Activation of Soluble Splenic Cell Guanylate Cyclase by Prostaglandin Endoperoxides and Fatty Acid Hydroperoxides*

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Purified prostaglandin endoperoxides (PGG2 and PGH2) and hydroperoxides (15-OOH-PGE2) as well as fatty acid hydroperoxides (12-OOH-20:4, 15-OOH-20:4, and 13-OOH-18:2) were examined as effectors of soluble splenic cell guanylate cyclase activity. The procedures described (in the miniprint supplement) for the preparation, purification, and characterization of these components circumvented the use of diethyl ether which obscured effects of lipid effectors because of contaminants presumed to be ether peroxides which were stimulatory to the cyclase.

Addition of prostaglandin endoperoxides or fatty acid hydroperoxides to the reaction mixture led to a time-dependent activation of guanylate cyclase activity; 2.5- to 5-fold stimulation was seen during the first 6 min. The degree of stimulation and rate of activation were dependent on the concentration of the fatty acid effector; when initial velocities (6 min) were assessed half-maximal stimulation was achieved in the range of 2 to 3 μM. However, by extending the incubation time to 90 min similar maximal increases in specific activity could be achieved with 3 or 10 μM PGG2 or PGH2. Activation of guanylate cyclase upon addition of prostaglandin endoperoxides or fatty acid hydroperoxides was prevented or reversed by the thiol reductants dithiothreitol (3 to 5 mM) or glutathione (10 to 15 mM). Na2S2O4, not known as an effective reducing agent of disulfides, prevented but was relatively ineffective in reversing activation after it had been induced by PGG2.

Pretreatment of the enzyme preparation with increasing concentrations of N-ethylmaleimide in the range of 0.01 to 1.0 mm prevented activation by PGG2 without affecting basal guanylate cyclase activity. These observations indicate that fatty acid hydroperoxides and prostaglandin endoperoxides promote activation of the cyclase by oxidation of enzyme-related thiol functions. In contrast PGE2, PGF2α, hydroxy fatty acids (13-OH-18:2, 12-OOH-20:4) as well as saturated (18:0) monoenoic (18:1), dienoic (18:2), and tetraenoic (20:4) fatty acids were ineffective in promoting cyclase activation in the range of 1 to 10 μM.

Studies to identify the species of the rapidly metabolized prostaglandin endoperoxides that serve as effectors of the cyclase indicated that PGG2 but not 15-OOH-PGE2 (the major buffer-rearrangement product of PGG2) is most likely an activator. In the case of PGH2, a rapidly generated (30 s) metabolite of PGH2 was found which contained a hydroperoxy or endoperoxy functional group and was equally as effective as PGH2 as an apparent activator of the enzyme.

The combined effects of PGG2 and dehydroascorbic acid, another class of activator, exhibited additivity with respect to the rate at which the time-dependent activation was induced. These results suggest that activation of soluble guanylate cyclase from splenic cells can be achieved by the oxidation of sulfhydryl groups that may be associated with specific hydrophobic sites of the enzyme or a related regulatory component.

Activation of the soluble form of platelet guanylate cyclase (GTP pyrophosphatase-lyase (cyclizing), EC 4.6.1.2) by micromolar concentrations of n-6 polyunsaturated fatty acids indicates the presence of relatively selective sites for hydrophobic ligands on some forms of this class of cyclase (1).

Guanylate cyclase activity from splenic cells (2-4) and from several other sources has also been shown to be enhanced by a process that appears to involve oxidation of the enzyme or a closely related regulatory component. The oxidative activation was first shown to occur spontaneously upon incubation of soluble tissue or cell extracts in air (5-8). Subsequently, it was shown to result from oxidation by a mild oxidant, such as dehydroascorbic acid (2-4), by substances, such as nitroprusside, NaNO2, or NH2OH, from which oxidizing equivalents in the form of nitric oxide (9, 10) are generated, or by free radicals which are believed to arise from nitrosamines (11, 12) H2O2 (13) or ascorbic acid (4).

Since hydrophobic and oxidant properties are inherent characteristics of naturally occurring prostaglandin endoperoxides and fatty acid hydroperoxides, studies were initiated to examine the effectiveness of these lipid components to serve as activators of guanylate cyclase activity. In the initial stage of investigation a stimulatory effect of a prostaglandin endoperoxide (PGG2) was uncovered (14).

The results of more extensive investigations which comprise the present report show that the soluble form of guanylate cyclase from guinea pig splenic cells can be activated by micromolar concentrations of prostaglandin endoperoxides, PGG2 and PGH2, or their metabolic products containing

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oxidizing functions but not by the reduced, hydroxy-containing prostaglandins E₂ or F₂α. It is also demonstrated that the cyclase is activated by hydroperoxy, but not hydroxy, fatty acids deriving from linoleic acid and eicosatetraenoic acid. Evidence is also presented indicating that the activation by these lipid peroxides involves the oxidation of sulfhydryl groups associated with hydrophobic sites of a guanylate cyclase related component.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, GTP, cAMP, cGMP, dithiothreitol, bovine serum albumin, sodium borohydride, N-methyl-N-nitroso-p-toluenesulfonamide, N-ethylmaleimide, cystine, and glutathione were obtained from Sigma. Dehydroascorbic acid was purchased from ICN and Chemical Procurement Laboratories. Dehydroascorbic acid solutions were made in 2 mM sodium phosphate, pH 7.5, containing 0.83% ammonium chloride. All solutions were maintained for no longer than 60 min at 4°C under argon or nitrogen.

**Prostaglandin Endoperoxide Activation of Guanylate Cyclase**

Soluble guinea pig splenic cell guanylate cyclase, like the soluble form of the enzyme from lung (6, 8) and platelets (1, 5), undergoes a spontaneous increase in activity upon incubation of tissue extracts in air (Fig. 1). Spontaneous activation was suppressed when reactions were conducted in an argon atmosphere (and deactivated argon saturated reagent) (viz. Fig. 3). It has been suggested that this spontaneous activation in air results from an oxidative process (1, 5, 6); in the case of the splenic cell enzyme it has been reported to involve the oxidation of thiol functions of the enzyme or a closely associated component (2, 3). This is consistent with the effect that the thiol reductant, dithiothreitol, has to prevent (Fig. 1) or to reverse (Fig. 8) this activation of the splenic cell enzyme; 3 mM dithiothreitol was required to completely prevent activation while lower concentrations (i.e. 0.1 and 1.0 mM) were only partially effective (Fig. 1). Qualitatively similar results were obtained with glutathione but the effective concentration range was 10 to 15 mM (viz. Fig. 2). In addition to preventing spontaneous activation, dithiothreitol, at the highest concentration (3 mM) employed, also suppressed basal activity. This inhibition is apparent from the decreased velocities during the early (i.e. 2 to 6 min) course of the reaction when the rates of cGMP production are linear with respect to time (Fig. 1) with or without dithiothreitol. Because of this apparent inhibition of basal activity by the thiol reductant, velocities obtained in the presence of thiol reducing agents could not be used as a valid representation of control rates to compare with those obtained in the presence of lipid peroxides with which reductants could obviously not be used. Such a comparison would overestimate any stimulatory action of a lipid effector. As demonstrated by White et al. (6) with the enzyme in soluble lung extracts, the reaction rates with the splenic cell cyclase at 30°C are relatively linear for the first 5 to 6 min before activation becomes apparent. The early linear portion of the reaction velocity (i.e. at or before 6 min) therefore, was used when assessing alterations inducible by fatty acid derivatives in enzyme activity at a fixed time of reaction.

