Metabolites of procainamide and practolol inhibit complement components C3 and C4

Edith SIM,* Lesley STANLEY,† Edward W. GILL and Alison JONES

Department of Pharmacology, University of Oxford, South Parks Road, Oxford OX1 3QT, U.K.

Drug-induced systemic lupus erythematosus arises from toxic side-effects of administration of hydralazine, isoniazid, procainamide and practolol. Hydralazine and isoniazid are nucleophilic drugs and inhibit the covalent binding reaction of complement components, C3 and C4, an effect likely to lead to deposition of immune complexes (a feature of systemic lupus erythematosus). Procainamide and practolol do not themselves inhibit C3 and C4. A range of metabolites and putative metabolites of procainamide and practolol were synthesized, and tested for their ability to inhibit the covalent binding reactions of C3 and C4. The highly nucleophilic hydroxylamine metabolite of procainamide was strongly inhibitory in both tests, as was a putative hydroxylamine metabolite of practolol. These studies indicate a potential role for the hydroxylamine metabolites in mediating the toxic side-effects of procainamide and practolol, and emphasize the need for adequate measurements of hydroxylamine metabolites in human tissue.

INTRODUCTION

Effects on the immune system have been implicated in a wide range of adverse reactions to drug therapy. Among such adverse reactions is the condition resembling systemic lupus erythematosus (SLE) which occurs on long-term treatment with hydralazine, a hypotensive, and isoniazid, which is used in treatment of tuberculosis (Harpey, 1973). Both hydralazine and isoniazid are highly nucleophilic monosubstituted hydrazines and both are metabolized by the polymorphic *N*-acetyl transferase enzyme of the liver (Weber & Hein, 1985). For drug-induced SLE occurring as a side-effect of therapy with hydralazine (Perry *et al.*, 1970) and isoniazid (Godeau *et al.*, 1974) patients who develop the condition are almost exclusively slow acetylators. This suggests that the toxic side-effects are mediated by the drugs themselves and not by metabolites.

It has been demonstrated that hydralazine and isoniazid, but not their acetylated metabolites, inhibit the classical-pathway complement protein C4 (Sim *et al.*, 1984). C4, and the homologous complement protein C3, contain an activated thiol ester which, on activation of C3 and C4 by appropriate proteinases, becomes exposed and reacts rapidly with any nucleophile, including (a) OH or NH₂ groups on the surface of complement-activating particles, (b) water, or (c) soluble nucleophilic compounds present in the environment (for review and discussion see Davies & Sim, 1981; Sim *et al.*, 1981; Law, 1983). When the classical pathway of the complement system is activated by, e.g., immune complexes, component C1 (bound to the complexes) cleaves C4, and activated C4 may then bind covalently to the surface of immune complexes (as in *a* above) or may react with water. Only C4 bound to immune complexes participates in formation of the C4₂ enzyme, which activates C3 (for review see Reid, 1986). Strong nucleophiles present in

solution may form a covalent bond with activated C3 or C4, thus reducing the amount of C3 or C4 which binds to the immune complex. Covalent binding of hydralazine to activated C4 (Sim & Law, 1985), and of other nucleophiles to activated C3, has been documented (Sim *et al.*, 1981; Law, 1983).

C3 and C4 deposition is important in regulating clearance of immune complexes. C3 deposition promotes solubilization of immune complexes by disruption of the immune lattice (Takahashi & Takahashi, 1981), and also facilitates phagocytosis of complexes via macrophage C3 receptors (Fearon & Wong, 1983). SLE is regarded as a disorder of immune complex clearance (Schifferli & Peters, 1983) and individuals with deficiencies of the early complement components (C1, C4, C2) are at increased risk of developing idiopathic SLE (Thompson, 1987). It seems likely, therefore, that inhibition of C4 deposition by isoniazid and hydralazine may be an important factor in development of drug-induced SLE.

Procainamide is also associated with development of drug-induced SLE as a toxic side effect. It is an aromatic amine, but is not a strong nucleophile, and is not an effective inhibitor of C4 binding (Sim *et al.*, 1984). Procainamide, an anti-arrhythmic drug, is also metabolized mainly by *N*-acetylation (Kark *et al.*, 1983). Both fast and slow acetylators can develop drug-induced SLE as a result of procainamide therapy, but slow acetylators are more prone to the condition (Reidenberg, 1981). As an alternative to *N*-acetylation, *N*-oxidation of procainamide occurs (Uetrecht *et al.*, 1984), forming a highly nucleophilic hydroxylamine metabolite of the drug.

