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Effects of sodium fluoride and cobalt chloride on the enantioselectivity of microsomal and cytosolic esterases in rat intestinal mucosa

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Abstract—The effects of sodium fluoride (NaF) and cobalt chloride ($CoCl_2$) on the enantioselective hydrolysis of racemic oxazepam 3-acetate (rac-OXA) by microsomal and cytosolic esterases in rat intestinal mucosa were studied. Microsomal and cytosolic esterases hydrolyzed *S*-OXA and *R*-OXA in approximately 1:19 and 4:1 ratios, respectively. The hydrolysis of *R*-OXA by microsomal esterases was inhibited by NaF with an IC_{50} of 13.4 ± 1.5 mM. Hydrolyses of both *S*-OXA and *R*-OXA by cytosolic esterases were inhibited by NaF with a similar IC_{50} value (~3 mM). The hydrolysis of *S*-OXA by cytosolic esterases was inhibited by $CoCl_2$ (IC_{50} = ~5 mM), whereas the hydrolysis of *R*-OXA by cytosolic esterases was stimulated by ~10% in the presence of 1 mM $CoCl_2$. In comparison, the hydrolysis of *R*-OXA by microsomal esterases was stimulated by ~55% in the presence of 1 mM $CoCl_2$. These results not only revealed the effects of NaF and $CoCl_2$ on the catalytic activities of enantioselective cytosolic and microsomal esterases, but also indicated that microsomal and cytosolic esterases that selectively hydrolyzed *R*-OXA were distinctly different protein entities.

Carboxylesterases/amidases (generally known as *esterases*) are proteins that have catalytic activities in the hydrolysis of a wide variety of endogenous and exogenous chemicals containing ester, thioester, carbamate, or amide linkages [1–3]. Esterases play important roles in the biotransformation and detoxification of toxic environmental chemicals such as organophosphorous insecticides and some ester compounds used as drugs in humans [1–3]. There are multiple forms of esterases in the intestines of human and experimental animals [4–10]. Microsomal and cytosolic esterases play different but equally important roles in the hydrolysis of ester drugs [11, 12].

Fluorides including NaF are known to interfere with many enzyme systems, including glycolytic enzymes, cholinesterases, and enzymes in which magnesium and manganese are present [13]. Various esterase preparations showed different sensitivities to the inhibitory effects of NaF [6, 14–16]. Cobalt is an essential trace metal incorporated in vitamin B_{12}, which plays an important role in the prevention of pernicious anemia. Cobalt and its salts are useful in paint dryers, as catalysts, and in permanent magnets. In the 1960s, cobalt was added to some beers as a foam restorative and stabilizer, which resulted in epidemic cardiomyopathy among heavy beer drinkers [17].

We recently reported [18] that microsomal and cytosolic esterases in rat intestinal mucosa possess opposite enantioselectivity in the hydrolysis of racemic oxazepam 3-acetate (rac-OXA)* to form oxazepam (OX). The enantiomers of OX undergo facile racemization in aqueous solutions [19]. In the hydrolysis of rac-OXA, microsomal esterases hydrolyzed predominantly the *R*-OXA, whereas cytosolic esterases hydrolyzed *S*-OXA and *R*-OXA in greater than 3:1 ratio [18]. The esterases in cytosol and microsomes that hydrolyzed *R*-OXA appeared to be more sensitive to the inhibitory effect of paraoxon than the esterases that hydrolyzed *S*-OXA [18]. We concluded that most esterases in cytosolic fraction were catalytic proteins distinctly different from membrane-bound esterases in microsomes. However, the minor *R*-OXA-selective esterases in cytosol may be derived from the *R*-OXA-selective membrane-bound esterases by autolysis and retain the enantioselectivity.

* Abbreviations: OX, oxazepam; OXA, 3-*O*-acyloxazepam (oxazepam 3-acetate); rac-OXA, racemic oxazepam 3-acetate; NDZ, *N*-desmethyldiazepam (nordiazepam); and CSP, chiral stationary phase.

