Biochemical Pharmacology, Vol. 36, No. 22, pp. 3847–3850, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

BIOLOGICALLY-SIGNIFICANT SCAVENGING OF THE MYELOPEROXIDASE-DERIVED OXIDANT HYPOCHLOROUS ACID BY SOME ANTI-INFLAMMATORY DRUGS

MOHAMMAD WASIL,*† BARRY HALLIWELL,* CHRISTOPHER P. MOORHOUSE,* DUNCAN C. S. HUTCHISON and HAROLD BAUM‡

*Department of Biochemistry, King's College (KQC), Strand Campus, London WC2R 2LS, †Department of Thoracic Medicine, King's College School of Medicine and Dentistry (KQC), Denmark Hill, London SE5 8RX, and ‡Department of Biochemistry, King's College (KQC), Kensington Campus, London W8 7AH, U.K.

(*Received* 16 *April* 1987; *accepted* 9 *June* 1987)

Abstract—Neutrophils contain the enzyme myeloperoxidase, which oxidizes Cl^- ions into the powerful oxidant hypochlorous acid (HOCl). HOCl inactivates α_1-antiprotease, permitting uncontrolled protease activities. Most anti-inflammatory drugs tested are capable of reacting with HOCl, but the reactions seem insufficiently rapid under physiological conditions to protect α_1-antiprotease against inactivation by HOCl. However, rapid scavenging of HOCl might contribute to the anti-inflammatory effects of penicillamine, gold sodium thiomalate, phenylbutazone and primaquine.

Activated neutrophils produce the oxygen-derived species superoxide (O_2^-) and H_2O_2 and, possibly, the hydroxyl radical (·OH) [1, 2]. They also release the enzyme myeloperoxidase, which uses H_2O_2 to oxidise Cl^- ions into a powerful oxidant that has been identified as hypochlorous acid (HOCl) [3–5]. It has been proposed that phagocyte-generated O_2^-, H_2O_2 and ·OH [2, 6, 7] as well as HOCl [3] are important in producing tissue damage at sites of inflammation, such as the inflamed joints of patients suffering from rheumatoid arthritis [6–9]. HOCl has also been implicated in the pathogenesis of emphysema [3].

Inflammatory diseases such as rheumatoid arthritis can be treated with a wide range of drugs, including steroids, non-steroidal anti-inflammatory drugs such as aspirin, diclofenac sodium or ibuprofen, so-called "disease-modifying drugs" such as penicillamine and gold sodium thiomalate, immunosuppressive compounds, and antimalarials such as chloroquine and quinacrine [10–17]. Each of these drugs probably has multiple mechanisms of action. For example, non-steroidal anti-inflammatory drugs not only inhibit cyclooxygenase, but might also act as scavengers of ·OH radicals [18, 19]. Many of the above drugs have been reported to influence the production of O_2^- and H_2O_2 by activated neutrophils or monocytes [10–15, 20]. They may also bind transition metal ions [7] in ways that affect the reactivity of such metal ions in stimulating free radical reactions (Aruoma and Halliwell, submitted for publication).

It has been suggested that a number of anti-inflammatory drugs, especially those containing sulphur (e.g. penicillamine) might scavenge HOCl generated by myeloperoxidase [21–23]. Pekoe *et al.* [24] found that almost every anti-inflammatory drug tested could inhibit the chemiluminescence of a system containing HOCl, suggesting that all these drugs could act as HOCl scavengers.

However, HOCl is a powerful oxidant that can attack a wide range of oxidizable biomolecules [4, 5, 25–28]. Scavenging of HOCl by a drug will only be physiologically significant if, at the concentration of drug that can be achieved *in vivo*, its reaction with HOCl is fast enough to protect important biological targets from attack by HOCl. For example, dimethylsulphoxide reacts with HOCl [5, 25, 28], but this reaction is probably too slow to account for the biological effects of dimethylsulphoxide [28].

The most important biological target that can be attacked by HOCl is thought to be the α_1-antiprotease protein. HOCl inactivates this protein, so permitting uncontrolled activity of proteases, especially elastase [3, 29]. In the present paper, we have examined the ability of a wide range of anti-inflammatory drugs to protect α_1-antiprotease against attack by HOCl. HOCl itself was used rather than the myeloperoxidase–Cl^-–H_2O_2 system, to avoid confusion arising from the ability of some drugs to react with H_2O_2 or to affect myeloperoxidase activity directly [21, 22].

