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EVIDENCE FOR H_2O_2 MEDIATING THE IRREVERSIBLE ACTION OF ACETYLENIC INHIBITORS OF PROSTAGLANDIN BIOSYNTHESIS

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ABSTRACT

Oxidizing intermediates formed during prostaglandin biosynthesis can be detected by ferrocytochrome c and epinephrine. Different intermediates were responsible for the oxidative colorimetric changes with epinephrine and ferrocytochrome c, and submicromolar amounts of oxidant were detectable.

Catalase diminished the absorbance change with epinephrine, but it did not stop the conversion of arachidonate to prostaglandins. This result indicates that small amounts of H_2O_2 were formed when producing the colorimetric change, and these had no apparent effect upon the enzyme stability. No colorimetric changes were detected during the time-dependent loss of oxygenase activity caused by various acetylenic acids, indicating that negligible amounts of H_2O_2 were formed. Nevertheless, the destructive action of the acetylenic acid was prevented by catalase, and it thereby appeared due to small amounts of H_2O_2 generated *in situ* as a result of a metastable complex of enzyme, oxygen and the acetylenic substrate analog.

INTRODUCTION

Formation of prostaglandins from fatty acid precursors occurs by way of a dioxygenase activity that apparently activates oxygen and inserts it into arachidonate to form the hydroperoxy endoperoxide intermediate (see reviews, refs. 1,2). The precise nature of the activated oxygen participating in the oxygenase reaction is unknown, and further useful information regarding the appearance and action of highly reactive oxidants formed in the oxygenase process may be found using cooxidants that presumably react with the oxygenase intermediates. For example, a quantitative assay for prostaglandin "synthetase" activity involving the oxidation of epinephrine to adrenochrome has been described (3). The mechanism of epinephrine oxidation appears complex, involving chain reactions and intermediate radicals such as superoxide (4) or oxygen radicals generated by the interaction of Fe^{++} and hydrogen peroxide (5). A complication in interpreting the epinephrine assay method is the recognized autoxidation of epinephrine (6) that can provide unlimited amounts of

oxidation independent of enzymic action. Nevertheless, the system can detect small amounts of oxidant, and a direct correlation between prostaglandin "synthetase" activity and epinephrine oxidation was reported (3). The values observed might reflect some stoichiometric relationship in response to hydrogen peroxide, superoxide radical or some other oxidant that occurred in the system as it forms prostaglandin.

Earlier work has demonstrated a progressive inactivation of the fatty acid oxygenases during the oxygenation reaction (7,8), and a highly reactive metastable intermediate was proposed to cause the destructive action. Certain acetylenic analogs of substrate acids also caused a time-dependent inactivation (9,10) of the cyclooxygenase with, however, no detectable hydroperoxide products formed or detectable oxygen consumed. Nevertheless, the inactivation process, like the typical reaction system, required oxygen and was prevented by added glutathione peroxidase (10) which removes the peroxide activators that are required for the oxygenase. With these features in mind, we set out to search for more evidence of an intermediate oxidant in the mechanism of prostaglandin synthesis and see if it corresponded to that promoting enzyme inactivation.

MATERIALS AND METHODS

Materials

An acetone powder of sheep vesicular gland was kindly supplied by the Upjohn Company. Phenol-activated vesicular gland oxygenase was prepared as previously described (10). Catalase, L-epinephrine and glutathione were purchased from Sigma. Ferrocytochrome c was prepared by dithionite reduction of cytochrome c from horse heart (Type III, Sigma) (11). Soybean lipoxygenase was obtained from Worthington Biochemical Corporation and bovine erythrocuprein (superoxide dismutase) from Miles Laboratories. Arachidonic acid was purchased from Applied Science Laboratories, 8,11,14-eicosatrienoic acid was a gift from Hoffmann-LaRoche and [1-^{14}C]8,11,14-eicosatrienoic acid was obtained from New England Nuclear Corp. The 5,8,11,14-eicosatetraynoate (5a,8a,11a,14a-20:4) was obtained from Hoffmann-LaRoche Inc., and the octadecynoate isomers (9a-18:1, 10a-18:1 and 9a,12a-18:2) were from Dr. F. D. Gunstone, St. Andrews. Silica gel G was purchased from Brinkman Industries and all other chemicals were reagent grade from commercial sources.

Oxygen Absorption Measurement

Oxygen absorption measurements were carried out with a Yellow Springs Instrument Co. Model 53 Oxygen Monitor as previously described (10). The oxygen electrodes were calibrated by the method of Robinson and Cooper (12) using N-methyl-phenazonium methosulfate and NADH. The oxygen concentration of 0.1 M Tris buffer (pH 8.5) was found to be 232 μM at 30°. Reaction mixtures were normally 3.0 ml, and additions were made as described in the text and figure legends.

