A Carbon-centered Free Radical Intermediate in the Prostaglandin Synthetase Oxidation of Arachidonic Acid

SPIN TRAPPING AND OXYGEN UPTAKE STUDIES*

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The ESR spin-trapping technique has been used to identify a free radical involved in the oxygenation of arachidonic acid by ram seminal vesicle microsomes. The ESR spectrum of the radical adduct indicates that a carbon-centered arachidonic acid free radical has been observed. The formation of this species is inhibited by indomethacin, but not by phenol, and it is probably the first intermediate formed during the prostaglandin synthetase-catalyzed oxidation of arachidonic acid. The chemical identity of the trapped radical was substantiated with an independent synthesis of a closely related radical adduct.

The prostaglandin synthetase-catalyzed oxidation of arachidonic acid is a specialized type of lipid peroxidation that has long been presumed to form free radical intermediates (1-3). The radical nature of the enzymatic oxidation of AA, suggested several years ago after the observation of a free radical ESR signal, although the chemical structure of the free radical(s) could only be inferred from the structure of the products (1). Egan et al. (4) also observed a free radical ESR signal from a reaction mixture containing AA and seminal vesicle microsomes that had been freeze-quenched to -196°C. Since accurate g value measurements could not be made, the radical's identity was not determined, but the authors concluded that the free radical either contained oxygen or was derived from an oxygen-centered species, and may be the hydroxyl radical formed by the cleavage of PGG₂.

De Groot et al. (5) have clearly demonstrated the involvement of a different type of free radical in the oxidation of linoleic acid by soybean lipoxygenase. These investigators used a water-soluble spin trap, 2-methyl-2-nitroso-propanoate, to detect the rather reactive carbon-centered conjugated dienyl linoleic acid free radical. Since the mechanism of soybean lipoxygenase is formally similar to that of prostaglandin synthetase, we used a similar spin trap, NB, to detect a free radical involved in the prostaglandin synthetase-catalyzed conversion of AA to PGG₂. Our spin-trapping results suggest that a carbon-centered arachidonic acid free radical is trapped during the enzymatic oxidation of AA. This species would result from the hydrogen abstraction of one of the methylene hydrogens of AA, which is presumably followed by a rearrangement of the double bonds (Scheme 1). The chemical identity of this free radical intermediate was further verified by independently synthesizing similar radical adducts from AA and NB. This investigation indicates that the first free radical intermediate formed by prostaglandin synthetase is a carbon-centered conjugated fatty acid free radical.

MATERIALS AND METHODS

NB and AA were purchased from Aldrich Chemical Co. and NuChek, respectively. NB (1 mg/ml) was prepared in 150 mM Tris-HCl (pH 7.5) in the dark, by stirring overnight at room temperature. ESR measurements were made at room temperature with a Varian Century series E-109 spectrometer, equipped with a TM₄ cavity. All ESR spectra were obtained with a calibrated modulation amplitude of 0.33 G, a microwave power of 20 milliwatts, a scan time of 30 min, a time constant of 4 s, and a receiver gain of 1.25 x 10⁶.

RSV were obtained from a local slaughterhouse and stored at -80°C. The microsomal protein was prepared from RSV as described previously (6), stored at -80°C and used within 1 week. The incorporation of oxygen into arachidonic acid by prostaglandin synthetase was monitored by using an oxygen electrode (Yellow Springs Instrument). RSV microsomes were added to 1.9 ml of Tris buffer (0.2-saturated, pH 7.5) at room temperature to give a concentration of 2 mg of protein/ml in 2 ml of solution. The reaction was initiated by the addition of AA in ethanol (0.25% of total volume) to give a final concentration of 400 μM. Indomethacin, purchased from Sigma Chemical Co., was added at a fixed concentration to the buffer before the addition of the microsomal protein and preincubated with the enzyme for 2 min prior to AA addition. ESR and oxygen uptake experiments were done simultaneously on incubation mixtures which were identical, except that NB was omitted from the oxygen consumption studies. The inhibition of oxygen uptake by NB and nitrosoozenzene was examined in air-saturated buffers.

