Phorbol Myristate Acetate Stimulates the Formation of 5-Oxo-6,8,11,14-Eicosatetraenoic Acid by Human Neutrophils by Activating NADPH Oxidase

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We have shown previously that human neutrophil microsomes contain a highly specific dehydrogenase which, in the presence of NADP⁺, converts 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5S-HETE) to its 5-oxo metabolite, 5-oxo-ETE, a potent agonist of these cells. However, intact neutrophils convert 5S-HETE principally to its ω-oxidation product, 5,20-diHETE, and to only small amounts of 5-oxo-ETE. Phorbol myristate acetate (PMA) dramatically shifts the metabolism of 5S-HETE by intact cells so that 5-oxo-ETE is the major metabolite. The object of this investigation was to determine the mechanism for the stimulatory effect of PMA on 5-oxo-ETE formation. The possibility that oxidants released in response to PMA nonenzymatically oxidized 5S-HETE was ruled out, since PMA did not appreciably stimulate the formation of 5-oxo-ETE from 5R-HETE. On the other hand, inhibition of NADPH oxidase either by diphenylene iodonium or by mild heating nearly completely prevented the stimulatory effect of PMA on the formation of 5-oxo-ETE. The possibility that this effect was mediated by superoxide seems unlikely, since it was still observed, although somewhat attenuated, in the presence of superoxide dismutase. Moreover, superoxide generated by another mechanism (xanthine/xanthine oxidase) did not appreciably affect the formation of 5-oxo-ETE by neutrophils. However, phenazine methosulfate, which can nonenzymatically convert NADPH to NADP⁺, mimicked the effect of PMA on 5-oxo-ETE formation by intact neutrophils. It is concluded that PMA acts by activating NADPH oxidase, resulting in conversion of NADPH to NADP⁺, which enhances the formation of 5-oxo-ETE and reduces the formation of 5,20-diHETE. Serum-treated zymosan has an effect on the metabolism of 5S-HETE similar to that of PMA in that it also stimulates the formation of 5-oxo-ETE and inhibits that of 5,20-diHETE.

Human neutrophils metabolize arachidonic acid almost exclusively by the 5-lipoxygenase pathway to give leukotriene B₄ (LTB₄) and 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5S-HETE) as the major initial metabolites (1, 2). LTB₄ is a potent activator of neutrophils and induces a variety of responses by these cells, including elevated cytosolic calcium levels (3), chemotaxis (4, 5), and adherence to endothelial cells (6). 5S-HETE also activates human neutrophils but only at relatively high and probably nonphysiological concentrations (7, 8).

We have shown recently that neutrophils convert 5S-HETE to a 5-oxo metabolite, 5-oxo-ETE (9), which, like LTB₄, is a potent stimulus of cytosolic calcium levels and chemotaxis in these cells (10). 5-oxo-ETE has also been shown to stimulate degranulation of neutrophils, which was enhanced by priming with tumor necrosis factor-α (11). The actions of 5-oxo-ETE are clearly independent of the LTB₄ receptor, since they are not prevented by prior treatment with either LTB₄ (i.e. no cross-desensitization) or the LTB₄ antagonist LY355583 (10, 11). Although not quite as potent as LTB₄ in stimulating neutrophils, 5-oxo-ETE is much more potent than LTB₄ as a chemotactic agent for eosinophils (12) and in stimulating changes in cell volume in guinea pig intestinal epithelial cells. LTB₄ is rapidly biologically inactivated in human neutrophils by conversion to 20-hydroxy-LTB₄ and ω-carboxyl-LTB₄ by the ω-oxidation pathway (13, 14). 5S-HETE is also metabolized by this pathway to 5,20-diHETE (15) and can be converted to other dihydroxy metabolites by 12-lipoxygenase (16) and 15-lipoxygenase (17) as shown in Fig. 1. The distribution of dihydroxy products formed is dependent on the enzymes that are present as well as whether or not the cells have been activated (18). In addition, 5-HETE can be esterified into triglycerides and phospholipids in neutrophils (19, 20) as well as in other cells (21, 22). As noted above, 5-HETE is also converted to 5-oxo-ETE by a microsomal dehydrogenase, which requires NAD⁺ as a cofactor and is highly specific for a substrate with a 5-hydroxyl group in the S configuration followed by a 6-trans double bond (9). Thus the three diHETE metabolites of 5-HETE shown in Fig. 1 are all converted to the corresponding 5-oxo derivatives by this pathway (9). The oxidation of 5S-HETE by microsomes is reversible, since 5-oxo-ETE is stereospecifically reduced to the former product in the presence of NADPH (9). It is not clear whether the reverse reaction is also catalyzed by 5-hydroxyicosanoid dehydrogenase or whether this is accomplished by a distinct reductase in neutrophil microsomes.

