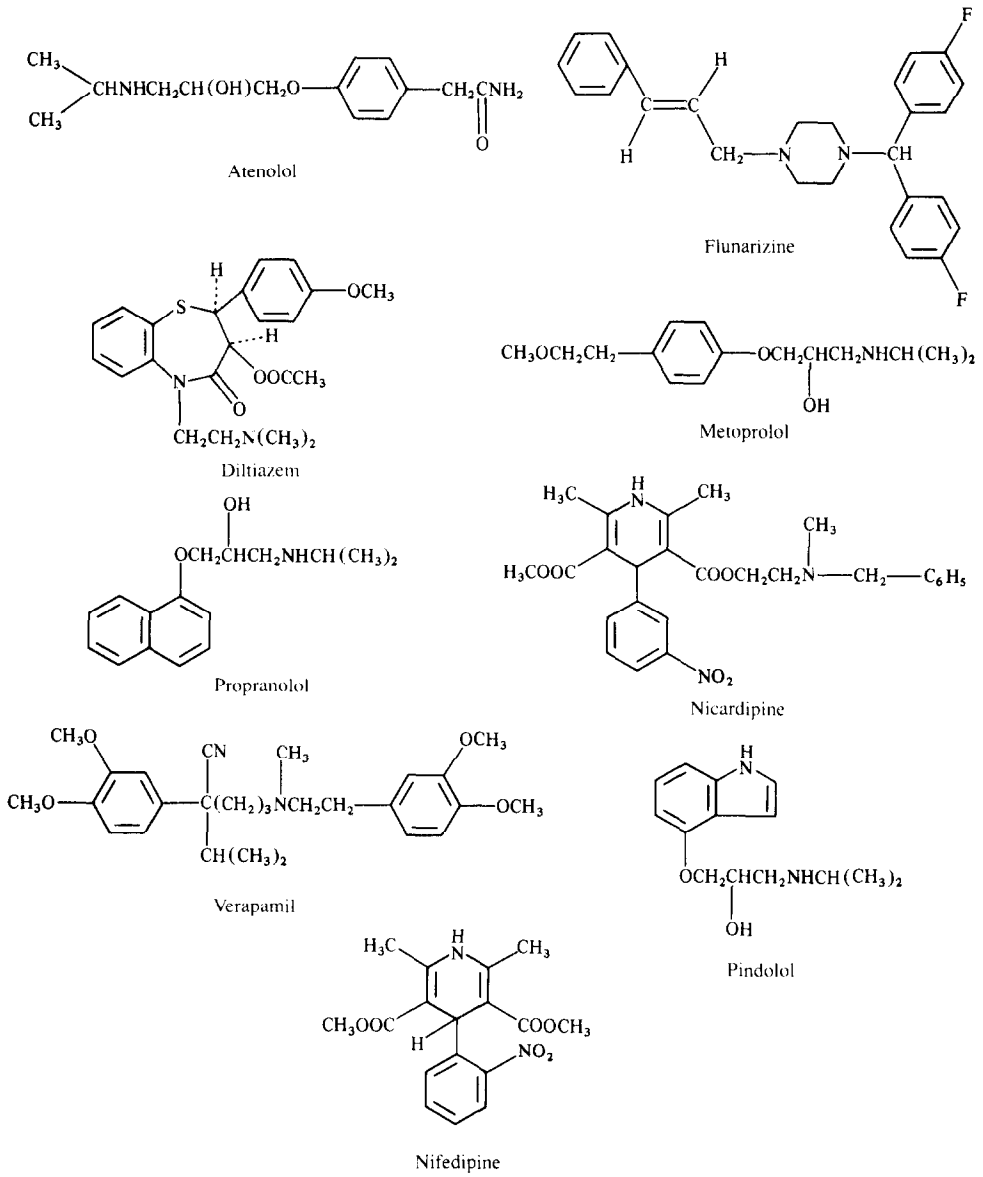

Fig. 1. Chemical structures of the drugs used in this study.

the atherosclerotic lesion. Indeed nifedipine has been reported to have antiatherogenic effects both in animals [23, 24] and in humans [24, 25].