MATERIALS AND METHODS

Reagents. α_1-Antiprotease (type A9024) was from Sigma. Porcine pancreatic elastase, NaOCl and other reagents used were from BDH Chemicals Ltd. Drugs were provided by their respective manufacturers.

Assays. Elastase and α_1-antiprotease were assayed at pH 7.4 essentially as described in [26] and [27]; full details are given in the legend to Table 1. HOCl was obtained immediately before use by adjusting NaOCl (BDH Chemicals Ltd) to pH 6.2 with dil. H_2SO_4, and its concentration measured as in ref. 25.

Table 1. Effect of anti-inflammatory drugs on the inactivation of α_1-antiprotease by HOCl

Drug added	Type of drug	Elastase activity (% maximum rate) Column A (simultaneous drug addition)	Column B (pre-incubation of HOCl with drug)
None	—	100	98
(Omit HOCl)	—	3	3
Paracetamol	Analgesic	99	3
Acetylsalicylic acid	Salicylic acid	98	42
Salicylic acid	Salicylic acid	99	12
Mefenamic acid	Fenamic acid	95	11
Flufenamic acid	Fenamic acid	98	5
Diflunisal	Salicylic acid	92	83
Naproxen	Propionic acid	94	60
Ibuprofen	Propionic acid	96	74
Ketoprofen	Propionic acid	100	97
Chloroquine	Anti-malarial	92	2
Diclofenac sodium	Phenylacetic acid	98	15
Indomethacin	Heterocyclic	51	1
Primaquine	Anti-malarial	5	1
Penicillamine	Sulphur-containing	3	2
Phenylbutazone	Pyrazolone	1	1
Piroxicam	Oxicam	38	7

α_1-antiprotease (final concentration 1.0 mg/ml) was mixed with the final concentration of drug stated and then HOCl was added to a final concentration of 75 μM. The final reaction volume was 35.1 μl, and also contained phosphate-buffered saline at pH 7.4 [26]. This reaction mixture (reaction mixture I) was incubated at 25° for 1 hr. 3 ml of buffer was then added, followed by pancreatic elastase. After further incubation for 30 min, elastase substrate was added and the elastase activity remaining was assayed as a rise in absorbance at 410 nm [26]. Results are expressed as a % of maximum elastase activity (100% is an A_{410} of 0.060/min), being the mean values of duplicate experiments that agree to within 5%. *Column A* Experiment as above. *Column B* The HOCl and drug were *pre-incubated* for 5 min *before* adding α_1-antiprotease, followed by incubation for a further 60 min. Elastase and buffer were then added, as above. Drugs were included in the reaction mixture at a final concentration of 1.0 mM. Where necessary, the pH of drug solutions was adjusted to pH 7.4 immediately before use.

Drugs were dissolved in phosphate-buffered saline or, where necessary, in 0.1% (w/v) Na_2CO_3 and the pH of reaction mixtures adjusted to 7.4, if required.

RESULTS

Preliminary experiments showed that none of the drugs, at the concentrations tested, had any effect on elastase itself. α_1-Antiprotease inhibits elastase, and a concentration sufficient to inhibit by approximately 97% was used in these experiments. This concentration (1.0 mg/ml) is close to the reported human serum concentration of α_1-antiprotease in normal subjects (1.2–1.3 mg/ml [31]). None of the drugs tested affected the ability of α_1-antiprotease to inhibit elastase (data not shown). If α_1-antiprotease is exposed to HOCl, its elastase-inhibitory capacity is lost (Table 1, first line). A concentration of 75 μM HOCl, within the range of concentrations likely to be produced adjacent to activated neutrophils and to released myeloperoxidase *in vivo* [5, 30], was used in these experiments.

In agreement with the report of Pekoe *et al.* [24] that most anti-inflammatory drugs can react with HOCl, pre-incubation of the HOCl with 1 mM concentrations of most drugs for 5 min decreased inactivation of α_1-antiprotease added subsequently, so that its elastase-inhibitory capacity was preserved (Table 1, column B). However, the protective effects were weak or absent in the cases of aspirin, naproxen, ibuprofen, ketoprofen and diflunisal (Table 1, column B).

