rapid communication

Misidentification of prostamides as prostaglandins

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Abstract Prostaglandins and endogenous cannabinoid metabolites share the same lipid backbone with differing polar head groups at exactly the position through which a large molecule is attached to provide antigenicity and thus raise antisera. Hence, we hypothesized that antisera raised against prostaglandins linked to a large molecule such as BSA at the carboxyl functional group would also recognize endogenous cannabinoid metabolites and lead to highly misleading interpretations of data. We found major cross-reactivity of commercial antisera raised to prostaglandins with endocannabinoid metabolites. Furthermore, in a well-characterized cell line (WISH) or primary amnion tissue explants, endocannabinoid treatment led to increased production of endocannabinoid metabolites as opposed to primary prostaglandins. This was apparent only after separation of products by thin-layer chromatography, because they measured as prostaglandins by radioimmunoassay. These findings have major implications for our interpretation of data in situations in which these prostaglandin-like molecules are formed, and they stress the need for chromatographic or spectrometric confirmation of prostaglandin production detected by antibody-based methods.—Glass, M., J. Hong, T. A. Sato, and M. D. Mitchell. **Misidentification of prostamides as prostaglandins.** ***J. Lipid Res.*** **2005.** 46: **1364–1368.**

Supplementary key words ethanolamides • prostamides • endocannabinoids • anandamide

The arachidonic acid derivatives anandamide (1), 2-arachidonyl-glycerol (2AG) (2, 3), virodhamine (4), and 2-arachidonyl-glyceryl ether (5) are exciting not only for their isolation as putative endogenous cannabinoids but also because they represent a novel class of signaling molecules (6). Although all endocannabinoids can bind to and activate cannabinoid receptors, there is increasing evidence of nonreceptor-mediated actions. Recent studies have demonstrated that anandamide and 2AG can be metabolized by cyclooxygenase-2 (COX-2) into prostaglandin-like molecules (7, 8) that consist of a prostaglandin with a polar head group, ethanolamide or glycerol, respectively. The prostaglandin-ethanolamides have been termed prostamides. The activity of prostaglandin E_2 (PGE_2)-ethanolamide at prostaglandin receptors has been investigated, and it possesses ~100- to 1,000-fold lower affinity and potency than PGE_2 itself, making this an unlikely target (9); likewise, this compound has little affinity for the cannabinoid receptors (10) or endocannabinoid-metabolizing enzymes (11). Hence, prostamides and glycerol equivalents may represent a novel class of mediators with separate receptors/transduction pathways. Prostamides are produced in vivo (12) and reduce intraocular pressure (13); indeed, bimatoprost (Lumigan™), one compound in a new class of highly efficacious ocular hypotensive agents, is a close analog of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-ethanolamide (13). Moreover, the glyceryl ester of PGE_2 can mobilize calcium and activate signaling pathways (14).

Given that it is unlikely that prostamides and glycerol equivalents produce their biological actions through existing prostaglandin receptors, and therefore are likely to generate a novel set of signaling pathways, it is essential that studies differentiate between the prostaglandins and the prostamides. However, many recent studies demonstrating prostaglandin production use a radioimmunoassay to detect and quantify the prostaglandin. The antibodies used in these studies are generally produced by the addition of an antigenic head group, thereby possibly conferring a similar conformation to the prostaglandin as it would adopt with the addition of an ethanolamide or glycerol. Therefore, we investigated the ability of PGE_2 and $PGF_{2\alpha}$ antibodies to recognize PGE_2-ethanolamide and $PGF_{2\alpha}$-ethanolamide, respectively. To determine the possible magnitude and thus significance of the problem, we investigated the relative production of these two prostamides by stimulated amnion-derived WISH cells and the production of PGE_2-ethanolamide by primary human amnion explants.

MATERIALS AND METHODS

Materials

DME-199 and Ham's/F12 culture media were obtained from Irvine Scientific (Santa Ana, CA). Fetal calf serum (FCS), antibi-

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otics, and streptavidin-alkaline phosphatase were purchased from Invitrogen (Auckland, New Zealand). Bovine γ-globulin and lipopolysaccharide (serotype 055:B5) were purchased from Sigma (St. Louis, MO). Human interleukin-1β (IL-1β) was a generous gift from the Immunex Corporation (Seattle, WA). Anandamide, 2AG, PGE_2, PGE_2-ethanolamide, $PGF_{2\alpha}$, $PGF_{2\alpha}$-ethanolamide, and methanandamide were purchased from Cayman Chemicals (Ann Arbor, MI). Tritiated PGE_2 and $PGF_{2\alpha}$ were purchased from Amersham-Pharmacia Biotech (Aylesbury, UK). WISH cells were obtained from Dr. L. Myatt (University of Cincinnati, OH).

