PGH Synthase and Lipoxygenase Generate Superoxide in the Presence of NADH or NADPH

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Purified PGH synthase when acting on arachidonic acid in the presence of reduced nicotinamide-adenine dinucleotide or reduced nicotinamide-adenine dinucleotide 3'-phosphate generated superoxide in burst-like fashion. In eight experiments using different batches of enzyme, the mean ± SE rate of superoxide generation from 100 U of enzyme measured as the superoxide dismutase-inhibitable reduction of cytochrome c was 5.06 ± 0.19 nmol/min in the first minute and 0.35 ± 0.03 nmol/min subsequently. Optimum rates of superoxide were seen at concentrations of reduced nicotinamide-adenine dinucleotide in excess of 80 μM and reduced nicotinamide-adenine dinucleotide 3'-phosphate in excess of 100 μM. Using prostaglandin G2 or linoleic acid as substrate rather than arachidonate also resulted in superoxide generation. When prostaglandin G2 was used as substrate, no superoxide was generated. The rate of superoxide generation was markedly inhibited by cyclooxygenase inhibitors. Superoxide generation was also observed during the action of lipoxygenase on either linoleic acid or arachidonic acid in the presence of reduced nicotinamide-adenine dinucleotide or reduced nicotinamide-adenine dinucleotide 3'-phosphate but not in their absence. Indomethacin had no effect on superoxide generation from lipoxygenase. We conclude that PGH synthase and lipoxygenase produce superoxide via a side-chain reaction dependent on the presence of suitable reducing cosubstrate. This mechanism is analogous to that described for peroxidases in general. (Circulation Research 1986;59:612–619)

RECENT studies showed that certain experimental interventions, including acute severe hypertension,1 fluid-percussion brain injury,2 and topical application of arachidonate or bradykinin,3 generate superoxide in the brain of anesthetized cats. This radical enters the cerebral extracellular space where it dismutates to hydrogen peroxide. Since cerebrospinal fluid (CSF) contains micromolar concentrations of free iron,4 superoxide and hydrogen peroxide interact via the iron-catalyzed Haber-Weiss reaction to produce hydroxyl radical.5

Acute hypertension,6 fluid-percussion brain injury7 and topical application of arachidonate6,8 cause a spectrum of functional, morphological, and biochemical cerebral arteriolar abnormalities. These consist of sustained dilation, reduced responsiveness to vasoconstrictor and vasodilator influences, focal destructive lesions of the endothelium and vascular smooth muscle, and reduced oxygen consumption of the vessel wall. Superoxide anion and other reactive agents derived from it, such as hydrogen peroxide and the hydroxyl radical, are the likely mediators of the cerebral arteriolar abnormalities seen in these conditions, since the latter are inhibited by scavengers of superoxide anion, hydrogen peroxide, and of the hydroxyl radical.6,8 Superoxide anion, hydrogen peroxide, and the hydroxyl radical have all been shown to dilate cerebral arterioles9 and sustained exposure of cerebral arterioles to these agents for more than a few minutes causes vascular injury.

The enzymatic source of superoxide anion in the experimental conditions noted above is not firmly established. The following evidence suggests strongly that superoxide is produced via the action of the prostaglandin hydroperoxidase. First, the cerebral arteriolar abnormalities noted above can be reproduced by topical application of prostaglandin G2 (PGG2), or 15-hydroperoxy-eicosatetraenoic acid (15-HPETE),11 which are good substrates of the prostaglandin hydroperoxidase. The cerebral arteriolar abnormalities are not produced by prostaglandin H2 (PGH2), the product of the reaction catalyzed by prostaglandin hydroperoxidase. Second, the vasodilatation and cerebral arteriolar abnormalities due to hypertension, brain injury, and arachidonate application are also minimized or prevented by cyclooxygenase inhibitors8,11 which block the production of PGG2 from arachidonate and hence deprive the enzyme of substrate resulting in reduced activity of prostaglandin hydroperoxidase. In contrast to this evidence implicating the cyclooxygenase pathway of arachidonate metabolism in superoxide anion production, in vitro studies with microsomal cyclooxygenase, showed no superoxide anion production.13 This discrepancy can be resolved by considering certain features of the PGH synthase (prostaglandin endoperoxide synthase) which is responsible for the metabolism of arachidonate to prostaglandins. This enzyme is a hemoprotein which has two activities; it is responsible for the oxidation of arachidonate to the hydroperoxide PGG2 (cyclooxygenase activity) and for the peroxidation of the 15-hydroperoxy group of PGG2 to a 15-hydroxy group, thus producing PGH2 (hydroperoxidase activity).14 Both activities are attributable to the same enzyme molecule and have not been separated.

