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SUPEROXIDE IS AN ANTAGONIST OF ANTI-INFLAMMATORY DRUGS THAT INHIBIT HYPOCHLOROUS ACID PRODUCTION BY MYELOPEROXIDASE

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Abstract—Myeloperoxidase, the most abundant enzyme in neutrophils, catalyses the conversion of hydrogen peroxide and chloride to hypochlorous acid. This potent oxidant has the potential to cause considerable tissue damage in many inflammatory diseases. We have investigated the ability of dapsone, diclofenac, primaquine, sulfapyridine and benzocaine to inhibit hypochlorous acid production by stimulated human neutrophils. The drugs were also tested against purified myeloperoxidase using xanthine oxidase to generate hydrogen peroxide and superoxide. The inhibitory effects of the drugs on hypochlorous acid production, either by cells stimulated with phorbol myristate acetate or by myeloperoxidase and xanthine oxidase, were significantly less than those determined with myeloperoxidase and reagent hydrogen peroxide. Comparable potency was observed only when superoxide dismutase was present to remove superoxide. We also observed that with the xanthine oxidase system, inhibition of hypochlorous acid production by dapsone decreased markedly as the concentration of myeloperoxidase increased. Dapsone was a poor inhibitor of hypochlorous acid production by neutrophils stimulated with opsonized zymosan, regardless of the presence of superoxide dismutase. With this phagocytic stimulus, catalase inhibited hypochlorous acid formation by only 60%, which indicates that a substantial amount of the hypochlorous acid detected originated from within phagosomes. Thus, it is apparent that dapsone is unable to affect intraphagosomal conversion of hydrogen peroxide to hypochlorous acid. All the drugs inhibit myeloperoxidase reversibly by trapping it as its inactive redox intermediate, compound II. We propose that superoxide limits the potency of the drugs by reducing compound II back to the active enzyme. Furthermore, under conditions where the activity of myeloperoxidase exceeds that of the hydrogen peroxide-generating system, which is most likely to occur in phagosomes, partial inhibition of myeloperoxidase need not affect hypochlorous acid production. We conclude that drugs that inhibit myeloperoxidase by converting it to compound II are unlikely to be effective against hypochlorous acid-mediating tissue damage.

With the realization that neutrophils contribute to tissue damage in many inflammatory pathologies [1], considerable effort is being directed at blocking their destructive potential. Much of this effort is focused on stopping the production of reactive oxidants. When stimulated, neutrophils discharge superoxide, which is converted to secondary oxidants including hydrogen peroxide and hypochlorous acid [2]. Hypochlorous acid is the most reactive oxidant produced by neutrophils in appreciable amounts [3]. It is highly damaging to proteins [1, 4] and reacts with unsaturated fatty acids to form chlorohydrins that are likely to destabilize cell membranes [5]. Thus, hypochlorous acid could be responsible for much of the inflammatory tissue damage inflicted by neutrophils.

Hypochlorous acid is produced from hydrogen peroxide and chloride by myeloperoxidase [6]. This is the most abundant enzyme of neutrophils, constituting 2–5% of their dry weight [3]. It plays a central role in host defence, but it is not absolutely essential for microbial killing since myeloperoxidase-deficient individuals are generally in good health [7]. In direct contrast, people with chronic granulomatous disease have serious infections due to the inability of their neutrophils to generate superoxide [8]. Therefore, targeting myeloperoxidase has the potential to eliminate much of the inflammatory damage inflicted by reactive oxidants without compromising host defence.

In the last few years a wide range of anti-inflammatory drugs have been shown to inhibit the formation of hypochlorous acid [9–14]. However, they may be less effective at inhibiting hypochlorous acid production by neutrophils as compared to purified myeloperoxidase. We found that dapsone and sulfapyridine inhibit myeloperoxidase by greater than 90% at a concentration of 5 μM [15], yet at 100 μM these drugs are unable to completely inhibit halogenation or hypochlorous acid-dependent cytotoxicity by neutrophils [16–18]. Recently we showed that several anti-inflammatory drugs are potent inhibitors of myeloperoxidase [15]. The drugs act by trapping myeloperoxidase at compound II, which is an inactive redox intermediate of the enzyme [2]. We have also shown that superoxide reduces compound II back to the active enzyme and boosts production of hypochlorous acid [19, 20]. In this paper we investigate whether the interaction of

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superoxide with myeloperoxidase impairs the ability of anti-inflammatory drugs to block hypochlorous acid formation by the purified enzyme and by stimulated human neutrophils.