In our recent report, we noted that the *R*-OXA-selective esterases in cytosol are thermally more stable than the *R*-OXA-selective esterases in microsomes [18]. Hence, the *R*-OXA-selective esterases in microsomal and cytosolic fractions may be uniquely different proteins. In this communication, we describe inhibition profiles by NaF and $CoCl_2$ which provide additional evidence that the *R*-OXA-selective microsomal and cytosolic esterases in rat intestinal mucosa are distinctly different protein entities.

Materials and Methods

Chemicals and animals. Demoxepam (7-chloro- 1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide; Ro 5-2092) and *N*-desmethyldiazepam (NDZ) were provided by Hoffmann-La Roche Inc. (Nutley, NJ). Rac-OXA was prepared from demoxepam according to Bell and Childress [20]. OX was provided by Wyeth-Ayerst Research (Princeton, NJ).

Esterase preparation and in vitro *incubation.* S9, cytosol, and microsomal fractions were prepared from small intestinal mucosae of male Sprague–Dawley rats (250–300 g body weight) as previously described [18]. Solubilized microsomal esterases were prepared by treatment of microsomes with Triton X-100 as described [18]. Many esterase preparations with similar catalytic and enantioselective activities were used during the course of this study. Hydrolysis studies were performed in 1-mL incubation mixtures. Incubation mixtures (each in triplicate) contained 100 nmol of rac-OXA (added in 20 μL acetonitrile) and various concentrations of NaF (or $CoCl_2$) in buffer A [0.05 M Tris–HCl (pH 7.5)/0.25 M sucrose:glycerol (87.5:12.5, v/v)] and an appropriate amount of protein equivalent of an esterase fraction prepared from rat intestinal mucosa. Reaction mixtures were incubated at 37° for 10, 20, or 30 min, depending on the esterase preparation used. At the end of incubation, 1 mL acetone was added, followed by the addition of chloroform (2 mL). NDZ (10 nmol added in 0.1 mL of acetonitrile) was added to serve as an internal standard for chromatography. The mixture was vortexed for ~20 sec and subsequently centrifuged to separate the organic and aqueous phases. A 1-mL aliquot of the organic phase was removed and evaporated to dryness with a gentle stream of nitrogen at ~50°. The residue was dissolved in 0.2 mL of acetonitrile for either reversed-phase or chiral stationary phase (CSP) HPLC analysis [18]. In all HPLC analyses, not more than 10 μL of a sample was injected.

HPLC. Each sample underwent both reversed-phase

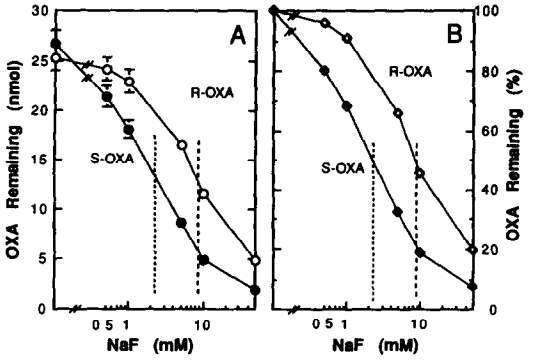

Fig. 1. Effects of NaF on the hydrolysis of rac-OXA by S9 fraction prepared from rat intestinal mucosa. In addition to other components (see Materials and Methods), each incubation mixture (in triplicate) contained 0.25 mg protein equivalent of S9 and the indicated concentrations of NaF. Reaction mixtures were incubated for 20 min at 37°. Panel A indicates the net amounts (in nmol) of *R*-OXA and *S*-OXA hydrolyzed per mL of incubation mixture. Panel B indicates normalized values of *R*-OXA and *S*-OXA hydrolyzed. The IC_{50} values (means ± SD, N = 3) were determined from data in panel B and are marked by vertical lines.