Determination of Products

Incubations with radioactive fatty acid were stopped by the addition of 10 ml of chloroform-methanol (3:1) and 0.1 ml of formic acid. The suspension was centrifuged and the phases were separated. The aqueous phase was further

extracted with 10 ml of chloroform. After centrifugation and phase separation, the organic phases were combined, followed by the addition of about 5 μmoles of antioxidant, butylated hydroxytoluene (BHT). The samples were then evaporated with either a stream of nitrogen or with a rotary evaporator. The residue was dissolved in a small volume of chloroform, and subsequent thin-layer chromatography (TLC) and assays for radioactivity were carried out as previously described (7).

Absorbance Measurements

Adrenochrome formation (4) was followed by the change in absorbance at 480 nm (ε = 4 $mM^{-1}cm^{-1}$) and was recorded on a Gilford Model 2000 multiple sample absorbance recorder.

Ferrocytochrome c oxidation was determined from the decrease in absorbance at 550 nm (11) relative to the fully reduced sample ($\Delta\varepsilon$ = 18 $mM^{-1}cm^{-1}$) using a Zeiss PMQ II Spectrophotometer.

RESULTS

Detection of Oxidants by Ferrocytochrome c and Epinephrine during Prostaglandin Biosynthesis

When ferrocytochrome c was added to the phenol-activated oxygenase preparation, a relatively slow oxidation of the cytochrome was observed as a decreased absorbance at 550 nm that was proportional to the amount of enzyme preparation added. This "basal" rate was approximately 0.1-1.2 μM/min depending on the cytochrome content. Upon the addition of arachidonic acid, a large (>10-fold) increase in the rate of cytochrome oxidation occurred (to 15 μM/min) and the rate then gradually diminished to a value (0.3 μM/min) less than that initially observed before arachidonate was added. Both the rate and the extent of cytochrome oxidation that occurred subsequent to fatty acid addition increased with increased amounts of oxygenase preparation used, and heat-denatured preparations gave no increased oxidation upon adding substrate.

When 200 μM epinephrine was added to enzyme preparations, the systems exhibited an irregular course of absorbance change in the first few minutes (results not shown) and then stabilized at an oxidation rate equivalent to less than 0.1 μM adrenochrome/min. At this stage, addition of arachidonic acid results in a very rapid oxidation of epinephrine to adrenochrome, which eventually stopped within 15 minutes after the addition of substrate. The adrenochrome was not stable, however, and there was a progressive loss of chromophore with time that appeared to be proportional to the maximum amount that had been formed. The extent of epinephrine oxidation was limited by the amount of active vesicular gland oxygenase used in a manner similar to that seen earlier for the extent of oxygen consumption (7) and described above for oxidation of cytochrome.

Cytochrome Oxidation in Relation to Oxygen Consumption and Fatty Acid Oxidation

When phenol-activated oxygenase and cytochrome c were mixed in a Thunberg tube under anaerobic conditions, the rate of cytochrome c oxidation was negligible. In addition, very little (0.08 μM/min) ferricytochrome c

formation was observed upon the anaerobic addition of substrate relative to that seen with the aerobic system (8.5 μM/min). When oxygen was then allowed to mix with the reactants, oxidation of cytochrome proceeded at a rapid rate that was comparable to the usual aerobic system.

A quantitative relationship between oxygen consumption, fatty acid oxidation, and ferricytochrome c formation was determined using radioactive 8,11,14-eicosatrienoic acid. Table I shows that under the conditions used, two moles of oxygen were consumed with each mole of fatty acid oxidized regardless of whether or not reduced cytochrome c was present. In addition, thin-layer chromatographic results showed that the presence of reduced cytochrome c did not significantly alter the relative amounts of radioactive products formed by the system.

TABLE I

RELATIONSHIP BETWEEN OXYGEN CONSUMPTION, FATTY ACID OXIDATION AND FERRICYTOCHROME C FORMATION

Phenol activated oxygenase (1.6 mg) was added to a reaction mixture containing 0.67 mM phenol, 1 mM sodium azide with or without ferrocytochrome c in 2.66 ml of 0.1 M Tris buffer (pH 8.0). After 1 min, [1-^{14}C] 8,11,14-eicosatrienoic acid (58 μM) was added to initiate the reaction. After 10 min, the mixture was worked up as described in Methods, and the values shown are for a representative experiment. Averaged values for oxygen consumption and ΔA^{550} measurements are set in the last two rows, and the figures in parentheses indicate the number of experiments included.

Ferrocytochrome C		
Initial (μM)	--	67
Oxidized (μM)	--	41
O_2 Consumed (μM)	58	60
20:3 Reacted (μM)	29	31
Product Distribution (%)		
Hydroxy Acids	23	26
PGD	17	18
PGE	50	48
PGF	2	3
Origin	8	5
Averaged Ratios		
O_2 Consumed/20:3 Reacted	2.0 (10)	2.0 (9)
O_2 Consumed/Cytochrome Oxidized	--	1.4 (9)

There was a cessation (data not shown) of the rapid rates of oxidation of cytochrome c and epinephrine when fatty acid oxidation terminated suggesting a transient presence of the oxidizing agent associated with prostaglandin biosynthesis. The reaction system was tested at different times to estimate the stability of that agent. Addition of ferrocytochrome c to the reaction mixture up to 120 minutes (Fig. 1A) after the fatty acid oxygenation had ceased (as evidenced by the oxygen electrodes) showed that the active agent present during the reaction with fatty acid was not stable for more than 5-10 minutes, even in the absence of reduced cytochrome c. Similarly, addition of epinephrine to the vesicular gland oxygenase system after the self-limited oxygenation had ceased resulted in a very small amount (<1 μM) of adrenochrome formation (Fig. 1B). The degree of chromophore formation with both detection systems closely paralleled the degree of prostaglandin synthesis measured by oxygen uptake with three different enzyme preparations of different specific activities.