MATERIALS AND METHODS

Materials. Sulfapyridine was a gift from Pharmacia (North Ryde, Australia). Dapsone, diclofenac, primaquine and benzocaine were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Stock solutions of these chemicals were prepared daily in either water, dilute HCl or dilute NaOH. Acetaldehyde was redistilled daily. Hydrogen peroxide solutions were prepared by dilution of a stock solution and concentrations were determined using ε_{240} 43.6 M^{-1} cm^{-1} [21]. Myeloperoxidase was isolated from human neutrophils and had a purity index of greater than 0.72 [19]. Its concentration was determined using ε_{430} 91,000 M^{-1} cm^{-1} [22]. Bovine erythrocyte superoxide dismutase, phorbol myristate acetate, taurine, cytochrome *c* (type III), bovine liver catalase, zymosan and xanthine oxidase were purchased from Sigma. Neutrophils were isolated from the blood of healthy donors by Ficoll-Hypaque centrifugation, dextran sedimentation and hypotonic lysis of contaminating red cells [23]. Zymosan was boiled for 20 min and washed twice in 10 mM phosphate buffer (pH 7.4) containing 138 mM NaCl and 10 mM KCl (phosphate-buffered saline, PBS). It was then incubated at 5 mg/mL in 30% human plasma with end over end rotation for 30 min at 37°. The opsonized zymosan was washed twice in PBS and suspended at 50 mg/mL.

Determination of the activity of purified enzymes. The amount of hypochlorous acid produced by purified myeloperoxidase and xanthine oxidase/acetaldehyde in PBS was determined by measuring the conversion of taurine to taurine chloramine. After 5 min reactions were stopped by adding 100 μM allopurinol and 20 μg/mL of catalase. The taurine chloramine formed was then assayed using 2-nitro-5-thiobenzoate (ε_{412} 13,600 M^{-1} cm^{-1}) [24]. Hydrogen peroxide consumption by purified myeloperoxidase was monitored continuously with a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) as described previously [25]. The activity of xanthine oxidase was monitored by measuring either the rate of hydrogen peroxide production with the peroxide electrode, or the superoxide dismutase-inhibitable rate of reduction of cytochrome *c* ($\varepsilon_{550\ (reduced-oxidized)}$ 21,000 M^{-1} cm^{-1}) [26]. The peroxidase activity of myeloperoxidase was assayed by measuring the oxidation of 1.6 mM tetramethylbenzidine at 655 nm in 0.5 mL of 50 mM acetate buffer (pH 5.4), with 300 μM hydrogen peroxide and 8% dimethylformamide [27]. Reactions were performed at room temperature and stopped after 10 min by adding 30 μg/mL of catalase and 1.75 mL of cold 200 mM acetate buffer (pH 3.0). Oxidation of tetramethylbenzidine was linear over this period, and the extent of the reaction was directly proportional to the concentration of myeloperoxidase.

Production of hypochlorous acid by human neutrophils. Neutrophils were incubated in PBS containing 10 or 20 mM taurine, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg/mL of glucose. The cells were stimulated with either phorbol myristate acetate or opsonized zymosan, and at the end of the incubation period chlorination of taurine was stopped by adding catalase and placing tubes in melting ice. Neutrophils were then pelleted by centrifugation and the taurine chloramine in the supernatant was assayed as described above. Superoxide production by cells was measured as superoxide dismutase-inhibitable cytochrome *c* reduction [26].

Statistics. One-way analysis of variance was used to determine if there were significant differences between means of treatment groups. A significant level of 0.05 was selected and the Tukey multiple comparison test was used to evaluate which means were significantly different from each other [28]. To calculate the concentration of drug that inhibited hypochlorous acid production by 50% (I_{50}), a rectangular hyperbola was fitted to dose–response curves using non-linear regression.

RESULTS

Effects of anti-inflammatory drugs on production of hypochlorous acid by myeloperoxidase and xanthine oxidase

The anti-inflammatory drugs, dapsone, diclofenac, primaquine and sulfapyridine, and the anaesthetic, benzocaine, are excellent inhibitors of hypochlorous acid production by myeloperoxidase [9, 15]. To determine if superoxide is able to influence inhibition by these drugs, we investigated their effects on the purified enzyme. Xanthine oxidase and acetaldehyde were employed as the source of hydrogen peroxide and superoxide. Xanthine was not used because its oxidation product, uric acid, reduces compound II and masks the effect of superoxide [19]. The concentrations of drugs used have been found to inhibit the initial rate of conversion of reagent hydrogen peroxide to hypochlorous acid by at least 80% [15]. However, in the presence of superoxide they were poor inhibitors of myeloperoxidase (Table 1). For example, primaquine and benzocaine inhibited by only 27% and 8%, respectively. When superoxide dismutase was added to the xanthine oxidase system, all the drugs were significantly more effective at blocking production of hypochlorous acid. In contrast, superoxide dismutase alone increased the production of hypochlorous acid by approximately 20%.