Unlike neutrophil microsomes, intact cells convert 5S-HETE to only small amounts of 5-oxo-ETE, and the major product is 5,20-diHETE (23). However, phorbol myristate acetate (PMA), a highly active activator of neutrophils, has no effect on the metabolism of 5S-HETE, and it is not clear whether this is due to the presence of a 5S-HETE dehydrogenase or whether this is accomplished by a distinct reductase in neutrophil microsomes.

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The abbreviations used are: LTB₄, leukotriene B₄; 5S-HETE, 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5R-HETE, 5(R)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; 5,20-di-HETE, 5,20-dihydroxy-6,8,11,14-eicosatetraenoic acid; PMA, phorbol 12-myristate 13-acetate; DPI, diphenylene iodonium; PMS, phenazine methosulfate; RP-HPLC, reversed-phase high pressure liquid chromatography.
has a dramatic effect on the metabolism of 5S-HETE, strongly stimulating the formation of 5-oxo-ETE and inhibiting the formation of 5,20-diHETE (23). The effects of PMA were completely prevented by the addition of staurosporine, suggesting that they were mediated by protein kinase-dependent protein phosphorylation (23). It was not clear from our previous study (23) whether the effects of PMA on the metabolism of 5S-HETE were due to direct effects on the enzymes metabolizing 5S-HETE or whether they were mediated indirectly by the activation of other cellular processes, such as arachidonate or activation of the respiratory burst. The objective of the current study was to determine the mechanism for the effects of PMA on the metabolism of 5S-HETE by human neutrophils.

**EXPERIMENTAL PROCEDURES**

**Materials**—5S-HETE was synthesized by incubation of arachidonic acid (NuChek Prep Inc., Elysian, MN) with porcine neutrophils in the presence of 5,8,11,14-eicosatetraenoic acid (16, 24). Diphénylene iodine monosulfate (DPI) was synthesized from 2-iodobiphenyl (Pfaltz and Waterbury, CT) as described by Collette et al. (25). It was quantitated with 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM Na,HPO₄ at a pH of 7.4. Prior to incubation, Ca²⁺ and Mg²⁺ were disrupted by sonication (model 4710 ultrasonic disruptor, Branson). Zymosan A was obtained from Vicemo (Vicemo, MI).

**Preparation of Microsomal Fractions from Neutrophils—**Purified human neutrophils (25 x 10⁶ cells/ml) in phosphate-buffered saline supplemented with 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA were disrupted by sonication (model 4710 ultrasonic homogenizer; Sonics and Materials, Danbury, CT) for 2 x 10 s at a setting of 40 cycles/s. The disruptate was centrifuged at 1,500 x g at 4 °C for 10 min to remove unbroken cells and nuclei. The postnuclear supernatant was centrifuged at 10,000 x g at 4 °C for 10 min, and the supernatant was centrifuged at 200,000 x g at 4 °C for 60 min. The pellet was resuspended in phosphate-buffered saline containing 1.5 mM CaCl₂ and 1 mM MgCl₂ at a concentration equivalent to 20 x 10⁶ cells/ml, unless otherwise indicated.