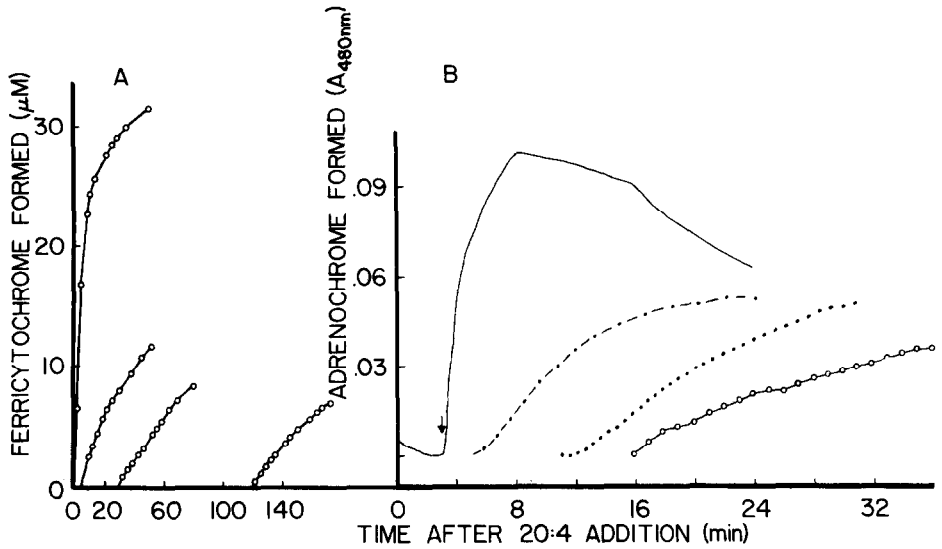

Fig. 1: Stability of Oxidative Intermediate Responsible for Ferrocytochrome c and Epinephrine Oxidation by Sheep Vesicular Gland Fatty Acid Oxygenase.

A: Arachidonic acid (103 μM) was added to 2.0 ml of a mixture of phenol activated acetone powder (0.8 mg) preparation in 0.1 M Tris buffer (pH 8.5) containing 0.66 mM phenol. Oxygen consumption was followed with an oxygen electrode (not shown). Ferrocytochrome c (60 μM) was added after 5, 30 and 120 min, the mixture was transferred to a UV cuvette and absorbance measurement monitored at 550 mμ. Another run (0 min) was monitored only at 550 mμ upon the addition of arachidonic acid. B: The procedure was identical to that described in A except 2 mg oxygenase preparation was used in 2.9 ml of 0.1 M Tris phenol buffer with 80 μM arachidonic acid. Absorbance readings at 480 mμ were monitored after the addition of 200 μM epinephrine. ·-·-·, epinephrine added after 5 min; ·····, 20 min; o—o, 50 min; ———, epinephrine added before arachidonic acid (0 min).

Participation of H_2O_2 in Chromophore Generation

Oxidation of epinephrine during the dioxygenase reaction decreased progressively with increasing amounts of added catalase (Fig. 2). Similarly, progressively increased levels of superoxide dismutase gave decreased amounts of epinephrine oxidation (data not shown). On the other hand, the rapid oxidation to ferricytochrome c by the oxygenase was not affected by added catalase (130 units) suggesting that different radical intermediates were responsible for the production of ferricytochrome c compared to those yielding adrenochrome. Added catalase had little effect on the oxygenation of fatty acid, and only a 20% decrease in oxygen consumption was observed with 6800 units of catalase. Addition of 400 μM glutathione to a mixture of phenol-activated oxygenase with either ferricytochrome or epinephrine decreased the oxidation of either detector system (data not shown) to less than 25% of that in the standard system.

