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Anti-aggregatory and contractile activity of analogues of prostaglandins E_1, D_1 and H_1

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The prostaglandins (PGs) are a family of potent biological compounds derived from arachidonic acid (AA; all *cis*-5,8,11,14-eicosatetraenoic acid). The naturally occurring PGs, the two-series metabolites, include PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 (prostacyclin) and TXA_2 (thromboxane), and are derived from the endoperoxide intermediate PGH_2. The production of unique PGs from novel AA analogues has permitted the study of how subtle differences in agonist structure affect their interactions with PG receptors located in different tissues. It has been reported previously that the TX analogues derived from four fatty acids differing in carbon chain length and position of the fatty acids' double bonds could be used to distinguish between vascular and platelet TX receptors [1]. Of interest to this study was that PGDs, PGEs and PGHs have been shown to have an effect on both platelets and smooth muscle [2, 3]. The possibility exists that PG analogues that would be potent anti-aggregatory agents could be synthesized, but they would be less active in stimulating smooth muscle. Such analogues could be potentially useful as anti-thrombotic agents. The present study describes the relative potencies, and the possible selectivity, of one series PGH and PGE analogues for inhibition of platelet aggregation and stimulation of smooth muscle contraction. The effects of the corresponding PGD_1 analogues on platelet aggregation are also presented.

Materials and methods

Synthesis of fatty acids and PG analogues. Fatty acids (19 : 3ω5, 20 : 3ω7, 21 : 3ω6, and 21 : 3ω7) were prepared by total organic synthesis [4], e.g. 20 : 4ω6, is AA, a 20 carbon fatty acid with 4 unsaturations starting 6 carbons removed from the terminal methyl group. The identical compounds labeled in position 1 with [^{14}C] were also synthesized (except 20 : 3ω6). Unlabeled 20 : 3ω6 and 20 : 4ω6 were purchased from NuChek Prep., Elysian, MN. The [1-^{14}C] compounds of 20 : 3ω6 (57 Ci/mole) and [1-^{14}C]20 : 4ω6 (56 Ci/mole) were obtained from new England Nuclear, Boston, MA. PG standards were a gift from Dr. John Pike, Upjohn Co., Kalamazoo, MI. 2′,5′-Dideoxyadenosine was purchased from P.L. Biochemicals Inc., Milwaukee, WI.

Endoperoxide analogues (PGHs) were formed from the corresponding fatty acids using sheep seminal vesicle (SSV) microsomes [homogenized in fatty acid free bovine serum albumen (BSA)] as a source of cyclooxygenase [5]. Approximately 1 μCi of the respective [1-^{14}C]fatty acid was added to monitor recovery and assist in the identification of products formed during incubation. Purification of the PGHs was performed by the method of Gorman *et al.* [6]. The PGH analogs were identified by monitoring their respective R_f values during TLC chromatography and their spontaneous breakdown to the corresponding PGEs and PGDs after incubation of the product in 0.1 M KPO_4 buffer, pH 7.4 [7]. Additionally, the PGEs were identified by HPLC using retention times and radioactivity as markers [8]. For the PGE_1 analogues, a single u.v. band at 192 nm was observed corresponding to between 84 and 94% of the injected radioactivity.

PGE analogues were prepared by incubating the corresponding fatty acids with BSA treated SSV microsomes in the presence of 1 mM epinephrine and 1 mM reduced glutathione at 37° for 30 min. After isolation of the PGEs from unreacted fatty acid using an open bed silicic acid column [7], products were isolated by TLC in solvent systems containing $CHCl_3$–CH_3OH–acetic acid–H_2O (90 : 8 : 1 : 0 : 8, v/v) [9] and the aqueous phase of ethyl acetate–isooctane–acetic acid–H_2O (110 : 50 : 20 : 100, v/v) [10].