However, a huge number of compounds has been suggested to act as antioxidants *in vivo*, and many of these suggestions are not feasible *in vivo* [26]. In evaluating the likelihood that a compound acts as an antioxidant, it is essential to know whether the rate at which it reacts with biologically-important reactive oxygen species would allow it to compete with biological molecules for such species *in vivo* (discussed in Ref. 26). In addition, some antioxidants which inhibit free radical damage to lipids have *pro-oxidant* effects in other systems [26–28].

In the present paper, we have used established methods (reviewed in Ref. 26) to study the reactions of β-blockers (propranolol, atenolol, metoprolol, and pindolol) and of calcium antagonists (diltiazem, verapamil, flunarizine, nicardipine, and nifedipine) with these reactive oxygen species (O_2^-, H_2O_2, HOCl and ·OH) that are known to be formed in the reoxygenated myocardium [1–4]. In addition, the effects of these compounds on ·OH generation from H_2O_2 in the presence of iron ions and upon lipid peroxidation stimulated by iron ions or by the myoglobin/H_2O_2 system [8–10] have been examined. The results obtained allow us to evaluate the likelihood of the proposals [15, 16, 18–20] that these compounds are capable of exerting antioxidant effects *in vivo*. Figure 1 shows the structures of the drugs we have tested.

MATERIALS AND METHODS

Hypochlorous acid and pig pancreatic elastase

Table 1. Action of drugs on the ability of HOCl to inactivate the elastase-inhibitory capacity of α_1-antiproteinase

Addition to first reaction mixture	Elastase activity in final reaction (A_{410}/sec × 10^{-3})	Activity of α_1-AP in inhibiting (100 − % of elastase activity detected)
Buffer, elastase	5.76	—
Buffer, α_1AP elastase	0	100
Buffer, α_1AP, HOCl	4.45	23
+metoprolol (2 mM)	4.66	19
+diltiazem (2 mM)	3.44	40
+propranolol (2 mM)	4.43	23
+atenolol (2 mM)	4.76	17
+pindolol (2 mM)	0.83	86
+pindolol (0.50 mM)	3.80	34
+pindolol (1.0 mM)	2.24	61
+pindolol (1.5 mM)	1.67	71

α_1-Antiproteinase (0.25 mg/mL), HOCl (210 μM) and drug (2 mM unless otherwise stated) were incubated in a final volume of 1.0 mL in phosphate-buffered saline at pH 7.4 (full details in [29]) for 20 min at 25° to allow HOCl to inactivate α_1-AP. Then 2 mL of buffer and 0.05 mL of pig pancreatic elastase solution [29] were added, followed by incubation at 25° for a further 20 min to allow any active α_1AP remaining to inhibit elastase (any HOCl remaining is diluted to a point at which it cannot affect elastase itself). The uninhibited elastase is then measured by adding elastase substrate, which is hydrolysed with a rise in A_{410}. Concentrations of drug quoted were those present in the first (1.0 mL) reaction mixture. Drugs were mixed with α_1AP and buffer immediately before adding HOCl.

