Stimulation of Aortic Smooth Muscle Prostacyclin by Serotonin: Role of Distinct Receptors in Contractile and Synthetic States

Dominique Demolle, Anne Van Coevorden, and Jean-Marie Boeynaems

It has been shown previously that serotonin stimulates the production of prostacyclin by bovine aortic smooth muscle cells in culture, via 5-HT₂ receptors (Coughlin SR, Moskowitz MA, Antoniades HN, Levine L. Proc Natl Acad Sci USA 1981;78:7134–7138). These cells express a synthetic phenotype, whereas the majority of the smooth muscle cells in the media from adult arteries are in a contractile state. We have now compared 5-HT stimulated prostacyclin production in bovine aortic media explants, a preparation of contractile smooth muscle, with cultured smooth muscle cells derived from the explants. In the 1–10 μM range, serotonin stimulates the release of prostacyclin from the explants of bovine aortic media, cultured for a short period. In the presence of cocaine (30 μM), 1 μM was sufficient to produce a maximal effect. The stimulatory action of serotonin was sustained with time and did not induce a lasting desensitization. The effect of serotonin on the explants was inhibited only partially (±30%) by ketanserin, a selective and potent 5-HT₂ antagonist. It was mimicked by 5-carboxamidotryptamine, a 5-HT₁ agonist, but was only weakly inhibited by methiothepin, a 5-HT₃ antagonist. As expected, in cultured smooth muscle cells, 5-carboxamidotryptamine was only a weak agonist in stimulating prostacyclin production. In conclusion, it appears that the serotonin effect on prostacyclin production is mediated by different receptors in media explants from bovine aortic media and cultured cells obtained by outgrowth from these explants: a 5-HT₂ receptor in the smooth muscle cells in culture and a receptor presenting some similarities with 5-HT₃ receptors in the explants. (Circulation Research 1989;64:806–813)

Prostacyclin (PGI₂), produced by the blood vessel wall,¹ is a potent inhibitor of platelet aggregation. The in vivo production of PGI₂ is increased in patients suffering from severe atherosclerosis with evidence of intravascular platelet activation.² In vitro studies have demonstrated that several factors stored or synthesized in platelets and released during aggregation stimulate vascular PGI₂ production. Endothelial cells from human umbilical vein³ and bovine aorta⁴ are able to use the platelet endoperoxides as substrates for their PGI₂ synthase. ATP and ADP stimulate the release of PGI₂ from porcine⁵ and bovine⁶ aortic endothelial cells. In bovine aortic smooth muscle cells, synthesis of PGI₂ is stimulated by 5-hydroxytryptamine (5-HT)⁷,⁸ and by platelet-derived growth factor.⁷,⁹ The stimulatory effect of 5-HT has been investigated previously in bovine aortic smooth muscle cells obtained by outgrowth from media explants.⁷,⁸ It is known that these cells have lost their contractility and undergone a modulation to a synthetic phenotype, characterized morphologically by a decrease in myofilaments and a more-developed endoplasmic reticulum and functionally by the capacity to replicate and synthesize components of the extracellular matrix.¹⁰–¹² These properties are characteristic of the smooth muscle cells in atheroma lesions,¹³ whereas the contractile phenotype is expressed by the vast majority of smooth muscle cells in normal adult arteries.¹¹ Therefore, we have reexamined the action of 5-HT on the production of PGI₂, using as a model of contractile arterial smooth muscle a preparation previously used for other types of biochemical studies,¹⁴ namely, explants of media cultured for short periods.
Materials and Methods