**Analysis of 5-HETE Metabolites—Suspensions (1 ml) of neutrophils or neutrophil microsomes were incubated with 5R-HETE or 5S-HETE.** The incubations were terminated by the addition of methanol (0.6 ml) and cooling to 0 °C. Prior to analysis, the concentration of methanol in the samples was adjusted to 15% by the addition of water, and 5-HETE metabolites were quantitated by precolumn extraction/reversed-phase high pressure liquid chromatography (RP-HPLC) (28) using a Waters Millipore gradient controller, WISP automatic injector, WAVS automated switching valve, model 991 diode array detector, and a model 600 solvent delivery system. The mobile phase was a linear gradient between a mixture of 80% solvent A (water/acetonitrile/acetic acid (80:20:1.02) and 20% solvent B (acetonitrile/methanol/water/cetic acid (38.5:54.7:5.0:0.2)) and a mixture of 5% solvent A and 95% solvent B over 45 min unless otherwise indicated. The flow rate was 1 ml/min. The stationary phase was a column of Novapak CN (3.9 x 150 mm; Waters Millipore). Products were quantitated by comparing the areas of their peaks of UV absorbance with that of the internal standard, prostaglandin B₂. The extinction coefficients used were: 5-HETE (23,000) (25), 5-oxo-EETE (19,600) (23), 5,20-diHETE (23,000), 5-oxo-20-hydroxy-ETE (19,600), and prostaglandin B₂ (28, 680). The identities of the products measured were confirmed by examination of their complete UV spectra. Measurement of Superoxide—Neutrophils (1.5 x 10⁶; 5 x 10⁶ cells/ml) were preincubated in a cuvette at 37 °C for 3 min in the presence or absence of superoxide dismutase (10 μg/ml), cytochrome c (50 μg/ml), Ca²⁺ (1.8 mM), and Mg²⁺ (1 mM). Various amounts of PMA in 4.5 μl Me₂SO were added, and UV absorbance at 550 nm was monitored using a Beckman model DU-64 spectrophotometer. The amount of cytochrome c reduced was calculated using a molar extinction coefficient of 21,000.

**RESULTS**

**PMA Enhances the Enzymatic Oxidation of 5S-HETE to 5-Oxo-ETE by Intact Neutrophils**

We have shown previously that PMA strongly stimulates the conversion of 5S-HETE to 5-oxo-EETE and inhibits the formation of 5,20-diHETE by neutrophils. One possible explanation for these effects could be that PMA-induced degranulation results in the release of myeloperoxidase which, via the formation of hypochlorous acid, could nonenzymatically oxidize 5S-HETE. To determine whether this is the case, neutrophils were incubated with 5-HETE and PMA in the presence of sodium azide, which inhibits myeloperoxidase, or methionine, which is a scavenger of hypochlorous acid (Table I). Although azide appeared to inhibit the formation of 5,20-diHETE somewhat, neither azide nor methionine was able to inhibit the stimulatory effect of PMA on the conversion of 5S-HETE to 5-oxo-EETE.

We have shown previously that 5R-HETE is not nearly as good a substrate for the microsomal 5-hydroxyeicosanoid dehydrogenase as 5S-HETE (23). If the oxidation of 5-HETE by intact cells stimulated with PMA is catalyzed by the same enzyme, then 5R-HETE should not be converted to appreciable amounts of 5-oxo-EETE under these conditions. Fig. 2 shows high pressure liquid chromatograms of the products obtained after incubation of 5S-HETE and 5R-HETE with intact neutrophils in the presence or absence of PMA (30 min). In the absence of PMA, 5S-HETE was converted principally to 5,20-diHETE, with only a small amount of 5-oxo-EETE being formed (Fig. 2A). However, in the presence of PMA, the situation was reversed, and the major product was 5-oxo-EETE (Fig. 2B). Like 5S-HETE, 5R-HETE was converted to substantial amounts of 5,20-diHETE in the absence of PMA (Fig. 2C). As in the case of 5S-HETE, PMA inhibited the ω-oxidation of 5R-HETE to 5,20-diHETE. However, in contrast to 5S-HETE, 5R-HETE was converted to only very small amounts of 5-oxo-EETE in the presence of PMA (Fig. 2D).
PMA Does Not Act by Directly Stimulating 5-Hydroxyecoanoid Dehydrogenase

As would be expected, the addition of PMA directly to microsomal fractions did not affect the rate of conversion of 5s-HETE to 5-oxo-ETE in the presence of NADPH (data not shown). However, the possibility remained that PMA could stimulate the phosphorylation of the dehydrogenase in intact cells, thus increasing its activity. To determine whether this could be true, neutrophils were incubated with PMA for 15 min at 37 °C in the absence of any substrate. The cells were then cooled to 0 °C in an ice-water bath, sonicated, and microsomal fractions prepared. The amount of 5-oxo-ETE formed by microsomes from PMA-treated neutrophils was slightly higher than the amount formed by control microsomes (p < 0.05) (Table II). However, it would seem very unlikely that this modest 16% increase in enzyme activity could explain the dramatic effect of PMA on the formation of 5-oxo-ETE by intact cells. Pretreatment of neutrophils with PMA appeared to slightly reduce the amount of 5,20-diHETE formed by microsomes, but this difference was not significant (Table II).