Practolol, a cardio-selective β -blocker, has also been associated with a condition like SLE (Raftery & Denman, 1973), although it has a more severe adverse side-effect known as the oculomucocutaneous syndrome (Wright, 1975). Practolol is an *N*-acetylated aromatic amine. De-

Abbreviation used: SLE, systemic lupus erythematosus.

* To whom correspondence and reprint requests may be addressed.

† Present address: Department of Biochemistry, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh EH8 9XD, U.K.

acetylated and *N*-oxidized forms may also occur. We have investigated the effect of procainamide, practolol and their chemically synthesized metabolites or putative metabolites on C3 and C4 deposition *in vitro*, to determine whether nucleophilic metabolites can inhibit the covalent binding of C3 or C4 in a manner similar to hydralazine and isoniazid.

MATERIALS AND METHODS

Proteins

C1 (the activated C1 component) was purified from pooled human serum as described by Sim (1981). Trypsin (treated with L-1-tosylamido-2-phenylethylchloromethyl ketone, type XIII) was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). C3 and C4 were isolated from pooled human plasma using the method described by Sim *et al.* (1981) and Sim *et al.* (1986) respectively. C3 and C4 were radiolabelled with ^{125}I using Iodobeads (Pierce, IL, U.S.A.) as catalyst as described previously (Sim *et al.*, 1985). C1 and trypsin were separately coupled to CNBr-activated Sepharose (Pharmacia) at pH 8.5 in sodium bicarbonate buffer according to the manufacturer's instructions to give 300–750 μg of enzyme/ml of packed Sepharose.

Covalent binding of C3 or C4

Covalent binding of activated C3 or C4 can be studied conveniently using an activating enzyme (proteinase) bound to Sepharose. ^{125}I -labelled C3 or C4 activated by the proteinase can then form ester bonds to OH groups on Sepharose (Sim *et al.*, 1981, 1984). Sepharose–C1 or Sepharose–trypsin was used as the combined activating and binding surface for C4 and C3 respectively. Incubation of ^{125}I -labelled C4 with Sepharose–C1 (30 min, 37 °C) was done as described previously (Sim *et al.*, 1984). Binding of C3 was studied similarly, in that Sepharose–trypsin [100 μl of a 1:1 (v/v) slurry in 25 mM-potassium phosphate/140 mM-NaCl, pH 7.4 (phosphate-buffered saline)] was incubated at 25 °C for 1 h in a total volume of 250 μl of phosphate-buffered saline containing 1 μg of ^{125}I -labelled C3 (5×10^5 c.p.m./ μg), and with or without the test compounds. The reaction was stopped by addition of 100 μl of 0.2 M-Tris/HCl/2% (w/v) SDS/8 M-urea (pH 6.8) and of 200 μl of Sephadex G-25 [1:1 (v/v) slurry in phosphate-buffered saline] as 'carrier' resin for centrifugation. Duplicate 20 μl samples of the total mixture were removed for determination of radioactivity. The rest of the mixture was centrifuged (2 min, 14000 *g*) and samples of supernatant were retained to determine the percentage of radioactivity unbound. Pellets were washed twice with 1 ml of each of the following solutions to remove non-covalently bound material: 0.1% (w/v) Triton X-100 in water; 2 M-NaCl/10 mM-sodium acetate, pH 5.5; 0.2 M-Tris/HCl/2% (w/v) SDS, 8 M-urea, pH 8.8. The pellet was transferred to a clean tube for estimation of covalently bound radioactivity. In each experiment, 100% inhibition was determined in the presence of 40 mM-NH₂OH which inhibits completely covalent binding of C3 and C4. All additions of potential inhibitors were made from stock solutions adjusted to pH 7.4 and freshly made up each day. In each experiment, the extent of inhibition was measured in triplicate for each concentration of inhibitor.

Synthesis of metabolites

***N*-Acetylprocainamide.** Procainamide base, obtained from the hydrochloride by treatment with NaOH solution and extraction into chloroform, was treated, in chloroform solution, with an excess of acetyl chloride, with cooling. The crystalline product was recrystallized from propan-2-ol, and its m.p. was confirmed as 187 °C.

***N*-Hydroxyprocainamide (hydroxylamine procainamide).** This was prepared using the method described by Uetrecht *et al.* (1984).

