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EFFECTS OF TENIDAP ON SUPEROXIDE-GENERATING ENZYMES

NON-COMPETITIVE INHIBITION OF XANTHINE OXIDASE

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Abstract—The anti-rheumatic drug tenidap has been shown previously to attenuate superoxide production by activated neutrophils. Given the importance of leukocyte as well as endothelial cell derived superoxide in mediating inflammatory responses, the effects of tenidap on mammalian enzymes capable of generating superoxide were determined. Tenidap had no effect on the generation of superoxide by NADPH oxidase reconstituted from fractionated neutrophil lysates. However, significant inhibition of superoxide production by mixtures of hypoxanthine and xanthine oxidase was observed in the presence of 3–30 μg/mL tenidap. The kinetics of xanthine oxidase inhibition by tenidap were non-competitive; the K_i of tenidap for xanthine oxidase was 11 μg/mL (34 μM). No inhibition of xanthine oxidase was observed in the presence of other known inhibitors of cyclooxygenase. Inhibition of xanthine oxidase may be a heretofore unrecognized mechanism of the anti-rheumatic effects of tenidap.

Key words: xanthine oxidase; tenidap; superoxide; NADPH oxidase; adenosine deaminase; nucleoside phosphorylase

Superoxide-derived oxidants are important mediators of inflammatory response and tissue injury. Mammalian cells generate superoxide by activation of an NADPH oxidase or during the xanthine oxidase-catalyzed conversion of purine metabolites (hypoxanthine and xanthine) to uric acid. Superoxide produced by endothelial cell-associated xanthine oxidase may facilitate endothelial adhesion responses required for recruitment of leukocytes into sites of inflammation [1, 2]. During activation of neutrophils (PMN¶) and macrophages, superoxide-derived oxidants such as HOCl are generated, which facilitate tissue degradation by leukocyte proteases [3].

Tenidap sodium, an anti-rheumatic drug currently used in clinical trials for the treatment of rheumatoid arthritis, has been shown previously to attenuate superoxide production by human neutrophils stimulated with formylated peptides (f-met-leu-phe) or SAIgG *in vitro* [4]. In clinical studies of patients with rheumatoid arthritis, subjects treated with tenidap have been observed to experience significant reductions in synovial fluid leukocyte counts [5]. Whether these *in vivo* effects are due to decreased production of leukocyte chemotactic factors within the joint or modulation of neutrophil/synovial endothelial interactions required for migration of leukocytes into the joint has not been determined. Since generation of superoxide is pivotal to the production of oxidants mediating tissue injury and may also be important in mediating leukocyte–endothelial cell adhesion functions, the mechanisms whereby tenidap attenuates superoxide production are of interest. Accordingly, we performed studies to determine the effects of tenidap on NADPH oxidase and purine-metabolizing enzymes.

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¶ Abbreviations: PMN, polymorphonuclear leukocytes (neutrophils); SAIgG, surface-associated IgG; ADA, adenosine deaminase; PIPES, 1,4-piperazinediethanesulfonic acid; PMA, phorbol myristic acetate; TNFα, tumor necrosis factor-α; and C5a, complement factor 5a.

MATERIALS AND METHODS

Materials and reagents

Xanthine oxidase, nucleoside phosphorylase, cytochrome *c*, adenosine, guanosine, hypoxanthine, naproxen, ibuprofen, diclofenac, indomethacin and other chemical reagents were obtained from the Sigma Chemical Co. (St. Louis, MO). Tenidap was obtained from the Central Research Division, Pfizer Inc. (Groton, CT).

Preparation of leukocytes

Neutrophils were prepared from normal donors as previously described [6]. The neutrophil preparations contained less than 1% contaminating mononuclear cells, and cell viability by trypan blue exclusion consistently exceeded 98%.

