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Isolation and Structure of Two Prostaglandin Endoperoxides That Cause Platelet Aggregation

(15-hydroperoxy endoperoxide/15-hydroxy endoperoxide/platelet aggregation/ contraction of rabbit aorta)

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ABSTRACT **Incubation for a short time of arachidonic acid with the microsomal fraction of a homogenate of the vesicular gland of sheep in the presence of 1 mM *p*-mercuribenzoate followed by extraction and silicic acid chromatography yielded two prostaglandin endoperoxides. The structures of these compounds, i.e., 15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (prostaglandin G_2) and 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid (prostaglandin H_2), were assigned mainly by a number of chemical transformations into previously known prostaglandins. The new prostaglandins were 50–200 times (prostaglandin G_2) and 100–450 times (prostaglandin H_2) more active than prostaglandin E_2 on the superfused aorta strip. The half-life of the prostaglandin endoperoxides in aqueous medium (about 5 min) was significantly longer than that of "rabbit aorta-contracting substance" released from guinea pig lung, indicating that none of the prostaglandin endoperoxides is identical with this factor. Addition of 10–300 ng/ml of the endoperoxides to suspensions of washed human platelets resulted in rapid aggregation. Furthermore, platelet aggregation induced by thrombin was accompanied by release of material reducible by stannous chloride into prostaglandin $F_{2\alpha}$, thus indicating the involvement of endogenous prostaglandin endoperoxides in platelet aggregation.**

Direct evidence for the formation of an endoperoxide during prostaglandin (PG) biosynthesis was recently obtained (1). In these experiments a compound, reducible to $PGF_{2\alpha}$, could be detected in incubations of arachidonic acid for a short time with the microsomal fraction of a homogenate of the vesicular gland of sheep. Preincubation with *p*-mercuribenzoate and other SH blocking agents reduced the rate of formation of PGE_2 as well as the rate of disappearance of the intermediate. This compound was also isolated by solvent extraction and thin-layer chromatography and was found to be a 15-hydroxy prostaglandin endoperoxide (1).

The present paper is concerned with isolation and determination of the structure of an additional prostaglandin endoperoxide having a hydroperoxy group at C-15, as well as the effect of the 15-hydroperoxy and 15-hydroxy endoperoxides on platelet aggregation and rabbit aorta strip.

MATERIALS AND METHODS

[*1*-^{14}C]Arachidonic acid, specific radioactivity about 11 Ci/mol, was prepared from 1-chloro-4,7,10,13-nonadecatetraene (kindly provided by Dr. W. Stoffel, Cologne, Germany, see ref. 2) and $Na^{14}CN$ followed by hydrolysis of the nitrile. The chemical and radiochemical purity was in excess of 98%, as judged by thin-layer radiochromatography. Part of the labeled acid was diluted with unlabeled material to make a preparation with specific radioactivity of 0.77 Ci/mol, which was used for incubations with vesicular gland microsomes.

Thin-Layer Chromatography (TLC) was carried out with plates coated with chloroform–methanol-washed Silica gel G and the following solvent systems (when not otherwise indicated): ethyl acetate–2,2,4-trimethylpentane (55:45, v/v) (TLC of methyl esters) and ethyl acetate–2,2,4-trimethylpentane–acetic acid (50:50:0.5, v/v/v) (TLC of free acids). A Berthold Dünnschichtscanner II was used for determination of radioactivity on TLC plates.

Enzyme Preparation and Incubations. Rinsed microsomal pellets obtained from 8 g of sheep vesicular gland (1) were suspended and homogenized in 20 ml of 92 mM potassium phosphate buffer pH 7.4 containing 7.2 mg (1 mM) of sodium *p*-hydroxymercuribenzoate (Sigma Chemical Co.). The mixture was kept at 37° for 2 min and subsequently incubated for 30 sec with 1.2 mg of [*1*-^{14}C]arachidonic acid in 0.1 ml of ethanol. The mixture was rapidly poured into a separatory funnel containing 100 ml of 25 mM HCl and extracted twice with diethyl ether. The combined ether phases were washed twice with water and dried over 75 g of anhydrous $MgSO_4$. The residue obtained after evaporation of the solvent was immediately dissolved in about 3 ml of diethyl ether–light petroleum (2:8, v/v) and subjected to silicic acid chromatography.