### Table I

| PMA | Inhibitor | Products | 5,20-DiHETE | 5-Oxo-ETE |
|-----|-----------|----------|-------------|-----------|
| -   | -         | 667 ± 81 | 45 ± 4      |
| -   | Azide     | 510 ± 17 | 36 ± 2      |
| +   | Methionine| 621 ± 96 | 46 ± 6      |
| +   | Azide     | 251 ± 57 | 327 ± 37    |
| +   | Methionine| 175 ± 38 | 397 ± 12    |

The Effects of PMA on the Metabolism of 5-HETE Are Mediated by NADPH Oxidase

The NADPH Oxidase Inhibitor DPI Inhibits the Effects of PMA on 5-Oxo-ETE Formation—To test the hypothesis that the effects of PMA on the metabolism of 5-HETE are dependent on its stimulatory effect on NADPH oxidase, neutrophils were treated with various concentrations of DPI prior to the addition of 5-HETE. Fig. 3 shows concentration-response curves for the effects of DPI on the conversion of 5-HETE to its major metabolites in the absence and presence of PMA (30 nM). In the absence of PMA, DPI had a modest stimulatory effect on the formation of 5-oxo-ETE but strongly inhibited the formation of 5,20-diHETE (IC50, about 100 nM) (Fig. 3A). In contrast, in the presence of PMA, DPI strongly inhibited the formation of 5-oxo-ETE (IC50, 50 nM) (Fig. 3B). DPI also inhibited the formation of 5,20-diHETE in the presence of PMA but was not quite as potent as in its absence. In the absence of PMA, 80 nM DPI inhibited the formation of 5,20-diHETE by 44% (p < 0.025; paired t test) (Fig. 3A), whereas in the presence of PMA this concentration of DPI had no effect on 5,20-diHETE synthesis (Fig. 3B). Formation of the ω-oxidation product of 5-oxo-ETE, 5-oxo-20-hydroxy-ETE was inhibited by DPI, both in the presence and absence of PMA.

Mild Heating of Neutrophils Inhibits NADPH Oxidase As Well as the Effects of PMA on the Metabolism of 5-HETE—It has been shown previously that heating of neutrophils at 46 °C prevents the activation of NADPH oxidase by PMA by inactivating p67phox, one of the cytosolic components of this enzyme (30). To determine whether this treatment can also prevent the effects of PMA on the metabolism of 5-HETE, neutrophils were heated at 46 °C for 9 min and then cooled rapidly on ice. This treatment completely prevented the ability of PMA to stimulate superoxide production (Fig. 4A), consistent with the reported inhibitory effect of mild heating on NADPH oxidase as discussed above. Incubation of the heated cells with 5-HETE resulted in the formation of a single major metabolite, 5,20-diHETE (data not shown), and only a small amount of 5-oxo-ETE, as was the case with control, nonheated cells (Fig. 4B). However, preincubation of the heated cells with PMA (30 nM) had no effect on the subsequent metabolism of 5-HETE, in contrast to the stimulatory and inhibitory effects of PMA on the synthesis...
of 5-oxo-ETE and 5,20-diHETE, respectively, by the control cells (Fig. 4B). To demonstrate that the inhibitory effect of heating on the synthesis of 5-HETE by PMA-stimulated neutrophils was not due to inactivation of 5-hydroxyeicosanoid dehydrogenase, the activities of this enzyme in microsomal fractions from heated and control neutrophils were compared. As shown in Fig. 4C, microsomal fractions from heated cells were just as active as the corresponding fractions from control cells in converting 5-HETE to 5-oxo-ETE.

Heating at 46 °C had a slight inhibitory effect on the formation of 5,20-diHETE by unstimulated neutrophils (33% inhibition; p < 0.02, data not shown). PMA inhibited the formation of this compound by heated cells by only 21% (p < 0.05), in contrast to the 69% inhibition observed with control cells (p < 0.001) (data not shown).