RESULTS AND DISCUSSION

The ESR spectrum (Fig. 1A) of the spin adduct observed in an incubation mixture containing RSV microsomes and AA clearly shows an unpaired electron interacting with the nitroxide nitrogen and the attached β proton shown in Scheme 1. The ESR parameters (a₁ = 15.7 G, a₂ = 2.5 G) are similar to the reported values of the carbon-centered linoleic acid radical adduct (a₁ = 16.0 G, a₂ = 2.0 G) (5). The relatively low magnitude of the nitrogen hyperfine coupling constant and the high stability of the nitroxide radical adduct preclude any possibility of having trapped oxygen-centered radicals of lipid peroxidation, such as alkoxy or peroxy radicals. The previously reported a₂ coupling constants for alkoxy and peroxy NB radical adducts are greater than 27 G (7, 8). Moreover, it has been shown that the alkyl peroxy NB radical adducts are too unstable to observe at room temperature and that they decompose to form the t-butyl radical which in turn reacts with NB to form di-t-butyl nitroxide (8, 9). After the first 30-min scan, the spectrum seen in Fig. 1A is distorted by a second signal, which was shown to be di-t-butyl nitroxide by the direct comparison with the ESR spectrum of commercial di-t-butyl nitroxide. Although the t-butyl radical can be formed in many ways, this very stable radical would be
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Concomitant with the appearance of the ESR signal in Fig. 1A, there is an increased rate of oxygen uptake upon the addition of AA to the incubation mixture containing RSV microsomes (control, Fig. 2). Indomethacin, a classic synthetase inhibitor, considerably decreases the ESR signal intensity at a concentration of 100 μM (Fig. 1B). No ESR signal was detected at a concentration of 400 μM indomethacin (Fig. 1C). A similar dose dependency for indomethacin is also seen in the AA-initiated oxygen consumption (compare the two curves (----- and ---) with the control in Fig. 2). The presence of a small ESR signal (Fig. 1D) from a mixture of microsomes and NtB and NtB alone corresponds to the incorporation of oxygen into endogenous unsaturated fatty acids present in the microsomes (Fig. 2). The four sharp lines seen in Fig. 1D are due to the hydrogen atom adduct of NtB and have previously been observed in rat hepatic microsomal incubations (10).

Although the ESR signal intensities in Fig. 1 are more closely related to steady state values rather than rates, the above results indicate that the ESR spectrum shown in Fig. 1A probably arises from a free radical intermediate of prostaglandin synthetase. Furthermore, it appears that the rate of oxygenation of AA by the microsomes, which is an index of prostaglandin formation (4), increases with increasing amounts of carbon-centered AA free radical.

In the absence of enzyme, no ESR signal is observed (Fig. 1E). With heat-denatured RSV microsomes, only the ESR spectrum of di-t-butyl nitroxide is observed. After a prolonged time or at higher concentrations of NtB and AA, a chemical reaction between AA and NtB occurs giving a spectrum (Fig. 3) very similar to that of Fig. 1A. The ESR spectrum of this species (αH = 15.5 G, αG = 2.0 G) appears under anaerobic or aerobic conditions and, therefore, cannot be due to the trapping of a free radical formed by AA autoxidation.

Sullivan (11) has previously shown that a stable alkenylnitroxide nitroxide can be synthesized through a novel pseudo Diels-Alder reaction by reacting nitrosobenzene with an olefin. Floyd et al. (12) have reported that a similar reaction occurs

**Scheme 1.** The mechanism of the first phase of prostaglandin biosynthesis according to Nugteren et al. (1) showing the incorporation of the first molecule of oxygen and how the NtB radical trap would react with the C12 carbon-centered free radical.

produced by the decomposition of AA peroxy radical adducts by the following mechanism (8).

\[
\text{AAO}_2 + \text{NtB} \rightarrow [\text{AAO}_2 \text{NtB}] \rightarrow \text{tBu-N}^+ + \text{tBu-N}^+ + \text{AAONO}_2
\]

![FIG. 1. ESR spectra of prostaglandin synthesis incubations. A, ESR spectrum of the carbon-centered AA-NtB radical adduct obtained upon the addition of 400 μM AA to an incubation mixture (0.25% ethanol) containing 2.0 mg/ml of RSV microsomal protein in a 1 mg/ml NtB solution of Tris buffer (pH 7.5). B, ESR spectrum obtained from an incubation mixture (0.3% ethanol) like that described above, but which had been preincubated for 2 min with 100 μM indomethacin. C, ESR spectrum obtained from the incubation of 2.0 mg/ml of RSV microsomal protein in the NtB solution of A. D, ESR spectrum obtained from mixing 400 μM AA with a 1 mg/ml NtB solution of Tris buffer (pH 7.5, 0.25% ethanol).
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![FIG. 2. Oxygen consumption tracings corresponding to Fig. 1A (---, after the addition of AA), Fig. 1B (-----), Fig. 1C (----), and Fig. 1D (----, before the addition of AA).
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FIG. 3. The ESR spectrum, taken as described under "Materials and Methods," of the nitroxide(s) formed by the reaction of equal volumes of AA (50 mg/ml) in absolute ethanol with NtB (1 mg/ml) in Tris buffer (150 mM, pH 9.0) under nitrogen at 22 C. Note that this concentration of AA is over 200-fold greater than 400 μM.

between 2-nitrosofluorene and oleic acid. A related reaction between NtB and AA could give an AA-NtB radical adduct identical with that thought to be formed by the enzymatic reaction (Scheme 2). Considering the addition reaction which appears to be taking place, up to eight radical adducts are possible where both β substituents are C4 or longer. Either one radical adduct predominates or the radical adducts formed have indistinguishable ESR spectra, as the literature on similar radical adducts suggests (13). This chemical reaction appears to provide a convenient method of independently synthesizing unsaturated lipid-NtB radical adducts, although further investigation is required.