PG antibodies

The "in-house"-generated antibody against PGE_2 used in these studies has been characterized extensively (15). Commercial antisera used to test cross-reactivity were obtained from Assay Design (Ann Arbor, MI; catalog number 90525), Cayman Chemicals (catalog number 41403), and ICN (Irvine, CA; catalog number 613551) for PGE_2 and from Perceptive Diagnostics (Cambridge, MA) for $PGF_{2\alpha}$.

PGE_2 and $PGF_{2\alpha}$ immunoassay

For cross-reactivity assays, standards were dissolved in serum-free medium and assayed for PGE_2 or $PGF_{2\alpha}$ by direct RIA as described previously (15, 16). Media from cell stimulations were diluted (1:3 to 1:50) before analysis to ensure that they fell within the range of the linear portion of the standard curve. Samples or standards were incubated overnight with [^{3}H]PG tracer (~5,000 cpm/tube) and antiserum (sufficient to give ~25% maximal binding) at 4°C. Unbound radiolabel was removed with cold dextran-coated charcoal, and the supernatant was mixed with Starscint scintillation fluid (Perkin-Elmer, Boston, MA) and counted in a Rack-β scintillation counter (Perkin-Elmer). Curve fitting and data extrapolation were performed using Ultraterm II software (Wallac Oy, Turku, Finland).

Medium containing the drugs (no cells/tissue) was included for each treatment to ensure that they did not contribute to cross-reactivity in the assay. Statistical differences were assessed by two-tailed *t*-test using InStat (Graph Pad, Inc., San Diego, CA).

Thin-layer chromatography

TLC protocols were developed to separate prostaglandins from prostamides. For separation of PGE_2 from PGE_2-ethanolamide, 200 μl of medium from treated cells was extracted with 1 ml of 90:10 chloroform-methanol, dried down, and spotted onto silica gel 60 (Merck) TLC plates without prewashing. Compounds were separated in a solvent mixture of 90:10 ethyl acetate-methanol. This resulted in relative mobilities of 0.15, 0.36, and 0.46 for PGE_2-ethanolamide, PGE_2, and anandamide, respectively, when 10 μg of each standard was visualized by iodination. $PGF_{2\alpha}$ and $PGF_{2\alpha}$-ethanolamide were extracted from 200 μl of medium with 1 ml of 80:20 chloroform-methanol acidified to pH 3.5 by citric acid before separation on the same TLC solvent system described above, with relative mobilities of 0.12 and 0.30 for $PGF_{2\alpha}$-ethanolamide and $PGF_{2\alpha}$, respectively, when visualized by iodination of 10 μg standards. The assays were validated through extraction of 3–10 ng/ml of the relevant compounds from media and subsequent separation by TLC, followed by quantitative RIA of the relevant region of the TLC plate. For all standards, regions of the plate corresponding to both the prostaglandin and the prostamide were scraped and analyzed; no signal above background was detected in the nonrelevant regions of the plate. For experimental samples and standards, the regions of the TLC plate corresponding to the prostaglandin and the prostamide were scraped and eluted in 1 ml of 9:1 chloroform-methanol, 800 μl of this was removed and dried down in a SpeedVac, and the residue was resuspended into serum-free medium; the PG RIA was then repeated on these isolated products. Recovery of samples was found to be equivalent for all compounds investigated and to be ~60%. In all cases, after the relative yield was taken into account, the combination of prostaglandin and prostaglandin-ethanolamide accounted for 100% of the prostaglandin measured from the original sample.