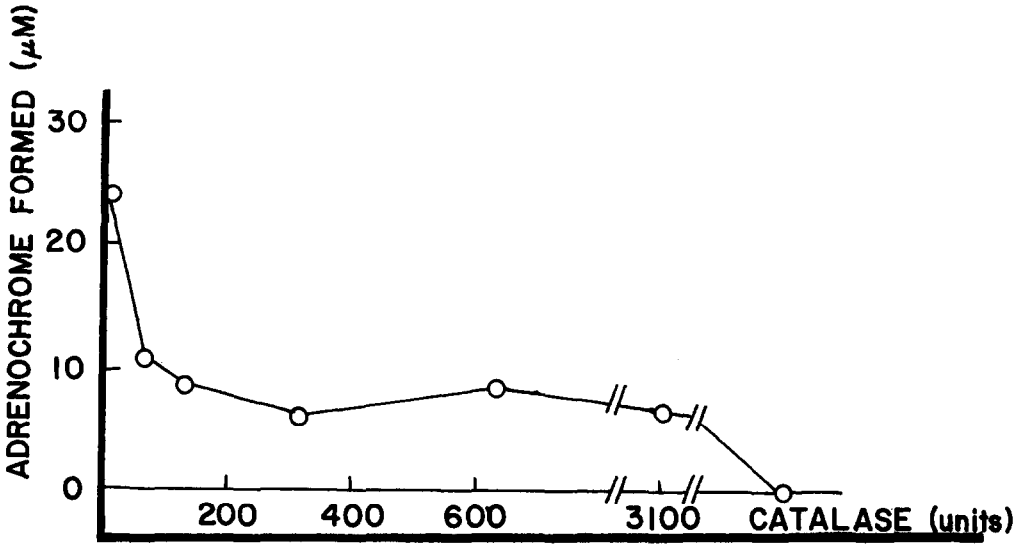

Fig. 2: Effect of Added Catalase on Epinephrine Oxidation by Sheep Vesicular Gland Prostaglandin Synthetase Complex.

Phenol activated prostaglandin synthetase enzyme preparation (2 mg) was added to 2.9 ml of a solution of epinephrine (1 mM) in 0.1 M Tris buffer (pH 8.5) containing 0.66 mM phenol. After 30 min, the indicated amount of catalase was added and arachidonic acid (48 μM final concentration) was added after an additional 30 min. Absorbance changes were continuously monitored at 480 mμ and the maximum absorbance change recorded was converted to μM adrenochrome formed.

Addition of catalase (100 units) nearly eliminated the time-dependent destruction of cyclooxygenase by the acetylenic acid (Δ9a,12a-18:2) as shown in Fig. 3. Intermediate amounts of catalase gave proportionally decreased destructive effects. The lack of catalase effect on oxygen consumption was supported by measurements of prostaglandin formation with radioactive eicosatrienoic acid as substrate. Added catalase (51 units) had no effect

on the fatty acid consumed (32 nmoles). When 85 μM substrate was added immediately after the addition of the 20 μM acetylenic inhibitor (no catalase present), 9.8 nmoles of the fatty acid reacted. This amount of

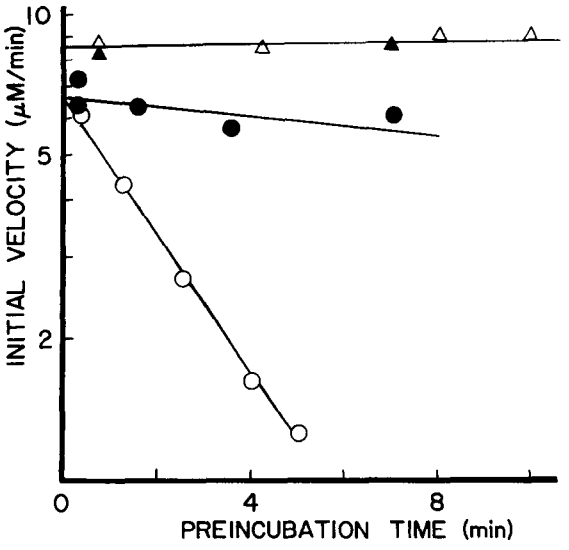

Fig. 3: Effect of Catalase on the Time-dependent Destruction of Sheep Vesicular Gland Fatty Acid Oxygenase by Δ9a,12a-18:2.

The phenol-activated enzyme preparation (3 mg) was added to 2.9 ml of solution of 0.66 mM phenol in 0.1 M Tris buffer (pH 8.5). After 1 min, catalase (100 units) and/or Δ9a,12a-18:2 (1 μM final concentration) was added. After the indicated preincubation times, arachidonic acid (85 μM final concentration) was added and oxygen consumption was followed with an oxygen electrode. Remaining active enzyme is indicated by the initial velocity which is plotted on a logarithm scale versus preincubation time of the oxygenase with catalase and/or 9a,12a-18:2:-Δ-,no additions;-▲-,100 units of catalase;-●-,1μM inhibitor plus 100 units catalase; -○-, 1μM inhibitor.

reaction is expected if only the reversible, competitive interaction of the inhibitor was manifested. After 7.5 min pre-incubation in the presence of 20 μM eicosatetraynoic acid and catalase, 6.8 nmoles of the substrate were consumed compared to only 2.5 nmoles in the absence of catalase. The protection by catalase suggests that the time-dependent loss of oxygenase was due to generated hydrogen peroxide. However, no direct evidence was found for hydrogen peroxide formation using ferrocytochrome c and added cytochrome peroxidase.

