Chlorpromazine Inhibition of Granulocyte Superoxide Production

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Superoxide production by granulocytes is a result of the activation of an NAD(P)H-dependent oxidase present in the plasma membrane. Chlorpromazine (5–50 μM) prolongs the time necessary for activation of the superoxide generating system and inhibits the extent of activation. When chlorpromazine is added after activation, there is an inhibition of further superoxide production. These effects are seen with digitonin, phorbol myristate acetate, and opsonized zymosan washing. Incubation of granulocytes with chlorpromazine results in decreased activation of the plasma membrane plasma membrane. Chlorpromazine (5–50 mM) similar effects. Lidocaine (1–10 mM) inhibits superoxide production but has no effect on the rate of activation. The effect of chlorpromazine on the rate of activation is reversible, but its effect on extent of activation is unaffected by extensive washing. Incubation of granulocytes with chlorpromazine results in decreased activation of the plasma membrane superoxide generating NADPH oxidase. Chlorpromazine also competes with NADPH for the membrane oxidase. These data and previously published results provide the basis of a model for the activation of the superoxide generating system.

PHAGOCYTIC CELLS in the presence of soluble1,2 and particulate3 stimulators generate and release superoxide (O₃). In granulocytes (PMN), this production is due to the activation and activity of a plasma membrane-bound reduced pyridine nucleotide oxidase.4,5 This enzyme is inactive in resting cells and increases in activity in parallel with the increase in cell O₂ production.6 Thus, there are two separable processes involved in its function: activation, the conversion from the inactive to the active state, and activity, the production of O₂.

In order to investigate the nature of the activation process for this enzyme, we examined the effects of chlorpromazine (CPZ) and other anesthetic drugs known to interact with plasma membranes6 on PMN O₂ production. We report that CPZ, and other local anesthetics at concentrations similar to those that affect nerve cell function7 and erythrocyte hemolysis,8 inhibit O₂ production by PMN by slowing the rate of activation, limiting the extent of activation, and inhibiting the activity of the plasma membrane NAD(P)H oxidase.

MATERIALS AND METHODS

Cytochrome c (type VI), superoxide dismutase (SOD), FAD, FMN, nitroblue tetrazolium (NBT), tetraacaine, NADPH, CPZ, xanthine, adenosine triphosphate (ATP), hexokinase, glucose-6-phosphate dehydrogenase, and xanthine oxidase were purchased from Sigma Chemical Co., St. Louis, Mo.; digitonin, from Fisher Scientific Co., Medford, Mass.; phorbol myristate acetate (PMA), from Consolidated Midland Corp., Brewster, N.Y.; NADH, from CalBiochem, San Diego, Calif.; zymosan, from ICN Pharmaceuticals, Cleveland, Ohio; and Dextran T500 and Ficoll-Paque, from Pharmacia Fine Chemicals, Piscataway, N.J. Trifluoperazine and prochlorperazine were gifts from Smith, Kline and French Laboratories, Philadelphia, Pa.

Digitonin solutions (250 μg/ml) in water were made fresh daily, PMA (2 mg/ml in dimethyl sulfoxide) was kept desiccated at −20°C and diluted daily to 20 μg/ml in phosphate-buffered saline (PBS). Zymosan was washed with normal saline and then opsonized by incubating with fresh human or guinea pig serum (20 mg/ml) for 30 min. The opsonized zymosan (OpZ) was centrifuged and resuspended at 15 mg/ml in Krebs-Ringer's phosphate buffer (KRP), pH 7.4. CPZ and other local anesthetics were dissolved in water and stored at ~70°C. The results reported are for single experiments. Each experiment was repeated 2–4 times with qualitatively similar results. Except where noted, each point is average of duplicate samples differing by less than 10%.