Primary explant and WISH cell cultures

All procedures involving human placentas were approved by the Auckland Ethics Committee. Placentas were obtained with informed consent from women undergoing elective cesarean section at term before the onset of labor. Amnion was removed manually. Tissue explants (6 mm disks) were excised with a cork borer as described previously (15). Explants were pooled and distributed randomly onto 12-well plates (three explants per well, three wells per treatment) containing medium supplemented with 10% FCS and antibiotics (0.2 mg/ml kanamycin, 0.087 mg/ml gentamycin, 0.065 mg/ml penicillin, and 1 mg/ml streptomycin). The explants were allowed to equilibrate over-

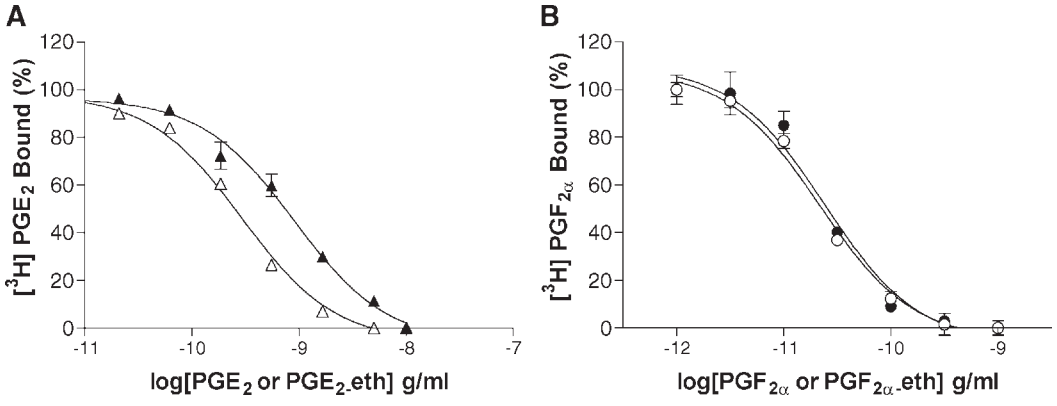

Fig. 1. Cross-reactivity of prostamides in standardized prostaglandin radioimmunoassays was determined by the ability of prostamides to displace the tritiated prostaglandin from the antibody compared with the parent compound. A: Displacement of [^{3}H]prostaglandin E_2 (PGE_2) from in-house-generated antibody by PGE_2 (closed triangles) or PGE_2-ethanolamide (PGE_2-eth; open triangles). B: Displacement of [^{3}H]prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from anti-$PGF_{2\alpha}$ antibody by $PGF_{2\alpha}$ (open circles) or $PGF_{2\alpha}$-ethanolamide ($PGF_{2\alpha}$-eth; closed circles). Error bars represent SEM.

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TABLE 1. EC_{50} values for the displacement of [^{3}H]PGE_2 by several commercial antibodies

Antibody Source	EC_{50}	
	PGE_2	PGE_2-Ethanolamide
	pg/ml	
ICN	543 ± 3	240 ± 2
Cayman Chemicals	744 ± 5	563 ± 5
Assay Design	520 ± 4	218 ± 3
In house	847 ± 4	167 ± 3

Values shown are means ± SD. A significantly higher affinity was observed for PGE_2-ethanolamide versus PGE_2 (two-tailed *t*-test; n = 3; $P < 0.0001$) for all antibodies tested.

night at 37°C in a humidified atmosphere of 5% CO_2. WISH cells were cultured in Ham's/F12 with 10% FCS and antibiotics (as described above). On the day of the experiment, the medium was replaced with serum-free medium containing 0.1% bovine γ-globulin. Explants or cells were then treated with the test substances or appropriate vehicle for 16 h. PGE_2 concentration in the medium (in picograms) was normalized to the wet weight of the explants in each well or to cell number.

RESULTS

We have examined the cross-reactivity of PGE_2-ethanolamide against our in-house-raised PGE_2 antibody as well as all of the available commercial antibodies against PGE_2. Our hypothesis was proven correct in a surprisingly consistent manner, because all antibodies tested not only cross-reacted with the endocannabinoid metabolite but also possessed a significantly higher affinity for PGE_2-ethanolamide than for PGE_2 (**Fig. 1A**, **Table 1**). Likewise, standard curves for $PGF_{2\alpha}$ and $PGF_{2\alpha}$-ethanolamide were indistinguishable from each other, indicative of 100% cross-reactivity in a RIA using a commercially available antibody (Fig. 1B).