It is noteworthy that some fatty acid-dependent oxidation of cytochrome c was observed after 25 min (7.5 μM at a rate of 1.2 μM/min) in the presence of the acetylenic inhibitor even though oxygen consumption measurements gave no evidence of fatty acid oxygenase activity remaining. On the basis of 4 cytochrome per O_2, the corresponding amount and rate of oxygen consumption would then be 1.9 μM and 0.3 μM/min, respectively. These values lie outside

the sensitivity of the oxygen electrodes used in these experimental conditions, and it appears that the ferrocytochrome reagent may be a more sensitive probe than the oxygen electrode for monitoring the fatty acid oxygenase reaction.

Comparison of Reversible and Irreversible Acyl Chain Interactions

When the fatty acid oxygenase was preincubated with the 5,8,11,14-acetylenic acid and epinephrine for increasing preincubation times, a progressive decrease was observed in the A_{480} produced after addition of arachidonic acid (Fig. 4A). When adrenochrome formation was investigated for the fatty acid oxygenase system that was reversibly inhibited by 9a-18:1 (Fig. 4B, dashed line), both the extent and rate of epinephrine oxidation were lower than that of the uninhibited control, and did not change greatly after different preincubation times. On the other hand, with the Δ10a-18:1, a rapid decrease was observed in the absorbance at 480 mμ that could be generated upon addition of arachidonic acid. These results again confirm the irreversible inhibition by the 10a-18:1 (10), and eicosatetraynoic acids, which is quite different from that of the reversible competitive inhibition with 9a-18:1 and other fatty acids.

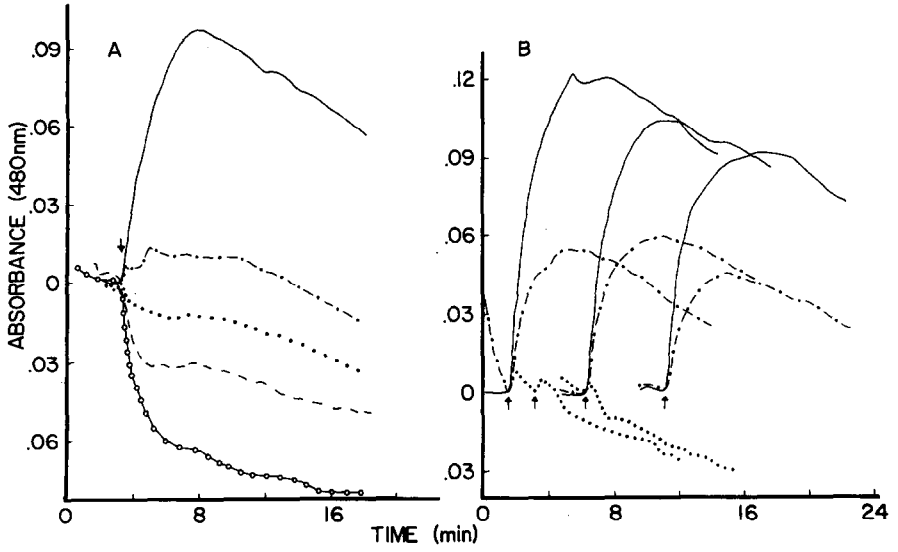

Fig. 4: Effect of Acetylenic Acids on Epinephrine Oxidation by the Prostaglandin Synthetase Complex.

A: A phenol-activated preparation (2 mg) was added to 2.9 ml of a solution of epinephrine (200 μM final concentration), and phenol (0.66 mM) with or without (——) eicosatetraynoic acid (9.3 μM) in 0.1 M Tris buffer (pH 8.5). Absorbance changes at 480 mμ were monitored and arachidonic acid (80 μM final concentration) was added after incubation for: •—•—•, 0.3 min; •••••, 1.0 min; - - -, 2.0 min; o—o—o, 7.0 min. B: Δ9a-18:1 and Δ10a-18:1. The procedure was identical to that described above except a final concentration of 150 μM of either Δ9a-18:1 or Δ10a-18:1 and 50 μM of arachidonic acid were used. ——, no acetylenic acid; •—•—•, Δ9a-18:1; •••••, Δ10a-18:1.

The reversibly competitive inhibitor, 20:3 (n-3), was investigated as another control in which time-dependent changes were not expected. The rates of ferrocytochrome c oxidation in the presence of 20:3 (n-3) prior to, during, and after the addition of arachidonic acid agreed with those obtained in the presence of 9a-18:1. There was no observable time-dependent effect, and both the rate and extent of chromophore formation decreased with increased concentration of 20:3 (n-3). Similar results were obtained with epinephrine.

Inhibition by Indomethacin

Indomethacin has also been reported to destroy the oxygenase activity in a time-dependent, concentration-dependent manner (13). We therefore investigated the effect that exposure of the fatty acid oxygenase to indomethacin had upon the oxidation of ferricytochrome c and epinephrine. The basal rate of cytochrome c oxidation prior to the addition of arachidonic acid was not appreciably affected by indomethacin (Fig. 5A, 0.52 vs 0.45 μM/min for the control). After exposure to indomethacin, the observed basal rate barely increased upon the addition of substrate (0.50 vs 3.3 μM/min for the control) which correlated with the lack of oxygenase activity indicated by oxygen consumption measurements.