Platelet Aggregation. Blood of healthy donors who had not taken aspirin for 1 week was collected with 7.5% (v/v) of 77 mM sodium EDTA and centrifuged at 200 × *g* for 15 min. The platelet-rich plasma thus obtained (280,000–400,000 platelets per μl) was centrifuged at 650 × *g* for 15 min. The platelet pellet from 12 ml of platelet-rich plasma was suspended in 5 ml of 0.15 M NaCl–0.15 M Tris·HCl buffer pH 7.4–0.077 M sodium EDTA (90:8:2, v/v/v) and subsequently recentrifuged. The pellet of washed platelets was suspended in 4 ml of Krebs–Henseleit medium (3) not containing calcium. Platelet aggregation was monitored with a Born aggregometer (4). To 1 ml of platelet suspensions at 37° were added prostaglandin endoperoxides (10–1000 ng in 1–5 μl of acetone) or 5 units of thrombin (Topostasin®, Hoffman-La Roche Co.) in 33 μl of 0.9% NaCl.

Abbreviations: PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; Me_3Si, trimethylsilyl; MO, *O*-methyloxime.

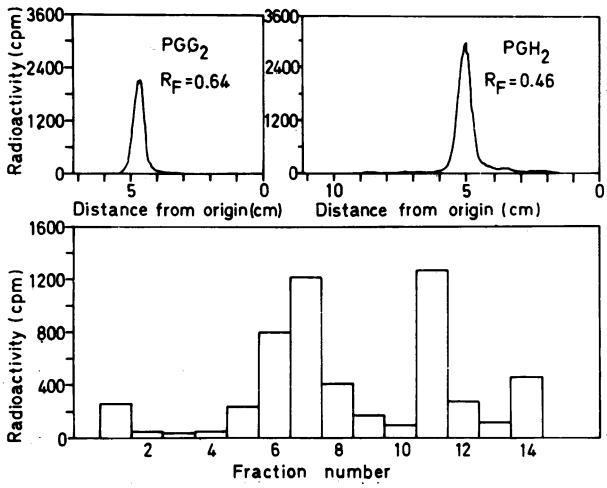

FIG. 1. *Lower panel:* Silicic acid chromatography of labeled material obtained after incubation of 1.2 mg of [*1*-^{14}C]arachidonic acid with the microsomal fraction from 8 g of vesicular gland in the presence of 1 mM *p*-mercuribenzoate. The column (1 g of silicic acid) was eluted with diethyl ether–light petroleum (2:8, v/v; fractions 1–4), diethyl ether–light petroleum (4:6, v/v; fractions 5–10), diethyl ether–light petroleum (6:4, v/v; fractions 11–13), and ethyl acetate (fraction 14). Fraction volume, 10 ml. *Upper panel:* TLC of labeled material present in fractions 6–8 of silicic acid chromatography (16% of incubated radioactivity, *left*) and material present in fractions 11–12 (11% of incubated radioactivity, *right*). Solvent system: ethyl acetate–2,2,4-trimethylpentane–acetic acid (50:50:0.5, v/v/v).

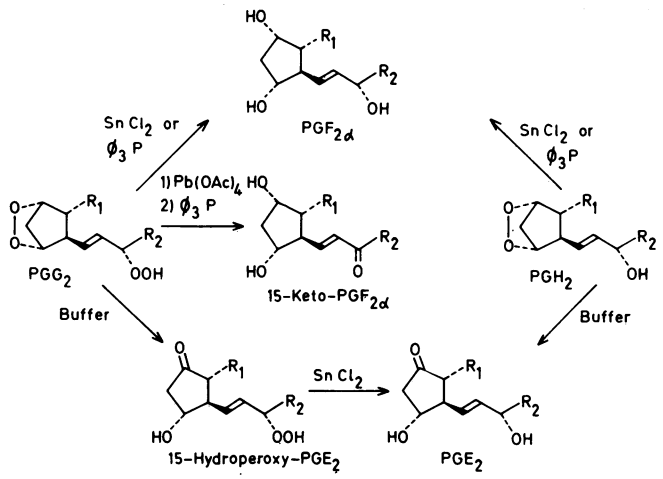

FIG. 2. Reactions performed on PGG_2 and PGH_2. R_1 = CH_2—CH=CH—$(CH_2)_3$—COOH; R_2 = $(CH_2)_4$—CH_3; ϕ = phenyl.