The Effects of PMA on 5-Oxo-ETE Formation Are Not Mediated by Superoxide

The Addition of Superoxide Dismutase Does Not Prevent the Effects of PMA on the Metabolism of 5-HETE—Activation of NADPH oxidase in neutrophils results in the production of large amounts of superoxide, and it is possible that this substance could mediate the effects of PMA on the metabolism of 5-HETE. To degrade the superoxide produced in the incubations, we added superoxide dismutase to neutrophils prior to the addition of PMA and 5-HETE. Fig. 5A shows the amounts of superoxide produced by neutrophils under conditions identical to those used to study the metabolism of 5-HETE. Since the amount of cytochrome c in these incubations was limiting, we could only measure superoxide production for the first few minutes in the absence of superoxide dismutase. Superoxide dismutase strongly inhibited the accumulation of superoxide by neutrophils in response to PMA (30 nM) under the conditions of our assay. However, it did not alter the inhibitory effect of PMA on the formation of 5,20-diHETE from 5-HETE (Fig. 5B). PMA stimulated the formation of 5-oxo-ETE in the presence of superoxide dismutase by about 5-fold (p < 0.01, paired t test), but the degree of stimulation was only about one-half that observed in the absence of this enzyme (p < 0.02) (Fig. 4B).

Generation of Superoxide by Xanthine/Xanthine Oxidase Does Not Stimulate the Formation of 5-Oxo-ETE from 5-HETE—To determine whether superoxide generated from a source other than NADPH oxidase could mimic the effects of PMA, neutrophils were incubated with 5-HETE in the presence of a mixture of xanthine oxidase (10, 30, or 100 milliunits/ml) and xanthine (400 μM). The addition of the two higher concentrations of xanthine oxidase resulted in the generation of superoxide at a rate in excess of that observed with PMA (30 nM), whereas the addition of 10 milliunits of xanthine oxidase resulted in a slightly lower rate of formation of superoxide (Fig. 6, inset). As shown in Fig. 6, xanthine in the presence of xanthine oxidase (100 milliunits) had only a slight and nonsignificant stimulatory effect on the formation of 5-oxo-ETE from 5-HETE, in contrast to the strong stimulatory effect of PMA. The addition of xanthine/xanthine oxidase significantly inhibited the conversion of 5-HETE and 5,20-diHETE (p < 0.05), but the magnitude of this effect was less than that of PMA (p < 0.05). Similarly, lower concentrations of xanthine oxidase (10 and 30 milliunits/ml) in the presence or absence of xanthine had no significant effects on the formation of 5-oxo-ETE by neutrophils (data not shown).

The Formation of 5-Oxo-ETE Is Stimulated by an Agent That Increases Intracellular Levels of NADPH

PMS Stimulates the Formation of 5-Oxo-ETE—Another consequence of activation of NADPH oxidase in neutrophils would be an increase in the ratio of NADPH to NADP+. We attempted to mimic the effects of PMA by using an agent, PMS, which is known to promote the nonenzymatic conversion of NADPH to NADP+. Low concentrations (10 μM) of PMS completely inhibited the ω-oxidation of 5-HETE and stimulated the formation of 5-oxo-ETE by about 2.4 fold (Fig. 7A). The modest stimulatory effect of low concentrations of PMS on
5-oxo-ETE formation may have been due to its inhibitory effect on the ω-oxidation of 5-oxo-ETE, combined with a diversion of substrate (i.e. 5-HETE) to the ω-oxidation pathway to the dehydrogenase pathway. Higher concentrations of PMS (EC₅₀ about 75 μM) had a much stronger stimulatory effect on the formation of 5-oxo-ETE, presumably due to conversion of intracellular NADPH to NADP⁺.

The Effects of PMS Are Not Mediated by NADPH Oxidase—To determine whether the effects of PMS could have been mediated by stimulation of NADPH oxidase, similar experiments were conducted in the presence of the NADPH oxidase inhibitor DPI (400 nm) (Fig. 7B). This concentration of DPI strongly inhibited the PMS-stimulated conversion of 5-oxo-ETE but had no effect on the response to PMS.