The generation of hydrogen peroxide by the xanthine oxidase system and its conversion to hypochlorous acid were also followed by monitoring the concentration of hydrogen peroxide using a peroxide electrode (Fig. 1). With the xanthine oxidase system alone there was a steady accumulation of hydrogen peroxide. In the presence of myeloperoxidase, there was an initial accumulation to a steady state level where all the hydrogen peroxide generated by xanthine oxidase was being consumed. The difference between the concentration of hydrogen peroxide in the presence and absence of myeloperoxidase represents the hydrogen peroxide

Table 1. Effects of anti-inflammatory drugs on the production of hypochlorous acid by xanthine oxidase and purified myeloperoxidase in the presence or absence of superoxide dismutase

Reaction system	Production of HOCl (% control) Dapsone (1 μM)	Diclofenac (15 μM)	Primaquine (1 μM)	Sulfapyridine (2.5 μM)	Benzocaine (1 μM)
XO/MPO	100 ± 11	100 ± 1	100 ± 2	100 ± 4	100 ± 4*
+Drug	79 ± 8	65 ± 6	73 ± 4	78 ± 2	92 ± 5*
+Drug/SOD	30 ± 6	34 ± 7	39 ± 13	33 ± 3	36 ± 6
+SOD	122 ± 6	118 ± 1	120 ± 2	122 ± 6	119 ± 5

Reactions were started by adding 20 mM acetaldehyde to xanthine oxidase (XO) and 25 nM myeloperoxidase (MPO) in PBS containing 10 mM taurine and 100 μM diethylenediaminepentaacetic acid. To this control system was added each drug at the concentration given in parentheses and/or 20 μg/mL of superoxide dismutase (SOD). After 5 min at 25° reactions were stopped by adding 20 μg/mL of catalase and 100 μM allopurinol. The concentration of taurine chloramine produced from the hypochlorous acid (HOCl) formed was then measured. Superoxide and hydrogen peroxide production was 5.0 and 8 μM/min, respectively.

Data are means ± SD of four experiments. For each drug, all means were significantly different from each other ($P < 0.05$), except where indicated by *.

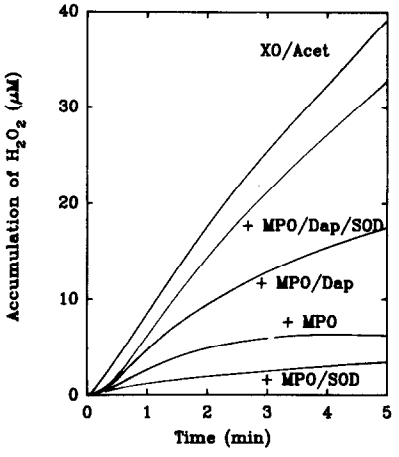

Fig. 1. The effects of dapsone and superoxide dismutase on the utilization of hydrogen peroxide by myeloperoxidase. The concentration of dapsone was 1 μM. Other conditions were as described in Table 1. XO, xanthine oxidase; Acet, acetaldehyde; MPO, myeloperoxidase; Dap, dapsone; HOCl, hypochlorous acid; SOD, superoxide dismutase.

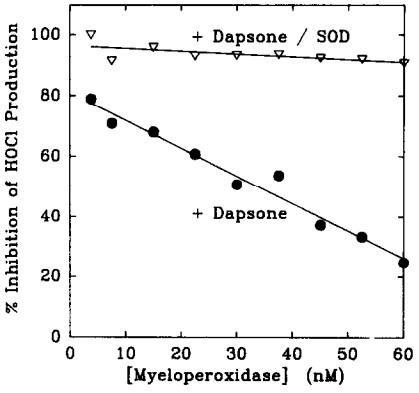

Fig. 2. The effect of the concentration of myeloperoxidase on its inhibition by dapsone and superoxide dismutase. Control reactions were started by adding 20 mM acetaldehyde to varying concentrations of myeloperoxidase, with xanthine oxidase and 100 μM diethylenediaminepentaacetic acid, in the presence (▽) or absence (●) of 20 μg/mL of superoxide dismutase (SOD). Dapsone (5 μM) was added to these systems and its degree of inhibition was calculated by comparing hypochlorous acid (HOCl) production with that of the respective control. Reactions were carried out at 37° in PBS containing 10 mM taurine, and stopped after 5 min by adding 20 μg/mL of catalase and 100 μM allopurinol. Hypochlorous acid production was determined by the amount of taurine chloramine formed. Xanthine oxidase produced superoxide at 7.2 μM/min.