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The prostaglandin hydroperoxidase, like other peroxidases, is capable of oxidizing a large number of reducing cosubstrates. These oxidations frequently follow chain reactions involving the formation of free radicals. Of particular interest is the finding that peroxidases are capable of oxidizing NADH and NADPH in a series of reactions which involve the formation of the radicals -NAD and -NADP. In the experiments described below, we tested whether purified PGH synthase produces superoxide in the presence of NADH or NADPH. We also tested the superoxide-producing capability of soybean lipoxygenase (lipoxidase) under similar conditions.

Materials and Methods

Cytochrome c (Type VI), nicotinamide-adenine dinucleotide, reduced form (NADH), nicotinamide-adenine dinucleotide 3'-phosphate, reduced form (NADPH), linoleic acid (sodium salt), indomethacin, aspirin, soybean lipoxygenase, superoxide dismutase (SOD) (from bovine blood 3,000 U/mg protein) and glutathione were obtained from Sigma Chemical Company, St. Louis, Mo. PGH synthase, arachidonic acid (sodium salt), hematin, phenol, and 0.1 M Tris buffer (pH 8.3) were obtained from Oxford Biochemical Research, Inc., Oxford, Mich. AHR 5850 (2-amino-3-benzoylbenzeneacetic acid) was a gift from the A. H. Robins Company, Richmond, Va. PGG₂ and PGH₂ were biosynthesized from radiolabelled arachidonic acid as described previously, using the methods described by Hamberg et al. Briefly, the source of cytochrome c was microsomes obtained from 30 g of defatted ram seminal vesicles (Pel-Freeze, Rogers, Ark.). The microsomes were preincubated for 5 minutes at 37°C in 80 ml of 0.1 M KH₂PO₄, 1 mM NaEDTA containing phenol (2 mM), L-tryptophan (1 mM), hemoglobin (0.1 mg/ml) and p-hydroxymercuribenzoate (1 mM). While the enzyme suspension was being stirred, the sodium salt of arachidonic acid (6 mg, 27 μCi) was added and the mixture incubated for 40 seconds. The incubate was then immediately quenched by pouring it into a -20°C solution containing 300 ml of diethyl ether, 40 ml of methanol and 10 ml of 0.2 M citric acid. After shaking for 1 minute, the ether phase was removed and dried with 60 g of anhydrous MgSO₄. The extract was then filtered and the ether removed with a vacuum evaporator. The residue was then dissolved in 1:9 diethyl ether:petroleum ether and applied to a 0.5 g column of silicic acid (CC-4, Mallinckrodt, St. Louis, Mo.). As described by Hamberg et al., PGG₂ elutes with 4:6 and PGH₁ with 6:4 diethyl ether:petroleum ether (v:v), respectively; in addition, PGH₂ in aqueous solution isomerizes to PGE₂, while PGG₂ isomerizes to 15-hydroperoxy-PGE₂, which is then reduced to PGE₂ with SnCl₂, then identified by gas chromatography/mass spectrometry. These features were used for distinguishing the two endoperoxides. Each endoperoxide containing eluate was then dried with nitrogen, redissolved in acetone and stored at -70°C until used.

