Biochemical Pharmacology, Vol. 34, No. 8, pp. 1337–1338, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

SHORT COMMUNICATIONS

Involvement of leukocyte peroxidases in the metabolism of tenoxicam

(*Received* 6 *August* 1984; *accepted* 23 *October* 1984)

During the course of our investigation on the mechanism of action of tenoxicam [4-hydroxy-*N*-(2′-pyridyl)-2-methyl-2H-thieno-⟨2,3e⟩-1,2-thiazine-3-carboxamide-1,1-dioxide] a potent anti-inflammatory agent, we have examined the metabolic fate of tenoxicam in leukocytes, which participate in the promotion of inflammation. The leukocyte extract (which contained both myeloperoxidase and eosinophil peroxidase) enzymatically converted this drug to three metabolites which were previously postulated to be formed via a C-3 oxidation of the thiazine ring by us [1]. The present paper will discuss the possible involvement of peroxidases in the metabolic conversion of tenoxicam and the pharmacological meaning of the reaction in the anti-inflammatory activity of this drug.

Materials and methods

Non-radioactive tenoxicam, [4-^{14}C]tenoxicam (specific activity, 84 μCi/mg) labelled at C-4 position of thiazine ring, and 3-(*N*-methylsulfamoyl)-2-thiophenecarboxylic acid (thiophenecarboxylic acid) were synthesized in F. Hoffmann-La Roche (Basle) and kindly supplied to us. 2-Methyl-thieno-⟨2,3d⟩-isothiazole-3(2H)one-1,1-dioxide (*N*-methylthiophenesulfimide) was prepared by an anodic oxidation of tenoxicam [1] and *N*-(2-pyridyl)-oxamic acid (pyridyloxamic acid) was synthesized according to the procedure described by Ohta and Masaki [2]. Cetyltrimethylammonium bromide and guaiacol were purchased from Wako Pure Chemical Industry Co. (Tokyo). Other reagents used in the present study were of analytical special grade commercially available.

Leukocytes were collected from the thighbone marrow of male rats (Sprague–Dawley strain, 7-week-old, 170–180 g) at 4° by the usual way.

Peroxidases (i.e. myeloperoxidase and eosinophil peroxidase) in the leukocytes were solubilized with 0.3% of cetyltrimethyl ammonium bromide in 10 mM potassium phosphate buffer (pH 7.0) according to the procedure described by Desser *et al.* [3]. Peroxidase activity was determined at 25° by following the increase in absorbance at 470 nm resulting from the oxidation of guaiacol. The 3-ml reaction mixture contained 13 mM guaiacol, 0.33 mM H_2O_2, enzyme solution, 10 mM phosphate buffer (pH 7.0). A unit of enzyme activity was defined as amount of enzyme that produces an increase of 1 absorbance unit in 1 min.

Ten micrograms (29.7 nmole) of [^{14}C]tenoxicam (0.5 μCi/mg) dissolved in 10 μl of dimethylsulfoxide was incubated with 1.6 units of leukocyte peroxidases in the presence of 0.1 mM H_2O_2 and 10 mM NaCl in a final volume of 1 ml of 50 mM potassium phosphate buffer, pH 6.0, for 10 min at 37°. After termination of the reaction by an addition of 100 μl of 1 N HCl in an ice-chilled waterbath, non-radioactive tenoxicam, thiophenecarboxylic acid, and N-methyl-thiophenesulfimide (200 μg each) were added to the mixture as internal standards. Each compound was extracted with 6 ml of ethyl acetate followed by evaporation of the organic solvent under reduced pressure. The residue dissolved in an appropriate volume of methanol was applied to a silica gel t.l.c. plate (Kiesel Gel, 60F_{254}, 0.25 mm thick, Merck, Darmstadt) and developed with a solvent system of chloroform/ethyl acetate/methanol/HCOOH (30:15:5:1, v/v). Radioactive substances with R_f values of 0.86, 0.64, and 0.50 were located under u.v. light and extracted from the zone of silica gel with ethylacetate (for the compound with R_f of 0.86) and methanol (for the two compounds with R_f of 0.64 and 0.56). The extract was completely dried under nitrogen stream and subsequently the residue was dissolved in 3.0 ml of methanol. A portion of the extract (2.0 ml) was used for determination of ^{14}C-radioactivity in a liquid scintillation spectrophotometer (LSC-700, Aloka, Tokyo) and another portion (0.5 ml) for monitoring the recovery of added non-radioactive carriers by measuring u.v. absorbance (UV-190 Spectrophotometer, Shimadzu Seisaku Co., Tokyo) at 365, 280, and 254 nm for tenoxicam, *N*-methylthiophenesulfimide, and thiophenecarboxylic acid, respectively.

