REFERENCES

1. Nebert DW, Gonzalez FJ, Coon MJ, Estabrook RW, Feyereisen R, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Nelson DR, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences characterized, gene mapping, and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991.
2. Gonzalez FJ, Molecular genetics of the P-450 superfamily. *Pharmacol Ther* **45**: 1–38, 1990.
3. Tamburini PP, Masson HA, Bains SK, Makowski RJ, Morris B and Gibson GG, Multiple forms of hepatic cytochrome P-450. Purification, characterisation and comparison of a novel clofibrate-induced isoenzyme with other forms of cytochrome P450. *Eur J Biochem* **139**: 235–246, 1984.
4. Hardwick JP, Song S, Huberman E and Gonzalez FJ, Isolation, complementary DNA sequence, and regulation of rat hepatic lauric acid ω-hydroxylase (Cytochrome P-450LAω). Identification of a new cytochrome P-450 gene family. *J Biol Chem* **262**: 801–810, 1987.
5. Gibson GG, Comparative aspects of the mammalian cytochrome P-450 IV gene family. *Xenobiotica* **19**: 1123–1148, 1989.
6. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. A mechanistic inter-relationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
7. Makowska JM, Anders C, Goldfarb PS, Bonner F and Gibson GG, Characterization of the hepatic responses to the short-term administration of ciprofibrate in several rat strains. Co-induction of microsomal cytochrome P-450 IVA1 and peroxisome proliferation. *Biochem Pharmacol* **40**: 1083–1093, 1990.
8. Dirven HAAM, de Bruijn AAGM, Sessink PJM and Jongeneelen FJ, Determination of the cytochrome P-450 IV marker, ω-hydroxylauric acid, by high-performance liquid chromatography and fluorimetric detection. *J Chromatogr* **564**: 266–271, 1991.
9. Reddy JK and Lalwai ND, Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
10. Rao MS and Reddy JK, Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis* **8**: 631–637, 1987.
11. Elcombe CR and Mitchell AM, Peroxisome proliferation due to di(2-ethylhexyl)phthalate: species differences and possible mechanisms. *Environ Health Perspect* **70**: 211–219, 1986.
12. Lake BG, Evans JG, Gray TBJ, Körösi SA and North CJ, Comparative studies on Nafenopin-induced hepatic peroxisome proliferation in the rat, Syrian Hamster, Guinea pig, and marmoset. *Toxicol Appl Pharmacol* **99**: 148–160, 1989.
13. Makowska JM, Bonner FW and Gibson GG, Comparative induction of cytochrome P-450 IVA1 and peroxisome proliferation by ciprofibrate in the rat and marmoset. *Arch Toxicol* **65**: 106–113, 1991.
14. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* **239**: 2379–2385, 1964.
15. Peters WHM and Jansen PLM, Immunocharacterization of UDP-glucuronoyltransferase isoenzymes in human liver, testine and kidney. *Biochem Pharmacol* **37**: 564–567, 1988.
16. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **131**: 248–254, 1976.
17. Kimura S, Hardwick JP, Kozak CA and Gonzalez FJ, The rat clofibrate-inducible CYP4A subfamily II. cDNA sequence of IVA3, mapping of the Cyp4a locus to mouse chromosome 4, and coordinate and tissue-specific regulation of the CYP4A genes. *DNA* **8**: 517–525, 1989.

Biochemical Pharmacology, Vol. 42, No. 9, pp. 1844–1847. 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00
Pergamon Press plc

The antihypertensive compounds hydralazine, dihydralazine and cadralazine and their metabolites inhibit myeloperoxidase activity as measured by chemiluminescence

(*Received* 23 *November* 1990; *accepted* 16 *June* 1991)

Antineutrophil cytoplasma antibodies (ANCA) now called C-ANCA were described in 1985 [1]. They have become a marker of systemic vasculitis, especially Wegener's granulomatosis and are directed against the elastinolytic enzyme, Proteinase 3.