The work on the reaction mechanism of the oxygenation of linoleic acid by potato tuber lipoxygenase and soybean lipoxygenase has demonstrated that the initially formed carbon-centered free radical isomerizes to a cis-trans conjugated diene prior to the oxygenation (5, 14). A similar mechanism was adopted in the present work (Scheme 1). Although AA has three methylene carbon atoms, it has been thought that the hydrogen abstraction takes place at the C10 position to give a carbon-centered radical, which is followed by isomerization of the 11 cis double bond to a 12 trans double bond. Oxygenation of this conjugated radical then occurs at C11 (1). This is consistent with the 11-peroxy-8,12,14-eicosatrienoic acid radical being the first oxygen-containing intermediate in the conversion of 8,11,14-eicosatrienoic acid into the corresponding endoperoxide (2, 3). Cyclization, oxygenation, and hydrogen abstraction by the peroxy AA radical in Scheme 1 would lead to the formation of PGG2.

Specifically deuterated AA is necessary to identify spectroscopically this free radical as a particular secondary carbon-centered free radical of AA. For example, the peroxy radical in Scheme 1 will, upon cyclization, form an oxygen-containing secondary carbon-centered free radical at C10 which can react either with a second molecule of oxygen or with a nitroso spin trap. The trapping of the C10 carbon-centered radical species is precluded under anaerobic conditions.

A spectrum similar to Fig. 1A, but indicating the presence of more di-t-butyl nitroxide, was observed when the incubation was purged with N2 for 5 min prior to the addition of AA. In contrast to the signal observed in oxygen-saturated incubations, this signal was not observed until after a delay of 40 min. In the absence of oxygen, hydroperoxides may replace oxygen in the enzymatic cycle of prostaglandin synthetase, as is the case with soybean lipoxygenase (15). Note that the endogenous oxygen uptake seen in Fig. 2 implies the formation of hydroperoxides before the incubation was purged with nitrogen. The delay in the appearance of the signal seen under nitrogen may be associated with hydroperoxides acting as the electron acceptor. In any case, Scheme 1 implies that a larger fraction of the carbon-centered AA free radicals will be trapped in the absence of oxygen (i.e. less peroxy radical will be formed).

Under aerobic conditions, carbon-centered free radicals of AA have been proposed to be formed as a consequence of RSV peroxidase activity acting nonspecifically upon AA (16). This reaction would also cause the reduction of hydroperoxides. A strong oxidant, possibly the oxygen-centered free radical (4, 17), could form the carbon-centered AA radical in a side reaction, but phenol (0.5 mM), which prevents the formation of the oxygen-centered free radical (4, 17) and is a well known substrate for peroxidases (18), has no significant effect

FIG. 4. Oxygen consumption tracings showing the NtB (-----) and nitrosobenzene inhibition (--------) of the oxygenation of AA by RSV.

SCHEME 2. The addition of NtB to the C11 double bond of AA according to a pseudo Diels-Alder mechanism as adapted from known reactions of nitrosobenzene (11) and nitrosofluorene (12).
on the amplitude of the carbon-centered AA radical signal.

Interestingly, the di-t-butyl nitroxide spectrum does not accumulate in the presence of phenol. The known reaction of peroxy radicals with phenol to form hydroperoxides and the phenoxy radical \((k \approx 10^4 \text{ M}^{-1} \text{ s}^{-1})\) may account for this observation (19).

The strongest evidence that the carbon-centered free radical of AA is an obligatory intermediate in the formation of PGG_2 is that NtB and nitrosobenzene are inhibitors of the oxygenation of AA. As illustrated in Scheme 1, the presence of nitroso spin traps (radical scavengers) should inhibit the oxygenation of AA, as is indeed the case (Fig. 4). The carbon-centered linoleic acid radical intermediate of lipoxygenase has already been found to react competitively with either oxygen or nitrosobenzene (20), and we find that nitrosobenzene is a much stronger inhibitor of the oxygenation of AA than is NtB (Fig. 4). The fact that nitrosobenzene is better than NtB as an inhibitor of oxygen uptake is consistent with nitrosodurene reacting with secondary alkyl radicals 6.7 times faster than NtB (13). Although nitroso compounds are good inhibitors of the oxygenation of AA by RSV, the well known toxicity of these compounds probably precludes their use as anti-inflammatory agents.

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