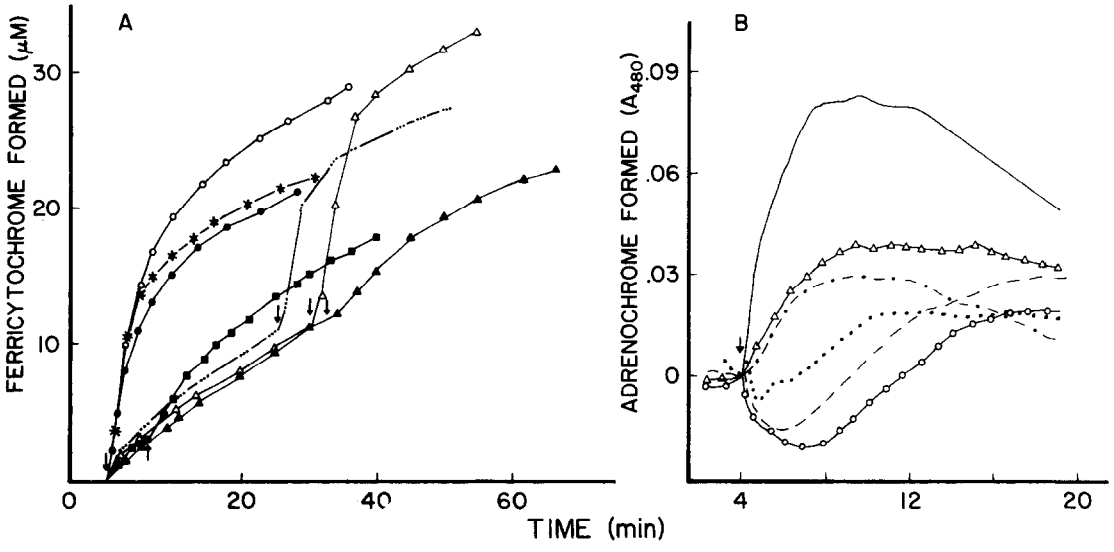

Fig. 5: Effects of Added Indomethacin and 20:3 (n-3).

A: On Ferrocytochrome c Oxidation: Procedures for indomethacin (20 μM final concentration) and 20:3 (n-3) (48 μM final concentration) were identical to that described for Fig. 1 except the final concentrations of arachidonic acid used were 105 and 35 μM respectively. Preincubation with no additions: o—o, 0.5 min; Δ—Δ, 30 min with 20 μM indomethacin; ●—●, 0.5 min; ■—■, 6.5 min; ▲—▲, 33 min and with 48 μM 20:3 (n-3): *—*—*, 0.5 min; •••—•••, 25 min.
B: On Epinephrine Oxidation. Procedures for indomethacin (20 μM final concentration) and 20:3 (n-3) (50 μM final concentration) were identical to that described for Fig. 4 except the final concentrations of arachidonic acid used were 84 and 36 μM respectively. Indomethacin: •—•—•, 0.3 min preincubation; •••••, 1.0 min; - - -, 5 min; o—o—o, 10 min; 20:3 (n-3) Δ—Δ.

When epinephrine oxidation by fatty acid oxygenase in the presence of indomethacin was monitored after the addition of arachidonic acid (Fig. 5B), there was an initial decrease in absorbance at 480 mμ followed by an increase in chromophore formation. The decrease in absorbance was not observed during a 0.3 min preincubation of the enzyme and indomethacin, but seemed due to added substrate and thus resembled that seen in anaerobic systems. When the enzyme and indomethacin were preincubated from 1-10 min, the time of minimum absorbance shifted progressively towards longer times after substrate addition.

DISCUSSION

Both systems used in these studies to detect intermediate oxidants showed a rapid response to added fatty acid substrate that was related to the amount of functioning oxygenase activity present. This rapid colorimetric response stopped several minutes after it had been initiated. The colorimetric changes were correlated with the oxygenase specific activities rather than with the amounts of non-enzymic components in the preparations (such as iron or copper or protein), and were proportional to the steady state concentration of biosynthetic intermediates. The absence of color changes in the anaerobic studies indicates that the oxidant was not present in the fatty acid or enzyme preparation, and that the color changes were due to oxygen-mediated events.

Ferrocytochrome c interfered with the epinephrine oxidation presumably by reducing the initiator or intermediates in the oxidative chain reaction. Another reducing agent, glutathione, has been reported to inhibit the cooxygenation of benzo(a)pyrene and 1,3-diphenylisobenzofuran by sheep vesicular gland prostaglandin synthetase (14). We found that glutathione also blocked the formation of both adrenochrome (as reported by Sih and Takeguchi (3)) and ferricytochrome c, but it did not interfere with prostaglandin formation. Apparently the cooxidation event that forms either chromophore reflects events initiated by some oxidant(s) in a manner that does not limit the rate of reaction. This could occur if the oxidant reacting with glutathione occurred subsequent to the cyclooxygenation reaction.