Assay for NADPH oxidase activity

Fluid phase, reconstituted NADPH oxidase was prepared by sonicating neutrophils, and then separating the cell membrane fraction from the cytosol and subcellular granules by ultracentrifugation of the sonicate through Percoll (400,000 *g*). The cytosol and cell membrane fractions were saved, and the membranes were resuspended in Borregard relaxation buffer (ATP, EGTA, PIPES, $MgCl_2$, NaCl, and KCl, pH 7.3) [7]. Just prior to performing the assays, oxidase was extracted from the membranes by incubating equal volumes of the membrane preparation with glycine-buffered (pH 8.0) sodium deoxycholate (2.33% in 50% glycerol) for 30 min at 4°, followed by ultracentrifugation (435,000 *g*). Reaction mixtures contained 150 μL of the resultant supernatant (containing dormant oxidase), 150 μL of the saved cy-

tosol fraction, and 500 μL of assay buffer (ATP, NADPH, 0.7 mg/mL cytochrome *c*, EGTA, and PIPES, pH 7.3), with or without 0–30 μg/mL tenidap [7]. The reaction was started by adding 7.5 μL of 15 mM SDS, and the change in extinction (550 nm) of cytochrome *c* during a 15-min incubation at 37° was measured spectrophotometrically; superoxide production was determined using extinction coefficients for non-reduced (0.89×10^4 M^{-1} cm^{-1}) and reduced cytochrome *c* (2.99×10^4 M^{-1} cm^{-1}) [8].

Assay for purine-metabolizing enzymes

ADA activity was determined using the direct spectrophotometric assay. Using cytosolic extracts of Jurkat cells (ATCC T1B 152) as a source for ADA, 10 μL of cell extract was added to mixtures of 50 mM potassium phosphate buffer (pH 7.4, 25°) with 60 and 20 μM adenosine ± 15 μg/mL tenidap, and the extinction at 265 nm was recorded [9].

Purine nucleoside phosphorylase activity was determined in a similar fashion by adding aliquots of Jurkat cell extract to mixtures of 100 mM potassium phosphate (pH 7.4, 25°) with 50 and 25 μM guanosine ± 15 μg/mL tenidap and recording the $\Delta_{extinction}$ at 257 nm [10].

Effects of tenidap on xanthine oxidase-mediated superoxide production were determined by recording the extinction at 550 nm of reaction mixtures containing 0.04 IU xanthine oxidase, 0.35 mg cytochrome *c*, and 1.5 to 20.0 μM hypoxanthine in 0.5 mL of 20 mM K_2HPO_4 (pH 7.3), ± 0–30 μg/mL tenidap over a 1-min incubation at 25°. Kinetic constants were determined using the EZ Fit program (E.I. DuPont de Nemour).

RESULTS

Effects of tenidap on activity of NADPH oxidase

In the presence of NADPH, ATP, and dilute detergent (SDS), NADPH oxidase components residing in the cytosol and plasma membrane fractions of sonicated neutrophils can be reconstituted to generate superoxide. To determine whether activity of NADPH oxidase is attenuated in the presence of tenidap, cell fractions of neutrophil lysates were incubated with the above cofactors and SDS in the presence or absence of tenidap. In triplicate experiments, superoxide generated by reconstituted NADPH oxidase derived from human neutrophils (392 ± 67 pmol/min) was not attenuated significantly in the presence of 30 μg/mL tenidap (376 ± 48 pmol/min). No reduction of cytochrome *c* was observed when tenidap was incubated with cytochrome *c* alone or with reaction mixtures in which either SDS, NADPH, or the cellular fractions had been deleted (data not shown).

Effects of tenidap on superoxide generation by purine-metabolizing enzymes

To determine the effects of tenidap on enzymes in the purine metabolism pathway, activities of ADA, purine nucleoside phosphorylase (PNP), and xanthine oxidase were determined in the presence or absence of tenidap. ADA activity, measured using the direct spectrophotometric assay of adenosine, was not altered in the presence of 15 μg/mL tenidap (data not shown). Similarly, tenidap had no effect on the activity of purine nucleoside phosphorylase (data not shown).