Antibodies against the lysosomal enzyme myeloperoxidase (MPO) were recently reported in patients with idiopathic necrotizing and cresentic glomerulonephritis [2]. We have found antimyeloperoxidase antibodies (anti-MPO) in patients with genuine systemic lupus erythematosus (SLE) [3]. We have also recorded circulating anti-MPO in the hydralazine-induced SLE-like syndrome [3], and in isolated hydralazine-induced kidney damage [4].

Hydralazine (1-hydrazinophthalazine) is an antihypertensive agent, which has been in clinical use since 1950. Cadralazine, (2,3-[6-(2-hydroxypropyl)ethylamino]-pyridazinyl)ethylcarbazate (ISF 2469) is structurally related to hydralazine and with the same pharmacological principles [5, 6]. Myeloperoxidase is a heme enzyme localized in azurophil granula in neutrophil granulocytes and monocytes [7]. Its main function is the killing of microorganisms taken up via phagocytosis. Since a connection between hydralazine-induced autoimmunity and circulating anti-MPO has been found [3], it was of interest to investigate the interaction between MPO and hydralazine and its relevant metabolites, and their influence upon enzymatic

activity, and furthermore, to compare with dihydralazine and the new antihypertensive agent cadralazine.

Materials and Methods

Myeloperoxidase (MPO). Human myeloperoxidase (oxidoreductase; EC 1.1.11.7) was purified according to Olsson *et al.* [8] and Matheson [9] with slight modifications using 1-day-old buffy coats. Its purity was checked on a sodium dodecylsulphate–polyacrylamide gel.

Chemicals. Hydralazine, dihydralazine, cadralazine and four hydralazine metabolites *N*-acetylhydrazinophthalazine (NAC-HP2) BA 14184, 3-hydroximethyltriazolophthalazine (3-OH-MTP) CGP 10601, methyltriazolophthalazine (MTP) BA 7114, triazolophthalazine TP BA 7127 and CGP 22639 the major metabolite to cadralazine were a kind gift from Ciba-Geigy (Basel, Switzerland). Luminol was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals used were of highest analytical purity.

Experimental design. Chemiluminescence measurements: The three main compounds were dissolved in 0.1 M phosphate buffer, pH 6.1. The following final concentrations were run: 13, 1.3 and 0.13 mM; 13, 1.3 and 0.13 μM; 13, 1.3 and 0.13 nM. Three separate runs with controls in parallel were performed for each of the agents. The metabolites BA 7114, BA 7127 and CGP 22639 were dissolved in 0.1 M phosphate buffer, pH 6.1. CGP 14184 and CGP 10601 were dissolved in a mixture of ethanol and phosphate buffer. Controls were run in parallel with the corresponding concentrations of ethanol as in incubations with the respective metabolite. The following final concentrations were run for BA 7114, BA 7127 and CGP 22639: 20, 2 and 0.2 mM; 20, 2 and 0.2 μM; 20 and 2 nM; for CGP 10601: 10, 2 and 0.2 mM; 20, 2 and 0.2 μM; 20 and 2 nM; for CGP 14184: 1 and 0.2 mM; 20, 2 and 0.2 μM; 20 and 2 nM).

Fifteen microlitres of MPO (3.6 mg/mL) was pre-incubated shortly with the same volume of the respective compound and transferred to a cuvette for luminometric recording. One hundred microlitres of luminol (5.6×10^{-4} M) and 50 μL NaBr (0.4 mM) were added and the content thoroughly mixed. The reaction was started by adding 50 μL H_2O_2 (17.6 mM) through a dispenser. Recording was carried out by a LKB-1250 luminometer and at 22°. The output signal was recorded on a LKB-recorder. Full deflection corresponded to 1000 mV.

Statistical analysis. Student's *t*-test was used for statistical calculations.

Results

Hydralazine, dihydralazine and cadralazine caused a reduction in myeloperoxidase enzyme activity at high concentrations. At a concentration above 1.3 mM all three compounds caused a total inhibition. Dihydralazine was found to be the most potent inhibitor of the three. On the other hand, cadralazine inhibited MPO activity more efficiently than hydralazine. At a concentration of 1.3 mM hydralazine caused an average reduction of 91%, dihydralazine 98.5% and cadralazine 95.5%. The corresponding figures at 0.13 mM were 57% for hydralazine, and 75% for cadralazine. Dihydralazine differed from the other two with a more pronounced inhibition of about 97%. At 0.1 and 0.05 mM inhibition was greater at 93.5% and 82%, respectively, as compared to hydralazine and cadralazine. No inhibition was found for hydralazine, dihydralazine or cadralazine in the range 1.3 μM to 0.13 nM as seen in Fig. 1.