consumed by this enzyme. Under all conditions, this difference was accounted for by the amount of hypochlorous acid produced. On average, 78% of the hydrogen peroxide produced was converted to hypochlorous acid, which increased to 93% when superoxide dismutase was added. Dapsone prevented about half the hydrogen peroxide from reacting with myeloperoxidase, while most of the hydrogen peroxide accumulated in the presence of dapsone and superoxide dismutase. Similar results were obtained for all the other drugs examined. The agreement between the concentrations of hydrogen peroxide consumed and hypochlorous acid produced confirmed that the drugs acted directly on myeloperoxidase, and not by scavenging hypochlorous acid or by inhibiting xanthine oxidase.

As the concentration of myeloperoxidase was increased, inhibition by dapsone became less effective (Fig. 2). However, the combination of dapsone and superoxide dismutase blocked production of hypochlorous acid by at least 90%, regardless of the concentration of myeloperoxidase.

To determine whether inhibition of myeloperoxidase by dapsone is irreversible, we incubated myeloperoxidase with xanthine oxidase and acetaldehyde in the presence and absence of superoxide

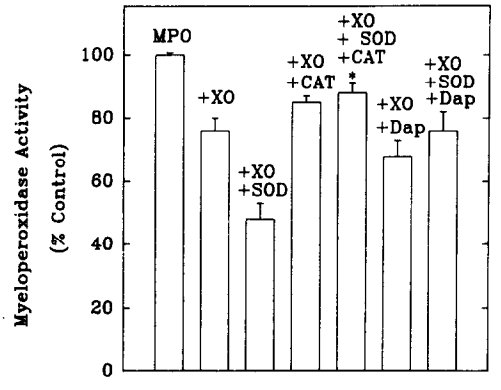

Fig. 3. Effects of the xanthine oxidase system and dapsone on the irreversible inactivation of myeloperoxidase. Myeloperoxidase (20 nM) was either incubated alone or with xanthine oxidase and 20 mM acetaldehyde (XO). Where indicated other additions were 20 μg/mL of superoxide dismutase (SOD), 30 μg/mL of catalase (CAT), and 5 μM dapsone (Dap). After 5 min of incubation at 37° in PBS containing 10 mM taurine and 100 μM diethylenediaminepentaacetic acid, samples were withdrawn and assayed for peroxidase activity. Xanthine oxidase generated 5 μM/min of superoxide. Data are means ± SD of four experiments. All means were significantly different to the control ($P < 0.05$) except where indicated by *. All means were significantly different to that for the system containing xanthine oxidase and superoxide dismutase.

dismutase and/or dapsone (Fig. 3). After 5 min samples were withdrawn and assayed for peroxidase activity using tetramethylbenzidine. Since tetramethylbenzidine readily reduces compound II, it detects all the myeloperoxidase present except any that has been irreversibly inactivated. Xanthine oxidase, acetaldehyde or dapsone alone did not significantly affect the peroxidase activity of myeloperoxidase. However, xanthine oxidase and acetaldehyde decreased the activity to 76% of the control. Adding superoxide dismutase exacerbated this irreversible inactivation. Catalase afforded significant protection of myeloperoxidase with the xanthine oxidase system and superoxide dismutase, which points to the involvement of hydrogen peroxide in the inactivation mechanism. In the absence of superoxide dismutase, dapsone did not significantly increase inactivation, whereas in its presence, dapsone provided significant protection. Under similar conditions, we also looked at the effect of the reagent hydrogen peroxide on the irreversible inactivation of myeloperoxidase. In the presence of chloride, 100 μM hydrogen peroxide inactivated about 40% of the enzyme in 10 min, with dapsone having no additional affect. In the absence of chloride, hydrogen peroxide gave 55% inactivation, which was unaffected by dapsone. Thus, we conclude that irreversible inactivation of myeloperoxidase was due to hydrogen peroxide and not dapsone.