Rabbit Aorta Strip. Spirally cut thoracal aortas, 3.5–4 cm from male rabbits weighing about 2000 g were suspended and superfused (5, 6) with 2 ml/min of Krebs–Henseleit bicarbonate medium (3) gassed with 96.5% O_2–3.5% CO_2. The isometric contractions were recorded by a Grass model FT.03C force-displacement transducer connected with a Grass model 79C polygraph. Prostaglandin endoperoxides (100–2000 ng in 1–20 μl of acetone) were dissolved in 1–9 ml of Krebs medium and either added to the strip immediately or incubated at 37° for different times before addition to the strip (about 400 μl added by drops during 15 sec).

RESULTS

Fig. 1 shows a silicic acid chromatography of the material isolated after incubation of [*1*-^{14}C]arachidonic acid with the microsomal preparation. The fractions corresponding to the major peaks of radioactivity were pooled and taken to dryness. The residue was dissolved in 0.5 ml of dry acetone, and aliquots were subjected to TLC (Fig. 1). The two compounds isolated appeared to be stable in dry diethyl ether and acetone at −20°. However, the presence of a sensitive peroxide group in both compounds was indicated by the rapid degradation induced by addition of water or ethanol. The less-polar compound (yield from [*1*-^{14}C]arachidonic acid, 15–20%; R_F = 0.64, Fig. 1) will be referred to as prostaglandin G_2 (PGG_2) and the more-polar compound (yield, 10–15%; R_F = 0.46, Fig. 1) as prostaglandin H_2 (PGH_2).

Structure of prostaglandin G_2 (Fig. 2)

Reduction by $SnCl_2$ and Triphenylphosphine. PGG_2 (50 μg) was treated with buffered $SnCl_2$ (25 mg of $SnCl_2$ in 5 ml of ethanol and 1 ml of 92 mM potassium phosphate buffer pH 7.4; ref. 1) at room temperature for 2 min. The material isolated by extraction with diethyl ether was treated with diazomethane and analyzed by TLC. A major peak of radioactivity (about 90% of the radioactivity applied) with R_F = 0.05 was observed (reference, methyl ester of $PGF_{2\alpha}$, R_F = 0.05). Part of this material was rechromatographed in a more-polar solvent system (water-saturated ethyl acetate; R_F = 0.35; reference, methyl ester of $PGF_{2\alpha}$, R_F = 0.35). Another part was converted into the Me_3Si derivative and analyzed by gas-liquid chromatography (GLC)–mass spectrometry (LKB 9000 instrument; column, 1% SE 30 on Gaschrom Q). A single peak with a retention time equivalent to C-24.1 appeared (reference, Me_3Si derivative of the methyl ester of $PGF_{2\alpha}$, C-24.1; ref. 1). The mass spectrum was identical with that of the Me_3Si derivative of the methyl ester of $PGF_{2\alpha}$.

Treatment of PGG_2 with $SnCl_2$ in nonaqueous medium (methanol–diethyl ether–light petroleum) gave a mixture of comparable amounts of $PGF_{2\alpha}$ and 12-hydroxy-5,8,10-heptadecatrienoic acid. The latter component was identified by analysis of the methyl ester-Me_3Si derivative by GLC-mass spectrometry. The retention time corresponded to C-19.3, and the mass spectrum showed ions of high intensity at m/e 366 (M), 335 (M-31; loss of $\cdot OCH_3$), 295 [M-71; loss of $\cdot(CH_2)_4CH_3$], 276 (M-90; loss of Me_3SiOH), 225 [M-141; loss of $\cdot CH_2$—CH=CH—$(CH_2)_3$—$COOCH_3$], and 173 [Me_3Si—O^+=CH—$(CH_2)_4CH_3$].

Treatment of PGG_2 (50 μg) with 1 mg of triphenylphosphine in 0.1 ml of diethyl ether at room temperature for 60 min gave a product containing 80–90% of $PGF_{2\alpha}$, as judged by TLC and identification as described above by GLC–mass spectrometry.