At the highest concentrations, 20–10 mM inhibition of MPO enzyme activity was seen for the following three hydralazine metabolites: BA 7114, BA 7127 and CGP 10601, 87, 35 and 54%, respectively, compared to controls. The metabolite CGP 14184 was tested at 1 mM because it was difficult to keep this substance in solution at concentrations above 1 mM. The most potent inhibitor was the cadralazine metabolite (CGP 22634), which at 20 mM caused a 100% inhibition, and at 0.2 mM caused a pronounced inhibition of myeloperoxidase enzyme activity (87%). For further details, see Table 1.

Discussion

This study shows that hydralazine, dihydralazine and cadralazine exert an inhibitory effect upon MPO activity. Hydralazine and dihydralazine have a similar molecular structure, and differ only in the number of hydrazine groups, hydralazine having one and dihydralazine two. The results show clearly that the number of hydrazine groups are important for blocking MPO enzyme activity. The new antihypertensive cadralazine was found to possess an intermediate position with a stronger inhibitory potency as compared to hydralazine, but weaker as compared to dihydralazine.

Cadralazine is chemically related to hydralazine and dihydralazine, but differs by an ethoxycarbonyl group attached to the hydrazine group. The underlying reason has been to "hide" the hydrazine group and thereby

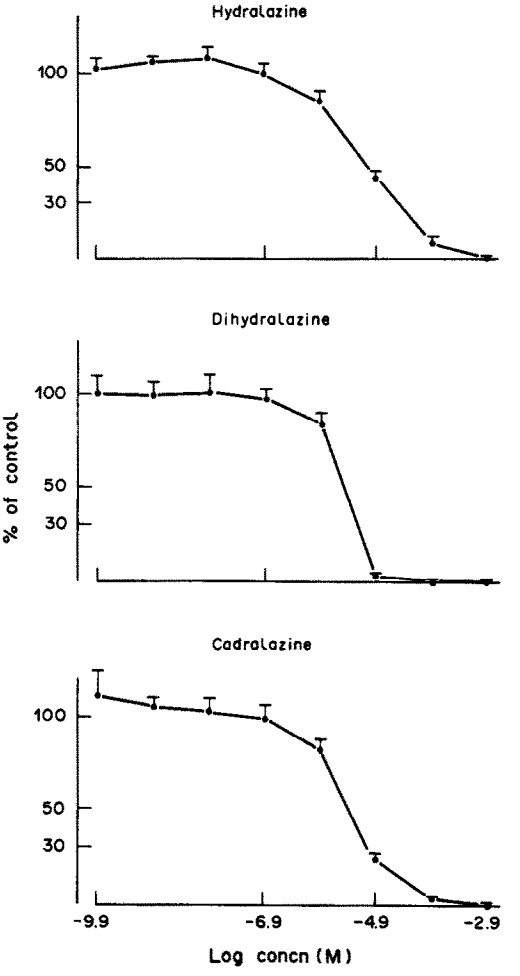

Fig. 1. Inhibition curves for the three antihypertensive compounds hydralazine, dihydralazine and cadralazine in the concentration range 13 mM to 0.13 nM. For all three compounds a statistical significance was found in reduction of MPO enzyme activity at the concentration 1.3 μM ($P < 0.001$).