Treatment of amnion-derived WISH cells with 0.2 ng/ml IL-1β and 10 μM anandamide resulted in a dramatic synergistic stimulation of PGE_2 production measured by RIA of ~3.75-fold above the predicted additive response (**Fig. 2**). Subsequent studies showed this to be a concentration-dependent response (**Fig. 3**); however, significant cell death occurred above 30 μM, limiting full concentration response curves. One possible cause of this synergy is that anandamide may be metabolized by fatty acid amide hydrolase to arachidonic acid and then used as substrate in the production of PGE_2. Arachidonic acid itself, however, did not display synergy with IL-1β (data not shown). Moreover, methanandamide, a hydrolysis-resistant analog of anandamide, produced an equivalent stimulation of PGE_2 production to anandamide at all concentrations (Fig. 3). Studies with the fatty acid amide hydrolase inhibitor arachidonyl serotonin were inconclusive, as the inhib-

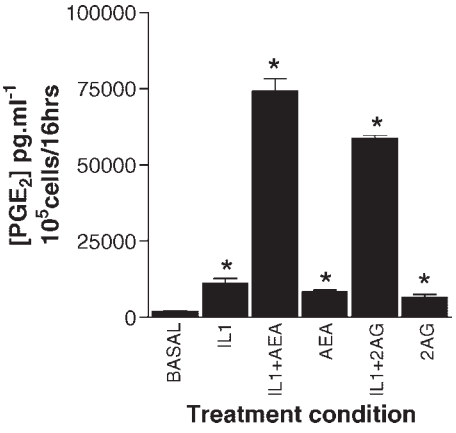

Fig. 2. Apparent PGE_2 production by WISH cells stimulated with 0.2 ng/ml interleukin-1β (IL-1β) in the presence and absence of 10 μM anandamide (AEA) or 2-arachidonyl-glycerol (2AG) as determined by RIA with in-house antibody raised to PGE_2. * Significantly different from basal ($P < 0.001$; n = 3). Error bars represent SEM.

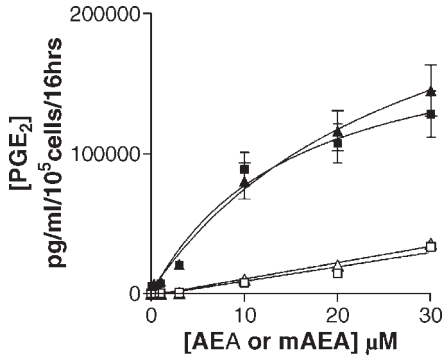

Fig. 3. PGE_2 stimulation by WISH cells in response to treatment with either anandamide (AEA; squares) or methanandamide (mAEA; triangles) in the absence (open symbols) or presence (closed symbols) of 0.2 ng/ml IL-1β as determined by RIA with in-house antibody raised to PGE_2. Error bars represent SEM.

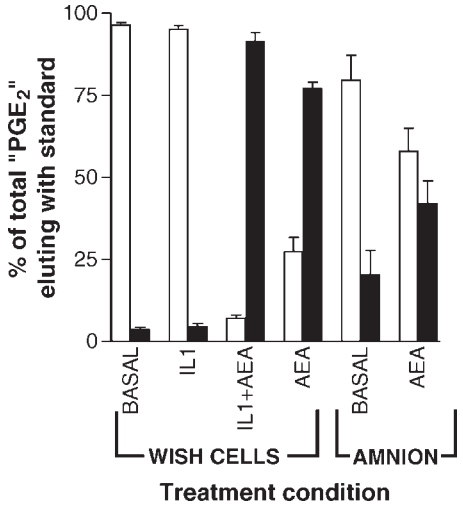

Fig. 4. Relative proportions of prostaglandin to prostamide production by stimulated WISH cells or primary amnion explants. Results of TLC separation of PGE_2 and PGE_2-ethanolamide followed by RIA with in-house antibody raised to PGE_2 on WISH cells and primary amnion explants. White bars represent the percentage of the total "PGE_2" signal that eluted with PGE_2 standards; black bars indicate the percentage that eluted with PGE_2-ethanolamide standards on the TLC plate. AEA, anandamide. Error bars represent SEM.

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itor itself resulted in significant cell death (data not shown). Furthermore, treatment of WISH cells with 0.2 ng/ml IL-1β and 10 μM 2AG resulted in a similar synergistic stimulation of PGE_2 production measured by RIA of ~3.5-fold above the predicted additive response (Fig. 2).

Next, we examined whether PGE_2-ethanolamide could contribute to the apparent PGE_2 production after anandamide stimulation. After stimulation of WISH cells with 10 μM anandamide in the presence or absence of 0.2 ng/ml IL-1β for 16 h, the lipids were extracted from the medium and separated by TLC, and the relevant areas of the TLC plates were reevaluated in the PGE_2 RIA. Although the stimulation of PGE_2 by IL-1β alone appears to be predominantly PGE_2 (97 ± 2%), the stimulation observed in the presence of anandamide and IL-1β eluted almost entirely with PGE_2-ethanolamide (95 ± 2%); stimulation of anandamide alone also resulted in 77 ± 2% PGE_2-ethanolamide production (**Fig. 4**). Consistent with this result, we found that $PGF_{2\alpha}$-ethanolamide is produced in an ~2:1 ratio with $PGF_{2\alpha}$ upon stimulation of WISH cells with anandamide and IL-1β cotreatment, in a manner exactly analogous to PGE_2-ethanolamide and PGE_2 production (data not shown).