Effects of superoxide dismutase and anti-inflammatory drugs on production of hypochlorous acid by human neutrophils

Neutrophils were stimulated with either phorbol myristate acetate or opsonized zymosan, and production of hypochlorous acid was detected by the formation of taurine chloramine in the supernatant. This accounts for about 90% of the chloramine formed, with the remainder associated with the cells [24]. When cells were stimulated with phorbol myristate acetate they produced approximately 33 μM hypochlorous acid/10^6 cells/hr. Based on the rate of superoxide production, and assuming all of it dismutated to hydrogen peroxide, about 17% of the hydrogen peroxide was converted to hypochlorous acid. This value is in good agreement with those obtained previously [24, 29]. Dapsone, diclofenac, primaquine, sulfapyridine and benzocaine were poor inhibitors of hypochlorous acid production by neutrophils (Table 2). They inhibited by only 35–60% at concentrations that have been shown to block the conversion of reagent hydrogen peroxide to hypochlorous acid by purified myeloperoxidase by more than 80% [15]. Inhibition by each drug was significantly greater in the presence of superoxide dismutase. With superoxide dismutase alone, hypochlorous acid production was increased by approximately 20%.

We also determined the effect of the concentration of dapsone on inhibition of hypochlorous acid production by neutrophils stimulated with phorbol myristate acetate. In the absence of superoxide dismutase, inhibition approached 90%, with 50% inhibition (I_{50}) at 5 μM (Fig. 4A). To determine the I_{50} for dapsone in the presence of superoxide dismutase, we compared the hypochlorous acid formed to that produced by cells in the presence of superoxide dismutase alone. The I_{50} of 1 μM from Fig. 4A is in good agreement with 0.5 μM obtained for the purified enzyme and reagent hydrogen peroxide [15]. We therefore conclude that superoxide limits the ability of these drugs to block hypochlorous acid production by neutrophils stimulated with phorbol myristate acetate.

We also determined the effect of dapsone on neutrophils stimulated with opsonized zymosan (Fig. 5). Using 1 mg/mL of opsonized zymosan, 54 ± 2 μM ($N = 3$) superoxide and 20 ± 2 μM hypochlorous acid were produced in 20 min by 10^6 neutrophils. Assuming all the superoxide dismutated to hydrogen peroxide, 74% of the extracellular hydrogen peroxide was accounted for by the hypochlorous acid formed. Superoxide dismutase increased the production of hypochlorous acid by 40%, so that the efficiency of conversion of hydrogen peroxide increased to 107%. At a concentration of 5 μM, dapsone inhibited hydrochlorous acid production by only 30%, whether or not superoxide dismutase was present. However, when compared to hypochlorous acid production in the presence of superoxide dismutase, the combination of dapsone and superoxide dismutase inhibited by 56%. Increasing the concentration of dapsone, increased the degree of inhibition (Fig. 4B). Either in the absence or presence of superoxide dismutase, inhibition did not reach 100%, plateauing

Table 2. Effects of anti-inflammatory drugs on the production of hypochlorous acid by neutrophils stimulated with phorbol myristate acetate in the presence or absence of superoxide dismutase

Reaction system	Production of HOCl (% control)				
	Dapsone (5 μM)	Diclofenac (50 μM)	Primaquine (5 μM)	Sulfapyridine (5 μM)	Benzocaine (10 μM)
PMN + PMA	100 ± 5	100 ± 5	100 ± 2	100 ± 3	100 ± 2
+Drug	55 ± 5	62 ± 3	39 ± 3	65 ± 5	54 ± 1
+Drug/SOD	17 ± 2	27 ± 2	24 ± 1	28 ± 3	22 ± 1
+SOD	127 ± 4	127 ± 4	118 ± 3	118 ± 5	118 ± 3

Reactions were started by adding 100 ng/mL of phorbol myristate acetate (PMA) to 2×10^6 neutrophils (PMN) in PBS containing 10 mM taurine. To this control system was added each drug at the concentration given in parentheses and/or 20 μg/mL of superoxide dismutase (SOD). After 1 hr at 37°, reactions were stopped by adding 20 μg/mL of catalase and placing tubes in melting ice. The taurine chloramine produced from the hypochlorous acid (HOCl) formed was then measured. The average rate of superoxide production was 5.9 μM/min/10^6 neutrophils.

Data are means ± SD of three experiments. For each drug, all means were significantly different from each other ($P < 0.05$).