Results

Superoxide generation was detected during the action of PGH synthase in the presence of NADH or NADPH but not in their absence. Figure 1A shows a typical example of the time course of absorbance at 550 nm following initiation of the reaction of PGH synthase with arachidonate in the presence of an optimal concentration of NADH. It is seen that superoxide generation occurred in burst-like fashion with a very high rate in the first 15-20 seconds followed by continued production at a much slower rate. Figure 1B shows the minute by minute superoxide generation calculated from absorbance data in the presence and absence of superoxide dismutase. Most of the superoxide was generated in the first minute of the reaction. Continued production at a rate of approximately 10% of the first
Time course of superoxide generation from PGH synthase. (A) Changes in absorbance at 550 nm in the presence and absence of superoxide dismutase (SOD) during the action of PGH synthase (100 U) on arachidonate (100 μM) in the presence of 80 μM NADH. Note the large superoxide production in the first few seconds after initiation of the reaction. (B) Minute by minute superoxide generation during the action of PGH synthase (100 U) on arachidonate (100 μM) in the presence of 80 μM NADH.

minute production rate continued for the remainder of the experiment (up to 25 minutes). The decline in superoxide production was due to the well-known self-deactivation of the enzyme and not due to lack of substrate, since subsequent additions of arachidonate did not increase superoxide production (Figure 2). In eight experiments using different batches of enzyme the mean ± SE rate of superoxide generation was 5.06 ± 0.19 nmol/min in the first minute and 0.35 ± 0.03 nmol/min subsequently. Oxygen consumption during the action of PGH synthase followed a similar time course (Figure 3). Oxygen consumption was markedly reduced in the presence of indomethacin (Figure 3).

Superoxide production was also observed during the action of lipoxygenase on either linoleic or arachidonic acid in the presence of NADH or NADPH, but not in their absence. The effect of concentration of NADH or NADPH on superoxide generation from lipoxygenase was similar to that seen with PGH synthase. The time course of the generation of superoxide by this enzyme was similar to that seen with PGH synthase, but declined more rapidly and reached zero in less than 10 minutes (Figure 9, left). Subsequent additions of substrate showed that the decline in superoxide production was due to both substrate limitations and deactivation of the enzyme (Figure 9, right). Superoxide generation was linearly related to the amount of enzyme used in the reaction mixture (Figure 10). When arachidonate (100–200 μM) was used as substrate, superoxide production was 39% of the rate seen with linoleic acid as substrate. Addition of hematin (1 μM) to the reaction mixture reduced the first minute rate of superoxide production by 30%.

Figure 6 shows that superoxide generation from PGH synthase was inhibited markedly by cyclooxygenase inhibitors, including indomethacin, AHR 5850, and aspirin. The inhibitory effect of aspirin required longer incubation than the other two drugs, whose effect was substantial even after 1 minute of incubation. Using PGG₂ as substrate for PGH synthase rather than arachidonate also resulted in production of superoxide anion (Figure 7). When PGG₂ was used, no superoxide anion was generated. This shows that superoxide generation is dependent on the hydroperoxidase reaction. Substituting linoleic acid for arachidonic acid also resulted in superoxide production from PGH synthase (Table 1). Superoxide anion production with linoleic acid as substrate was also inhibited by indomethacin (Table 1). Figure 8 shows the effect of substrate concentration (either arachidonate or linoleate) on superoxide production. The decrease in superoxide production at the higher substrate concentrations is probably due to a detergent effect of the fatty acids on the enzyme.
Discussion

The principal finding of these experiments is that PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH but not in their absence. It is clear that superoxide is not a direct product of the cyclooxygenase, prostaglandin hydroperoxidase, or lipoxygenase reactions. It is rather a product of a side-chain reaction, dependent on the presence of suitable reducing co-substrates. Such reducing co-substrates must possess two features to produce superoxide. First, they must be capable of being oxidized by the prostaglandin hydroperoxidase or lipoxygenase by a mechanism which gives rise to a radical. Second, the radical so generated must be capable of reacting with molecular oxygen to produce superoxide at a rate which is considerably faster than the rate with which the radical dismutates. These conditions are fulfilled by NADH and NADPH, but not by glutathione. The possibility exists, however, that other naturally occurring reducing agents may act like NADH and NADPH.