PGD analogues were synthesized by incubating the appropriate fatty acid with BSA treated SSV microsomes and a rat basophilic leukemia (RBL-1) cell supernatant (a rich source of PGH → PGD isomerase) [11]. Conversion of the SSV-generated PGHs to PGEs was minimized by inhibition of the PGH → PGE isomerase present in the SSV with 0.3 μM parahydroxymercuribenzoate [5]. PGDs were separated by TLC as described above and were eluted from the silica gel with chloroform–methanol (2 : 1). HPLC confirmed the presence of one major peak representing between 57 and 78% of the total injected radioactivity.

Assay of biological activity. Anti-aggregatory activity of the generated PG and endoperoxide analogues was tested against AA-induced aggregation of 0.5 ml human platelet-rich plasma (PRP) as previously described [12]. The contractile activity of the PGH_1 analogues was tested on superfused rabbit aorta, whereas PGE_1 analogues were studied on rat stomach strip using the method described by Leduc *et al.* [1]. Spiral strips of the rabbit aorta or strips of rat stomach fundus were superfused at 37° with oxygenated Krebs–Henseleit buffer at a flow rate of 10 ml/min. To assess whether the PGE_1 and PGD_1 analogues inhibited aggregation by elevating platelet cyclic AMP levels, the PRP was preincubated with 100 μM 2′,5′-dideoxyadenosine, and inhibitor of adenylate cyclase, for 1 min before addition of the AA.

Results

The biological activities of several analogues of PGE_1, PGH_1 and PGD_1 were tested as inhibitors of AA-induced platelet aggregation (Table 1). The most potent anti-aggregatory compounds tested were the PGE_1s derived from 19 : 3ω5, 20 : 3ω6 and 21 : 3ω7. The common feature of these compounds is the presence of double bonds at positions, 8, 11 and 14 in the fatty acid substrate. Other PGEs tested were less active.

In contrast to the PGEs, the anti-aggregatory activity of the PGDs was not dependent on the presence of the bonds at positions 8, 11 and 14. The most potent PGD_1 tested was generated from 20 : 3ω5. Unfortunately, no other ω5-PGDs were available for testing. The PGH_1 compounds were considerably less potent than their corresponding PGE_1 metabolites though the rank order of potency was generally maintained (Table 1). In all cases, the analogues were found to produce their anti-aggregatory effects via a cyclic AMP mediated mechanism since pretreatment with 2′,5′-dideoxyadenosine (100 μM), 2 min prior to the addition of

Table 1. Inhibition of platelet aggregation rate by endoperoxides and prostaglandins synthesized from various fatty acid substrates

Fatty acid	ED_{50} (ng product/incubation) PGH	PGE	PGD
19 : 3ω5		3 ± 1	
20 : 3ω5	3000 ± 150	50 ± 6	30 ± 5
20 : 3ω6	50 ± 10	10 ± 3	150 ± 15
20 : 3ω7	3000 ± 400	500 ± 18	80 ± 10
21 : 3ω6	8000 ± 200	150 ± 25	250 ± 13
21 : 3ω7	40 ± 6	8 ± 2	300 ± 13
20 : 4ω6 (AA)	35*	300*	6 ± 13

Values represent the amount of product required to produce a 50% inhibition of the platelet aggregation rate. Results are means ± SEM obtained from dose–response curves of three to four individuals. PG metabolites were preincubated with the platelet preparations (0.5 ml) for 1 min before addition of 125 μg AA to induce platelet aggregation.

*From Ref. 12.

Table 2. Effect of trienoic fatty acid analogues on smooth muscle contractile activity

Trienoic Fatty acid	ED_{50} (ng prostaglandin) PGH*	PGE†
19 : 3ω5		100 ± 10
20 : 3ω5	2000 ± 100	900 ± 100
20 : 3ω6	4000 ± 150	70 ± 7
20 : 3ω7	5000 ± 150	1000 ± 20
21 : 3ω6	N/A‡	300 ± 20
21 : 3ω7	1500 ± 200	30 ± 3
20 : 4ω6 (AA)	150 ± 10	40 ± 3

Contractile activity of the PGH analogues was tested on the superfused rabbit aorta, whereas activity of the PGE analogues was studied on rat stomach strip.

*Values (mean ± SEM; N = 3–4) represent the amount of PG required to produce a 50% maximum contraction of rabbit aorta strip.