Another potential source of error that was identified during the initial phase of this work related to the conventional procedures used to extract, purify, and store fatty acid metabolites examined in these studies as well as the evidence obtained to document their chemical structure is presented in the miniprint supplement (Figs. 1S through 14S).

**RESULTS**

**Guanylate Cyclase Preparation**—Spleens were removed from decapitated 200- to 250-g male Hartley guinea pigs and homogenized in 10 ml of phosphate (100 mM)-buffered saline, pH 7.4 (NaCl/P). Connective tissue was removed by sedimentation and the red blood cells were disrupted by hypotonic shock for 10 min at 37°C in 1 mM Tris-HCl, pH 7.5, containing 0.83% ammonium chloride. All solutions were equilibrated with argon. The white cells were sedimented and washed three times by successive centrifugation and resuspension in NaCl/P. The cells were finally suspended at a density of 200 three times by successive centrifugation and resuspension in NaCl/P. The soluble fraction was removed and disruption was determined to be approximately 95%. The cell lysate was centrifuged at 105,000 x g for 60 min to separate the soluble and particulate enzyme fractions. The soluble fraction was removed and maintained for no longer than 60 min at 4°C under argon or nitrogen until assayed.

**Guanylate Cyclase Assay**—Activity of the enzyme was measured by a modification of the method of Kimura and Murad (16). Unless otherwise noted, final concentrations of the components in the cyclase assay (30 µl) were 1 mM GTP, 2 mM MnCl₂, 5 mM 3-isobutyl-1-methylxanthine, 15 mM creatine phosphate, and 0.2 mg/ml of creatine phosphokinase in 50 mM Tris-HCl, pH 7.5. Fatty acids and prostaglandins tested as possible effectors of guanylate cyclase were added prior to addition of the reaction mixture to the enzyme to reaction tubes which were stoppered and maintained on dry ice. Solvent, containing the lipid component, was evaporated under vacuum (10 to 20 s) before addition of reaction mixture followed by the enzyme extract (10 µl containing 10 to 12 µg of protein); addition of the reaction mixture preceded the enzyme by 5 s. Appropriate volumes of the solvent alone representative of those used to transfer the lipid effector to the reaction mixture were added separately to these reaction vessels with a Hamilton Syringe and samples were withdrawn from stopped vessels by syringe and transferred to tubes in which reactions were terminated as described above.

The protein concentration of the extract was determined by the method of Bradford (17) and adjusted to 1.0 to 1.2 mg/ml prior to assay. The cGMP formed was converted to the 3′-O-acetyl derivative (18) and assayed by radioimmunoassay as previously described (1). The binding reaction (300 µl) was conducted in 50 mM sodium acetate, pH 4.0, containing 20 mM CaCl₂, 0.5 mg of γ-globulin, 0.75 mg of bovine serum albumin, 25,000 cpm of 125I-tagged-cGMP tyrosine methyl ester, and antibody sufficient to bind 40% of the total counts per min added. Incubation at 4°C was terminated after 14 h or more by ethanol precipitation and the precipitated antigen-antibody complexes were counted in a gamma counter. Standard curves were prepared in glass-distilled water and in the presence of all the reaction components (including boiled enzyme) to control for any possible interference by the reactants in the acetylation or binding reactions.

**Isolation and Characterization of Fatty Acid and Prostaglandin Metabolites**—A detailed description of the methods used for the isolation and purification of the fatty acids and prostaglandin components examined in these studies as well as the evidence obtained to document their chemical structure is presented in the miniprint supplement (Figs. 1S through 14S).

**PROSTAGLANDIN ENDOPEROXIDE ACTIVATION OF GUANYLATE CYCLASE**

Portions of this paper (including Figs. 1S to 14S) are presented in miniprint at the end of this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-410, cite author(s), and include a check or money order for $4.05 per set of photocopies.
The activity of the soluble fraction of guanylate cyclase from splenic cell lysates was assayed as described under "Experimental Procedures" in the absence or presence of dithiothreitol at the concentrations indicated. The values represent the mean of duplicate determinations.

**Fig. 1.** Prevention of spontaneous activation in air of soluble splenic cell guanylate cyclase by increasing concentrations of diithioretil (DTT). The activity of the soluble fraction of guanylate cyclase from splenic cell lysates was assayed as described under "Experimental Procedures" in the absence or presence of diithioretil at the concentrations indicated. The values represent the mean of duplicate determinations.

**Fig. 2.** Activation of guanylate cyclase by a nonvolatile component from diethyl ether. Panel A, diethyl ether (60 ml) from a freshly opened can was evaporated under vacuum (-70°C) and guanylate cyclase reaction mixture without or with glutathione (GSH) (10 mM) followed by enzyme extract was added. In reactions not containing glutathione at the onset the reducing agent (10 mM) was added where indicated 5 min (5') after initiation of the reaction and the cGMP formed thereafter was determined (--.--). Panel B, diethyl ether (60 ml) from either a freshly opened can (designated Stock), after storage for 7 days at room temperature in a clear glass container (designated Aged) or after exposure to a short wave ultraviolet (Mineralite) hand lamp for 24 h (designated UV-exposed) was evaporated to dryness under vacuum. Guanylate cyclase reaction mixture was added followed by the addition of cell extract to initiate the reaction and the cGMP formed after 10 min determined as described under "Experimental Procedures." Each value represents the mean of duplicate determinations; the range is indicated by the vertical bars.

Because of earlier reports (1) that soluble guanylate cyclases (i.e. from platelets) can be stimulated by (n-6) polyunsaturated fatty acids, it was judged essential to eliminate endogenous lipid components (i.e. nonradioiodelabeled) and possible oxidized lipid products deriving from freshly prepared microsomes or acetone powder preparations of microsomes which are ordinarily used to generate the prostaglandin endoperoxides. The precaution taken to eliminate this potential source of error was to lipid deplete (22) the sheep vesicular gland microsomes used for the enzymic generation of PGG<sub>2</sub> and PGH<sub>2</sub>. From 300 mg of microsomes prepared by this procedure (i.e. the amount used in a standard reaction mixture to generate prostaglandin endoperoxides) there was no lipid detectable upon thin layer chromatography of pooled extracts obtained from three successive extractions with chloroform/methanol (2:1). It was also determined that fractions from silicic acid chromatography corresponding to those containing PGG<sub>2</sub> and PGI<sub>2</sub>, had no detectable effect on guanylate cyclase activity when extracts of lipid depleted microsomes without added arachidonic acid were chromatographed (not shown).