To ensure that this was not a unique characteristic of the WISH cells, we stimulated human amnion explants (n = 4) with anandamide and then performed TLC separation, followed by a RIA for PGE_2. As for the WISH cells, basal "PGE_2" production primarily identified PGE_2 (76 ± 17%), whereas after anandamide stimulation (16 h), a greater percentage of the "PGE_2" measured was actually PGE_2-ethanolamide (42 ± 12%; Fig. 4).

DISCUSSION

We have demonstrated for the first time that PGE_2-ethanolamide is a major product under conditions of evoked COX-2 activity and anandamide release; by extension, other prostamides may be formed. Indeed, a similar synergy in PGE_2 production was observed for 2AG and IL-1β in this study, suggestive of the production of PGE_2-glycerol by WISH cells. Moreover, prostamides E_2 and $F_{2\alpha}$ cross-react in their respective prostaglandin radioimmunoassays. Therefore, the results of this study strongly suggest that chromatographic or mass spectrometric methods are required to confirm the specificity of compounds identified as prostaglandins by antibody-based methodologies.

This finding raises the intriguing possibility that prostamide production may have been misidentified in previous studies as prostaglandin production under conditions in which anandamide may be released and COX-2 induced. Breakdown of anandamide into arachidonic acid does not appear to be a major factor in these studies, as the hydrolysis-resistant form of anandamide, methanandamide, produced identical responses, and no ethanolamide production was observed in response to arachidonic acid treatment. Anandamide treatment in the absence of an inflammatory stimulus was sufficient to produce PGE_2-ethanolamide in WISH cells and primary amnion tissue; however, the highly synergistic response observed in the presence of IL-1β and anandamide in the WISH cells suggests that the induction of COX-2 by IL-1β (17) is necessary for the efficient generation of prostamides, despite a previous finding that anandamide stimulation itself stimulates COX-2 induction (18). Furthermore, the relative lack of ethanolamide production in the absence of added anandamide suggests that in this system the exogenously applied anandamide provides a substrate for prostaglandin-ethanolamide production, indicating that anandamide is not being released by these cells in sufficient quantities to produce detectable ethanolamides. However, it is likely that physiological conditions exist in which anandamide will be released at times of COX-2 induction. Anandamide release has been described in response to hemorrhagic shock (19), lipopolysaccharide treatment of macrophages (20), and lipopolysaccharide challenge of human peripheral lymphocytes (21). Likewise, COX-2 is induced by a range of inflammatory stimuli, such as IL-1β (17) and lipopolysaccharide. This raises a concern that the responses to COX-2 inhibitors may include some effects that would be unforeseen because of the suppression of endocannabinoid metabolite formation and actions. Until we know the full range of metabolites formed and their activities, the resulting effects could be quite unpredictable and possibly of pathological concern. Although mass spectrometric confirmation of the identity of metabolites would be useful, the metabolites produced in this study clearly both migrate with PG-ethanolamide and cross-react with the highly specific PG antiserum, suggesting a very close identity with the PG-ethanolamides. Moreover, the identity of the product is less important than the crucial fact that it is clearly not a PG and that it is the major product formed under these stimuli.

Excitingly, these findings suggest that at the site of inflammatory/infectious challenges, both anandamide and COX-2 may be increased to synergistically induce PGE_2-ethanolamide. Hence, the potential exists for the action of this and similar derivatives to have roles in an extraordinary range of pathologies. Similar ethanolamide metabolites of the other prostaglandins (F, D, etc.) as well as oxidative products (lipoxygenase, cytochrome P450) have also been described, as have metabolites of the other endocannabinoids (e.g., glycerol derivatives) (22). Thus, a new series of pathways, products, and mechanisms of action await elucidation, and with them new therapeutic targets. It will be critical to establish substrate affinities and enzyme capacities to elucidate the range and proportion of different products, which will include native eicosanoids as well as endocannabinoid metabolites, and likely myriad of integrated responses attributable to differential affinities for various receptors.

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