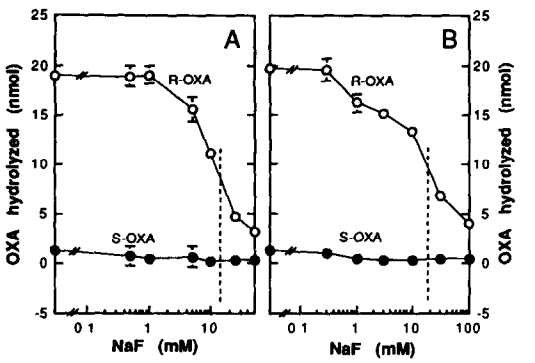

Fig. 2. Effects of NaF on the hydrolysis of rac-OXA by microsomes (panel A) and solubilized proteins derived from microsomes (panel B) prepared from rat intestinal mucosa. In addition to other components (see Materials and Methods), each incubation mixture (in triplicate) contained 0.1 mg protein equivalent of either microsomes or solubilized microsomal proteins and the indicated concentrations of NaF. Reaction mixtures were incubated for 10 min at 37°. The IC_{50} values (means ± SD, N = 3) were determined in a manner similar to that described in the legend of Fig. 1 and are each marked by a vertical line.

and CSP HPLC analyses as described in detail in an earlier report [18]. Reversed-phase HPLC analysis quantified the amount of OX formed in the hydrolysis of rac-OXA. CSP HPLC analysis quantified the percentages of both *S*-OXA and *R*-OXA remaining in the incubation mixture. Results of both reversed-phase and CSP HPLC analyses gave the amounts of *S*-OXA and *R*-OXA hydrolyzed in each incubation mixture.

Results and Discussion

Esterases contained in S9 hydrolyzed both *S*-OXA and *R*-OXA (Fig. 1). NaF inhibited the hydrolysis of *S*-OXA and *R*-OXA with an IC_{50} of 2.3 mM (2.5 ± 0.6 mM, N = 3) and 8.5 mM (8.7 ± 0.5 mM, N = 3), respectively. The IC_{50} values are more clearly seen in the normalized plot (Fig. 1B).

We had established previously that the *R*-OXA-selective esterases in S9 were primarily associated with the microsomal fraction [18]. The highly *R*-OXA-selective membrane-bound esterases were found to be inhibited by NaF with an IC_{50} of ~13 mM (13.4 ± 1.5 mM, N = 3) (Fig. 2A). Because only a very small amount of *S*-OXA was hydrolyzed (Fig. 2A), the IC_{50} value for *S*-OXA-selective esterases in microsomes could not be determined.

Esterases in cytosolic fraction hydrolyzed *S*-OXA substantially faster than *R*-OXA (Fig. 3), and these results were consistent with those in the earlier report [18]. NaF inhibited the hydrolysis of *S*-OXA with an IC_{50} of 3.1 mM (3.5 ± 0.6 mM, N = 3). This IC_{50} value was similar to that found for the *S*-OXA-selective esterases contained in S9 fraction (Fig. 1). The NaF inhibition profile of *R*-OXA hydrolysis in cytosol yielded an IC_{50} of 2.3 mM (2.6 ± 0.5 mM, N = 3). A similar IC_{50} value for *R*-OXA-selective esterases by NaF was obtained when the experiment described in Fig. 3A was conducted using a higher concentration of cytosolic proteins.

The IC_{50} (13.4 mM) for the *R*-OXA-selective esterases in microsomes was 5-fold higher than the IC_{50} (2.6 mM) for the *R*-OXA-selective esterases contained in cytosol (Figs. 2 and 3). We had demonstrated previously that soluble esterase proteins derived from membrane-bound

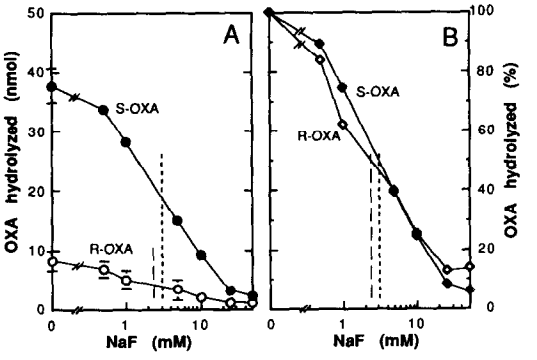

Fig. 3. Effects of NaF on the hydrolysis of rac-OXA by cytosolic fraction prepared from rat intestinal mucosa. In addition to other components (see Materials and Methods), each incubation mixture (in triplicate) contained 0.25 mg protein equivalent of cytosol and the indicated concentrations of NaF. Reaction mixtures were incubated for 30 min at 37°. Panel A indicates the net amounts (in nmol) of *R*-OXA and *S*-OXA hydrolyzed per mL of incubation mixture. Panel B indicates normalized values of *R*-OXA and *S*-OXA hydrolyzed. The IC_{50} values (means ± SD, N = 3) were determined from the data in panel B and are marked by vertical lines.