The changes with both colorimetric systems were measured with precision, and the total equivalents of oxidized reagents (in terms of O_2) were about one fifth of those consumed during fatty acid oxygenation. The results suggest that the cyclooxygenase product, PGG, or an enzyme-PGG complex provides the oxidant. A peroxidase reaction of the hydroperoxide group could readily initiate subsequent cooxidations (15,16) while providing PGH to form other prostaglandins. The overall reaction provides three of the four atoms of oxygen consumed for prostaglandin formation and one fourth of the oxygen for peroxidase-like cooxidations. Peroxidation reactions may be accompanied by chemiluminescence (17,18) and the appearance of radical signals in a manner described for cyclooxygenase action (14,18,19). Our results would indicate that the peroxidase activity has broad specificity and is capable of transferring electrons from either cytochrome c or epinephrine to the hydroperoxide.

A Role for Hydrogen Peroxide in Enzyme Destruction

We observed cytochrome c peroxidase activity in our crude cyclooxygenase preparations (unpublished results) and expected that ferrocytochrome c oxidation would give a sensitive measure of any H_2O_2 (20) formed in the

system. Added catalase, however, had no effect on ferricytochrome formation, and an oxidative intermediate other than H_2O_2 appears responsible for the oxidation of ferricytochrome c. Presumably PGG may form PGH and oxidize the cytochrome c without any H_2O_2 involved. The lack of a catalase effect also suggests that high concentrations of H_2O_2 are not normally an obligatory intermediate in prostaglandin biosynthesis as was proposed earlier (21). The ability of catalase to reduce adrenochrome formation suggests that the first oxidized form of epinephrine produces H_2O_2 before eventually forming the observed chromophore. In our studies, catalase did not appreciably inhibit oxygen consumption or the formation of oxidized fatty acid products from arachidonic or 8,11,14-eicosatrienoic acids. Thus our results with the enzyme from sheep vesicular gland differ from those for bovine preparations described by Panganamala et al. (21).

We previously reported that certain acetylenic acids destroy the sheep oxygenase in an irreversible time-dependent manner (10). The marked specificity of this process for certain positional isomers suggests that those analogs may bind to the oxygenase and mimic the substrate in inducing some reactive form of oxygen intermediate. The analogs, however, were incapable of complete reaction, and, the intermediate active oxygen degenerated instead through reactions which led to enzyme inactivation. Since we find that catalase is able to prevent the destructive effect of the acetylenic acids, it appears that H_2O_2 is an important reactant in this process. We have searched for evidence that the H_2O_2 was formed by an oxidase ($RH + H_2O + O_2 \rightarrow R\text{-}OH + H_2O_2$) or by peroxidase action on trace peroxide contaminants ($ROOH + H_2O \rightarrow ROH + H_2O_2$). If the acetylenic analogs cause or facilitate the generation of H_2O_2, we thought that it might be detected by oxidation of either epinephrine or ferrocytochrome c. However, no colorimetric evidence was obtained for H_2O_2 formation with the acetylenic acids. On the contrary, a decreased absorbance of adrenochrome was observed, and we believe that the added unsaturated acid participated in reactions that terminated the oxidative chain reactions.

Paradoxically, the basal rate of epinephrine oxidation appeared to produce more hydrogen peroxide than did the acetylenic acids, but H_2O_2 did not destroy the oxygenase activity. One must consider that the hydrogen peroxide generated during inactivation by acetylenic fatty acids might be formed so close to the active site of the oxygenase that very small amounts could destroy it without being adequate to initiate the autocatalytic chain reaction of adrenochrome formation. This concept of compartmentalization is supported by the previously described "cryptic" nature of the E_b form of cyclooxygenase (22) that allowed the tetrayne acid to inactivate the enzyme even in the presence of an active glutathione peroxidase system. A concern for compartmentalization was also expressed by Marnett et al. (14) in noting a lack of evidence for superoxide or singlet oxygen in the cyclooxygenase system.

Both catalase and glutathione diminished the cooxidation of epinephrine, showing an ability to reduce oxidative intermediates before they can form the chromophores. Nevertheless, glutathione did not prevent the time-dependent destruction of the oxygenase by the acetylenic acids. Thus we note again that the destructive oxidant which inactivates the enzyme is not equivalent to that oxidizing the chromogens. In the presence of active glutathione peroxidase, glutathione completely prevented loss of enzyme (E_a) activity. This result further emphasizes the role of hydrogen peroxide or a lipid

hydroperoxide in the irreversible inactivation by the acetylenic acids. The exact role of such a peroxide is not known at present, however, the mechanism of these oxygenases seems to involve binding to a peroxide activator (7,13) as well as oxygen and substrate acid. Such a complex may form destructive hydroxyl radicals (13,14). This would be similar to a mechanism proposed by Hodgson and Fridovich (23) for the irreversible inactivation of superoxide dismutase by hydroxyl radical or its ionized equivalent generated from hydrogen peroxide.