**Activation by Prostaglandin Endoperoxides and Fatty Acid Hydroperoxides—**Micromolar concentrations of prostaglandin endoperoxides PGG<sub>2</sub> and PGH<sub>2</sub>, were found to be effective activators of the soluble form of splenic cell guanylate cyclase (Fig. 3). Guanylate cyclase velocity was increased over 4-fold upon the addition of PGG<sub>2</sub> (10 μM) and over 3-fold when PGH<sub>2</sub> (10 μM) was added to the reaction. Activation was apparent with the inclusion of either prostaglandin endoperoxide when the reaction was conducted in room air or in an argon atmosphere with argon-equilibrated reagent. Because spontaneous activation was suppressed in reactions conducted in argon, the relative increase in activity attributable to PGG<sub>2</sub> or PGH<sub>2</sub> under these conditions was slightly greater (Fig. 3). The stimulatory effectiveness of these prostaglandin endoperoxides in an argon atmosphere indicates that they are not merely accelerating the process by which the undefined spontaneous activation is promoted and that their stimulatory effect is not dependent upon the presence of molecular oxygen. Furthermore, it was established that the activation of PGG<sub>2</sub> or PGH<sub>2</sub> (6 or 10 μM) was independent of protein concentration over a 4-fold range (3 to 12 μg/mg).

The fatty acid hydroperoxides, 15-OOH-20:4, 12-OOH-20:4, and 13-OOH-18:2 (Fig. 4 B, C, and D), also serve as effective enhancers of the cyclase activity; the stimulatory effect is concentration-dependent with respect to the lipid effector. The time course of the reaction (in room air) in the presence of 1, 3, or 6 μM PGG<sub>2</sub> (Fig. 4A) or the fatty acid hydroperoxides.
stimulatory activity; half-maximal activation is achieved at 2 to 3 μM and increases in activity of 300 to 400% at 10 μM under the experimental conditions employed. The hydroperoxy fatty acids, 13-OOH-18:2, 12-OOH-20:4, 15-OOH-20:4, and PGH₂ produced more than a doubling of activity at concentrations between 5 and 10 μM in these experiments when rates were determined at 6 min. The extent to which enzyme activity is enhanced by these stimulatory fatty acids varies among experiments and can range from 250% as shown here for PGH₂ and three of the hydroperoxy fatty acids, to 300 to 500% (viz. Figs. 3 and 4). However, under the conditions employed the concentration dependence of the effect of the stimulatory lipids remains relatively constant between 2 and 3 μM for half-maximal activation. Considering the time-dependent nature of the activation, additional complications of spontaneous activation and the presence of other components in the crude cell extract with which these effectors probably interact, these values should be considered as only first approximations of effective activator concentrations.

The relative ineffectiveness of 12-OOH-20:4 compared to its hydroperoxy-containing counterpart (i.e. 12-OOH-20:4) is also apparent (Fig. 6A): the hydroxy fatty acid was ineffective until a concentration of 50 μM was achieved. Similarly, the 13-OH analogue of the stimulatory 13-OOH-18:2 was totally ineffective at concentrations of 1, 3, and 10 μM (not shown). In Fig. 6B, 18:1 and 18:0 and 5,8,11,14-eicosatetraenoic acid are relatively ineffective in the 1 to 10 μM range; only 20:4 and 18:2 produce any stimulatory effect but maximum activation by these two fatty acids is no more than 50% at concentrations between 50 and 100 μM. The splenic cell-soluble guanylate cyclase, therefore, differs from the soluble platelet enzyme with respect to eicosatetraenoic acid stimulability since the activity of the platelet enzyme is enhanced significantly by eicosatetraenoic acid at concentrations in the range of 1 to 10

(Fig. 4B, C, and D) indicates that the stimulatory effect of these lipid metabolites is time-dependent. This progressive increase in enzyme activity is apparent with all concentrations of the activators tested. With the lowest concentration (1 μM) little or no increase in activity is detectable until some time between 2 and 4 min of the reaction. With higher concentrations of the fatty acid effectors (3 and 6 μM (Fig. 4) or 10 μM (Fig. 3)) activation is apparent at the earliest time (i.e. 2 min) examined and velocities become progressively greater with time. A more extensive examination of the characteristics of the time-dependent activation is shown in Fig. 5 where the changes in guanylate cyclase specific activity are plotted with respect to time over a period of 90 min; this extended incubation period permitted apparent completion of the activation that occurred spontaneously or in the presence of PGG₂ or PGH₂. The specific activity increased over 3.5-fold from 163 to 600 pmol/min/mg of protein as a result of spontaneous activation and approximately 8.5-fold to values between 1,300 to 1,500 pmol/min/mg of protein in the presence of PGG₂ or PGH₂. The maximal increase in specific activity was comparable with either of the prostaglandin endoperoxides, but the time required for maximal activation in the presence of 10 μM PGG₂ was considerably shorter (about 15 min) than with 10 μM PGH₂ (about 45 min). Furthermore, virtually the same maximum specific activity was achieved with 10 μM as with 3 μM concentrations of either prostaglandin endoperoxide, but the rate at which activation proceeded was faster with the higher concentration of each.

The concentration dependence of guanylate cyclase activation by the various fatty acid hydroperoxides and prostaglandin endoperoxides determined after a 6 min reaction in room air is shown in Fig. 6A. PGG₂ and 15-OOH-PGE₂ appear to be among the most potent of the components exhibiting

![Fig. 3. Time course of PGG₂ and PGH₂ activation of soluble splenic cell guanylate cyclase in air or argon. The reactions were conducted as described under "Experimental Procedures." The concentration of PGG₂ and PGH₂ was 10 μM. The values represent the means of duplicate determinations and the vertical bars represent the range.](https://example.com/fig3.png)

![Fig. 4. Time course of the activation of soluble splenic cell guanylate cyclase by various concentrations of PGG₂, 13-OOH-18:2, 15-OOH-20:4, and 12-OOH-20:4. Conditions and procedures are described under "Experimental Procedures." The values represent averages of triplicate determinations ± standard error.](https://example.com/fig4.png)
It is noteworthy that \( \text{H}_2\text{O}_2 \) at concentrations as high as 100 \( \mu \text{M} \) had no effect on splenic cell cyclase while the more hydrophobic benzoyl peroxide stimulated activity 70% at a concentration as low as 2 \( \mu \text{M} \) and as much as 300% with increasing concentrations from 10 to 100 \( \mu \text{M} \) (not shown).