were from BDH Chemicals Ltd (Poole, U.K.). Drugs and other reagents, including myoglobin (horse-heart), xanthine oxidase (EDTA-free), superoxide dismutase (bovine erythrocyte enzyme) and α_1-antiproteinase (type A9024) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Assays. Elastase and α_1-antiproteinase were assayed essentially as described in Ref. 29: full details are given in the legend to Table 1. Hypochlorous acid was produced just before required by adjusting NaOCl to pH 6.2 with dilute H_2SO_4. Generation of O_2^- by the hypoxanthine–xanthine oxidase system was carried out essentially as described in [30]. Reaction of drugs with H_2O_2 was measured by a peroxidase-based assay system [30]. Rat liver microsomes were prepared by differential pelleting and their peroxidation in the presence of 100 μM $FeCl_3$ and 100 μM ascorbate was measured by the thiobarbituric acid (TBA) test in the presence of 0.02% (w/v) butylated hydroxytoluene (BHT) in the TBA reagents to suppress peroxidation during the assay itself [31]. Peroxidation of arachidonic acid by the myoglobin/H_2O_2 system was studied in reaction mixtures containing the following reagents at the final concentrations stated: KH_2PO_4–KOH buffer, pH 7.4 (25 mM), arachidonic acid (0.4 mM), myoglobin (50 μM), H_2O_2 (0.5 mM) and diethylenetriaminepenta-acetic acid (100 μM) to bind any metal ions released [32]. Tubes were incubated at 37° for 10 min and peroxidation was measured by the TBA test as described above. Hydroxyl radical formation was measured in the presence of ascorbate, H_2O_2 and $FeCl_3$ (±EDTA) by the deoxyribose method, as described in Ref. 33. The bleomycin assay was conducted essentially as described in Ref. 27. Xanthine oxidase activity was measured either spectrophotometrically, by monitoring conversion of xanthine to uric acid [20], e.g. by mixing xanthine oxidase (0.03 units/mL) with variable xanthine concentrations (up to 25 μM) at each of three different propranolol concentrations 0, 100 and 250 μM in 50 mM KH_2PO_4–KOH buffer pH 7.4 containing 10 μM EDTA, or by measuring oxygen uptake in similar reaction mixtures using a Hansatech O_2 electrode (Hansatech Ltd, Kings Lynn, Norfolk, U.K.). For the latter, rates were usually around 0.04 μmol O_2 consumed per min. The assay system contained, in a final volume of 1.0 mL, 0.4 units of xanthine oxidase, 4 mM hypoxanthine and drug at the final concentration stated using phosphate saline buffer pH 7.4 (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4). Enzyme was incubated with drug for 3 min before adding hypoxanthine to start the reaction.

RESULTS

Action of drugs on systems generating hydroxyl radicals

A mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbic acid at pH 7.4 generates ·OH radicals, which can be detected by their ability to degrade the sugar deoxyribose into fragments that, on heating with thiobarbituric acid at low pH, generate a pink chromogen [33].

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad (1)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + \cdot OH + OH^- . \quad (2)$$

Table 2. Scavenging of hydroxyl radical, and inhibition of iron ion-dependent hydroxyl radical generation, by flunarizine, DL-propranolol, verapamil, diltiazem, atenolol and metoprolol

Compound	Concentration tested (mM)	Rate constant for reaction with ·OH (M^{-1} sec^{-1})	Inhibition of site-specific ·OH generation (%)
Verapamil	0.25–1.50	1.4×10^{10}	—
	0.25	—	10
	0.50	—	15
	1.00	—	22
	1.50	—	26
Flunarizine	0.25–1.50	Pro-oxidant action	—
	0.25	—	+13
	0.50	—	+14
	1.00	—	+22
	1.50	—	+29
Propranolol	0.25–1.50	1.9×10^{10}	—
	0.25	—	18
	0.50	—	25
	1.00	—	32
	1.50	—	46
Diltiazem	0.4–5.0	8.3×10^{9}	—
	0.42	—	13
	0.83	—	19
	1.67	—	22
Atenolol	0.4–5.0	2.7×10^{10}	—
	0.42	—	17
	0.83	—	32
	1.67	—	59
	3.33	—	62
Metoprolol	0.4–4.0	1.4×10^{10}	—
	0.42	—	9
	0.83	—	21
	1.67	—	26

Reaction mixtures contained in a final volume of 1.2 mL, the following reagents at the final concentrations stated: 10 mM KH_2PO_4–KOH buffer pH 7.4, 2.8 mM H_2O_2, 2.8 mM deoxyribose, 20 μM $FeCl_3$ (pre-mixed with 100 μM EDTA before addition to the reaction mixture unless otherwise stated). 0.1 mM ascorbate was added to start the reaction and tubes were incubated at 37° for 1 hr. Products of ·OH attack upon deoxyribose were measured as in [33]. Drugs were added to the reaction mixtures to give the final concentrations stated. Drugs were dissolved in water containing minimum NaOH to ensure solubilization where necessary. Control experiments showed that this practice did not interfere with the outcome of the assay. The pH of each drug solution was adjusted to close to 7.4 before addition to the reaction mixture. Drugs soluble only in organic solvents cannot be tested in the deoxyribose assay. For studies of site-specific ·OH generation, no EDTA was added to the reaction mixture (see the text). Results are representative of three experiments, results from different experiments varied by no more than 10%.