Dehydration by Lead Tetraacetate. The methyl ester of PGG_2 (about 250 μg) was dissolved in 0.2 ml of dry benzene and treated with 2 mg of $KHCO_3$ and 6 mg of $Pb(OAc)_4$. After 5 min at room temperature and 5 min at +4°, 7 mg of triphenylphosphine was added and the mixture was left at room temperature for 30 min. TLC showed the presence of a minor (R_F = 0.05; methyl ester of $PGF_{2\alpha}$, R_F = 0.05) and a major component (R_F = 0.13; methyl 9α,11α-dihydroxy-15-ketoprosta-5,13-dienoate, R_F = 0.13). The O-methyl-oxime (MO)-Me_3Si derivative of the latter component (C-

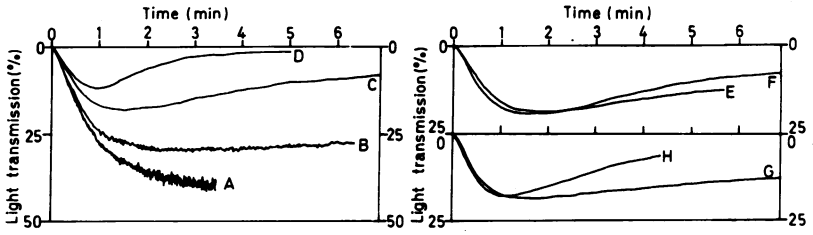

FIG. 3. Light transmission recordings in studies of platelet aggregation by prostaglandin endoperoxides. (*Left*) Platelet suspensions (1 ml, 37°) treated with 5 units of thrombin (*A*), 55 ng of PGG_2 (*B*), 55 ng of PGH_2 (*C*), and 11 ng of PGG_2 (*D*). (*Right*) Platelet suspensions (1 ml, 37°) treated with 18 ng of PGG_2 (*E,F*) and 55 ng of PGH_2 (*G,H*). Suspensions *F* and *H* were preincubated for 1 min with 50 μg of aspirin.

24.2) gave a mass spectrum identical to that of the MO-Me_3Si derivative of a sample of methyl 9α,11α-dihydroxy-15-ketoprosta-5,13-dienoate (7).

Isomerization in Aqueous Medium: Formation of 15-Hydroperoxy-PGE₂. PGG_2 (150 μg) in 0.5 ml of acetone was added to 10 ml of potassium phosphate buffer pH 7.4 and kept at room temperature for 4 hr. The product isolated by extraction with diethyl ether was treated with diazomethane and subjected to TLC. A major peak (60–70% of the applied radioactivity, R_F = 0.30) and several minor peaks of less polar derivatives appeared. The major compound was assigned the structure 11-hydroxy-15-hydroperoxy-9-ketoprosta-5,13-dienoic acid (methyl ester) on the basis of the following experiments. Reduction with 10 mg of $SnCl_2$ in 2 ml of methanol gave a single component that chromatographed with the methyl ester of PGE_2 (R_F = 0.10). This material was conclusively identified as the methyl ester of PGE_2 by analysis of the MO-Me_3Si derivative by GLC-mass spectrometry.

In another experiment, the isomerization product was treated with sodium borodeuteride (98% deuterium, Merck, Sharp, and Dohme, Quebec, Canada) and subsequently subjected to TLC (solvent: water-saturated ethyl acetate). Two peaks of radioactivity in proportions 1:1 appeared (R_F = 0.35 and 0.23, chromatographing with the methyl esters of $PGF_{2\alpha}$ and $PGF_{2\beta}$, respectively). The mass spectra recorded on the Me_3Si derivatives showed a molecular ion at m/e 585 (unlabeled derivatives, m/e 584), i.e., 1 atom of deuterium was incorporated in the borodeuteride reduction. The ion at m/e 218 ($Me_3SiO^+{=}C^2H{-}CH{=}CH{-}OSiMe_3$) supported the location of this deuterium at C-9.

Structure of prostaglandin H_2 (Fig. 2)

The identity of PGH_2 with the previously isolated 15-hydroxy endoperoxide (1) was suggested by TLC [solvent system: organic layer of ethyl acetate–2,2,4-trimethylpentane–water (75:75:100, v/v/v)]. The methyl esters of both compounds had an R_F = 0.49 (1). Proof of the identity of the two compounds was furnished by the following experiments.

Reduction by $SnCl_2$ and Triphenylphosphine. PGH_2 (50 μg) was treated with buffered $SnCl_2$, as described above for PGG_2. Analysis by TLC of the esterified product showed a major peak (about 90% of the applied radioactivity) with R_F = 0.05 (methyl ester of $PGF_{2\alpha}$, R_F = 0.05). The identity of the reduction product with the methyl ester of $PGF_{2\alpha}$ was proved by rechromatography in a more polar solvent system with the methyl ester of $PGF_{2\alpha}$ as reference as well as by GLC-mass spectrometric analysis of the Me_3Si derivative (see above and ref. 1). When PGH_2 was treated with $SnCl_2$ in nonaqueous medium (methanol–diethyl ether–light petroleum), comparable amounts of $PGF_{2\alpha}$ and 12-hydroxy-5,8,10-heptadecatrienoic acid were formed, i.e., the products formed by the same treatment of PGG_2 (see above). Treatment of PGH_2 (50 μg) with 1 mg of triphenylphosphine in 0.1 ml of diethyl ether at room temperature for 60 min gave a product containing about 90% $PGF_{2\alpha}$, as judged by TLC as well as GLC-mass spectrometric analysis of the Me_3Si derivative.