†Values (mean ± SEM; N = 3–4) represent the amount of PG metabolite required to produce a 50% contraction of a rat stomach preparation.

‡N/A = not active.

the compound, inhibited the response of the cell to the PG analogues.

It was possible that the spontaneous or enzymatic breakdown product of the 20 : 3ω6 derived PGH_1 in PRP could account for its potency. The half-life for the 20 : 3ω6 derived PGH_1 in aqueous solution has been reported to be 40 sec, with PGE_1 and PGD_1 found to be the principal breakdown products [3]. The other trienoic fatty acid derivatives tested in these studies may have longer half-lives (i.e. similar to 20 : 4ω6; 300 sec), suggesting some intrinsic platelet anti-aggregatory activity of these PGH_1 analogues.

It was of interest to determine if the 8,11,14 double-bond pattern of their fatty acid precursor could predict the biological activity of the PGEs and PGHs as smooth muscle contractile agents. The results shown in Table 2 demonstrate that the three 3ω-PGEs derived from 19 : 3ω5, 20 : 3ω6 and 21 : 3ω7 were the most potent PGEs tested in contracting the rat stomach strip. 21 : 3ω7 PGE_1 was the most potent, with an effective dose 50 (ED_{50}) of 30 ng.

The PGH_1 analogues were tested as agonists for the contraction of rabbit aorta strip. The prototypical endoperoxide, PGH_2 (derived from AA), demonstrated an ED_{50} of 150 ng. Although most of the PGH_1 analogues were full agonists in this system, they were all much less potent than PGH_2. The most active PGH_1 analogue was derived from 21 : 3ω7 and had an ED_{50} of 1500 ng. Thus, despite the presence of the 8,11,14 series in this molecule, it was one order of magnitude less active than PGH_2 derived from AA. The 21 : 3ω6 PGH_1 possessed no intrinsic activity in contracting rabbit smooth muscle, even when tested in doses as high as 10 μg.

Discussion

In the present study we have demonstrated that the one-series PG analogues possess biological activities. This is particularly striking for the PGE_1 analogues derived from precursors containing the 8,11,14 double bond. The PGE_1 analogue derived from 19 : 3ω5 had an ED_{50} of 3 ng as an anti-aggregatory substance compared to PGE_1 derived from 20 : 3ω6 which had an ED_{50} of 10 ng. It should also be noted that PGE_2 (derived from AA), which contains an additional double bond at C-5 is less active in this system [12]. While this suggests that there are rigid structural requirements for PGEs to elicit an effect, the structural requirements for activity of the PGD_1 analogues are not as obvious. Of this series, the most potent anti-aggregatory compound was the PGD_1 derived from 20 : 3ω5, which had an ED_{50} of 30 ng. Its 21-carbon congener (derived from 21 : 3ω6) was much less active. Furthermore, PGD_2 (derived from AA) was more active than any of the one-series PGD analogues. It has been reported that PGD_3 (derived from 20 : 5ω3) is an even more potent anti-aggregatory agent than is PGD_2 [2]. These data suggest that the presence of the C-5 double bond, absent in the PGD_1 analogues, may be critical for the anti-aggregatory activity of the PGDs. The differences in the structural requirements for activity observed between PGE and PGD analogues in these studies further suggest that two distinct platelet receptors mediated their biological effects [13].

The PGH_1 analogues have the same rank order of potency as their corresponding PGE_1 breakdown products, but they were less active. The activity of the PGH_1 analogues may be largely the result of their spontaneous breakdown into a mixture of PGE and PGD. However, since the PGH analogues have different half-lives, some of their activity is likely due to that of the parent PGH compound. In contrast, the activities of the PGH_1 analogue and its derivative PGE_1 product on smooth muscle contraction were found to be different. The one-series endoperoxides were essentially inactive in this system, contrasting with the potent activity of the PGH_2 (derived from AA) in contracting rabbit aorta. The one-series PGE analogues were all less potent than PGE_2 in contracting the rat stomach strip, with the exception of the PGE_1 derived from 21 : 3ω7, which was as potent as PGE_2. As in the platelet, the most potent PGE_1 derivatives were those synthesized from fatty acid precursors containing the 8,11,14 double-bond configuration.