Provided that an excess of EDTA is used, any ·OH generated by reaction (2) that escapes direct scavenging by EDTA enters "free solution" and is equally accessible to deoxyribose and to any other scavenger of ·OH added [33]. Thus the ability of a substance to inhibit (competitively) deoxyribose degradation under these reaction conditions is a measure of its ability to scavenge ·OH and can be used to calculate a rate constant for reaction of ·OH with the scavenger [33, 34].

All the drugs tested except flunarizine competitively inhibited deoxyribose degradation, suggesting that they are powerful scavengers of ·OH, reacting with it at diffusion controlled rates (second order rate constants $>10^9$ M^{-1} sec^{-1}). Table 2 shows a representative set of experimental results. Flunarizine actually *accelerated* deoxyribose damage in the reaction mixtures. Control experiments showed that none of the compounds affected the measurement of deoxyribose degradation (they had no effect when added with the TBA reagents), nor did they react with ·OH to give TBA-reactive material (omission of deoxyribose from the reaction mixtures completely abolished chromogen formation). Only the drugs that could be dissolved in aqueous solution or in dilute alkali solution to give sufficient concentrations could be tested in this assay system, since organic solvents are themselves

powerful scavengers of ·OH [35]. For this reason, pindolol, nicardipine and nifedipine were not tested in the deoxyribose assay.

When iron ions are added to the reaction mixture as $FeCl_3$ (not complexed to EDTA), some of the ions form a complex with deoxyribose [36]. This complex can be reduced by ascorbate to give Fe^{2+}, which can remain attached to deoxyribose [37] and can subsequently react with H_2O_2. This reaction appears to produce ·OH [26, 34, 36, 37] which immediately attacks the deoxyribose in a site-specific manner. The resulting deoxyribose degradation is not inhibited by ·OH scavengers at moderate concentrations [26]. The only molecules that can inhibit deoxyribose degradation are those that have iron ion-binding capacity and can withdraw the iron ions from the deoxyribose and render them inactive or poorly active in Fenton reactions [34]. Hence the ability of a substance to inhibit deoxyribose degradation at pH 7.4 in reaction mixtures containing $FeCl_3$, H_2O_2 and ascorbate (in the *absence of EDTA*) seems to be a measure of the ability of that substance to diminish iron ion-dependent ·OH generation by binding the necessary iron ions [26, 34].

None of the compounds tested (at low concentrations) showed any marked ability to inhibit ·OH generation by the $FeCl_3/H_2O_2$/ascorbic acid system. (Table 2, last column). At high concentrations (>1 mM) propranolol and atenolol had some inhibitory action. Flunarizine again stimulated the deoxyribose damage.

Do the drugs scavenge superoxide or hydrogen peroxide?

A mixture of hypoxanthine and xanthine oxidase at pH 7.4 generates O_2^-, which can be detected by its ability to reduce ferricytochrome *c* to ferrocytochrome *c*, [38]. Any added compound that is itself able to react with O_2^- should decrease the rate of reduction of cytochrome *c*. None of the drugs listed in Table 2, tested at concentrations up to 5 mM (which did not themselves reduce ferricytochrome *c* directly under our reaction conditions) had any significant inhibitory effect on the rate of reduction of 100 μM ferricytochrome *c*, whereas superoxide dismutase inhibited its reduction by >90%. Drugs soluble only in organic solvents (nicardipine, pindolol and nifedipine) were not tested here. At pH 7.4, cytochrome *c* reacts with O_2^- with a rate constant of about $2.6 \times 10^5\ M^{-1} sec^{-1}$ [39]. The inability of any of the drugs to inhibit cytochrome *c* reduction at concentrations 50 times greater than those of cytochrome *c* suggests that their reactions with O_2^-, if any, proceed with rate constants no greater than about $10^3\ M^{-1} sec^{-1}$. None of the drugs, tested at concentrations up to 250 μM, affected the activity of xanthine oxidase itself, as measured by the uptake of O_2 using an oxygen electrode (data not shown). This result contradicts a previous report in the literature [20] that propranolol is a xanthine oxidase inhibitor, in which an assay based on spectrophotometric measurement of uric acid formation was employed. The effect of propranolol on xanthine oxidase was also tested by this assay method [20]. Figure 2 shows that propranolol was not inhibitory up to a concentration of 250 μM. Use of higher concentrations was precluded by the high A_{290} of the drug which resulted in high signal: noise ratios and serious errors in spectrophotometric readings.