However, when HOCl is generated *in vivo*, both α_1-antiprotease and any drug ingested by the patient will be present simultaneously. Only if a drug can, at the concentrations present, combine more quickly with HOCl than does α_1-antiprotease will the elastase inhibitory capacity of this protein be preserved. Table 1 (column A) shows that the following drugs, tested at concentrations up to 1 mM, were *not* able to protect α_1-antiprotease against inactivation by 75 μM HOCl: paracetamol, aspirin, salicylate, mefenamic acid, flufenamic acid, diflunisal, naproxen, ibuprofen, ketoprofen, diclofenac sodium or chloroquine. However, five drugs *were* able to protect the α_1-antiprotease: indomethacin, primaquine, penicillamine, phenylbutazone and piroxicam. Table 2 shows the concentration-dependence of these protective effects. The protections by indomethacin and piroxicam were only seen at high ($\geq$400 μM) drug concentrations. Phenylbutazone and penicillamine were effective at concentrations comparable to those of the HOCl present, suggesting that, even in the presence of α_1-antiprotease, they preferentially scavenge HOCl on a 1:1 molar basis. Primaquine was protective at concentrations >100 μM. Table 2 shows that thiomalic acid, the thiol component of gold sodium thiomalate, could

Table 2. Effect of anti-inflammatory drugs on the inactivation of α_1-antiprotease by HOCl

Drug present	Concentration in reaction system 1 (μM)	Elastase activity % maximum rate
None	—	99
Omit HOCl	—	2
Phenylbutazone	50	52
	100	9
	200	1
Piroxicam	50	99
	400	80
	800	38
	1000	38
Indomethacin	200	98
	600	68
	800	56
	1000	51
Primaquine	50	98
	100	78
	200	48
	400	10
	600	3
Penicillamine disulphide	50	92
	100	21
	200	13
	400	2
Thiomalic acid	200	44
	800	21
	1000	0

Experiments were carried out as described in the legend to Table 1 (column A), i.e. drug, buffer and α_1-antiprotease were present together when HOCl was added. 100% elastase activity was a ΔA_{410} of 0.059/min.

also protect α_1-antiprotease against inactivation by HOCl.

DISCUSSION

In agreement with the results of Pekoe *et al.* [24], many of the drugs tested seemed able to react with HOCl, in that pre-incubation of the drug with HOCl for 5 min before adding α_1-antiprotease decreases inactivation of α_1-antiprotease added subsequently. However, when HOCl is generated *in vivo* by activated neutrophils, or by myeloperoxidase released from neutrophils, both α_1-antiprotease and any drug administered will be present simultaneously. α_1-Antiprotease is present in plasma at concentrations of 1.2–1.3 mg/ml [31], close to the concentration of 1.0 mg/ml used in our experiments. A physiological concentration of HOCl [30] was also used. However, most drugs, tested at concentrations up to 1 mM, were unable to protect α_1-antiprotease against HOCl when all reagents were present simultaneously in the reaction mixture. Hence, although these drugs *can* react with HOCl, they probably do not do so quickly enough for this to be a contribution to their biological activity. An alternative possibility is that reaction of the drug with HOCl produces a short-lived oxidant that can itself attack α_1-antiprotease [3]. However, this would not alter the conclusion that protection of α_1-antiprotease against attack by HOCl does not contribute significantly to the action of these drugs.

Of the drugs tested, six could offer protection to α_1-antiprotease, i.e. they could compete effectively with α_1-antiprotease for the HOCl added. For piroxicam and indomethacin, concentrations approaching 1 mM were required, which suggests that the biological relevance of these results is questionable. However, penicillamine was protective even at very low concentrations, which confirms the proposals of Cuperus *et al.* [21, 22] that scavenging of HOCl may contribute to the anti-inflammatory actions of penicillamine. Thiomalic acid was also very protective, which suggests that scavenging of HOCl might contribute to the actions of gold sodium thiomalate. Phenylbutazone was protective at physiological concentrations (50–100 μM). Indeed, the ability of leukocytes to chlorinate phenylbutazone, presumably by HOCl attack, has recently been described [32]. Whether the protection shown by primaquine is biologically relevant remains to be established; although blood concentrations of antimalarials remain low during therapy, they are highly concentrated in tissues [20], including neutrophils [33], and may reach concentrations in neutrophils [33] at which scavenging of HOCl might become significant. This could protect the neutrophil from attack by its own HOCl [34] and preserve it in an activated state for a longer time.

Acknowledgements—We are very grateful to the Arthritis and Rheumatism Council, CIBA-Geigy Pharmaceuticals and the Joint Research Committee of King's College Hospital for financial support. The trustees of the Rayne Insti-

tute generously provided laboratory space. B.H. is a Lister Institute Research Fellow.