Superoxide anion generation via the action of PGH synthase was dependent on the hydroperoxidase activity of the enzyme. This is evident from the fact that superoxide anion was generated when PGG2 was used as substrate, but not when PGH2 was the substrate. The effect of the nonsteroidal anti-inflammatory drugs on superoxide generation by PGH synthase is consistent with this interpretation. Superoxide anion generation was inhibited by these drugs when arachidonate was used as substrate because the inhibitors reduced the amount of PGG2 produced, thereby depriving the prostaglandin hydroperoxidase of substrate. They had no effect on superoxide generation when PGG2 was used as substrate since they are known not to inhibit the hydroperoxidase reaction. Similarly, indomethacin had no effect on superoxide generation by lipoxygenase, a finding consistent with the absence of inhibition of lipoxygenase by nonsteroidal anti-inflammatory agents. These findings show that the effect of indomethacin was due to inhibition of PGH synthase and not due to a potential superoxide dismutase-like action or competition with superoxide for the reduction of cytochrome c. This conclusion is consistent with the absence of an effect of indomethacin on superoxide production by xanthine oxidase or activated polymorphonuclear cells, when assayed as the superoxide dismutase-inhibitable portion of cytochrome c reduction.30

The mechanism of generation of superoxide by PGH synthase or lipoxygenase in the presence of NADH and NADPH is analogous to what has been reported with...
peroxidases. In the case of peroxidases, it is known that the interaction of the enzyme with the hydroperoxide substrate produces intermediates known as compound I and compound II. These intermediates are enzyme-centered radicals whose exact identity is not fully established. Based on electron-spin-resonance studies, compound I of horseradish peroxidase contains two oxidizing equivalents per mole as oxoferryl iron and a porphyrin-$\pi$ cation-radical. While the intermediate product of cytochrome c peroxidase with substrate contains its two oxidizing equivalents in the form of a ferryl ion and a free radical of an amino acid in the protein part of the molecule. Recently, Lambeir et al showed that PGH synthase reacts with organic hydroperoxides and fatty acid hydroperoxides in milliseconds to generate an intermediate which is spectrally similar to compound I of horseradish peroxidase. Compound I of PGH synthase is then converted to compound II within 170 msec. Compound II decays to the resting enzyme in a few seconds. It therefore appears that PGH synthase in terms of its reaction cycle is a typical heme peroxidase. Generation of superoxide during the action of peroxidases depends on the interaction of the enzyme-centered radical intermediates with NADH or NADPH to produce the radical -NAD and -NADP, respectively, in accordance with the following reactions:

$$\text{P + ROOH} \rightarrow \text{P-I + ROH}$$

$$\text{P-I + NADH} \rightarrow \text{P-II + \cdot NAD}$$

$$\text{P-II + NADH} \rightarrow \text{P + \cdot NAD}$$

where P represents the peroxidase, P-I and P-II are the enzyme-centered intermediates, ROOH is the hydroperoxide substrate, and ROH the corresponding hydroxide product. Superoxide anion is generated by the interaction of the radicals -NAD or -NADP with molecular oxygen as follows:

$$\text{\cdot NAD + O}_2 \rightarrow \text{NAD}^+ + \text{\cdot O}_2^-$$

$$\text{\cdot NADP + O}_2 \rightarrow \text{NADP}^+ + \text{\cdot O}_2^-$$

These reaction sequences can be generalized to explain the generation of superoxide from PGH synthase and from lipoxygenase.

Several features of superoxide generation by PGH synthase are consistent with well-known properties of the activity of this enzyme as measured by oxygen consumption or by prostaglandin synthesis. These include the following:

1) The requirement for hematin is essential for the production of superoxide anion just as it is essential for the other activities of the enzyme. The acceleration of superoxide generation in the presence of phenol is probably due to protection of the enzyme from inactivation by hematin. Phenol also accelerates prostaglandin synthesis by PGH synthase.