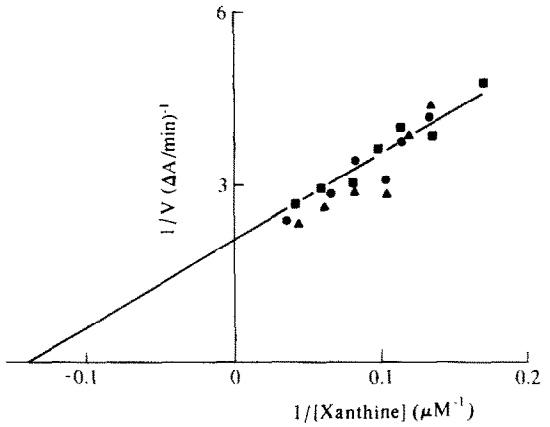

Fig. 2. Lineweaver–Burk plot for the steady state analysis of xanthine oxidase inhibition by propranolol: xanthine oxidase activity was determined spectrophotometrically as described in the Materials and Methods section. Propranolol was used at the final concentration shown. 0 μM (■); 100 μM (●); 250 μM (▲).

Similarly, none of the drugs, tested at concentrations up to 5 mM, showed any reactivity with H_2O_2 as tested by incubating them with 10 mM H_2O_2 and then measuring residual H_2O_2 by a peroxidase-based method [30].

Do the drugs scavenge hypochlorous acid?

One of the most important targets attacked by HOCl *in vivo* is α_1-antiproteinase, the major inhibitor in human body fluids of serine proteinases such as elastase [40]. α_1-Antiproteinase is rapidly inactivated by HOCl, losing its ability to inhibit elastase [40]. Although many drugs have been shown to react with HOCl *in vitro*, few do so sufficiently rapidly to protect α_1-antiproteinase against inactivation. A good test of whether a compound might be capable of scavenging HOCl at a biologically-significant rate is, therefore, to examine its ability, over the concentration range present *in vivo*, to protect α_1-antiproteinase against inactivation by HOCl [26, 41, 42].

Table 1 shows that α_1-antiproteinase inhibited elastase: a concentration just sufficient to inhibit completely was used. Treatment of the α_1-antiproteinase with 210 μM HOCl almost completely abolished its elastase-inhibitory capacity (Table 1, third line). Inclusion of most drugs in the reaction mixture at a final concentration of up to 2 mM failed to protect the α_1-antiproteinase significantly. The exception was pindolol, which protected when it was added at lower concentrations, although 0.5 mM was needed to give a significant inhibition. Control experiments showed that pindolol did not inhibit elastase directly, nor did it interfere with the ability of α_1-antiproteinase to inhibit elastase.

Table 3. Action of drugs on Fe^{3+}/ascorbate stimulated peroxidation in rat liver microsomes