In contrast, superoxide production by mixtures of hypoxanthine and xanthine oxidase was attenuated markedly in a concentration-dependent fashion by 3–30 μg/mL (10–100 μM) tenidap (Fig. 1). Since tenidap has known inhibitory effects on cyclooxygenase, identical experiments were performed in the presence of other cyclooxygenase-inhibiting anti-inflammatory drugs. No inhibition of xanthine oxidase activity was observed in

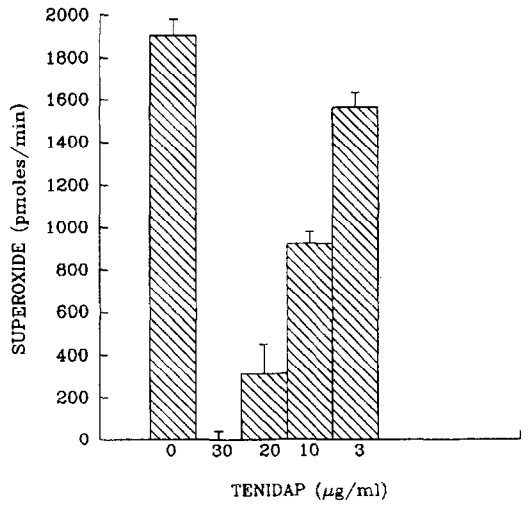

Fig. 1. Generation of superoxide by mixtures of hypoxanthine and xanthine oxidase in the presence of various concentrations of tenidap. Mixtures containing 0.04 IU/mL xanthine oxidase, 0.6 mg/mL cytochrome *c*, and 25 μM hypoxanthine in phosphate buffer were incubated (25°) with 0–30 μg/mL (0–100 μM) tenidap. Bar heights denote mean (N = 3 replicates) initial reaction velocity expressed as $\Delta_{extinction}$ at 550 nm/min; error bars denote SD.

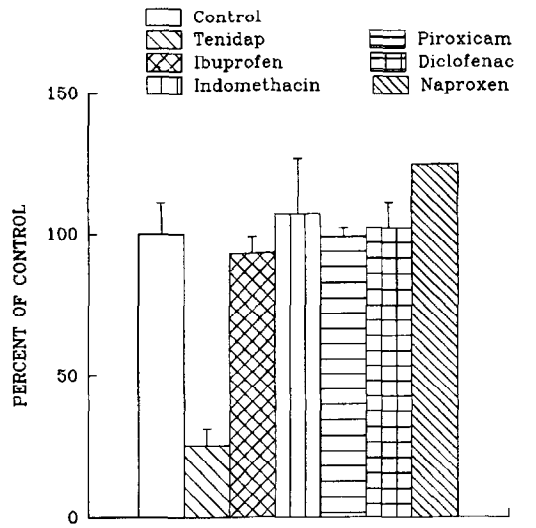

Fig. 2. Effects of tenidap and other inhibitors of cyclooxygenase on activity of xanthine oxidase. Mixtures containing 0.04 IU/mL xanthine oxidase, 0.6 mg/mL cytochrome *c*, and 25 μM hypoxanthine in phosphate buffer were incubated (25°) with 100 μM concentrations of tenidap, indomethacin, naproxen, ibuprofen, diclofenac, or piroxicam. Bar heights denote the mean (N = 3) rate of superoxide generated by mixtures expressed as percent of control mixtures incubated in the absence of drug; error bars denote SD. The mean rate of superoxide generation by control mixtures (without drug) was 798 ± 135 pmol/min.