**The Effector Species of Prostaglandin Endoperoxides**—A characteristic of the prostaglandin endoperoxides \( \text{PGG}_2 \) and \( \text{PGH}_2 \) is their lability in aqueous solution (i.e. \( T_{1/2} \) of 4.5 to 5.5 min) (19, 23). Both the \( \text{PGG}_2 \) and \( \text{PGH}_2 \) used in these experiments were found to undergo degradation in buffer with a half-time of 4 to 4.5 min determined by the loss of the characteristic biological action each has to promote aggregation of washed human platelets (not shown). To aid in establishing that the activation of guanylate cyclase observed with \( \text{PGG}_2 \) or \( \text{PGH}_2 \) added to the reaction mixture results from an action of the prostaglandin endoperoxides rather than from a product deriving from aqueous rearrangement, the effect of preincubating the endoperoxides in the reaction mixture before addition of the enzyme containing extract was examined (Table I). The stimulatory effectiveness of \( \text{PGH}_2 \) disappeared (\( T_{1/2} \) of 5.5 min) upon incubation in the aqueous solution. The major aqueous rearrangement product of \( \text{PGH}_2 \) was shown to be \( \text{PGE}_2 \) (see miniprint supplement) in confirmation of previous reports (19); \( \text{PGE}_2 \) at concentrations up to 10 \( \mu \text{M} \) has no effect on soluble splenic cell guanylate cyclase (Table II). In contrast to the loss of \( \text{PGH}_2 \) effectiveness upon its rearrangement in buffer, incubation of \( \text{PGG}_2 \) for as long as 60 min before addition of the enzyme did not result in any loss of stimulatory activity (Table I). The basis for this result was investigated by isolating the major products of \( \text{PGG}_2 \) rearrangement and examining their effect on guanylate cyclase activity. Four \(^{14} \text{C}-\) labeled rearrangement products were detectable by thin layer chromatography (viz. Fig. 4S,B). Of these four components isolated by chromatography on silicic acid one of the major products (Peak IV, Fig. 6S) was found to be as potent as \( \text{PGG}_2 \) with regard to activating guanylate cyclase. This component was conclusively identified as 15-\( \text{OOH-PGE}_2 \) (Figs. 7S and 8S). This agrees with the findings of Hamberg and Samuelsson (19) who reported 15-\( \text{OOH-PGE}_2 \) to be the major product of \( \text{PGG}_2 \) degradation in buffer. The characteristics of the stimulatory action of 15-\( \text{OOH-PGE}_2 \) with respect to the time course of enhancing enzyme activity

**Fig. 5.** Changes in guanylate cyclase specific activity during an extended incubation with different concentrations of \( \text{PGG}_2 \) and \( \text{PGH}_2 \). The enzyme reactions were conducted as described under "Experimental Procedures" in the presence or absence of 3 or 10 \( \mu \text{M} \) \( \text{PGG}_2 \) or \( \text{PGH}_2 \) as indicated and the mean specific activities during the time intervals shown determined the means of quadruplicate determinations ± standard error.

**Fig. 6.** Concentration dependence of the activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides, fatty acid hydroperoxides, and hydroxy fatty acids. cGMP generation after a 6-min incubation with the designated concentration of the lipid component was compared with that in control reactions containing equivalent volumes of the solvent which was evaporated before initiating the reaction as described under "Experimental Procedures." The values represent averages of 2 to 10 determinations.
The major metabolic product, is ineffective as an activator in the 1 to 10
PGG₂ probably serves as an activator of the cyclase. Also, since PGE₂,
was not due to the formation of 15-OOH-PGE₂. These findings help to
establish that activation of guanylate cyclase by PGG₂, is for by the chromatographic degradation product of PGG₂ plus
product formed
PGG₂ can stimulate the cyclase explains the persistence of
similarly to PGA₂ (Fig. 4S,B); it was similar in potency to
PGH₂, has not been accomplished, one characteristic of it that has
been uncovered is a positive reactivity with N,N-dimethyl-p-
phenylenediamine which indicates the presence of a peroxy
functional group. This is a characteristic of all of the other
metabolites of PGH₂ that has been reported to date (Fig.
7, F and G). Although the characterization of this component
has not been accomplished, one characteristic of it that has
been uncovered is a positive reactivity with N,N-dimethyl-p-
phenylenediamine which indicates the presence of a peroxy
functional group. This is a characteristic of all of the other
fatty acid and prostaglandin metabolites which have been
identified, progressive enhancement of activity) were found to be identical to those exhibited by PGG₂ (10 μM) (not shown); their relative potencies were comparable (viz. Fig. 6A). Guanylate cyclase stimulatory activity was also found with one of the minor components generated that migrated similarly to PGA₂ (viz. Fig. 4S,B); it was similar in potency to PGG₂. The component was not characterized. The demonstration that two of the hydrolytic rearrangement products of PGG₂ can stimulate the cyclase explains the persistence of stimulatory activity after PGG₂ degradation in aqueous solution and the presence of a hydroperoxy function on the major product formed (i.e. 15-OOH-PGE₂) provides a basis for explaining the stimulatory effectiveness of this component.

The possibility that the stimulation of guanylate cyclase activity seen upon addition of PGG₂ to the enzyme reaction mixture may derive from a rearrangement of PGG₂ to 15-
OOH-PGE₂ (or other hydroperoxy-containing products) was further examined by determining the steady state level of 15-OOH-PGE₂ achieved in the cell extract-containing reaction mixture.

As shown in Fig. 7 (Panels A to D), some of the products generated from PGG₂ upon incubation (1 and 6 min) in the cell extract-containing reaction mixture (Fig. 7, Panels B and C) differ from those formed upon incubation in buffer alone (Fig. 7, Panel D). (The products obtained in buffer alone were identical to those found to be generated in reaction mixture devoid of enzyme (not shown).) Some of the marked differences in the products generated in the presence or absence of cell extract is that 15-OOH-PGE₂ (RF = 0.39) is not detectable in the enzyme containing reaction mixture at 1 or 6 min (Fig. 7, R and C) or at 15, 30, or 120 s (not shown). In the presence of the soluble cell extract the major product generated (16% at 1 min and 32% at 6 min) chromatographed with authentic PGE₂. Also apparent is that PGG₂ is the major component present in the extract-containing reaction mixture at 1 and 6 min of incubation (64 and 34%, respectively) (i.e. accounted for by the chromatographic degradation product of PGG₂ plus degraded PGG₂ (see legend to Fig. 7)). These findings help to establish that activation of guanylate cyclase by PGG₂ is not due to the formation of 15-OOH-PGE₂. Also, since PGE₂, the major metabolic product, is ineffective as an activator in the 1 to 10 μM concentration range, a reasonable conclusion is that PGG₂ probably serves as an activator of the cyclase.

The possibility that other still unidentified metabolic products of PGG₂ which constitute no more than 35% of the total lipid present at 6 min may be stimulatory to guanylate cyclase has, however, not been eliminated.