Drug added	Final reaction mixture (mM)	Extent of peroxidation (A_{532})	% Inhibition of peroxidation
None	—	1.600	0
Propranolol	0.10	1.070	33
	0.15	0.782	51
	0.20	0.656	59
	0.40	0.346	78
	0.50	0.248	84
	0.60	0.202	87
	1.00	0.108	93
	2.00	0.050	97
Verapamil	0.10	0.948	41
	0.15	0.732	54
	0.20	0.528	67
	0.40	0.179	89
	0.50	0.099	94
	0.60	0.075	95
	1.00	0.039	98
Diltiazem	0.50	1.140	29
	1.00	0.940	41
	2.00	0.830	48
Pindolol	0.50	1.150	28
	1.00	0.630	61
	2.00	0.114	93
Nifedipine	0.50	1.280	20
	1.00	0.980	39
	2.00	0.240	85
Flunarizine	0.10	0.765	52
	0.20	0.410	74
	0.25	0.204	87
	0.30	0.151	91
	0.50	0.107	93
	1.00	0.090	94

Reaction mixtures contained, in a final volume of 1 mL, 0.57 mg of microsomal protein, 100 μM $FeCl_3$, 100 μM ascorbate, drug at the final concentration stated and 10 mM KH_2PO_4–KOH buffer pH 7.4. Reaction mixtures were incubated at 37° for 20 min before performing the TBA test. Results are those of a representative experiment, but the order of inhibitory capacity of different drugs was identical in four experiments.

Inhibition of lipid peroxidation by drugs

The rat liver microsomal system [43] is a well-established system for testing antioxidant activity. Several drugs were tested for their ability to inhibit microsomal lipid peroxidation in the presence of $FeCl_3$ and ascorbic acid. Propranolol, verapamil and flunarizine were especially inhibitory. Diltiazem, nifedipine and pindolol were inhibitory only at high concentrations (Table 3). None of the drugs tested interfered with the thiobarbituric acid (TBA) test used to measure peroxidation, since none of them inhibited the colour formation if they were added at the end of the incubation with the TBA reagents instead of being included in the reaction mixtures.

For comparison, the ability of the drugs to inhibit peroxidation induced by the myoglobin/H_2O_2 system was examined, Table 4 shows a representative set of results. Significant inhibitions were produced by diltiazem, propranolol, verapamil, flunarizine and nifedipine at final concentrations of 1 mM in the reaction mixtures, but atenolol or metoprolol showed slight stimulation. Pindolol appeared to *stimulate* peroxidation significantly. Table 5 shows the concentration-dependence of some of these effects. None of the drugs caused any peroxidation themselves if either myoglobin or H_2O_2 were omitted from the reaction mixtures.

DISCUSSION

It has been proposed that agents affecting Ca^{2+} metabolism, and β-blockers, might additionally have antioxidant properties [15–17, 19, 44]. In the present paper, we have investigated this proposal by examining the ability of several such drugs to react with different oxygen-derived species. All drugs except flunarizine were powerful scavengers of the highly-reactive hydroxyl radical, ·OH. However, even *in vitro*, drug concentrations of >250 μM were needed to significantly scavenge ·OH. Most biological molecules react with ·OH with equally-high rate constants, and so it is very unlikely that drug

Table 4. Action of drugs on myoglobin-dependent peroxidation of arachidonic acid in the presence of H_2O_2

Compound added	Amount of peroxidation (A_{532})	Inhibition of peroxidation (%)
None	0.208	0
Metoprolol	0.235	14*
Diltiazem	0.138	34
Propranolol	0.124	40
Verapamil	0.079	62
Atenolol	0.216	4*
Pindolol	0.315	51*
Nicardipine	0.141	32
Flunarizine	0.120	42
Nifedipine	0.113	46

* Stimulation of peroxidation.

Peroxidation was measured by the TBA test as described in the Materials and Methods section. Drugs were added to give a final concentration of 1 mM in the reaction mixtures. A representative experiment is shown, but the order of inhibitory action of the drugs (and the stimulation by pindolol) were reproducible in three experiments.

concentrations are ever high enough *in vivo* for scavenging of ·OH to be a protective mechanism [26]. In patients administered these drugs, concentrations in body fluids are rarely greater than the micromolar range (e.g. 10 mg flunarizine once daily gives peak plasma concentrations of 115 μg/dm^3, or 0.28 μM [45]). None of the drugs tested was significantly effective at interfering with iron ion-dependent ·OH generation *in vitro* except for atenolol and propranolol, again at unphysiologically-high (millimolar) concentrations. The reason for the observed *stimulation* of ·OH generation by flunarizine is unknown and further work is underway to examine this.