Desethylprocainamide. *N*-Benzyl-*N*-ethylethylenediamine was prepared by treating 2-bromoethylphthalimide with *N*-benzylethylamine and subsequent cleavage with hydrazine. Refractive index, n_D^{20} , was 1.5150. This compound was treated with nitrobenzoyl chloride to give *N*-benzyl-*N*-ethyl-*N'*-nitrobenzoyl-ethylenediamine hydrochloride, which was simultaneously debenzylated and reduced with hydrogen over 10% palladium charcoal in methanol to give *N*-ethyl-*N'*-(4-aminobenzoyl)ethylenediamine hydrochloride (desethylprocainamide) (m.p. 156 °C) from ethanol ether.

Desacetylpractolol and *N*-hydroxy-desacetylpractolol (hydroxylamine practolol). 1-(4-Nitrophenoxy)-2,3-propylene oxide was treated with isopropylamine in aqueous ethanol to give *N*-isopropyl-2-hydroxy-3-(4-nitrophenoxy)propylamine (compound A). Compound A was reduced in ethanol containing one equivalent of HCl using 10% palladium charcoal and H₂ at 101.325 kPa pressure to give desacetylpractolol hydrochloride (m.p. 170 °C).

Compound A was reduced catalytically using 10% palladium charcoal poisoned with 0.1% triethyl phosphite, according to the method described by Uetrecht *et al.* (1984). H₂ (2 mol) was taken up and h.p.l.c. analysis of the product showed a single peak, well-resolved from the corresponding nitro and amino compounds. The product *N*-hydroxy-desacetylpractolol (hydroxylamine practolol) was isolated as the oxalate salt dihydrate (m.p. 190 °C).

Structures of compounds discussed are shown in Figs. 1 and 3.

SDS/polyacrylamide-gel electrophoresis

Samples of the incubation supernatants of the pure radiolabelled proteins were subjected to SDS/polyacrylamide-gel electrophoresis after reduction and alkylation as described previously (Sim & Law, 1985) and then autoradiography of the dried gels was carried out (Sim *et al.*, 1981).

RESULTS

Procainamide and metabolites

A scheme for the metabolism of procainamide and the structures of the compounds discussed is shown in Fig. 1. The logarithmic dose-response curve for the inhibition by procainamide and its hydroxylamine metabolite of ^{125}I -labelled C3 binding to Sepharose–trypsin is shown in Fig. 2. The concentrations of compounds giving 50% inhibition of ^{125}I -labelled C3 binding to Sepharose–trypsin and ^{125}I -labelled C4 binding to Sepharose–C1 are shown in Table 1. For binding of both

Fig. 1. Scheme for generation of metabolites of procainamide

Route 1 is *N*-dealkylation (Taber *et al.*, 1979), route 2 is *N*-oxidation (Utrecht *et al.*, 1984) and route 3 is *N*-acetylation (Weber & Hein, 1985).

Fig. 2. Inhibition of binding of ^{125}I -labelled C3 to Sepharose-trypsin

^{125}I -labelled C3 (5×10^5 c.p.m.) was incubated with Sepharose-trypsin in the presence or absence of procainamide (●) or the hydroxylamine metabolite (○), and the amount of ^{125}I -labelled C3 covalently bound was measured after washing, as described in the text. The extent of inhibition was determined from 100% inhibition obtained in the presence of 40 mM- NH_2OH . Results are shown as the average of triplicate determinations and error bars indicate the range of values observed.

Table 1. Effect of drugs and metabolites on covalent binding of C3 and C4

The concentrations (mM) of drug or metabolite required to give 50% inhibition of binding of ^{125}I -labelled C3 to Sepharose-trypsin or of ^{125}I -labelled C4 to Sepharose-C1, determined as in Fig. 2, are shown. Where appropriate, values are expressed as the means $\pm$ S.E.M. The number of individual experiments is shown in parentheses. Abbreviation: ND, not done.

Compound	Concentration giving 50% inhibition of covalent binding (mM)	
	C3	C4
Procainamide	$14.4 \pm 3.3(6)$	$17.5 \pm 2.8(6)$
<i>N</i> -Acetylprocainamide	28.4	$37.6 \pm 2.5(3)$
Hydroxylamine procainamide	$1.7 \pm 0.3(4)$	$1.4 \pm 0.2(3)$
Desethylprocainamide	ND	> 20
Practolol	$29.2 \pm 4.4(3)$	$25.6 \pm 3.1(3)$
Deacetylated practolol	> 10	ND
Hydroxylamine practolol	$1.2 \pm 0.4(3)$	$1.3 \pm 0.2(5)$