Preparation of PMN

PMN were harvested from the peritoneal cavity of guinea pigs 18 hr after an intraperitoneal injection of casein and suspended at 5 × 10⁶/ml in KRP with 5 mM glucose as previously described.9 Human PMN were prepared from dextran-sedimented, Ficoll-Hypaque-centrifuged blood and suspended in PBS with 5 mM glucose at 5 × 10⁶/ml as previously described.10

PMN O₂ Production

O₂ production by PMN was monitored continuously in a double-beam spectrophotometer thermostatted at 37°C as previously described for digitonin,1 PMA,10 and OpZ.¹ In each assay, PMN (2.5 × 10⁶) were added to each of two 1-ml cuvettes containing KRP with 5 mM glucose, cytochrome c (50 nmole), and either digitonin (10 μg), PMA (1 μg) or OpZ (3 mg) with or without CPZ or other local anesthetics. SOD (10 μg) was also present in the reference cuvette, and the rate of O₂-dependent cytochrome-c reduction was monitored at 550 nm. The molar extinction coefficient for this change in absorption at 550 nm is 21,000.¹¹ The lag time for the activation was derived by extrapolating the linear rate back to the baseline absorbance, as previously described,¹ and as shown in Fig. 1.

NBT reduction to formazan by PMN, another assay for O₂ production,¹³ was quantitated by the technique of Baehner and Nathan.¹⁴ PMN (2.5 × 10⁶) were incubated for 15 min at 37°C with 0.1% NBT in 1 ml KRP with either 10 μg digitonin, 0.4 μg PMA, or 4.5 mg OpZ and with varying concentrations of CPZ. The reaction was terminated with 10 ml 0.5 N HCl, and the cells pelleted by
sucrose to a final concentration of 1 -2 mg protein/mI. These
also added. Forty microliters of digitonin (250 ug/ml) were then
added, and both the lag time and rate of O2 production was
measured. The CPZ effect was also seen in PMN stimulated with
OpZ or PMA. This direct inhibitory effect of CPZ was
independent of PMN concentration from 0.5 to 5 x
106 PMN/ml, but as can be seen in Table 1, was
inversely related to the concentration of digitonin or
PMA used to stimulate O2- production. This dose
dependence was not seen with OpZ. Inhibition of O2-
production by similar concentrations of CPZ was
found for human PMN stimulated by OpZ or PMA.
CPZ can act as an electron donor\textsuperscript{19} and thus may
compete with cytochrome-c for interaction with O2-.

**Fig. 1.** Effect of CPZ on PMN O2 production. PMN (2.5 x 10\textsuperscript{7}) were added to cuvettes in the presence and absence of 20 \mu M CPZ and assayed for the O2 production. The rate of O2 production is calculated from the linear change in absorbance. The lag time is calculated by the extrapolation of this linear rate to the baseline absorbance.

**Preparation of Membrane Particles**

Plasma-membrane-enriched particles were prepared by modification of a previously described procedure for macrophages.\textsuperscript{5,6} PMN (1.0 x 10\textsuperscript{8}) were incubated for 5 min at 37°C with 50 \mu g digitonin in 5 ml KRP with 5 mM glucose and varying concentrations of CPZ. The cell suspension was placed in a room temperature sonication bath for 6–8 sec, then on ice. After centrifugation at 70 g for 5 min, the resulting cloudy supernatant was centrifuged at 12,000 g for 10 min. The resulting pellet was suspended in 0.6–1.0 ml of 0.34 M sucrose to a final concentration of 1–2 mg protein/ml. These particles have been previously shown to be threecold enriched over homogenates for plasma membrane markers.\textsuperscript{5}

**NAD(P)H-Dependent O2- Production and NAD(P)H Oxidation**

O2- production by these particles was assayed for by adding cytochrome-c (50 nmole), NADPH or NADH (1.0 \mu mole, except were noted), NaCN (0.5 \mu mole), and CPZ (where noted) to both the sample and reference compartments of a double-beam spectrophotometer thermostatted at 37°C in a total volume of 0.9 ml of 0.05 M potassium phosphate, pH 7.5. In addition, SOD (20 \mu g) was added to the reference compartments. The assays were started with the addition of 0.1 ml of the membrane particles to both the sample and reference cuvettes and the absorbance change at 550 nm followed.

The oxidation of NAD(P)H to NAD(P) was monitored at 340 nm after the addition of 0.1 ml of membrane particles to cuvette containing NAD(P)H (0.1 \mu mole) and NaCN (0.5 \mu mole) in 0.9 ml of 0.05 M potassium phosphate, pH 7.5.