Similarly none of the drugs tested could react with O_2^- or H_2O_2 at detectable rates. Our studies with xanthine oxidase did not confirm the reported ability of propranolol to inhibit this enzyme [20]. No inhibition was detected in our experiments when the enzyme was assayed in three different ways: uptake of oxygen, O_2^--dependent reduction of cytochrome *c*, or conversion of xanthine to uric acid, although the established xanthine oxidase–inhibitor allopurinol was found to inhibit the enzyme completely in all these assays. None of the drugs (at concentrations below the millimolar range) appeared capable of offering significant protection to α_1-antiproteinase against inactivation by HOCl. The drug simply might not react sufficiently rapidly with HOCl to protect the α_1-antiproteinase. Alternatively, or additionally, the drugs might react with HOCl to form a product that also inactivates α_1-AP [26, 46]. Verapamil and propranolol had weak inhibitory effects (Table 5) against fatty acid peroxidation stimulated by the myoglobin/H_2O_2 system (which is thought to be relevant to the myocardium) whereas

Table 5. Action of verapamil, propranolol and pindolol on myoglobin-dependent peroxidation: concentration dependence

Drug	Concentration (mM)	Amount of peroxidation (A_{532})	Inhibition of peroxidation (%)
Verapamil	0	0.267	—
	0.10	0.230	14
	0.15	0.218	18
	0.20	0.204	24
	0.40	0.177	34
	0.60	0.133	50
	1.00	0.107	60
	2.00	0.064	76
Propranolol	0	0.270	—
	0.10	0.240	10
	0.20	0.239	10
	0.40	0.222	17
	0.50	0.201	25
	0.60	0.179	33
	1.00	0.154	42
Pindolol	0	0.177	—
	0.10	0.296	67*
	0.15	0.331	87*
	0.20	0.360	103*
	0.40	0.396	124*
	0.60	0.378	114*
	0.80	0.335	89*
	1.00	0.301	70*

* Stimulation of peroxidation.

Drugs were added to give the final concentrations in the reaction mixtures. Reaction conditions are as described in the legend to Table 4. Results of a representative experiment are shown, but were reproducible in three experiments.

pindolol reproducibly *stimulated* this peroxidation. By contrast, pindolol inhibited peroxidation of rat liver microsomes induced by $FeCl_3$ and ascorbic acid, as did propranolol, verapamil and flunarizine.

These inhibitory effects were evident at concentrations in the 100–200 μM range, which again seems high. However, it has been argued that the lipid solubility of these compounds means that they might accumulate within membranes *in vivo* and offer some degree of antioxidant protection [18, 47–49]. Nifedipine, propranolol and verapamil have already been shown to protect sarcolemmal membranes [21] or canine myocytes [44] against peroxidation induced by an iron-ion/dihydroxyfumarate system, which produces a complex mixture of oxygen-derived species including $O_2^{\cdot-}$, H_2O_2 and ·OH [50]. Dihydroxyfumarate oxidation is an extremely complex process [50] and the possibility that drugs affect free radical generation by dihydroxyfumarate as well as, or instead of, actually protecting the lipid target against free radical damage must not be ignored. Flunarizine, but not nifedipine, inhibited peroxidation of liposomes (made from rat myocardial lipids) induced by a hypoxanthine/xanthine oxidase/Fe^{3+}–ADP system [16]. It is clear from the published literature [16, 18, 19, 21, 44, 51] and from data in the present paper (compare Table 3 and Tables 4 and 5) that the relative inhibitory efficiencies of the drugs appear to depend on the lipid system examined and on how the peroxidation is induced.

Our results show that any *in vivo* antioxidant effects of the drugs tested are likely to be exerted only against lipid peroxidation, and then only if the drugs accumulate in membranes to a sufficiently-high concentration. The apparent *pro-oxidant* effects of flunarizine in the ·OH system (Table 2) and of pindolol in the myoglobin system (Table 5) are a further illustration of the complexity of the biological effects of alleged antioxidants [26].

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