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

Rakesh C. Kukreja, Hermes A. Kontos, Michael L. Hess, and Earl F. Ellis

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 H2 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 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)
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 involves the formation of the radicals ‘NAD’ and ‘NADP’. These radicals readily react with oxygen to form the superoxide anion.

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-benzoyl-benzenacetic acid) was a gift from the A. H. Robins Company, Richmond, Va. PGG2 and PGH2 were biosynthesized from radiolabeled 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 silica gel (CC-4, Mallinckrodt, St. Louis, Mo.). As described by Hamberg et al., PGG2 elutes with 4:6 and PGH2 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. PGE2 is 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 and 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
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). The effect of the concentration of NADH and NADPH on the rate of production of superoxide is illustrated in Figure 4, which shows superoxide production in the first minute after initiation of the reaction. Optimum production rates of superoxide anion were seen at concentrations of NADH in excess of 80 μM and at NADPH concentrations in excess of 100 μM. Superoxide anion generation was linearly related to the amount of enzyme used in the reaction mixture (Figure 5). Omission of the enzyme or of hematin resulted in no superoxide production. Omission of phenol resulted in a decrease in the rate of production of superoxide to 21% of the corresponding rate with phenol (from 4.52 to 0.93 nmol/min in the first minute of the reaction). Substitution of glutathione (80 μM) for NADH or NADPH resulted in no superoxide generation.

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 arachidonic or linoleic) 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.

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 60%.

Figure 2. Effect of repeated additions of arachidonate on superoxide production by PGH synthase (100 U) in the presence of 80 μM NADH. At zero time and at each arrow, sufficient arachidonate was added to the cuvette to give an increment in concentration of 100 μM.
generation from 4.21 nmol/min to 2.51 nmol/min. Addition of phenol (500 μM) increased the rate of superoxide production to 6.68 nmol/min. With hematin and phenol together the rate of superoxide generation was still higher than control, at 6.04 nmol/min. Indomethacin had no inhibitory effect on superoxide generation by lipoxygenase (Table 1).

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 PGH₂ 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.

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 oxyferryl iron and a porphyrin-π 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 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:

\[
P + ROOH \rightarrow P-I + ROH
\]

\[
P-I + NADH \rightarrow P-II + \cdot NAD
\]

\[
P-II + NADH \rightarrow P + \cdot NAD
\]

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:

\[
\cdot NAD + O_2 \rightarrow NAD^+ + \cdot O_2^-
\]

\[
\cdot NADP + O_2 \rightarrow NADP^+ + \cdot O_2^-
\]

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. 2) 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.

3) 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.

4) 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-
Superoxide from PGH Synthase and Lipoxygenase

Kukreja et al

7.00

a.

arachidonic acid
linoleic acid

SUBSTRATE CONCENTRATION

0.1 1 10 100 1000

RANGE OF SUPEROXIDE GENERATION (nmol/min)

0 1.0 2.0 3.0 4.0 5.0 6.0 7.0

FIGURE 8. Effect of arachidonic or linoleic acid concentration on the rate of superoxide generation in the first minute following initiation of the reaction. The reaction mixture contained 100 U of PGH synthase with 100 μM NADH. Note that the horizontal scale is logarithmic.

NADH concentration is 1.2 μM in the cytosol and 433 μM in the mitochondria. 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, 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, thereby showing that the metab-

FIGURE 9. Left: Time course of superoxide generation from 600 U of soybean lipoxygenase acting on 25 μM linoleic acid in the presence of 140 μM NADPH. Right: Effect of subsequent additions of linoleic acid on the rate of superoxide generation from lipoxygenase. At zero time and at each arrow, sufficient linoleic acid was added to the cuvette to give an increment in concentration of 25 μM. Note that each additional provision of substrate resulted in progressively less superoxide generation, indicating inactivation of the enzyme.
olism of arachidonate via PGH synthase is accelerated. 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 derived in association with accelerated arachidonate metabolism via PGH synthase. Recently, we showed that following fluid percussion brain injury superoxide appears in the cerebral extracellular space. 2

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. 6 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 11 suggests that this is a viable possibility.

Acknowledgment

We thank Dr. R. N. Pittman for assistance with the oxygen consumption measurements.

References

24. Miyamoto T, Ogino N, Yamamoto S, Hayaishi O: Purification of soybean lipoxygenase. The reaction mixture contained 25 μM linoleic acid and 140 μM NADPH.

![Figure 10. Relation of the rate of superoxide generation in the first minute after initiation of the reaction to the concentration of soybean lipoxygenase. The reaction mixture contained 25 μM linoleic acid and 140 μM NADPH.](http://circres.ahajournals.org/content/59/6/618)
35. Yonetani T, Schleyer H: Studies on cytochrome c peroxidase.

Key Words • superoxide • arachidonic acid • cyclooxygenase • NADH • NADPH • lipoxygenase • oxygen radicals
PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH.
R C Kukreja, H A Kontos, M L Hess and E F Ellis

Circ Res. 1986;59:612-619
doi: 10.1161/01.RES.59.6.612

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/59/6/612

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/