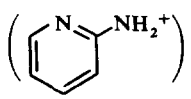
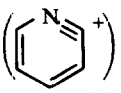
Results

Metabolism of tenoxicam by solubilized peroxidases. During the incubation of [4-^{14}C]tenoxicam with leukocyte peroxidases, 11.2 nmoles of *N*-methylthiophenesulfimide and a trace amount of thiophenecarboxylic acid (0.7 nmole) were formed. In a control incubation mixture run simultaneously with heat denatured enzymes (100°, 5 min), the formation of these two radioactive metabolites was virtually negligible. Omission of H_2O_2 or addition of 0.1 mM sodium azide (a potent inhibitor of peroxidases) abolished the formation of each metabolite and the parent compound was recovered without significant loss, whereas omission of NaCl, which has been considered to give a hypochlorite ion by a reaction with an active oxygen produced by peroxidases in the mixture [4], stimulated the formation of these two metabolites (i.e. *N*-methylthiophensulfimide, 18.9 nmole; thiophenecarboxylic acid, 1.0 nmole).

Identification of tenoxicam metabolites. For identification of each metabolite, the incubation was performed in a 10-times larger scale (the mixture contained 2 mg of the substrate and 100 units of the enzymes): the incubation conditions were the same as those described above except that (a) incubation time was 60 min and (b) 25 μl of 0.2 M H_2O_2 was added to the reaction mixture every 5 min during the course of the incubation. After termination of the reaction by an addition of 1 ml of 1 N HCl, radioactive compounds were extracted twice with 30 ml of ethyl acetate.

The organic solvent was evaporated under reduced pressure and t.l.c. of the residue with the aforementioned solvent system resulted in a separation of three radioactive compounds with R_f values of 0.86 (M-1), 0.64 (tenoxicam), and 0.50 (M-2). The EI-mass spectrum (70 eV, JMS-DX300 High Resolution Mass Spectrometer, JEOL, Tokyo) of compound M-1, which was further purified on a t.l.c. plate with dichloromethane (R_f = 0.45), gave a molecular peak at *m/z* 203 (a base peak, $C_6H_5NO_3S_2$), *m/z* 175 (M − CO), *m/z* 139 (M − SO_2), *m/z* 110 (M − SO_2NCH_3) and the fragmentation pattern was identical with that of authentic *N*-methylthiophenesulfimide. An EI mass spectral pattern of compound M-2 was very similar with that of synthesized thiophenecarboxylic acid: a protonated molecular peak was present at *m/z* 222 ($C_6H_7NO_4S_2$) together with fragment peaks including *m/z* 204 (M − OH), *m/z* 191 (M − $NHCH_3$), *m/z* 175 (M − $NHCH_3$ − OH + H), *m/z* 147 (M − $NHCH_3$ − COOH + H), *m/z* 139 (M − SO_2 − H_2O), *m/z* 128 (M − SO_2NHCH_3 + H), and *m/z* 111 (M − SO_2NHCH_3 − OH + H).

In addition to above two radioactive metabolites, a non-radioactive metabolite (M-3) was isolated from the remaining aqueous phase by the following method.

The aqueous phase was adjusted to pH 3.0, evaporated to about 3 ml under reduced pressure, and filtered through a membrane filter (0.8 μm, Millex-PF, Millipore Co., USA). The filtrate was applied to a preparative h.p.l.c. column (YMC SH-343, 15 μm, 250 × 20 mm, Yamamura Chemical, Osaka, Japan). The column was eluted with 1% acetic acid at a flow rate of 8 ml/min and a compound, which was detected at 280 nm and emerged at 26–29 min, was collected. After lyophilization, the residue was dissolved in 100 μl of methanol and a portion of the solution was subjected to a mass spectral analysis: the EI mass spectrum (20 eV) showed a molecular peak at m/z 166 ($C_7H_6N_2O_3$), m/z 121 (M—COOH), m/z 94  , and m/z 78  and was identical with that of synthetic pyridyloxamic acid.