The varied effectiveness reported for the acetylenic acid in inhibiting prostaglandin biosynthesis may occur because of the concomitant need for peroxide to get irreversible inactivation. The acetylenic analog will always bind competitively and reversibly at the substrate site, however, in some tissues, the oxygenase may somehow not form the abortive intermediate that yields H_2O_2. Alternatively, catalase and/or glutathione peroxidase levels in the tissue may be great enough to remove the H_2O_2 and leave the cyclo-oxygenase only reversibly inhibited. The present results indicate that the irreversible inactivation by acetylenic acids may be a sensitive indicator of the availability of peroxides ("peroxide tone") within the cells.

ACKNOWLEDGMENT

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REFERENCES

1. Samuelsson B, Granstrom E, Green K, Hamberg M, Hammarstrom S. Prostaglandins. Annual Review of Biochemistry 44: 669-695, 1975.

2. Lands WEM, Hemler ME, Crawford CG. Functions of polyunsaturated fatty acids: Biosynthesis of prostaglandins. Pp 193-228 in Polyunsaturated Fatty Acids. (WH Kunau, RT Holman eds) American Oil Chemists Society, Champaign, IL.

3. Takeguchi C, Sih CJ. A rapid spectrophotometric assay for prostaglandin synthetase: Application to the study of non-steroidal anti-inflammatory agents. Prostaglandins 2: 169-185, 1972.

4. Misra HP, Fridovich I. The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry 247: 3170-3175, 1972.

5. Mazur A, Green S, Shorr E. The oxidation of adrenaline by ferritin iron and hydrogen peroxide. Journal of Biological Chemistry 220: 227-255, 1956.

6. Trautner EM, Bradley TR. The early stages of the oxidation of adrenalin in dilute solution. Australian Journal of Science Research B 4: 303-343, 1951.

7. Smith WL, Lands WEM. Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular glands. Biochemistry 11: 3276-3285, 1972.

8. Lands WEM, LeTellier PR, Rome LH, Vanderhoek JY. Inhibition of prostaglandin biosynthesis. Advances in Biosciences 9: 15-28, 1973.

9. Ahern DG, Downing DT. Inhibition of prostaglandin biosynthesis by eicosa-5,8,11,14-tetraynoic acid. Biochimica et Biophysica Acta 210: 456-461, 1970.

10. Vanderhoek JY, Lands WEM. Acetylenic inhibitors of sheep vesicular gland oxygenase. Biochimica et Biophysica Acta 296: 374-381, 1973.

11. Yonetani T. Cytochrome oxidase from beef heart muscle. Biochemical Preparations 11: 14-20, 1966.

12. Robinson J, Cooper JM. Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode. Analytical Biochemistry 33: 390-399, 1970.

13. Smith WL, Lands WEM. Stimulation and blockade of prostaglandin biosynthesis. Journal of Biological Chemistry 246: 6700-6704, 1971.

14. Marnett LJ, Wlodawer P, Samuelsson B. Co-oxygenation of organic substrates by the prostaglandin synthetase of sheep vesicular gland. Journal of Biological Chemistry 250: 8510-8517, 1975.

15. Yang SF. Biosynthesis of ethylene: Ethylene formation from methional by horseradish peroxidase. Archives of Biochemistry and Biophysics 122: 481-487, 1967.

16. Chan HWS. Singlet oxygen analogs in biological systems. Coupled oxygenation of 1,3-dienes by soybean lipoxygenase. Journal of the American Chemical Society 93: 2357-2358, 1971.

17. Noguchi T, Nakauo M. Effect of ferrous ions on microsomal phospholipid peroxidation and related light emission. Biochimica et Biophysica Acta 368: 446-455, 1974.

18. Marnett LJ, Wlodawer P, Samuelsson B. Light emission during the action of prostaglandin synthetase. Biochemical and Biophysical Research Communications 60: 1286-1294, 1974.

19. Egan RW, Paxton J, Kuehl FA. Mechanism for irreversible self-destruction of prostaglandin synthetase. Journal of Biological Chemistry 251: 7329-7335, 1976.

20. Yonetani T. Studies on cytochrome c peroxidase. Journal of Biological Chemistry 240: 4509-4514, 1965.

21. Panganamala RV, Sharma HM, Sprecher H, Geer J, Cornwell DG. A suggested role for hydrogen peroxide in the biosynthesis of prostaglandins. Prostaglandins 8: 3-11, 1974.

22. Lands WEM, LeTellier P, Rome L, Vanderhoek JY. Regulation of prostaglandin synthesis. Pp 1-7 in Prostaglandin Synthetase Inhibitors. (HJ Robinson, JR Vane eds) Raven Press, New York, 1974.

23. Hodgson EK, Fridovich I. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: Inactivation of the enzyme. Biochemistry 14: 5294-5299, 1975.