**Xanthine Oxidase Activity**

Xanthine oxidase-catalyzed O2 production was monitored continuously in a double-beam spectrophotometer thermostatted at 37°C by following SOD inhibitable cytochrome-c\textsuperscript{16} Ten microliters of xanthine oxidase (1 mg/ml) was added to each of 2 cuvettes containing xanthine (0.15 \mu mole), cytochrome-c (0.05 \mu mole), and varying concentrations of phenothiazines in 0.05 M potassium phosphate, pH 7.5. The reference cuvette also contained SOD (10 \mu g). The absorbance change at 550 nm was monitored continuously.

**Other Assays**

PMN ATP content was measured using the hexokinase, glucose-6-phosphate dehydrogenase method as previously described,\textsuperscript{17} and protein was measured by the Lowry technique\textsuperscript{18} using bovine serum albumin as a standard.

**RESULTS**

**Effect of CPZ on PMN O2- Production**

To determine the direct effect of CPZ on digitonin-stimulated O2- production, CPZ was added to assay cuvettes prior to the addition of the PMN. The effects are seen in Fig. 1. In the presence of 20 \mu M CPZ there is an increase in the time necessary for activation of the O2- generating system (lag time) and an inhibition of the linear rate of O2- production. The magnitudes of both of these effects are dose dependent. As can be seen in Fig. 2, CPZ at concentrations between 5 and 50 \mu M produces a progressive decrease in the rate of O2- production with a corresponding increase in the lag time. When CPZ is added to PMN after the linear rate of O2- production is obtained (i.e., 90 sec after the interaction of cells and digitonin—Fig. 2, open squares), further O2- production is inhibited to the same extent as when CPZ is added prior to activation. The CPZ effect was also seen in PMN stimulated with OpZ or PMA. This direct inhibitory effect of CPZ was independent of PMN concentration from 0.5 to 5 x 10\textsuperscript{6} PMN/ml, but as can be seen in Table 1, was inversely related to the concentration of digitonin or PMA used to stimulate O2- production. This dose dependence was not seen with OpZ. Inhibition of O2- production by similar concentrations of CPZ was found for human PMN stimulated by OpZ or PMA.
We therefore examined the effect of CPZ on \( O_2^- \)-dependent cytochrome-c reduction by xanthine-xanthine oxidase. CPZ at concentrations up to 500 \( \mu M \) had no effect on \( O_2^- \) -dependent cytochrome-c reduction by xanthine-xanthine oxidase. Thus, CPZ does not act by scavenging \( O_2^- \). We also examined the effects of CPZ on NBT reduction by stimulated PMN, another assay for \( O_2^- \) production.\(^3\) As shown in Fig. 3, the addition of CPZ results in less NBT reduction by PMN stimulated with either digitonin, PMA, or OpZ. This is similar to the effect of CPZ on \( O_2^- \)-dependent cytochrome-c reduction. Thus CPZ neither competes with cytochrome-c for \( O_2^- \) nor depends on the presence of cytochrome-c for its effect.

**Effect of Other Phenothiazines and Local Anesthetics**

We studied two other phenothiazines, trifluoperazine and prochlorperazine, for their ability to inhibit \( O_2^- \) production by digitonin-stimulated PMN. The results, as shown in Fig. 4, demonstrate that trifluoperazine and prochlorperazine inhibit \( O_2^- \) production (Fig. 4A) and the rate of activation (Fig. 4B) at lower concentrations than CPZ. We also examined the effects of two local anesthetics, tetracaine and lidocaine (Fig. 5), and found that the former mimics the effects of CPZ but requires higher concentrations. At millimolar concentrations, lidocaine also inhibits \( O_2^- \) production but has no effect on the rate of activation of the \( O_2^- \) generating enzyme. We examined the effects of trifluoperazine (500 \( \mu M \)), prochlorperazine (500 \( \mu M \)), tetracaine (1 \( mM \)), and lidocaine (10 \( mM \)) on \( O_2^- \)-dependent cytochrome-c reduction by xan-
Irreversibility of CPZ Inhibition of $O_2$ Production