A similar assessment was made of the products generated from PGH₂ during incubations in reagent with and without cell extract. As shown in Fig. 7, F to H, some additional components are generated from PGH₂ in the cell extract-containing reaction. It is also apparent that PGH₂ (RF = 0.60) undergoes a much more rapid metabolic conversion than PGG₂ in reaction mixture containing splenic cell extract; by 0.5 min the small amount of PGH₂ remaining (approximately 10 to 15%) appeared as a small shoulder of a major component generated with an RF value of 0.71. The different chromatographic behavior of PGH₂ and this PGH₂-derived component could be established in two additional chromatographic systems (not shown). After 1 min (not shown), as in the example shown at 6 min (Fig. 7, Panel G), no detectable PGH₂ remained. It was pointed out above that the aqueous rearrangement products of PGH₂ are ineffective in stimulating guanylate cyclase (viz. Table I). This is consistent with the demonstration that the major product formed (40%) from PGH₂ in buffer is PGE₂ (Fig. 7H) and indicates that the minor product (19.3%) (shouder of PGH₂ with RF = 0.43) which migrated identically to authentic PGD₂ in this system (not shown) is also not a stimulatory product of PGH₂. Therefore, the additional major products of PGH₂ formed in extract-containing media are represented by PGF₂α (22.6% at 6 min) and the component with RF 0.71. Since PGF₂α was shown to be ineffective as a stimulator of the cyclase at a concentration of 10 μM (viz. Table III), the only remaining candidate of the major components generated, besides PGH₂, that might serve as an activator of the enzyme is the component with RF = 0.71 which represents 49 and 23.7% of the degraded PGH₂ at 0.5 and 6 min, respectively.

This PGH₂-derived component was isolated by silicic acid chromatography upon elution with 20% ethyl acetate in petroleum ether. When tested as a possible effector of guanylate cyclase activity it was found that this metabolite at a concentration of 10 μM stimulated cyclase activity 3.2-fold (Table II) compared to 10 μM PGH₂ which enhanced enzyme activity 2.95-fold in this experiment. The activation induced by the PGH₂-derived metabolite (PG, RF = 0.71) as well as PGH₂ could be prevented and reversed by dithiothreitol (Table II). This rapidly generated metabolite of PGH₂ does not correspond to any metabolite of PGH₂ that has been reported to date (Fig. 7, F and G). Although the characterization of this component has not been accomplished, one characteristic of it that has been uncovered is a positive reactivity with N,N-dimethyl-p-phenylenediamine which indicates the presence of a peroxy functional group. This is a characteristic of all of the other fatty acid and prostaglandin metabolites which have been identified.

### Table I

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<tr>
<th>Prostaglandin Endoperoxide (10 μM)</th>
<th>Time of rearrangement in buffer</th>
<th>Guanylate cyclase activity</th>
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<td></td>
<td>min</td>
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<tr>
<td></td>
<td>30</td>
<td>330 ± 3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>332 ± 30</td>
</tr>
<tr>
<td></td>
<td>103 ± 6</td>
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</tr>
<tr>
<td>PGH₂</td>
<td>0</td>
<td>25 ± 24</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>205 ± 4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>188 ± 11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>131 ± 9</td>
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<tr>
<td></td>
<td>30</td>
<td>123 ± 3</td>
</tr>
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</table>

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Without Dithiothreitol</th>
<th>Dithiothreitol, 0 min*</th>
<th>Dithiothreitol, 5 min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62 ± 4</td>
<td>42 ± 7</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>PGH₂ (RF = 0.71), 10 μM</td>
<td>199 ± 47</td>
<td>52 ± 12</td>
<td>68 ± 34</td>
</tr>
<tr>
<td>PGH₂, 10 μM</td>
<td>183 ± 22</td>
<td>34 ± 20</td>
<td>60 ± 11</td>
</tr>
</tbody>
</table>

* Dithiothreitol concentration was 2 mM.

* Dithiothreitol was added at 5 min and the velocity during the subsequent 3 min period was determined.

* Specific activity calculated during a 3-min interval representing the 5- to 8-min period of the incubation.
found to be stimulatory to soluble splenic cell guanylate cyclase.

The structural similarities of the prostaglandins E₂ and F₂₅ with PGG₂ and especially with 15-OOH PGE₂ prompted an examination of the effectiveness of these prostaglandins to serve as modifiers of guanylate cyclase activity, particularly with respect to an interaction with the enzyme that would interfere with PGG₂ or 15-OOH-PGE₂ promoted activation. It was found (Table III), as pointed out earlier, that neither PGF₂₀ nor PGE₂ alone had any significant influence on the cyclase activity at concentrations of 3 or 10 μM (or at concentrations as high as 50 μM (not shown)). PGF₂₀ (10 μM) also did not interfere significantly with activation induced by PGG₂ (4 or 6 μM) or by 15-OOH-PGE₂ (10 μM), nor did PGE₂ (3 or 10 μM) diminish the stimulatory effectiveness of 15-OOH-PGE₂ (10 μM). The apparent lack of competition between these structurally similar substances with contrasting stimulatory effectiveness supports the conclusion that an oxidizing function (i.e. endoperoxide or hydroperoxide groups) is required for activation of this enzyme by fatty acid metabolites.

Oxidative Mechanism of Guanylate Cyclase Activation—That the activation by these endoperoxide and/or hydroperoxy-containing fatty acids derives from their oxidizing potential is strongly suggested by the demonstration that thiol reductants such as diithiothreitol or glutathione not only prevent, but also reverse, the activation they induce. In Fig. 8, A and C, the effect of diithiothreitol to prevent and to reverse the activation 5 or 10 min after it occurs in the presence of either PGG₂ or 13-OOH-18:2 is shown. Identical results were obtained with glutathione and with either of the reductants when activation is promoted in the presence of PGH₂ (viz. Table II), or 12-OOH-20:4 and 15-OOH-20:4 (not shown). In contrast to this action of thiol reductants to reverse cyclase activation, Na₂S₂O₄ was relatively ineffective in reversing PGG₂ activation once it had been induced (Fig. 8B). Na₂S₂O₄ could, however, prevent PGG₂ activation when present at the onset of the reaction. The contrasting results with these two classes of reducing agents with regard to reversing PGG₂-induced activation is consistent with the concept that sulfhydryl functions associated with a hydrophobic site on the enzyme (or related component) undergo oxidation promoted by the lipid oxidants. The effect that Na₂S₂O₄ and the thiol reductants have to prevent activation probably derives from actions both have to reduce the oxidizing component (24). The prevention of spontaneous activation by Na₂S₂O₄ and diithiothreitol (Fig. 8) would also be consistent with rapid removal (i.e. by chemical reduction) of the oxidizing species which is generated (e.g. spontaneously). However, the reduction of transition metals or heme iron that may be involved in the oxidation-reduction reactions underlying the spontaneous activation is also possible. The involvement of sulfhydryl groups in the activation by PGG₂ is also indicated by the
effect that N-ethylmaleimide has to block activation by the prostaglandin endoperoxide (Fig. 9). Pretreatment of the soluble enzyme fraction with increasing concentrations of N-ethylmaleimide from 0.01 to 1.0 mM, which have little or no effect on basal guanylate cyclase activity, leads to a progressive loss of PGG₂ stimulability.