Isomerization in Aqueous Medium. PGH_2 (150 μg) in 0.5 ml of acetone was treated with 10 ml of potassium phosphate buffer pH 7.4 at room temperature for 4 hr. The esterified product was analyzed by TLC. A major peak (about 70% of the radioactivity applied, R_F = 0.10) was observed (reference, methyl ester of PGE_2, R_F = 0.10). The major compound was conclusively identified as the methyl ester of PGE_2 by GLC-mass spectrometric analysis of the MO-Me_3Si derivative (1).

Isomerization by Silica Gel. PGH_2 (50 μg) was treated with diazomethane and applied to a TLC plate. The plate was left at room temperature for 2 hr and subsequently developed with the organic layer of ethyl acetate–2,2,4-trimethylpentane–water (75:75:100, v/v/v) as solvent. The major part of the radioactivity applied (about 70%) appeared in two peaks with R_F = 0.07 and 0.17 (ratio, 1:5, reference: methyl ester of PGE_2, R_F = 0.07). Analysis of the MO-Me_3Si derivatives by GLC-mass spectrometry established the identity of the two compounds with the methyl esters of PGE_2 and 11-dehydro-$PGF_{2\alpha}$, respectively.

Platelet aggregation by PGG_2 and PGH_2

PGG_2 and PGH_2 induced aggregation of human blood platelets (Fig. 3). The extent as well as the reversibility of the aggregation was dependent upon the concentration of the endoperoxides, i.e., small amounts (about 10 ng/ml) gave a small and reversible aggregation whereas higher concentrations (50–300 ng/ml) gave a pronounced aggregation that was reversible only to a small extent. Linear relationships between *log* concentration and maximum aggregation were obtained for both compounds in the concentration range 10–300 ng/ml. The relative potency of PGG_2 and PGH_2 was about 3:1. Preincubation for 1 min with 50 μg/ml of aspirin gave no significant change in the maximum aggregation induced by PGG_2 or PGH_2. However, a somewhat greater initial slope as well as an increased reversibility were seen in the presence of aspirin (Fig. 3), suggesting stimulation of the first phase and inhibition of the second phase of aggregation (8).

In order to explore whether prostaglandin endoperoxides are released during platelet aggregation, the following experi-

ments were done. Four milliliters of platelet suspension were treated with 20 units of thrombin at 37°. One min after the addition of thrombin, the clumped platelets were removed by filtration under reduced pressure and samples of 1 ml of the filtrate were removed and added (2.5–3.5 min after addition of thrombin) to 15 ml of ethanol and to 15 ml of ethanol containing 75 mg of $SnCl_2$. $PGF_{2\alpha}$ was determined in these solutions by multiple-ion analysis with [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ as carrier (9). The amount in the nonreduced platelet medium was 11.7 ± 1.9 ng/ml (three experiments) and in the reduced medium 30.2 ± 6.3 ng/ml (three experiments), indicating the presence of PGG_2 and/or PGH_2.

Stimulation of rabbit aorta strip

The prostaglandin endoperoxides both stimulated the superfused aorta strip. Linear relationships between *log* concentration and contractile force were obtained in the concentration range 10–700 ng/ml. The following relative potencies were found: PGH_2/PGG_2, 2–4:1; PGG_2/PGE_2, 50–200:1; PGH_2/PGE_2, 100–450:1. Samples of PGG_2 and PGH_2 (670 ng/ml), incubated at 37° in the assay medium for 30–60 min, did not produce any significant response when tested on the strip, showing that PGG_2 and PGH_2 were rapidly degraded in aqueous medium into products with much less smooth muscle-stimulating activity. By assaying samples preincubated at 37° for different times a series of smooth muscle responses were obtained. These responses were converted into concentrations by a standard curve prepared by assaying known amounts of PGG_2 and PGH_2 without preincubation. By plotting *log* concentration against time, it was found that the half-life of the endoperoxides in aqueous medium was about 5 min.