2) The characteristic burst-like time course of superoxide generation followed by a rapid decline to a steady state level which is only a fraction of the initial rate is due to the well-known self-deactivation of the enzyme. The oxygen consumption associated with the activity of PGH synthase follows a similar time course.

3) The effect of cyclooxygenase inhibitors on superoxide generation with various substrates discussed above is also consistent with the well-known effects of these inhibitors on the enzyme.

It is appropriate to consider the potential physiologi-
and pathophysiological significance of generation of superoxide by PGH synthase and lipoxygenase. It is clear that for these enzymes to generate superoxide, they must be coupled with NADH and NADPH or with other suitable reducing co-substrates. It is possible that this may occur in some cells but not in others. The concentrations of NADH or NADPH influence superoxide generation strongly. It is therefore important to determine whether the cellular concentrations of NADH and NADPH are sufficient to allow generation of superoxide from PGH synthase and from lipoxygenase. Tischler et al.\textsuperscript{37} found that the concentration of NADH in rat liver cells averaged 270 \( \mu M \) in the cytosol and 638 \( \mu M \) in the mitochondria. The concentration of NADPH was 367 \( \mu M \) in the cytosol and 4,233 \( \mu M \) in the mitochondria. Although these concentrations would seem adequate to support relatively high rates of superoxide generation, the following additional considerations must be kept in mind. We do not know what the concentration of NADH and NADPH is at the exact cellular site where PGH synthase and lipoxygenase are located. PGH synthase appears to be localized in the endoplasmic reticulum and in the nuclear membrane.\textsuperscript{38} Also, the concentration of free NADH may be considerably lower than its total concentration. For example, in rat liver cells, the free NADH concentration is 1.2 \( \mu M \) in the cytosol and 433 \( \mu M \) in the mitochondria.\textsuperscript{37} This is due to preferential binding of NADH to enzymes. Therefore, the concentration of NADH available to the PGH synthase in lipoxygenase may be considerably lower. On the other hand, if NADH is bound to these enzymes, a very high local concentration might be achieved. More evidence is needed to resolve these issues.

As indicated in the introduction, acute severe hypertension, fluid percussion brain injury, and topical application of arachidonate on the brain surface of anesthetized cats cause similar cerebral arteriolar abnormalities which have been ascribed to oxygen radicals, derived from arachidonate metabolism via PGH synthase, because they are prevented by oxygen radical scavengers and by cyclooxygenase inhibitors. The most complete evidence supporting the origin of oxygen radicals in these conditions from the metabolism of arachidonate via PGH synthase was obtained in fluid percussion brain injury. In this condition, it was shown that the activity of brain phospholipase C increases,\textsuperscript{39} thereby providing an enzymatic mechanism for the generation of free arachidonate, the substrate of PGH synthase. It was also shown that the concentration of prostaglandins in the brain rises after fluid percussion brain injury,\textsuperscript{40} thereby showing that the metab-
The cerebral arteriolar abnormalities seen in this condition are prevented by pretreatment with cyclooxygenase inhibitors or by oxygen radical scavengers suggesting that they are mediated by oxygen radicals. Recently, we showed that following fluid percussion brain injury superoxide appears in the cerebral extracellular space. The cerebral arteriolar abnormalities seen in acute severe hypertension resemble those of fluid percussion brain injury and they are also inhibited by pretreatment with cyclooxygenase inhibitors. However, in this condition, there is no increase in brain phospholipase C activity and no significant rise in prostaglandin concentration. This may be due to the fact that the accelerated arachidonate metabolism in acute hypertension occurs only in the vessels and not in the brain parenchyma. The fact that exogenous arachidonate is capable of initiating radical production from the wall of cerebral arterioles in vitro suggests that this is a viable possibility. Our results support this hypothesis.

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