Possibility of Separate Hydrophobic and Hydrophilic Sites—It has been suggested that dehydroascorbic acid activates soluble guanylate cyclase from guinea pig splenic cells by an oxidative mechanism involving sulfhydryl-disulfide in terconversion at a site distinct from the catalytic site (2). At least two classes of oxidants are, therefore, represented by the agents which have thus far been shown to serve as activators of this cyclase; the hydrophobic fatty acid hydroperoxides and endoperoxides, and the hydrophilic dehydroascorbic acid. The possibility that there are separate hydrophobic and hydrophilic ligand sites associated with the enzyme was investigated by determining if the effects of the two classes of oxidants exhibit additivity (Fig. 10). With saturating concentrations of dehydroascorbic acid (5 μM) and 6 μM PGG₂ added at the onset of the reaction, the velocity measured during the first 4 min (502 pmol/min/mg) was equivalent to the sum of the rates at 4 min with dehydroascorbic acid (201 pmol/min/mg) or PGG₂ (302 pmol/min/mg) alone. In this experiment PGG₂ and dehydroascorbic acid alone stimulated the control rate (72 pmol/min/mg of protein), at 4 min, 4.2- and 2.8-fold, respectively, while the activation produced by the two activators together was 7-fold by 4 min. The addition at 4 min of PGG₂ or dehydroascorbic acid to reactions initially exposed to only dehydroascorbic acid or PGG₂, respectively, promoted additional enhancement of enzyme specific activity measured at 7 min; the specific activity of the enzyme in the reaction originally containing only dehydroascorbic acid increased from 291 to 458 pmol/min/mg of protein after addition of PGG₂ and the velocity of the PGG₂-containing reaction increased from 454 to 718 pmol/min/mg of protein after addition of dehydroascorbic acid. Although the complexities of the reaction in the inset of Fig. 10, the results of another experiment are shown in which the changes in specific activities of the cyclase are plotted with respect to the time of incubation with one or the other or a combination of the activators. In this experiment a concentration of PGG₂ (15 μM) was employed which produced near maximal stimulation within 6 min (442 pmol/min/mg (viz. Fig. 6)). Even under these conditions, the specific activities measured during the early course (2 min) of the reaction were substantially greater when dehydroascorbic acid (5 mM) was included with the prostaglandin endoperoxide. Although the complexities of the time-dependent enzyme activation preclude any unequivocal conclusions, these results suggest the presence of separate hydrophobic and hydrophilic regulatory sites. However, from the results obtained thus far it can be concluded only that

Table III

<table>
<thead>
<tr>
<th>Prostaglandin</th>
<th>Guanylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol cGMP/min/mg protein</td>
</tr>
<tr>
<td>PGG₂</td>
<td></td>
</tr>
<tr>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>139 ± 16</td>
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<tr>
<td>6</td>
<td>311 ± 7</td>
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<td>10</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>269 ± 15</td>
</tr>
<tr>
<td>6</td>
<td>340 ± 5</td>
</tr>
<tr>
<td>10-OOH-PGE₂</td>
<td></td>
</tr>
<tr>
<td>PGE₂</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>67 ± 5</td>
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<td>10</td>
<td>75 ± 3</td>
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<td>10</td>
<td>291 ± 28</td>
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<td>10</td>
<td>259 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>247 ± 33</td>
</tr>
<tr>
<td>15-OOH-PGE₂</td>
<td></td>
</tr>
<tr>
<td>PGE₂</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>67 ± 5</td>
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<td>10</td>
<td>86 ± 6</td>
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<tr>
<td>10</td>
<td>291 ± 28</td>
</tr>
<tr>
<td>10</td>
<td>306 ± 2</td>
</tr>
<tr>
<td>10</td>
<td>319 ± 22</td>
</tr>
</tbody>
</table>

Fig. 8. Effectiveness of dithiothreitol and relative ineffectiveness of Na₂S₂O₄ to reverse activation of soluble guanylate cyclase promoted upon the addition of PGG₂ or 13-OOH-18:2. A, soluble splenic cell guanylate cyclase was assayed in the presence or absence of 6 μM PGG₂. Dithiothreitol (DTT) (3 mM) was present when the reaction was initiated (0') or added at 5 min (5') or 10 min (10') as indicated by the origin of the dashed lines which represent cGMP generation in the presence of the reducing agent. B, same protocol as in Panel A except that 10 μM PGG₂ was employed and Na₂S₂O₄ (5 mM) was used as the reducing agent. C, same protocol as in Panel A except that 10 μM 13-OOH-18:2 (18:2-OOH) and 1 mM dithiothreitol were used. The values shown are the means of triplicate determinations which did not differ by more than 10%. The differences in specific activity of guanylate cyclase in the three experiments reflects the variation ordinarily encountered with the enzyme activity from this source.
plotted with respect to time of incubation. The reactions were conducted in the presence or absence of 15 μM PGG2 or 5 mM dehydroascorbic acid. The values on the ordinate represent the range of N-ethylmaleimide indicated and a 10-μl aliquot was then transferred to cyclase reaction mixture as described under "Experimental Procedures" with or without 10 μM PGG2. The guanylate cyclase reaction was conducted for 6 min at 30°C. The values represent the means of duplicate determinations; the vertical bars represent the range.

![Graph showing prevention of guanylate cyclase activation with PGG2 upon pretreatment of soluble cell extract with N-ethylmaleimide.](image)

The results of the present experiments indicate that purified fatty acid metabolites with oxidizing functions represented by hydroperoxy or endoperoxide groups are effective stimulators of splenic cell guanylate cyclase activity. The results also indicate that activation of the cyclase by these lipid components involves oxidation of the enzyme or a closely associated regulatory component.

**TABLE IV**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Enzyme activity</th>
<th>pmol cGMP/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PGG2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Ca2+</td>
<td>2.3 ± 0.5</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Mg2+</td>
<td>14.2 ± 1.3</td>
<td>88.3 ± 2.7</td>
</tr>
<tr>
<td>Mn2+</td>
<td>76.2 ± 8.0</td>
<td>377.0 ± 13.0</td>
</tr>
<tr>
<td>+ DHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Ca2+</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Mg2+</td>
<td>7.1 ± 0.2</td>
<td>13.2 ± 0.0</td>
</tr>
<tr>
<td>Mn2+</td>
<td>49.0 ± 6.0</td>
<td>194.1 ± 3.8</td>
</tr>
</tbody>
</table>

Each divalent cation was present at a concentration of 2 mM and the GTP concentration was 1 mM. A, reactions were conducted for 6 min in the presence or absence of 10 μM PGG2. B, a separate experiment in which reactions were conducted for 10 min in the presence or absence of 5 mM dehydroascorbic acid. Values are the means of duplicate determinations ± range.

**DISCUSSION**

The results of the present experiments indicate that purified fatty acid metabolites with oxidizing functions represented by hydroperoxy or endoperoxide groups are effective stimulators of splenic cell guanylate cyclase activity. The results also indicate that activation of the cyclase by these lipid components involves oxidation of the enzyme or a closely associated regulatory component.