Fig. 3. A scheme for generation of practolol metabolites

Details are given in the text.

proteins, the order of inhibitory potency of drug and metabolites is the same: the hydroxylamine metabolite is the most potent inhibitor whereas procainamide and *N*-acetylprocainamide are poor inhibitors. The desethyl metabolite is a poor inhibitor of C4 binding. By SDS/polyacrylamide-gel electrophoresis and autoradiography, it was observed that neither the cleavage of C4 by C1 nor the cleavage of C3 by trypsin, is inhibited by the hydroxylamine metabolite. Therefore the observed inhibition is due to inhibition of covalent binding to the Sepharose rather than inhibition of activation of the complement proteins.

Practolol and metabolites

A scheme for generation of the practolol metabolites which have been investigated is shown in Fig. 3. The

effects of practolol and its putative metabolites on ^{125}I -labelled C3 and ^{125}I -labelled C4 binding are shown in Table 1. Again the hydroxylamine metabolite is a potent inhibitor of C3 and C4 covalent binding activity.

DISCUSSION

It has previously been suggested that toxic metabolites of procainamide (Uetrecht *et al.*, 1984; Budinsky *et al.*, 1987) and practolol (Amos *et al.*, 1978) are generated by oxidation of these drugs. Our results show that the hydroxylamine derivatives of both drugs inhibit the covalent binding reaction of activated C4 and C3. Two other drugs, hydralazine and isoniazid, which are also associated with toxic side-effects giving rise to SLE-like symptoms, also inhibit C3 and C4 (Sim *et al.*, 1984). As discussed in the Introduction, inhibition of C3 and C4 binding may diminish clearance of immune complexes. Thus inhibition of C3 and C4 by these drugs or their metabolites is likely to contribute to the immune complex deposition characteristic of SLE.

Hydroxylamine procainamide is a strong nucleophile and will react slowly with 'bystander' proteins, becoming covalently bound with low efficiency to, e.g., ovalbumin over a period of 14 h (Uetrecht, 1985). However the reaction described in the present study is likely to occur essentially instantaneously once C3 and C4 are activated (Sim *et al.*, 1981).

At concentrations of 1–2 mM-hydroxylamine procainamide or hydroxylamine practolol 50% inhibition of C3 and C4 binding occurs. Inhibition is detectable at much lower levels (Fig. 2). In a previous study, slightly lower concentrations of hydralazine and isoniazid were found to cause 50% inhibition of C4 binding. It is not possible to relate these concentrations of drugs or metabolites to concentrations found in tissues during therapy with any of the four drugs named above, as measurements of these substances in human tissues, have not adequately been made. Many measurements are available for procainamide and its major identified metabolites (*N*-acetylprocainamide and *p*-aminobenzoic acid) in body fluids and urine after short-term (1–4 days) administration of the drug. Patients receiving 1–1.5 g of procainamide/day attain a therapeutic plasma concentration of up to approx. 70 μM , with *N*-acetylprocainamide levels of up to 170 μM . Procainamide and the *N*-acetyl form equilibrate rapidly from the plasma into tissue fluids and both forms of the drug are excreted in the urine. SLE-like side effects occur, however, only after long-term therapy with procainamide (1–1.5 g/day for several years) (Reidenberg, 1981; Qazi & Gerber, 1986), and the pattern of tissue accumulation of the drug and its metabolites is unknown. The hydroxylamine metabolite discussed here has only recently been detected (Uetrecht *et al.*, 1984; Uetrecht, 1985; Budinsky *et al.*, 1987), and its quantitative importance as a metabolite is as yet unknown. We would suggest, however, that this metabolite is likely to be of major importance in inducing SLE symptoms.

The toxic effects of practolol are associated mainly with the eye, and work with single high-dose (400 mg/kg) administration of practolol to hamsters, has clearly shown that the drug and its metabolites accumulate in the eye up to a level of approx. 200 μM (Rosenbaum

et al., 1986). In humans, long-term therapy with practolol may also result in accumulation of the drug and its metabolites in the eye. If the putative hydroxylamine metabolite accumulates in the eye, it may cause local inhibition of complement in the blood supply of the lachrymal glands, leading to deposition of immune complexes and reduction in tear secretion, as has been described in the oculomucocutaneous syndrome (Wright, 1975). Complement activity is also present in the cornea (Mondino & Brady, 1981) and lesions observed in this syndrome may also be associated with antibody deposition in the corneal/conjunctival area.

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