The Effects of PMS on the Conversion of 5-HETE to 5-Oxo-ETE by Microsomes—To determine whether the effects of PMS could be due to a direct effect on 5-hydroxyeicosanoid dehydrogenase, its effects on the conversion of 5-oxo-ETE to 5-oxo-ETE by microsomal fractions were investigated (Fig. 7C). Neutrophil microsomes alone or in the presence of NADPH were capable of forming only very small amounts of 5-oxo-ETE, whereas in the presence of NADP⁺, large amounts of the latter substance were formed. In the absence of any cofactors, PMS (100 μM) had a slight stimulatory effect on the formation of 5-oxo-ETE, but its effect was much less than that of NADP⁺. PMS had no effect on 5-oxo-ETE formation in the presence of NADP⁺ but strongly stimulated its formation in the presence of NADPH to levels comparable to those formed in the presence of NADP⁺ (Fig. 7C).

Serum-treated Zymosan Stimulates the Conversion of 5-HETE to 5-Oxo-ETE

In addition to PMA, we also investigated the effects of serum-treated zymosan on the metabolism of 5-HETE. Neutrophils were preincubated for 10 min in the presence or absence of serum-treated zymosan and then incubated with 5-HETE for a further 20 min. As shown in Fig. 8, serum-treated zymosan did
an effect very similar to that of PMA, although not quite as pronounced, on the metabolism of 5-HETE. Preincubation with azide, a myeloperoxidase inhibitor, and methionine, a scavenger of hypochlorous acid. This is unlikely, however, since preincubation of intact cells with PMA had only a very small effect on microsomal enzyme activity. The effects of PMA could also be mediated by its dramatic stimulatory effect on NADPH oxidase, as illustrated in Fig. 9. This enzyme complex is latent in unstimulated neutrophils, but becomes highly active in cells stimulated with activators of protein kinase C, such as PMA, due to phosphorylation of one of its cytosolic components (p47-phox) and translocation of p47-phox and p67-phox to the plasma membrane (35). Once activated, the enzyme rapidly oxidizes cytosolic NADPH to NADP+ and transfers the electrons to molecular oxygen, resulting in the formation of large amounts of superoxide (33).

To determine whether activation of NADPH oxidase was required for the stimulatory effect of PMA on 5-oxo-ETE formation, neutrophils were incubated with an inhibitor of this enzyme, DPI (34, 35), prior to the addition of PMA. DPI slightly stimulated the production of 5-oxo-ETE by intact neutrophils in the absence any other stimulus, presumably by inhibiting its conversion to its oxidation product, 5-oxo-20-hydroxy-ETE. In contrast, DPI strongly inhibited the formation of 5-oxo-ETE by cells stimulated with PMA. These results suggest that DPI does not directly affect 5-hydroxyicosanoid dehydrogenase, but only prevents the increased activity of this enzyme resulting from activation of NADPH oxidase. This was confirmed by the finding that DPI had no effect on the conversion of 5S-HETE to 5-oxo-ETE by neutrophil microsomes (data not shown) or by intact cells in the presence of PMS.

Although the results obtained with DPI are strongly suggestive of a role for NADPH oxidase in the PMA-induced stimulation of 5-oxo-ETE formation, the possibility that it could be acting by another mechanism cannot be excluded. For example, the data in Fig. 3 show that DPI inhibits the ω-oxidation of 5-HETE in unstimulated cells, indicating that this substance can inhibit enzymes other than NADPH oxidase. To attempt to confirm the involvement of NADPH oxidase in the stimulatory effect of PMA on 5-oxo-ETE formation we investigated the effects of inactivating this enzyme by another mechanism. Heating of neutrophils at 46°C has been shown to result in inactivation of NADPH oxidase but retention of other cellular functions such as phagocytosis and chemotaxis (36-39). This is due to the selective inactivation of the p67-phox component of the enzyme complex (30). The results in Fig. 4 clearly show that heating neutrophils at 46°C for 9 min blocked superoxide production in response to PMA and nearly completely prevented the stimulatory effect of PMA on the conversion of 5S-HETE to 5-oxo-ETE. The inhibitory effect of heating on 5-oxo-ETE formation was not due to inactivation of 5-hydroxyicosanoid dehydrogenase, since the activity of this enzyme in microsomal fractions prepared from heated cells was nearly identical to that in control cells.

Thus both heating and DPI, which inhibit NADPH oxidase by completely different mechanisms, prevent the stimulatory effect of PMA on 5-oxo-ETE formation by neutrophils. The inhibitory effects of both treatments were observed only in intact cells, since in both cases 5-hydroxyicosanoid dehydrogenase activity in microsomal fractions from the treated cells was the same as in microsomes from control cells. These results thus provide strong evidence that PMA acts by stimulating NADPH oxidase.