Of the fatty acid components tested, the fatty acid hydroperoxides represented by 12-OOH-20:4, 15-OOH-20:4, and 13-OOH-18:2, as well as the prostaglandin hydroperoxide, 15-OOH-PGE₂, activated the soluble splenic cell guanylate cyclase 250 to 500%. Half-maximal activation was achieved with concentrations of these lipid hydroperoxides in the 2 to 3 μM range. In contrast, the hydroxy-containing analogues of these fatty acids that were examined (e.g. 12-OH-20:4, 13-OH-18:2, or PGE₂ and PGF₂α), as well as the fatty acids 18:0, 18:1, 18:2,
and 20:4, were ineffective in a comparable concentration range (i.e. 1 to 10 μM). The relatively small stimulatory effect (about 50%) produced by 12-OH-20:4 and the fatty acids 20:4 and 18:2 at much higher concentrations (i.e. 50 to 100 μM) suggests that soluble splenic cell guanylate cyclase may be activable by a mechanism involving lipid interactions with hydrophobic enzyme sites. Fatty acid activation of guanylate cyclase has been demonstrated with the soluble enzyme from platelets (1, 25), and the particulate enzyme from fibroblasts (26) and adipocytes (97). Some specificity with regard to the fatty acid activator is suggested even with the minimally effective fatty acids (i.e. 20:4, 18:2, and 12-OH-20:4) since monoenoic (18:1) and polyunsaturated fatty acids were totally ineffective even in the high concentration range tested. A high degree of specificity for lipid structural determinants was exhibited by the reduced form of the soluble platelet guanylate cyclase (i.e. assayed with dithiothreitol) which was stimulated by microsomal levels of polyunsaturated fatty acids with n-6 structure (1). These observations suggest the presence of hydrophobic sites on the enzyme from splenic cells and from other cell and tissue sources.

From what is presently known about the metabolism of hydroperoxy fatty acids, it is unlikely that metabolites other than the hydroxy fatty acids are generated in tissue extracts. Since the hydroxy fatty acid analogues of the hydroperoxy fatty acids were found to be relatively ineffective in the 1 to 10 μM range, it is reasonable to conclude that hydroperoxy fatty acids represent effective activators in the low micromolar range while the non-hydroperoxy-containing component may affect the enzyme activity less specifically at higher concentrations. It has been suggested (28, 29) that fatty acid hydroperoxides may activate guanylate cyclase from studies in which a reaction mixture containing 20:4 or 18:2 and lipoxygenase led to activation of a partially purified soluble guanylate cyclase from platelets. The identity of the fatty acid products was, however, not established in these studies but a correlation was made between peroxide equivalents present and the extent of cyclase activation (28).

Although both PGH2 and PGG2, when introduced into the reaction, appeared to be half-maximally effective as activators of the cyclase in the range of 2 to 3 μM, and both brought about as much as a 250 to 500% stimulation of the enzyme during the first 6 min of the incubation, it is not certain to what extent the parent compound or metabolites, or both, of these relatively rapidly metabolized components may have contributed to the activation seen. The apparent activation by PGG2 or PGH2 did not require molecular oxygen and the effects induced by both prostaglandin endoperoxides were reversible and preventable by thiol reductants. This indicates that the stimulatory species of prostaglandin, whether represented by the parent compound or metabolites generated in the reaction mixture, are effective by virtue of their oxidizing potential. Some evidence in favor of PGG2 representing a species of prostaglandin that activates the cyclase is that: (a) although 15-OOH-PGE2 is a major chemical rearrangement product of PGG2 formed in aqueous solution, 15-OOH-PGE2 is not generated in the cell extract-containing reaction; (b) the major product of PGG2 metabolism in the extract is PGF2a which is nonstimulatory; and (c) 64 and 34% of the parent endoperoxide still remain at 1 and 6 min, respectively. It remains to be determined whether one or more of the presently unidentified components which together represent no more than 35% of the total constituents present in the PG2-containing reaction at 6 min may also be stimulatory to the enzyme.

Since PGG2 contains both hydroperoxy and endoperoxide functional groups, its effectiveness as an activator would seem predictable from the stimulatory effect all hydroperoxy-containing fatty acids were found to have on the cyclase. What effectiveness to attribute to the endoperoxyl function, which also possesses oxidizing potential, is not as clear from these studies. Although the addition of PGH2 led to activation of the cyclase, it was found that this prostaglandin endoperoxide was virtually all metabolized by 60 s in the reaction mixture containing cell extract. Although PGH2 remains a likely candidate as an activator species, evidence was obtained that a metabolite of PGH2 may represent an alternative or additional stimulator. PGE2 and a component that co-migrated with PGD2 are two of the major metabolites of PGH2 that were identified. Neither of these components could be considered effective metabolites because they are among the aqueous rearrangement products of PGH2 which are all nonstimulatory. The unidentified component to which approximately 50% of the PGH2 was converted by 30 s was found to be stimulatory to the cyclase. This PGH2-derived metabolite promoted enzyme activation that could be prevented or reversed by dithiothreitol, which suggests that it contains an oxidizing function. The presence of an oxidizing group on this component was confirmed by the positive reactivity it was found to have with N,N-dimethyl-p-phenylenediamine.

The involvement of protein sulphydryl groups and their conversion to disulfides as a result of an oxidation that appears to underlie the activation is also indicated by the relative ineffectiveness of Na2S2O4 compared to the effectiveness of dithiothreitol to reverse activation. The inhibition of PGG2-induced activation by pretreatment of the soluble preparation with the sulphydryl-reactive N-ethylmaleimide at concentrations of this reagent that did not affect basal enzyme activity also argues in favor of sulphydryl-disulfide interconversion in the activation. The fact that PGE2 and PGF2a, which were ineffective as activators, also did not interfere with PGG2 or 15-OOH-PGE2 activation indicates that the effectiveness of thiol reductants to reverse activation does not derive from the generation of reduction products (i.e. PGE2 and PGF2a) which may serve as inhibitors of the enzyme. The apparent lack of competition between PGE2 or PGF2a, and PGD2 or 15-OOH-PGE2 also underscores the importance of an oxidizing function for interaction with enzyme (or enzyme complex) but the ineffectiveness of H2O2 to activate also emphasizes the hydrophobic requirement for the ligand. The lack of effect of H2O2 to activate soluble guanylate cyclase from liver (13) has already been reported, although it has been suggested that H2O2 may underlie the spontaneous activation in air of the soluble cyclase from lung (6).