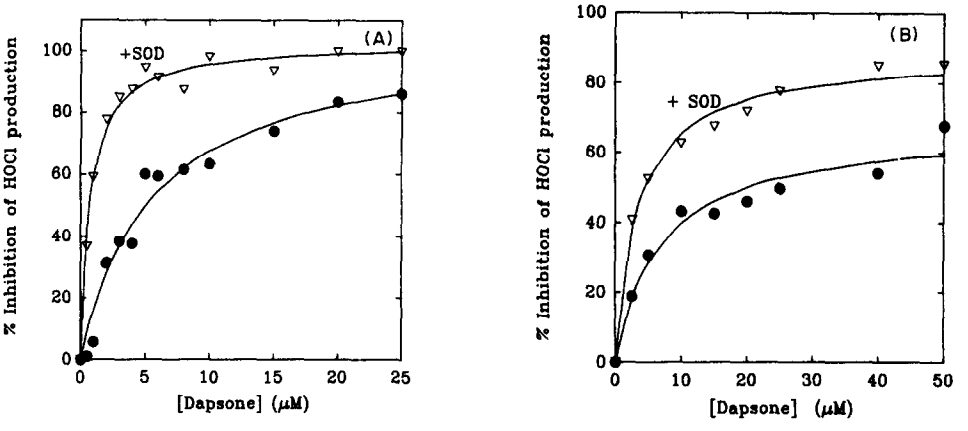

Fig. 4. The effect of dapsone on the inhibition of hypochlorous acid production by neutrophils. Neutrophils were stimulated with (A) phorbol myristate acetate or (B) opsonized zymosan in the presence (▽) or absence (●) of superoxide dismutase. Increasing concentrations of dapsone were added to these systems. To calculate the degree of inhibition, hypochlorous acid (HOCl) produced in the presence of dapsone was compared to that of the respective control. Conditions for cells stimulated with phorbol myristate acetate were as described in Table 2, and those for cells stimulated with opsonized zymosan are described in Fig. 5.

at 60% and 80%, respectively. Thus, superoxide decreased the ability of dapsone to inhibit hypochlorous acid production by cells stimulated with opsonized zymosan. However, regardless of the presence of superoxide dismutase, dapsone was an ineffective inhibitor. It should be noted that at 50 μM, dapsone inhibited the respiratory burst by 30%, so that part of its effect on hypochlorous acid production was due to the decreased generation of hydrogen peroxide. Interestingly, catalase inhibited hypochlorous acid production by only 60% (Fig. 5). With neutrophils stimulated with phorbol myristate acetate, or with purified myeloperoxidase and xanthine oxidase, catalase inhibited by 83% or 95%, respectively (not shown).

DISCUSSION

Numerous anti-inflammatory drugs have been proposed to attenuate inflammation by inhibiting the neutrophil enzyme myeloperoxidase [9–14]. For example dapsone, diclofenac, primaquine, sulfapyridine and benzocaine are all excellent inhibitors when added to myeloperoxidase, hydrogen peroxide and chloride [9, 15]. However, in the neutrophil, myeloperoxidase functions in the presence of a superoxide generating system that produces hydrogen peroxide as a secondary product [2]. We have shown that when superoxide is present, either with pure enzyme or neutrophils, the inhibitory effects of the drugs are much less than reported previously. Only when superoxide dismutase was added to remove superoxide did the I_{50} values approach those obtained when reagent hydrogen peroxide was used with myeloperoxidase. The synergistic action of superoxide dismutase with the drugs indicates that superoxide interacts with myeloperoxidase to overcome the inhibition of

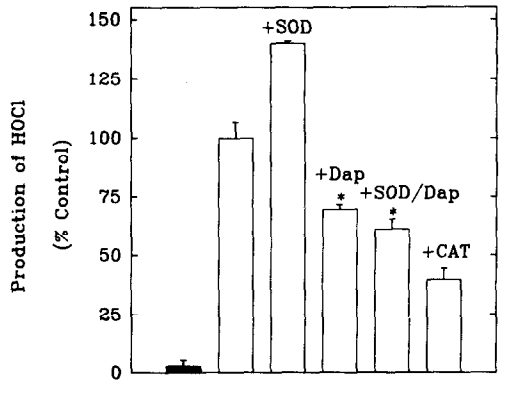

Fig. 5. Production of hypochlorous acid by neutrophils stimulated with opsonized zymosan. Neutrophils (PMN) at 2×10^6/mL were either incubated alone in PBS containing 20 mM taurine (■), or with 1 mg/mL of opsonized zymosan (□). Where indicated other additions to stimulated cells were 20 μg/mL of superoxide dismutase (SOD), 5 μM dapsone (Dap) and 20 μg/mL of catalase (CAT). After 20 min at 37° reactions were stopped by adding 20 μg/mL of catalase and placing tubes in melting ice. Production of hypochlorous acid was determined by measuring the formation of taurine chloramine. Data are means and SD of three experiments. All means were significantly different ($P < 0.05$) from each other except where indicated by *.