In results described in previous sections, the effects of CPZ and other local anesthetics were examined immediately after interaction with PMN. We also incubated PMN ($5 \times 10^7$/ml) with varying concentrations of CPZ for 10 min. The cells were then centrifuged and washed twice with PBS and then assayed for digitonin-stimulated $O_2$ production. The activity of these PMN was compared with unincubated cells assayed for $O_2$ production in the presence of similar concentrations of CPZ. As can be seen in Fig. 6A, the inhibition of digitonin-stimulated PMN $O_2$ production was similar for the incubated washed cells and the cells assayed in the presence of CPZ. As shown in Fig. 6B, inhibition of the rate of activation of the $O_2$-generating system occurs only if CPZ is present during the activation process. Similar results were obtained with trifluoperazine and prochlorperazine. The 10-min incubation in CPZ or other phenothiazines resulted in no clumping of the PMN and in no change in viability as measured by trypan blue exclusion.

Other Aspects of CPZ Inhibition

It has been shown that incubation of erythrocytes with CPZ results in ATP depletion.\textsuperscript{20} At high concentrations of CPZ ($>100 \mu M$), PMN ATP content has been shown to decrease.\textsuperscript{21} We have previously demonstrated inhibition of activation of the PMN $O_2$-generating system by depleting cells of ATP.\textsuperscript{22,23} At the concentrations of CPZ used in the present studies (5–100 $\mu M$), we found no change in PMN ATP content.

The effect of CPZ on membranes is believed to involve the displacement of calcium from the membrane.\textsuperscript{24} We have previously shown that maximum $O_2$ production by digitonin-stimulated guinea pig PMN requires calcium.\textsuperscript{22} However, we found that the degree of inhibition by CPZ of digitonin-stimulated $O_2$ production was independent of the calcium concentration in the extracellular buffer from 0 to 1 mM.

CPZ is known to interact with flavoproteins.\textsuperscript{25} The PMN $O_2$-generating enzyme is probably FAD dependent.\textsuperscript{26} We examined the effect of FAD and FMN on CPZ inhibition. Fifty micromolar FAD and FMN had no effect on CPZ inhibition of either activation or activity of the $O_2$-generating system.

Effect of CPZ on NAD(P)H-Dependent $O_2$ Production

$O_2$ production by PMN is the result of the activation and activity of an NAD(P)H-dependent $O_2$-generating enzyme present on the plasma membrane of guinea pig\textsuperscript{2} and human\textsuperscript{4} PMN. We investigated the mechanism of the above described inhibition of PMN $O_2$ by examining the effect of CPZ on this enzyme activity. Membrane particles were made from digitonin-stimulated PMN in the presence of 20 or 50 $\mu M$ CPZ and the absence of CPZ. The particles were then assayed for NADPH- and NADH-dependent $O_2$ production. Figure 7 shows that the presence of CPZ during interaction of PMN with digitonin results in less NADPH- or NADH-dependent $O_2$ production by membrane particles. We also examined the effect of CPZ incubation on the oxidation of NADPH and
NADH by membrane particles. As can also be seen in Fig. 7, there is a decrease in both NADPH and NADH oxidation in parallel with the inhibition of O₂ production.

It has previously been determined that the membrane enzyme responsible for O₂ production is a flavoprotein, a class of enzymes with which CPZ is known to interact. We therefore determined the direct effect of CPZ on NADPH-dependent O₂ production by membrane particles. Figure 8 shows a Lineweaver-Burk analysis of this inhibitory action. CPZ inhibits O₂ production by competing with NADPH for the enzyme. The apparent Kₘ for NADPH is 4.8 μM, and the apparent Kᵢ for CPZ is 3.5 μM. This concentration of CPZ is similar to that necessary for 50% inhibition of PMN O₂ production (~20 μM).