The ratio of amount of *N*-methylthiophenesulfimide over that of pyridyloxamic acid in the reaction mixture was found to be approximately 1:1 by measuring the ^{14}C-radioactivity of the former and u.v. absorbance of the latter.

Discussion

[4-^{14}C]Tenoxicam was metabolized to a nearly equimolar amount of *N*-methylthiophenesulfimide (M-1) and its counterpart, pyridyloxamic acid (M-3), together with a trace amount of thiophenecarboxylic acid (M-2) by a leukocyte extract which possessed a high activity of the two kinds of peroxidases (Fig. 1).

Several lines of evidence clearly demonstrated the participation of peroxidases in the present metabolic reaction: (1) H_2O_2 was an essential factor for the biotransformation of tenoxicam in the present incubation system, (2) sodium azide, a potent inhibitor of peroxidases, completely inhibited the biotransformation of tenoxican at a concentration of 0.1 mM, and (3) peroxidases are well known to catalyze an oxidation reaction in a pH range of 5–6 [4, 5] and, indeed, the reaction for tenoxicam was observed only under acidic conditions (pH 5 and 6) but not under the neutral nor basic ones (data not shown).

The oxidation reaction mediated by peroxidases has generally been recognized as proceeding via one of two reaction mechanisms (i.e. an oxidation reaction of substrates with an active oxygen species directly produced by peroxidases and with a hypochlorite ion generated by a reaction between a chloride anion and an active oxygen species) or both in the presence of a chloride anion [6]. The present results unequivocally indicate the participation of an active oxygen, which was directly produced by the enzymes, in the conversion of tenoxicam. The contribution of a hypochlorite ion to the conversion reaction could not, however, be ruled out in spite of the result that the omission of NaCl from the reaction mixture caused an increase in the amount of both *N*-methylthiophenesulfimide and thiophenecarboxylic acid in the mixture, because sodium hypochlorite was found to easily oxidize tenoxicam to give the same sorts of compounds identified in the present study (data not shown).

Formation of an equimolar amount of *N*-methylthiophenesulfimide and pyridyloxamic acid strongly indicated that the first step to produce *N*-methylthiophenesulfimide (M-1) was not a mere hydrolysis of the amido bond of tenoxicam, but a direct oxidation of C-3 position of the thiazine ring. Thus, as was discussed in our previous paper [11], the C-3 position of tenoxicam was metabolically vulnerable to yield a putative C-3 oxidation intermediate(s) which was subsequently converted to the above metabolites.

In summary, a leucocyte extract containing both myeloperoxidase and eosinophil peroxidase catalyzed a C-3 oxidation reaction of tenoxicam, a potent anti-inflammatory agent, to give nearly equimolar amount of 2-methyl-thieno-⟨2,3d⟩-isothiazole-3(2H)one-1,1-dioxide and *N*-(2-pyridyl)-oxamic acid together with a trace amount of 3-(*N*-methylsulfamoyl)-2-thiophenecarboxylic acid. These metabolites were likely to be formed by a direct attack of an active oxygen or a hypochlorite ion, or both, to C-3 position of tenoxicam. These results suggest that, at least, a part of anti-inflammatory activity of this drug is attributable to its action as scavenger of these reactive species.

Acknowledgements—We thank Ms T. Kiuchi for preparing this manuscript.

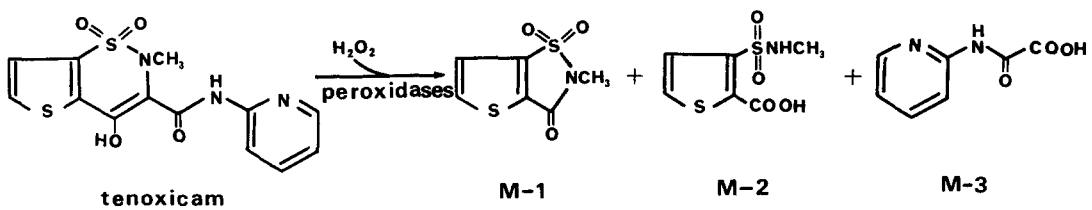


Fig. 1. Metabolic conversion of tenoxicam by leukocyte peroxidases.

Department of Biochemistry, Nippon Roche Research Center, 200-Kajiwara, Kamakura, Kanagawa pref, Japan.

SHIGEYASU ICHIHARA
HIROKI TOMISAWA
HIDEO FUKAZAWA
MITSURU TATEISHI

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