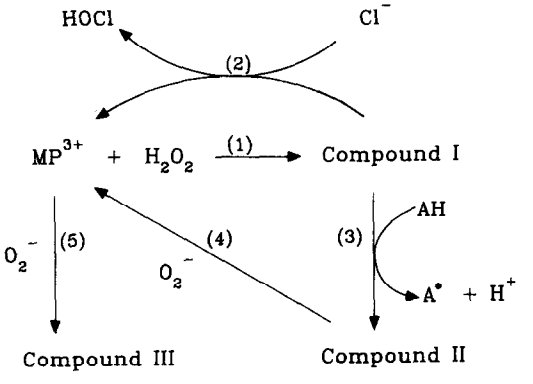

Fig. 6. Redox transformations of myeloperoxidase. MP^{3+}, ferric myeloperoxidase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; AH, reducing substrate; O_2^-, superoxide.

hypochlorous acid formation. Thus, previous findings that these drugs are potent inhibitors of myeloperoxidase, and proposals that they and related drugs can attenuate hypochlorous acid-mediated inflammatory tissue damage, must be re-evaluated.

The synergistic action of the drugs (AH) and superoxide dismutase is explained by their ability to affect the redox transformations of myeloperoxidase (MP^{3+}) (Fig. 6 reactions 1–5). Myeloperoxidase converts hydrogen peroxide and chloride to hypochlorous acid via reactions 1 and 2 [3]. Compounds with oxidizable functional groups, including some anti-inflammatory drugs, inhibit myeloperoxidase by promoting the accumulation of inactive compound II (reaction 3) [15]. Compound II is not a dead-end complex, since superoxide (O_2^-) reduces it back to the active enzyme (reaction 4) [19]. Therefore, in the presence of a flux of superoxide, the drugs would not trap myeloperoxidase as compound II, and the enzyme would be recycled to allow continued production of hypochlorous acid. However, faced with the combination of superoxide dismutase and drug, myeloperoxidase would get trapped at compound II, and its inhibition would be maximized.

Superoxide reacts with myeloperoxidase either by reaction 4, which enhances activity, or by reaction 5, which results in the formation of compound III and is inhibitory [19, 25]. Thus, superoxide boosts the activity of myeloperoxidase when compound II accumulates, as seen in the presence of the drugs. In the absence of drug, where compound II does not accumulate [15], the slight enhancement by superoxide dismutase is explained by its prevention of reaction 5.

Bozeman *et al.* [30] have proposed that inhibition of myeloperoxidase by dapsone is irreversible, and not due to accumulation of compound II. They suggested that a metabolite of dapsone was responsible for inactivating the enzyme. In agreement with them, we showed that when myeloperoxidase and dapsone are incubated with either xanthine oxidase and acetaldehyde, or with hydrogen peroxide, there is irreversible loss of peroxidase activity. In contrast to their results, but in agreement with the findings of Stendahl *et al.* [16] and van Zyl *et al.* [31], we found that dapsone did not contribute to the irreversible inactivation of myeloperoxidase. We conclude that under our experimental conditions hydrogen peroxide, not a metabolite of dapsone, irreversibly inactivated myeloperoxidase. Lactoperoxidase and chloroperoxidase are also inactivated by hydrogen peroxide [32, 33]. Hence, the effect of superoxide on inhibition of myeloperoxidase by dapsone and the related drugs is best explained by its reaction with compound II, and not by preventing irreversible inactivation of myeloperoxidase.

With myeloperoxidase and a superoxide generating system, we showed that dapsone is an efficient inhibitor of hypochlorous acid production at low concentrations of myeloperoxidase. However, the effect of dapsone decreases as the concentration of myeloperoxidase increases. This inverse relationship occurs because the rate of hypochlorous acid production is determined either by the concentration of myeloperoxidase or the flux of hydrogen peroxide. When myeloperoxidase is limiting, such that is unable to keep pace with the rate of hydrogen peroxide generation, inhibition of its activity will decrease formation of hypochlorous acid. However, when myeloperoxidase is present in excess, it will consume hydrogen peroxide as fast as xanthine oxidase produces it. Under these conditions, partial inhibition of myeloperoxidase need not affect the rate of hypochlorous acid production. Only when the activity of myeloperoxidase becomes limiting will hypochlorous acid production start to decrease.