DISCUSSION

Recently we reported the isolation and identification of a 15-hydroxy prostaglandin endoperoxide formed from arachidonic acid in the presence of the microsomal fraction of a homogenate of sheep vesicular gland (1). We have now extended this work by isolation and identification of the analogous endoperoxide having a hydroperoxy group at C-15. The two endoperoxides were found to be relatively stable in organic solvents such as diethyl ether and acetone, but very unstable in the presence of hydroxylic solvents such as ethanol and water. Accordingly, an incubation procedure for a short time with rapid extraction with diethyl ether and drying the ether phase with magnesium sulfate was developed for isolation of the two prostaglandin endoperoxides. Silicic acid chromatography carried out in the usual manner made it possible to obtain the compounds in pure form (Fig. 1). We suggest the trivial names prostaglandin G_2 (PGG_2) for the less-polar endoperoxide (15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid) and prostaglandin H_2 (PGH_2) for the more-polar endoperoxide (15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid). The conversion of 8,11,14-eicosatrienoic acid into analogous endoperoxides, i.e., prostaglandins G_1 and H_1, has recently been demonstrated.*

The structure of PGG_2 was established by three sets of experiments (Fig. 2). Treatment of PGG_2 with mild reducing agents such as $SnCl_2$ and triphenylphosphine gave $PGF_{2\alpha}$ as major product. This result showed the presence of a peroxide bridge between C-9 and C-11 but did not discriminate between a hydroxy and a hydroperoxy group at C-15 since the agents used would reduce the latter group into the former. In a second experiment, PGG_2 was treated with lead tetraacetate in benzene followed by triphenylphosphine. In this case, 15-keto-$PGF_{2\alpha}$ was the major product. Lead tetraacetate effects dehydration of hydroperoxides into ketones (10) and, therefore, formation of a 15-keto prostaglandin from PGG_2 by this treatment strongly indicated the presence of a hydroperoxy group at C-15. The isomerization of PGG_2 into 15-hydroperoxy-PGE_2 in aqueous medium gave independent evidence for a peroxide bridge between C-9 and C-11 and proved the presence of a hydroperoxy group at C-15.

PGH_2 was found to be identical with the previously isolated 15-hydroxy prostaglandin endoperoxide by several criteria, e.g., TLC, reduction with $SnCl_2$ and triphenylphosphine (yielding $PGF_{2\alpha}$), isomerization in aqueous medium (yielding PGE_2 as main product), and isomerization by silica gel of the methyl ester into the methyl esters of 11-dehydro-$PGF_{2\alpha}$ and PGE_2.

It seems likely that PGG_2 is the first stable compound formed from arachidonic acid by the "prostaglandin synthetase." By the isolation of PGG_2 it was demonstrated for the first time that introduction of the oxygen function at C-15 of the prostaglandins occurs by a dioxygenase reaction. We propose the name fatty acid cyclo-oxygenase for the enzyme(s) that catalyzes the conversion of arachidonic acid into PGG_2 by oxygenation at C-11 and C-15.

The high biological potency of the new prostaglandins was examplified by their stimulating activity on the rabbit aorta. PGG_2 was 50–200 times and PGH_2 100–450 times, more potent than PGE_2. "Rabbit aorta-contracting substance" released from guinea pig lung (11, 12) was recently found to have a half-life significantly shorter than that of PGG_2 and PGH_2 (about 5 min)†, indicating that none of the prostaglandin endoperoxides is identical with this factor (13).

PGE_1 inhibits platelet aggregation by stimulation of adenylate cyclase (14). PGE_2 has been reported to exert a double effect: a PGE_1-like effect on the first phase of aggregation (probably by stimulation of adenylate cyclase) followed by a stimulatory effect on the second phase of aggregation (15). In the absence of agents capable of inducing platelet aggregation, no effect is seen by either PGE_1 or PGE_2. The new prostaglandins PGG_2 and PGH_2, however, induced rapid aggregation when they were added to a platelet suspension. The relative potency of PGG_2 and PGH_2 was about 3:1. Our findings that PGG_2 and/or PGH_2 are formed during thrombin-induced aggregation and that they are powerful aggregating agents indicate that they play a role in platelet aggregation. Aspirin, an inhibitor of prostaglandin biosynthesis in platelets (16), has been reported to inhibit the second phase of platelet aggregation (8). It is suggested that this effect is due to inhibited formation of PGG_2 and PGH_2 from arachidonic acid within the platelet.

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