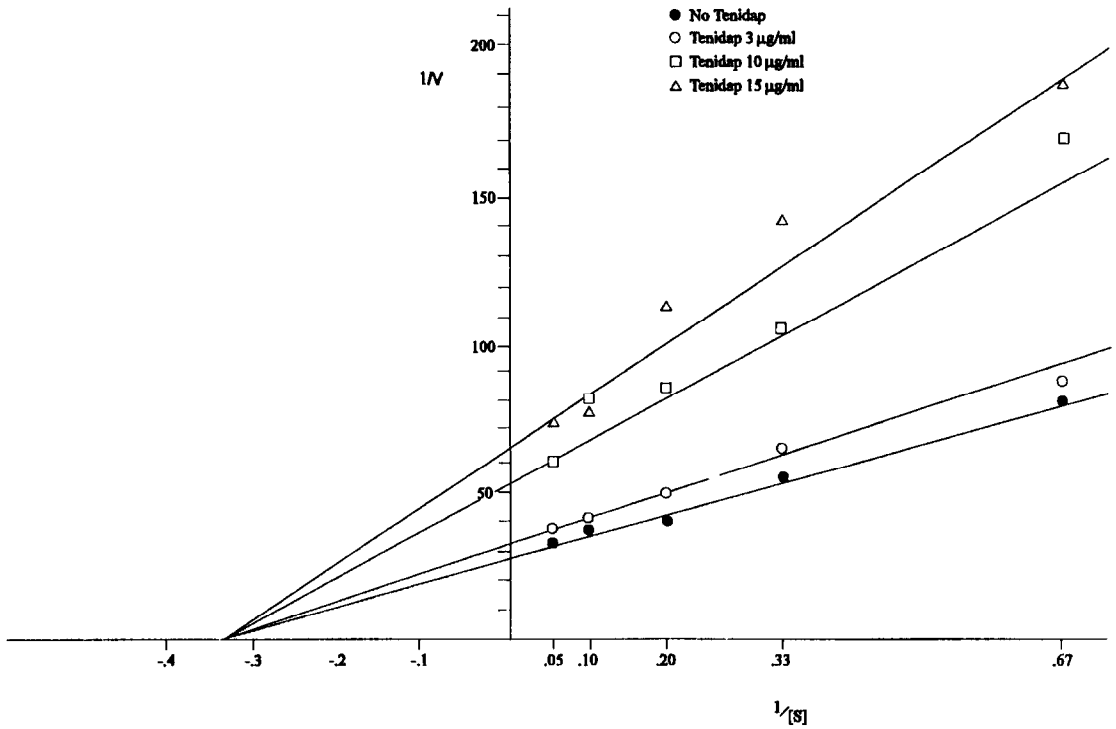

Fig. 3. Lineweaver–Burk plot of xanthine oxidase activity in the presence of various concentrations of tenidap. Mixtures of xanthine oxidase, hypoxanthine, cytochrome *c*, and 0–15 μg/mL tenidap were incubated at 25°. Initial reaction velocity (v) was determined from $\Delta_{extinction}$ at 550 nm during the initial 15 sec of incubation for each substrate/inhibitor combination. [S] = concentration of hypoxanthine (μM); ordinate values represent $1/(\Delta_{extinction}/min)$.

the presence of 100 μM concentrations of indomethacin, piroxicam, ibuprofen, or diclofenac (Fig. 2). Superoxide generated by mixtures of hypoxanthine and xanthine oxidase was actually enhanced in the presence of naproxen (Fig. 2).

Kinetics of xanthine oxidase inhibition by tenidap

To further characterize the inhibition of xanthine oxidase by tenidap, Lineweaver-Burk plots were generated from results of experiments in which 1.5–20 μM hypoxanthine and 3–15 μg/mL (10–46 μM) tenidap were incubated with a fixed amount of xanthine oxidase. Inhibition of enzymatic activity was still observed even when the substrate concentration (1000 μM) exceeded the concentration of tenidap by 10-fold (data not shown). Of the inhibition models tested, a non-competitive model best fit the data (Fig. 3), with the K_i of tenidap for xanthine oxidase being 11 μg/mL (34 μM).

DISCUSSION

In both animal models of inflammation and in the joints of patients with rheumatoid arthritis, tenidap has been shown previously to attenuate inflammatory responses significantly [5, 11]. Although tenidap has known inhibitory effects on cyclooxygenase and 5-lipoxygenase [12], it remains unclear whether attenuation in the generation of inflammatory eicosonoids accounts for all of the observed effects of tenidap *in vivo*. The heretofore unrecognized inhibitory effect of tenidap on xanthine oxidase suggests another mechanism whereby tenidap attenuates acute inflammatory responses.