The utility of any of these novel PG derivatives as effective anti-thrombotic agents would require a potent anti-platelet activity devoid of "musculotropic" activity. From the present studies, a potentially useful therapeutic agent might be the PGE_1 derived from 19 : 3ω5. This analogue was the most potent inhibitor of platelet aggregation tested and showed some selectivity of action since its anti-aggregatory and smooth muscle activities differed by a factor of 30. Other potential biological activities of this compound remain to be tested. Previous studies have shown that PGD_3 (derived from 20 : 5ω3) has potent anti-platelet properties, whereas it was less active than PGD_2 in con-

tracting muscle [12] and unpublished observation). Our results also suggest that the potent anti-aggregatory agent PGD_1 (derived from 20 : 3ω5) warrants additional study of its biological activity. These studies indicate that specifically tailored PG analogues may be synthesized which would exert specific anti-thrombotic effects without a concurrent indication of other biological activity.

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Elevated serum copper concentration in monocrotaline pyrrole treated rats with pulmonary hypertension

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Monocrotaline pyrrole (MCTP) is an active metabolite of the plant toxin, monocrotaline (MCT) [1]. When administered to rats, MCTP produces pulmonary vascular injury, pulmonary hypertension, and right ventricular enlargement by unknown mechanisms [2–5]. Because much of the pathophysiology of MCTP-induced pulmonary hypertension is similar to that observed in humans suffering from primary pulmonary hypertension, the MCTP-treated rat provides a useful animal model for studying this human disease.

It has been reported recently that the concentration of copper in the serum of patients with primary pulmonary hypertension is greater than in the normal population [6]. It was of interest, therefore, to determine whether serum copper concentration also increases in this animal model of pulmonary hypertension. Accordingly, changes in the serum concentration of copper were examined in MCTP-treated rats. The serum concentrations of a number of other elements were measured to determine whether any of these also changed with the development of pulmonary hypertension.

Materials and methods

Male, Sprague–Dawley rats (CF:CD(SD)BR) (Charles River Laboratories, Portage, MI) weighing 230–280 g were used in these studies. They were housed on corn cob bedding in plastic cages kept in an animal isolator (Contamination Control, Inc., Lansdale, PA) so that the rats breathed only HEPA*-filtered air. A 12-hr light/dark cycle and conditions of controlled temperature and humidity were maintained.

MCTP was synthesized from monocrotaline (MCT) (TransWorld Chemicals, Washington, DC) via an *N*-oxide intermediate, as described by Mattocks [7], and it was dissolved in *N*,*N*-dimethylformamide (DMF). Rats received either MCTP (3.5 mg/kg) or DMF vehicle via the tail vein on day 0, and then they were killed on day 3, 5, 8, or 14. Rats were anesthetized with sodium pentobarbital, and pulmonary arterial pressure (PAP) was measured as described previously [4]. A 3.5 French umbilical vessel catheter was introduced through the right jugular vein, carefully advanced into the right ventricle, and then gently manipulated into the pulmonary artery [8]. Pressure was measured with a Statham P23ID pressure transducer and was recorded on a Grass model 7 polygraph.

After determination of PAP, blood was collected from the abdominal aorta into glass syringes. The blood was allowed to clot at room temperature for approximately 2 hr, and then it was spun in a centrifuge (600 *g*, 10 min). The serum was collected and stored frozen (−4°) in plastic tubes until analysis for copper as described below.

Right ventricular enlargement (RVE) was assessed as an increase in the ratio of the weight of the right ventricle to the weight of the left ventricle plus septum [9].

The serum was prepared for determination of copper by mixing it with twice the volume of concentrated nitric acid (Baker instra-analyzed grade), and then the mixture was ashed overnight at 90–100°. The concentrations of copper and several other elements were determined in the samples by inductively-coupled argon plasma emission spectroscopy

* HEPA = high efficiency particulate air.