microsomal proteins retain the *R*-enantioselectivity in the hydrolysis of rac-OXA [18]. However, the sensitivity of membrane-bound *R*-OXA-selective esterases to the inhibitory effect of NaF may be altered when the proteins are solubilized. To determine if solubilized microsomal esterases retained the same sensitivity to the inhibitory effect of NaF, solubilized microsomal esterases were used to determine the NaF inhibition profile. Consistent with the results in the earlier report [18], solubilized proteins derived from microsomes not only retained the *R*-

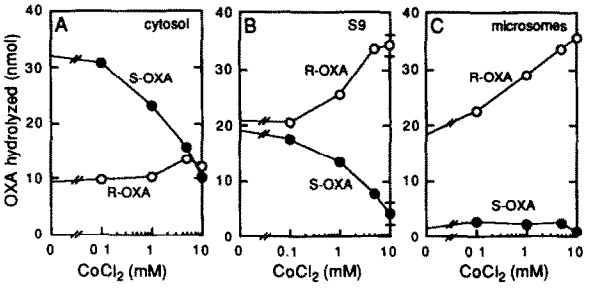

Fig. 4. Effects of $CoCl_2$ on the hydrolysis of rac-OXA by cytosolic (panel A), S9 (panel B), and microsomal (panel C) fractions prepared from rat intestinal mucosa. In addition to other components (see Materials and Methods), each incubation mixture (in triplicate) contained 0.25 mg protein equivalent of either cytosol or S9 (or 0.1 mg protein equivalent of microsomes), and the indicated concentrations of $CoCl_2$. Each reaction mixture was incubated for 30 min (cytosol), 20 min (S9), or 10 min (microsomes) at 37°.

enantioselectivity in the hydrolysis of rac-OXA (Fig. 2B), but also had a similar sensitivity to the inhibitory effect of NaF (Fig. 2B; $IC_{50} = 18 \pm 3$ mM, N = 3).

The results in Figs. 1–3 thus indicated that the *R*-OXA-selective esterases in cytosol were distinctly different from the *R*-OXA-selective esterases in microsomes; these two types of esterases had different sensitivities to the inhibitory effect of NaF. The possibility that the *R*-OXA-selective esterases contained in cytosol were autolysis products derived from microsomes can therefore be eliminated. *S*-OXA-selective and *R*-OXA-selective esterases in cytosol had similar sensitivities to the inhibitory effect of NaF. It remained to be established if the enantioselectivity was due to one or more protein entities.

$CoCl_2$ was found to inhibit *S*-OXA-selective esterases with an IC_{50} of ~5 mM (Fig. 4, A and B). In contrast, the membrane-bound microsomal *R*-OXA-selective esterases were stimulated by the presence of $CoCl_2$ (Fig. 4, B and C); 1 mM $CoCl_2$ stimulated microsomal esterases by ~55% (Fig. 4C). In comparison, the *R*-OXA-selective esterases in cytosol were only stimulated by ~10% by the presence of 1 mM $CoCl_2$ (Fig. 4A). These results not only revealed the interesting properties of $CoCl_2$, but also confirmed that the *R*-OXA-selective esterases in microsomes and cytosol were distinctly different proteins.

In conclusion, considerably greater insight into the properties of cytosolic and microsomal esterases in rat intestinal mucosa was gained by using rac-OXA as a substrate. Microsomal esterases were less sensitive to the inhibitory effect of NaF than cytosolic esterases. The *R*-enantioselectivity of microsomal esterases was stimulated by $CoCl_2$, whereas the *S*-enantioselectivity of cytosolic esterases was inhibited by $CoCl_2$. The *R*-OXA-selective esterases in cytosol were not autolysis products derived from the *R*-OXA-selective esterases in microsomes.

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