One possible mechanism for the stimulatory effect of activation of NADPH oxidase on 5-oxo-ETE formation could be that
superoxide somehow enhanced the activity of 5-hydroxyeicosanoid dehydrogenase. However, superoxide dismutase did not prevent the effects of PMA on the metabolism of 5-HETE, although its stimulatory effect on dehydrogenase activity was attenuated. Since the experiment with superoxide dismutase did not completely rule out the possibility that superoxide itself contributes to the effect of PMA on the production of 5-oxo-ETE, the effects of a superoxide generating system were also investigated. In contrast to the strong stimulatory effect of PMA on the formation of 5-oxo-ETE, addition of a mixture of xanthine and xanthine oxidase had only a small and nonsignificant effect on the formation of this substance. Thus it would appear very unlikely that the stimulatory effect of PMA is mediated by superoxide.

Another consequence of activation of NADPH oxidase by PMA in neutrophils would be conversion of NADPH to NADP⁺. This could have several consequences on the metabolism of 5-HETE: (i) oxidation to 5-oxo-ETE requires NADP⁺ (9); (ii) α-hydroxylation to 5,20-diHETE requires NADPH (40); and (iii) reduction of 5-oxo-ETE to 5,5'-HETE requires NADPH (9). Thus an increase in the ratio of NADP⁺ to NADPH would be
expected to result in increased formation of 5-oxo-ETE and a reduction in the formation of 5,20-diHETE. Although we were not able to test this hypothesis directly, we attempted to manipulate intracellular levels of these cofactors by the addition of PMS, which can stimulate the nonenzymatic oxidation of NADPH to NAPD+ (31, 32). This resulted in the formation of higher levels of 5-oxo-ETE than did the addition of a maximally stimulating concentration of PMA. As was the case with PMA, PMS did not appear to act directly on the dehydrogenase enzyme, nor could it replace NAPD+ as a cofactor, since it had relatively little effect on the formation of 5-oxo-ETE by neutrophil microsomes in the absence of other cofactors. Neither did it act by stimulating NAPDH oxidase, since its effects were not prevented by pretreatment of neutrophils with DPI. However, PMS strongly stimulated the formation of 5-oxo-ETE by microsomes in the presence of NADPH, presumably by conversion of this cofactor to sufficient amounts of NAPD+ to allow the reaction to proceed at maximal velocity. Thus manipulation of endogenous cofactor levels in the manner expected for PMA can mimic the effects of the latter substance on 5-oxo-ETE formation.

Both DPI and PMS are potent inhibitors of the ω-oxidation of 5-HETE to 5,20-diHETE (Figs. 3A and 7A). Pyocyanin, which is a derivative of phenazine, has previously been reported to inhibit the 20-hydroxylation of LTβ by human neutrophils (41), but in the present study we found it to be considerably less potent than PMS in this respect (data not shown). The effects of DPI and PMS on ω-oxidation were unlikely to be mediated by NAPDH oxidase, but rather were presumably due to direct inhibitory effects on the 20-hydroxylase enzyme, which is presumably the same enzyme as the one responsible for the conversion of LTβ to 20-hydroxy-LTβ by human neutrophils (15, 40). The fact that complete inhibition of ω-oxidation by PMS has only a modest stimulatory effect on the formation of 5-oxo-ETE indicates that diversion of substrate from the ω-oxidation pathway cannot explain its strong stimulatory effect, nor that of PMA, on the formation of 5-oxo-ETE.

It can be concluded that the synthesis of appreciable amounts of 5-oxo-ETE by neutrophils requires an increase in the level of cytosolic NAPD+. This can be accomplished by activation of NADPH oxidase by stimulation of protein kinase C, which appears to be the mechanism for the effect of PMA on 5-oxo-ETE formation. It is possible to short circuit this process by adding PMS, which can nonenzymatically convert neutrophil NAPDH to NAPD+. It would seem likely that alterations in cofactor levels as a result of stimulation of neutrophils during phagocytosis, as suggested by the experiment with zymosan, would also be important in regulating 5-oxo-ETE formation from endogenous substrate by these cells.

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