**DISCUSSION**

The onset of O₂ production by PMN stimulated by particulate or soluble stimuli is believed to be due to the activation of a plasma membrane reduced pyridine nucleotide oxidase. Some investigators have described other subcellular locations for the oxidase system. Patriarca et al. felt that NADPH oxidase is in the granule fraction. However, based on their method of preparation, the granules were most probably contaminated by membrane particles. Recently, Badwey and Karnovsky describe a soluble NADH oxidase in the cytoplasm of guinea pig PMN that they feel is responsible for O₂ production. However, this enzyme is found in resting PMN, and it is not clear what the relationship of this enzyme is to the oxidative burst since with the interaction of phagocytic cells with stimuli a change in rate of production of O₂ occurs. The mechanism for the activation of the O₂-generating system is not completely known. We have shown that for digitonin-stimulated O₂ production by guinea pig PMN, activation is calcium-dependent, N-ethyl-maleimide-sensitive, and requires on-going ATP synthesis. In addition, the temperature dependence shows a slope change at 30°C, possibly indicating a relationship between membrane fluidity and activation. Drugs such as CPZ and other local anesthetics have been shown to alter the fluidity of the plasma membrane of cells, usually making them more fluid. In order to examine aspects of activation of the plasma membrane oxidase we studied the effects of CPZ on the rate of activation, the extent of activation, and the activity of the O₂-generating system. CPZ inhibited both the rate and extent of activation.
when present in the assay medium. If PMN were incubated with CPZ and then washed free of extracellular CPZ, the extent of activation was still inhibited, but there was no longer an effect on the rate of activation. Thus, we could separate two different effects of CPZ on activation. We showed that the effect of CPZ was at least in part due to inhibition of the activation of a membrane-bound $O_2^\cdot$-generating NADPH oxidase.

CPZ has also been shown to interact with flavoproteins. Since the NADPH oxidase is an FAD-dependent enzyme, we tested the effect of CPZ on this enzyme. CPZ competes with NADPH for the enzyme with an apparent $K_1$ of 35 $\mu$M, similar to the concentration that inhibits $O_2^\cdot$ production in PMN. This competitive inhibition may also account in part for the effect of CPZ on PMN $O_2^\cdot$ production. It is, however, possible that the interaction of CPZ with the PMN plasma membrane alters its properties, resulting in an inactivation of the $O_2^\cdot$-generating enzyme. This would explain the ability of CPZ to inhibit $O_2^\cdot$ production even when added after activation. We have described three distinct effects of CPZ on PMN $O_2^\cdot$ production. CPZ affects the rate of activation, the extent of activation, and the activity of the NAD(P)H-dependent $O_2^\cdot$-generating system.

It has previously been shown that PMN have negatively charged membranes and that activation with PMA and other soluble stimuli to produce $O_2^\cdot$ is accompanied by a depolarization of the membrane. CPZ has no effect on the resting membrane potential of human PMN (Whitin, Simons and Cohen, unpublished observation), but does affect both the rate and extent of depolarization of human PMN when stimulated with PMA.

The effects of CPZ on $O_2^\cdot$ production were also seen with two other phenothiazines, trifluoperazine and prochlorperazine, and another anesthetic, tetracaine. The other phenothiazines were more active than CPZ, but tetracaine required 30-fold higher concentrations. This dose dependency is similar to that required to anesthetize nerves and to inhibit hemolysis, suggesting that CPZ acts on the PMN in a similar manner to its action on other cells. The effects on these cells are thought to be related to the ability of anesthetics to increase the volume of the membrane and to make it more fluid.

Based on our data and the demonstration that the $O_2^\cdot$-generating system involves a flavoprotein, a cytochrome-$b$, and lipid dependence for maximal activity of the soluble enzyme, we propose a model for activation. At rest, the PMN membrane is in a fluid state. After interaction with a soluble stimulus or an appropriately opsonized particle, the membrane depolarizes, resulting in a change in the membrane fluidity allowing the flavoprotein moiety of the oxidase to interact with lipid and a cytochrome moiety. This change produces an enzyme that is capable of oxidizing NAD(P)H and producing $O_2^\cdot$. CPZ and other anesthetic agents prevent activation, resulting in a membrane that remains negatively charged and more fluid. These agents also cause a loss of rigidity of previously formed enzyme complexes (perhaps by interacting with the lipid) and a return to a more fluid membrane state, accounting for inhibition when added after activation. We plan to test this hypothesis by examining the fluidity of the PMN plasma membrane as a function of temperature, activation, and interaction with anesthetics. Preliminary results indicate that during the resting state the PMN membrane lipid is in a fairly fluid environment (Berde and Cohen, unpublished observation). Such studies on the PMN membrane should increase our understanding of this complex activation process.

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