Table 1. Interaction of four hydralazine metabolites and the major metabolite to cadralazine which are detected in plasma, with myeloperoxidase (MPO) enzyme activity

	Hydralazine				Cadralazine
Concn (mM)	BA 7114 (%) N	BA 7127 (%)N	CGP 1060 1 (%) N	CGP 14184 (%) N*	CGP 22639 (%) N
20	−87 ± 7.2 (8)	−35.3 ± 5.1 (7)	—	—	−100 ± 0.0 (6)
10	—	—	−54.5 ± 6.3 (5)	—	—
2	−8.6 ± 4.7 (9)	1.5 ± 2.1 (7)	−4.0 ± 7.1 (5)	—	−99.2 ± 0.3 (6)
1	—	—	—	5.7 ± 5.5 (6)	—
0.2	−1.1 ± 5.3 (8)	−3.7 ± 4.3 (7)	−3.9 ± 2.9 (5)	−2.9 ± 7.0 (5)	−87.2 ± 3.3 (6)
2×10^{-2}	+3.6 ± 4.9 (5)	+1.9 ± 3.5 (7)	−2.7 ± 4.5 (5)	−6.6 ± 9.3 (5)	−6.4 ± 2.6 (12)
2×10^{-3}	5.1 ± 4.0 (4)	−2.4 ± 5.1 (7)	−2.3 ± 3.9 (5)	+1.4 ± 8.8 (5)	+6.1 ± 8.0 (9)
2×10^{-4}	+8.0 ± 2.4 (4)	−0.8 ± 3.6 (7)	10.2 ± 8.6 (5)	+14.9 ± 4.2 (5)	+1.0 ± 5.0 (9)
2×10^{-5}	+1.8 ± 6.1 (6)	−1.4 ± 4.3 (7)	1.0 ± 2.5 (5)	−2.7 ± 2.9 (5)	+0.8 ± 7.7 (8)
2×10^{-6}	+1.4 ± 5.7 (5)	−0.8 ± 5.3 (7)	1.7 ± 4.2 (5)	+2.6 ± 4.5 (4)	+2.3 ± 6.0 (8)

Controls were run in parallel. CGP 14184 and CGP 10601 were dissolved in ethanol/phosphate buffer. Controls with corresponding percentages of ethanol were run parallel to each drug concentration.
− Inhibition of MPO enzyme activity.
+ Stimulation of MPO enzyme activity expressed against controls.
* It was difficult to keep this substance in solution at concentrations above 1 mM.
Values are means ± SD. N, number of observations.

preventing interaction with macromolecules. However, this study shows that cadralazine binds to myeloperoxidase with the same strength as hydralazine. Hydralazine, as well as cadralazine inhibited MPO enzyme activity more efficiently than their metabolites, indicating a stronger binding to the MPO molecule.

Hydralazine may induce two kinds of autoimmune adverse effects. It may cause a SLE-like syndrome called also "later toxicity". This condition differs in a very significant fashion from idiopathic systemic lupus erythematosus, in that the kidney is characteristically spared in hydralazine lupus. In the idiopathic disease, however, renal failure is frequent. The underlying pathogenic mechanisms for, both the induced SLE-like syndrome [10, 11], as well as the induced isolated kidney damage [12, 13] are still unknown. It has been found previously that hydralazine is able to bind to macromolecules. Therefore, it has been speculated that the hydrazine group could be the chemical moiety responsible for the induced lupus-like syndrome, where a reaction between the hydrazine group and the pyrimidine of DNA could be a possible explanation for the immunological side-effects [14]. Hydralazine has also been reported to complex with soluble nucleo protein (a DNA–histone complex) and thereby change its physical properties [15]. Furthermore, it has also been shown that hydralazine binds covalently to the complement component C4 [16].

On the other hand, it has been found that several chemical substances bind to MPO and have been found to inhibit its enzyme activity. Diketones inhibit MPO activity [17]. They are used for probing for essential arginine residues in proteins [18]. It has been reported that chloride binds to MPO. By resonance Raman spectra it was evidenced that chloride binds to the central iron atom of the chlorine [19].

The study shows that hydralazine, dihydralazine and the new agent cadralazine bind to MPO. Evidently they bind to the enzymatic active site or very near to it. Spectral analysis and nuclear magnetic resonance (NMR) could help to identify the exact binding site.

In conclusion, from a theroretical point of view it could be assumed that the binding of hydralazine to MPO could contribute under certain circumstances, and in patients with a genetic predisposition, to production of antibodies against MPO.