REFERENCES

1. B. M. Babior, *New Engl. J. Med.* **298**, 659 (1978).
2. B. Halliwell, *Cell Biol. Int. Rep.* **6**, 529 (1982).
3. S. J. Weiss, *Acta physiol. scand.* Suppl. **548**, 9 (1986).
4. J. M. Albrich, C. A. McCarthy and J. K. Hurst, *Proc. natn. Acad. Sci. U.S.A.* **78**, 210 (1981).
5. C. C. Winterbourn, *Biochim. biophys. Acta* **840**, 204 (1985).
6. J. M. McCord, *Science* **185**, 529 (1974).
7. B. Halliwell and J. M. C. Gutteridge, *Mol. Asp. Med.* **8**, 89 (1985).
8. B. Halliwell, J. M. C. Gutteridge and D. Blake, *Phil. Trans. R. Soc. Lond. Ser. B* **311**, 659 (1985).
9. P. G. Winyard, D. R. Blake, S. Chirico, J. M. C. Gutteridge and J. Lunec, *Lancet* **i**, 69 (1987).
10. N. P. Hurst, J. K. French, A. L. Bell, G. Nuki, M. L. O'Donnell, W. H. Betts and L. G. Cleland, *Biochem. Pharmac.* **35**, 3083 (1986).
11. N. P. Hurst, A. L. Bell and G. Nuki, *Ann. Rheum. Dis.* **45**, 37 (1986).
12. P. Biemond, A. J. G. Swaak, J. M. A. Penders, C. M. Beindorff and J. F. Koster, *Ann. Rheum. Dis.* **45**, 249 (1986).
13. H. B. Kaplan, H. S. Edelesen, H. M. Korchak, W. P. Given, S. Abramson and G. Weissmann, *Biochem. Pharmac.* **33**, 371 (1984).
14. D. H. Nelson, A. R. Wennhold and D. K. Murray, *J. Steroid Biochem.* **14**, 321 (1981).
15. Y. Oyanagui, *Biochem. Pharmac.* **27**, 777 (1978).
16. L. Iannuzzi, N. Dawson, N. Zein and I. Kushner, *New Engl. J. Med.* **309**, 1023 (1983).
17. M. C. Grootveld, M. T. Razi and P. J. Sadler, *Clin. Rheumatol.* **3**, 5 (1984).
18. K. O. Hiller, P. L. Hodd and R. L. Willson, *Chem-Biol. Interact.* **47**, 293 (1983).
19. M. Grootveld and B. Halliwell, *Biochem. J.* **237**, 499 (1986).
20. Y. Miyachi, A. Yoshioka, S. Imamura and Y. Niwa, *Ann. Rheum. Dis.* **45**, 244 (1986).
21. R. A. Cuperus, A. O. Muijsers and R. Wever, *Arthritis Rheum.* **28**, 1228 (1985).
22. R. A. Cuperus, A. O. Muijsers and R. Wever, *Biochim. biophys. Acta* **749**, 18 (1983).
23. H. Vapaatalo, *Med. Biol.* **64**, 1 (1986).
24. G. Pekoe, K. Van Dyke, H. Mengoli, D. Peden and D. English, *Agents Actions* **12**, 232 (1982).
25. D. R. Green, J. H. Fellman and A. L. Eicher, *FEBS Lett.* **192**, 33 (1985).
26. M. Wasil, B. Halliwell, D. C. S. Hutchison and H. Baum, *Biochem. J.* **243**, 219 (1987).
27. M. Grootveld, B. Halliwell and C. P. Moorhouse, *Free Radical Res. Commun.* **4**, 69 (1987).
28. M. Wasil, B. Halliwell, M. Grootveld, C. P. Moorhouse, D. C. S. Hutchison and H. Baum, *Biochem. J.* **243**, 867 (1987).
29. J. Travis and G. S. Salvesen, *Annu. Rev. Biochem.* **52**, 655 (1983).
30. B. Kalyanaraman and P. G. Sohnle, *J. clin. Invest.* **75**, 1618 (1985).
31. M. I. Kamboh, *Dis. Markers* **3**, 135 (1985).
32. S. Ichihara, H. Tomisawa, H. Fukazawa, M. Tateishi, R. Joly and R. Heintz, *Biochem. Pharmac.* **35**, 3935 (1986).
33. J. K. French, N. P. Hurst, M. L. O'Donnell and W. H. Betts, *Ann. Rheum. Dis.* **46**, 42 (1987).
34. R. A. Clark and N. Borregaard, *Blood* **65**, 375 (1985).