It has recently been suggested (2) that oxidation of protein thiol functions is implicated in the activation of soluble splenic cell guanylate cyclase induced by another oxidant, dehydroascorbic acid. The evidence in support of that conclusion was similar to that obtained with the lipid oxidants, including the reversal of dehydroascorbic acid-promoted activation by thiol reductants and prevention of activation by N-ethylmaleimide. DeRubertis and Craven (12) have also recently demonstrated that the activation of soluble hepatic guanylate cyclase by the chemical carcinogen, N-methyl-N-nitro-N-nitroguanidine is reversed by dithiothreitol and that the stimulatory effects of N-methyl-N-nitro-N-nitroguanidine and nitroprusside on the soluble hepatic guanylate cyclase are prevented by N-ethylmaleimide. From these studies DeRubertis and Craven proposed that stimulation of hepatic guanylate cyclase by these activators may involve oxidation of tissue sulfhydryl groups (12). The process of oxidation as a mechanism of guanylate cyclase activation is also implied by studies showing that oxidants such as periodate (30) and a group of chemical agents represented by azide, nitroprusside, hydroxylamine, nitrite,
hydrazine (8, 10, 31-36), and numerous N-nitroso-containing compounds (11, 12, 37) either elevate cellular cGMP levels or stimulate guanylate cyclase activity or both, in a variety of cell-free systems. This latter group of agents are believed to form highly reactive nitroxides as a consequence of their cellular metabolism (10, 12). Activation of guanylate cyclase by nitric oxide has indeed been demonstrated (10). The possibility that hydroxyl free radicals may represent a species of oxidizing equivalents within the cell that stimulate the cyclase under certain conditions has also been suggested (13). The fact that thiol reductants have been shown to produce an effect opposite to that of oxidants on cGMP metabolism in intact cells (i.e. lower cGMP steady state levels) and to bring about a corresponding, reversible, suppression of the activity of guanylate cyclase from the same cells (2), lends credence to the possibility of an oxidative-reductive mechanism of modulating guanylate cyclase activity.

One characteristic of the lipid hydroperoxy- or endoperoxide-induced stimulation, or both, of soluble guanylate cyclase from splenic cell is that the activation is a time-dependent process. Although it is possible that one component of the time dependence may derive from the rate at which effective metabolites are generated from the parent compounds, this is probably not the only, or even the major, contributing factor since the hydroperoxy fatty acids, which probably do not generate secondary oxidizing metabolites, and dehydroascorbate, which was not found to undergo any detectable metabolic conversion (2), promote activation in a similar time-dependent manner. The rate at which activation occurs appears to increase with increasing concentrations of effector, but essentially the same maximal increases in specific activity can be achieved with 3 or 10 μM concentrations of either PGG2 or PGH2 if the incubation is extended for a time (60 to 90 min) to permit completion of the activation process. The demonstration that the rate of activation in the presence of near maximally effective concentrations of PGG2 is accelerated when another class of oxidant such as dehydroascorbic acid is also included underscores the time-dependent nature of the activation process and also raises the possibility that sulfhydryl groups associated with separate hydrophobic and hydrophilic sites may undergo oxidation. The time dependence of the activation process could be explained merely on the basis of time-dependent chemical oxidation of protein sulfhydryl groups at specific enzyme sites. It is equally likely that oxidation of the free protein thiol occurs relatively rapidly. If so, an initial alteration in the state of the enzyme or of a component in the enzyme complex could occur along with a corresponding increase in enzyme activity represented by the early stimulation seen. The progressively greater increase in activity could result from a secondary, time-dependent, conformational change that ultimately leads to a maximally activated enzyme state. The initial interaction with effector and degree of oxidation it may induce could determine the rate at which the secondary conformational change takes place.

Although the fatty acid and prostaglandin metabolites shown in this study to be effectors of the splenic cell guanylate cyclase are naturally occurring components (in contrast to chemical substances such as those cited above which may provide oxidizing equivalents though nitroxide radicals), the physiological significance of the activation that they produce remains obscure. PGG2 and PGH2 formation have been implicated with the enhanced accumulation of cyclic GMP levels that occurs when aggregation of human platelets is induced by collagen or thrombin (14, 38, 39). Enhanced cellular accumulation of cGMP in epidermis has also been associated with markedly increased levels of 12-OH-20:4 (40) and it would be expected that correspondingly greater amounts of its precursor, 12-OH-20:4, would be generated under such conditions. Since hormones and other cell membrane active substances which promote cellular accumulation of cyclic GMP are ineffective as activators of guanylate cyclase (41), other components which may be generated upon cell stimulation could be envisaged to serve as modulators of the enzyme. Although the compounds tested here or their metabolites are attractive candidates for coupling cell membrane generated signals with the modulation of guanylate cyclase activity, it is not possible at this time to determine whether the stimulatory effects of the lipid components described here play such a role in situ.

Acknowledgments—We would like to express our gratitude to Thomas P. Krick for her expert technical assistance and interest in the work and to Thomas P. Krick for performing the mass spectral analysis of the lipid components.

REFERENCES

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Additional references are found on p. 7678.
Prostaglandin Endoperoxide Activation of Guanylate Cyclase

**Supplement**

7674 Prostaglandin Endoperoxide Activation of Guanylate Cyclase by Prostaglandin D1 and Thromboxane A2.

**Methods and Materials**

**Materials**

- Prostaglandin D1 and thromboxane A2 were purchased from Sigma Chemical Co., St. Louis, Mo., and used without further purification.
- Guanylate cyclase was purified from guinea pig lung homogenates by sequential chromatography on DEAE-cellulose, Sephadex G-100, and CM-Sephadex C-25 columns.
- The reaction was initiated by the addition of 20 mg of thromboxane A2 to 0.5 ml of the enzyme preparation in 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EGTA, and was terminated by the addition of 0.1 N HCl.

**Methods**

- The reaction was monitored by measuring the formation of cyclic GMP using a specific antibody.
- The formation of cyclic GMP was quantified by radioimmunoassay using a specific antibody.
- The turnover number of the enzyme was determined by measuring the formation of cyclic GMP at various enzyme concentrations.
- The inhibition was determined by measuring the inhibition of the enzyme activity by various compounds.

**Results**

- The addition of thromboxane A2 to the enzyme preparation resulted in a significant increase in the formation of cyclic GMP.
- The turnover number of the enzyme was approximately 100000 mol/min/mol enzyme.
- The inhibition of the enzyme activity by various compounds was determined.

**Discussion**

- The results indicate that thromboxane A2 activates guanylate cyclase by binding to a specific site on the enzyme.
- The activation is specific and depends on the presence of calcium ions.
- The mechanism of activation is likely to involve an increase in the permeability of the cell membrane to calcium ions.

**Figure**

- The figure shows the effect of thromboxane A2 on the formation of cyclic GMP.
- The results are expressed as the percentage of the control value.

**Conclusion**

- The activation of guanylate cyclase by thromboxane A2 provides a new mechanism for the regulation of cyclic GMP levels in the cell.
- This mechanism may have important implications for the understanding of the role of cyclic GMP in cellular processes.

**References**


Prostaglandin Endoperoxide Activation of Guanylate Cyclase

This is a scientific article discussing the activation of guanylate cyclase by prostaglandins and endoperoxides. The article includes graphs and diagrams illustrating the activation process. The text is technical and focuses on the chemical and biological mechanisms involved in this process. The diagrams show the structure of the molecules and their interactions, providing a visual representation of the scientific concepts discussed in the text.
Activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides.

G Graff, J H Stephenson, D B Glass, M K Haddox and N D Goldberg


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