The failure of other cyclooxygenase inhibitors to attenuate xanthine oxidase activity suggests that the structural features of tenidap responsible for mediating inhibition of xanthine oxidase are likely distinct from those mediating the drug's known inhibitory effects on eicosonoid-metabolizing enzymes. Comparable to other

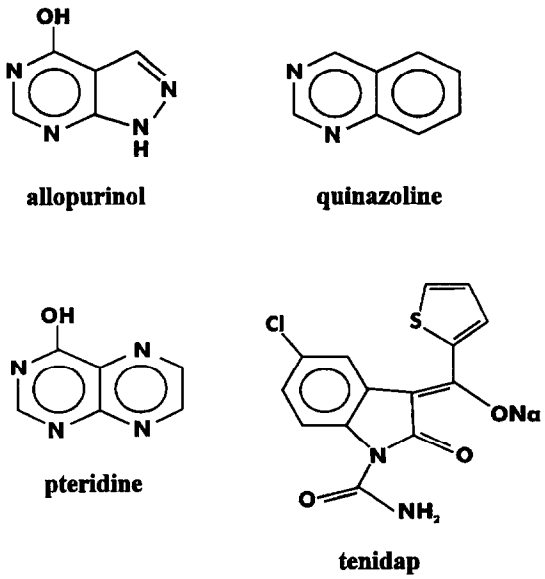

Fig. 4. Structures of tenidap and other inhibitors of xanthine oxidase.

known heterocyclic inhibitors of xanthine oxidase and unlike most cyclooxygenase inhibitors (an exception being indomethacin), tenidap contains two fused unsaturated rings containing at least one nitrogen atom (Fig. 4) [13–16]. However, unlike allopurinol and other purine analogs that exhibit substrate-competitive inhibition of xanthine oxidase [17], the inhibition of xanthine oxidase by tenidap is non-competitive. Although the binding sites involved and mechanism of inhibition remain to be determined, the finding of non-competitive inhibition suggests that tenidap interacts with xanthine oxidase domains distal to the substrate binding site, possibly resulting in allosteric effects that attenuate enzymatic activity.

The absence of a direct inhibitory effect of tenidap on NADPH oxidase activity is consistent with results of previous studies examining the effects of tenidap on neutrophil activation *in vitro*. As previously reported, tenidap significantly attenuates superoxide generated by neutrophils stimulated with SAIgG, but does not attenuate superoxide generated by neutrophils stimulated with direct activators of protein kinases such as PMA [4]. Previous studies have failed to demonstrate significant xanthine oxidase activity in neutrophils [18], and we have noted no inhibition of superoxide production by SAIgG- or PMA-stimulated neutrophils in the presence of allopurinol (W.W.C., unpublished observations). These findings indicate that tenidap attenuates PMN superoxide production via mechanisms independent of the effect of the drug on xanthine oxidase, and more likely interferes with ligand-triggered signal transduction events occurring prior to activation of protein kinases and NADPH oxidase.

However, with regard to endothelial cell functions, the effect of tenidap on xanthine oxidase activity may have important anti-inflammatory consequences. Mammalian endothelial cells constitutively express significant amounts of xanthine dehydrogenase which, in the presence of TNFα, C5a, or sequential hypoxia and re-oxygenation, is converted to xanthine oxidase [19, 20]. Studies of cytokine-stimulated endothelial cells and animal models of ischemia–reperfusion injury suggest that superoxide generation by endothelial cell-associated xanthine oxidase enhances leukocyte–endothelial cell adhesion responses [1, 2]. Inhibition of xanthine oxidase activity with attendant attenuation of PMN–endothelial cell interactions may, therefore, be a mechanism of tenidap's anti-inflammatory effects *in vivo*. Further studies to elucidate the mechanism whereby tenidap alters xanthine oxidase turnover and endothelial cell adhesion responses may provide useful insights into the anti-inflammatory effects of this compound *in vivo*.

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