Acknowledgements—This study was supported by grants from the Swedish Association against Rheumatism; the National Association of Kidney Patients, Stockholm, Sweden; Alfred Österlunds Foundation; and the Swedish Society for Medical Research, Stockholm. The author is grateful to Göran Englund and Mats Henning, Ciba-Geigy, Gothenburg, for valuable discussions during the study.

Department of Medical Microbiology
University of Lund
Lund, Sweden

LENNART NÄSSBERGER*

REFERENCES

1. van der Woude FJ, Lobatto S, Permin J, van der Giessen, Rasmussen N, Wiik A, van Es LA, van der Hem K and The TH, Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* **i**: 425–429, 1985.
2. Falk RJ and Jennette JC, Antineutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and cresentic glomerulonephritis. *N Engl J Med* **318**: 1651–1657, 1988.
3. Nässberger L, Sjöholm AG, Jonsson H, Sturfelt G and Åkesson A, Autoantibodies against neutrophil cytoplasm components in systemic lupus erythematosus and in hydralazine-induced lupus. *Clin Exp Immunol* **81**: 380–383, 1990.
4. Nässberger L, Johansson AC, Björck S and Sjöholm AG, Antibodies to neutrophil granulocyte myeloperoxidase and elastase. Autoimmune responses in glomerulonephritis due to hydralazine treatment. *J Intern Med* **229**: 261–265, 1991.
5. Semeraro C, Dorigotti L, Bunfi C and Carpi C, Pharmacological studies on cadralazine a new antihypertensive vasodilator. *J Cardiovasc Pharmacol* **3**: 455–467, 1981.
6. Catalano M, Parini J, Romano M and Libretti A, Controlled clinical trial of cadralazine as a second step

* Address for correspondence: Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

drug in the treatment of hypertension. *Eur J Clin Pharmacol* **28**: 135–138, 1985.
7. Bainton DF, Ullyot JL and Farquhar MG, The development of neutrophilic polymorphonuclear origin and content of azurophil and specific granules. *J Exp Med* **134**: 907–934, 1971.
8. Olsson I, Olofsson T and Odeberg H, Myeloperoxidase mediated iodination of granulocytes. *Scand J Haematol* **9**: 483, 1972.
9. Matheson WR, Wong PS and Travis I, Isolation and properties of human neutrophil myeloperoxidase. *Biochemistry* **20**: 325, 1981.
10. Perry HM, Late toxicity to hydralazine resembling systemic lupus erythematosus or rheumatoid arthritis. *Am J Med* **54**: 58–72, 1983.
11. Strandberg I, Boman G, Hassler L and Sjöqvist F, Acetylator phenotype in patients with hydralazine-induced lupoid syndrome. *Acta Med Scand* **200**: 367–371, 1976.
12. Ihle BU, Whitwork JA, Dowling IP and Kincaid-Smith P, Hydralazine and lupus nephritis. *Clin Nephrol* **22**: 230–238, 1984.
13. Björck S, Westberg G, Svalander C and Mulec H, Rapidly progressive glomerulonephritis after hydralazine. *Lancet* **ii**: 42, 1983.
14. Dubroff ML, Reid R and Papalian M, Molecular models for hydrazine-related systemic lupus erythematosus. *Arthritis Rheum* **24**: 1082–1085, 1981.
15. Tan EM. Drug-induced autoimmune disease. *Fed Proc* **33**: 1894–1897, 1974.
16. Sim E, Gill EW and Sim RB, Drugs that induce systemic lupus erythematosus inhibit complement component C4. *Lancet* **ii**: 422–424, 1984.
17. Willassen NP and Little C, Effect of 2,3-butanedione on human myeloperoxidase. *Int J Biochem* **21**: 755–759, 1989.
18. Aurebekk B and Little C, Functional arginine in phospholipase C of *Bacillus cereus*. *Int J Biochem* **8**: 757–762, 1977.
19. Ikede-Saito M, Argade PV and Rousseau DC, Resonance Raman evidence of chloride binding to the heme iron in myeloperoxidase. *FEBS* **184**: 52–55, 1985.