Thus, inhibition by dapsone and related drugs will depend to a large extent on the ratio of the concentration of myeloperoxidase to the flux of hydrogen peroxide, and will be significant only when the enzyme is saturated. This finding is crucial to understand the effects of drugs on the production of hypochlorous acid by neutrophils.

When neutrophils were stimulated with phorbol myristate acetate they converted only 17% of their hydrogen peroxide to hypochlorous acid, which indicates that myeloperoxidase was limiting. This is expected because phorbol myristate acetate is a poor stimulus for degranulation of myeloperoxidase but promotes large extracellular fluxes of superoxide and hydrogen peroxide [34]. As a consequence, even in the presence of superoxide, dapsone was an effective inhibitor at sufficiently high concentrations. In contrast to phorbol myristate acetate, opsonized zymosan promotes the degranulation of large amounts of myeloperoxidase [35]. We found that most of the extracellular hydrogen peroxide generated by cells given this phagocytic stimulus was accounted for by the formation of hypochlorous acid, which indicates that the activity of the myeloperoxidase released approached that of the NADPH-oxidase. This may explain why dapsone was a poor inhibitor of hypochlorous acid production compared to its effect on cells stimulated with phorbol myristate acetate.

However, inhibition of hypochlorous acid production by neutrophils stimulated with opsonized zymosan is complicated by the release of myeloperoxidase mainly into phagocytic vacuoles [35]. Although we detected hypochlorous acid in the supernatant, not all of it was produced extracellularly. This is apparent from our results that catalase inhibited hypochlorous acid production by only 60%, compared to almost complete inhibition with xanthine oxidase system and with cells stimulated with phorbol myristate acetate. Since catalase has limited access to phagosomes [36], we conclude that when cells are stimulated with opsonized zymosan a substantial amount of the hypochlorous acid detected is formed intracellularly. Thus, the poor inhibition of hypochlorous acid production by dapsone most likely reflects its inability to influence intraphagosomal activity of myeloperoxidase. Dapsone may have limited accessibility to phagosomes, or conditions within these vacuoles may render it ineffective against hypochlorous acid production. The former possibility is unlikely, since dapsone is very lipophilic and should cross cell membranes readily. Given the abundance of myeloperoxidase in neutrophils, it is conceivable that within phagosomes myeloperoxidase activity greatly exceeds that of the NADPH-oxidase. Under these conditions, partial inhibition of myeloperoxidase by dapsone need not affect the formation of hypochlorous acid.

The poor inhibitory effect of dapsone on hypochlorous acid production by cells stimulated with opsonized zymosan was only marginally increased by superoxide dismutase. Regardless of the concentration of myeloperoxidase, 5 μM dapsone in combination with superoxide dismutase should have inhibited hypochlorous acid production by about 90%, as occurred with xanthine oxidase and cells stimulated with phorbol myristate acetate. We suggest that this did not occur because superoxide dismutase has limited access to phagosomes [36]. Thus, dapsone and superoxide dismutase would inhibit just the extracellular formation of hypochlorous acid.

Although there is considerable indirect evidence for the formation of hypochlorous acid inside phagosomes [3], to date this has not been detected directly. It has even been suggested that hypochlorous acid may not be formed within these intracellular compartments in appreciable amounts [37]. Our finding that catalase only partially inhibits hypochlorous acid formation by cells stimulated with opsonized zymosan does not support this suggestion, but strengthens the argument that neutrophils produce hypochlorous acid within phagosomes.

In conclusion, we have shown that drugs that convert myeloperoxidase to compound II will be poor inhibitors of hypochlorous acid production when superoxide is present to recycle the inactive enzyme. Furthermore, these drugs will inhibit effectively only when the flux of hydrogen peroxide is sufficient to saturate myeloperoxidase. For the neutrophil this is most likely to occur in the extracellular environment, but inhibition will still be limited by superoxide. Within phagosomes, the superoxide effect should be augmented by the high concentration of myeloperoxidase which is unlikely to be saturated by hydrogen peroxide. Therefore, these drugs will have little effect on intracellular hypochlorous acid formation. To achieve more efficient inhibition, drugs that act by different mechanisms are needed. These could involve oxidation of a drug to free radicals that irreversibly inactivated myeloperoxidase, or conversion of the enzyme to compound III. Compound III formation occurs when myeloperoxidase oxidizes hydroquinone [38] and the anticancer drug amsacrine, which inhibits hypochlorous acid production [39].

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