Preparation and Culture of Bovine Aortic Media Explants

The thoracic aorta was excised from a freshly slaughtered cow, the adventitia and intima were removed, and the media was cut into small squares (1 or 2 mm). These explants were rinsed three times and put in Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 20% (vol:vol) Ham’s F₁₂ medium, and 20% (vol:vol) fetal calf serum (FCS). Approximately 50 mg tissue was incubated in 2.5 ml medium at 37°C under an atmosphere of 5% CO₂, 95% air. For the measurement of PGI₂ production, the explants were rinsed after removal of the culture medium and preincubated for 30 minutes in DMEM. Then, the medium was changed, and the tissue was incubated in DMEM with the tested agents. In some experiments, the incubation medium was Krebs-Ringer-HEPES (124 mM NaCl, 5 mM KC1, 1.25 mM MgSO₄, 1.45 mM or 0 mM CaCl₂, 1.25 mM KH₂PO₄, 25 mM HEPES, pH 7.4, and 8 mM glucose), and the incubation was performed at 37°C under air.

Preparation and Culture of Bovine Aortic Smooth Muscle Cells

Bovine aortic smooth muscle cells were obtained by outgrowth from explants of the bovine aortic media, as described by Ross. The explants were cultured in Petri dishes in the complete medium (including 20% FCS) and left undisturbed for 10 days before the medium was changed. When the cells were locally confluent around the explants, they were detached by a 10-minute incubation in a Ca- and Mg-free Hanks’ buffer containing trypsin (10 mg/100 ml) and EDTA (1 mM) and cultured in the complete medium. At confluency, the cells presented the typical “hills and valleys” pattern described as specific of the smooth muscle cells in culture. For the measurement of PGI₂, the cells (passages 3–8) were incubated for 24 hours in the complete medium but without FCS. Ascorbic acid (1 mM) and pargyline (10 μM) were included to prevent 5-HT degradation.

Preparation and Culture of Endothelial Cells

Endothelial cells from bovine aorta and from human umbilical vein were obtained by mild collagenase digestion, as described. They were cultured in the same medium described above for the smooth muscle cells. With both cell types, the experiments were performed using confluent monolayers (±10⁶ cells/dish) between passages 2 and 5.

Prostaglandin Radioimmunoassay

The production of PGI₂ was measured by the radioimmunoassay (RIA) of its stable degradation product, prostaglandin 6-keto-F₁₂(6-K-PGF₁₂), performed directly in the incubation medium, without extraction or chromatography. A rabbit antiserum was raised against 6-K-PGF₁₂, coupled to bovine serum albumin, as described: the limit of detection was 16 pg, and the cross-reactions were 1.2% with PGF₂α, 0.3% with PGE₂, and 0.1% with thromboxane B₂. One hundred microliter aliquots of incubation media, [³H]6-K-PGF₁₂ (11,000 dpm), anti-6-K-PGF₁₂ antiserum (final dilution, 10⁻⁴), and bovine γ-globulins (0.25 g/dl) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 minutes at room temperature. Then, 0.4 ml of a cold 25% (wt/wt) solution of polyethylene glycol was added to separate bound and free antigen.

Materials

DMEM, Ham’s F₁₂, glutamine, streptomycin, penicillin, and fungizone were purchased from Flow Laboratories, Irvine, UK. Fetal calf serum was obtained from Gibco, Grand Island, New York. Collagenase type II was obtained from Cooper Biomedical, Freehold, New Jersey. 5-Hydroxytryptamine creatine sulfate and hydrochloride, tryptamine, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, histamine, noradrenaline, pargyline, and ascorbic acid were purchased from Sigma Chemical Co., St. Louis, Missouri. Cocaine was given by Dr. J. Fontaine. 5-Carboxamidotryptamine and 2-ChF₅-hydroxytryptamine were received, respectively, from Dr. W. Feniuk (Glaxo, Ware, UK) and Dr. R. Römer (Sandoz, Basel, Switzerland). Ketanserin was obtained from Janssen Pharmaceutica, Beerse, Belgium; methiothepin was a gift from Hoffman La Roche, Basel, Switzerland. [³H]6-K-PGF₁₂ was purchased from Amersham Corp, Brussels, Belgium, and 6-K-PGF₁₂ from Upjohn Diagnostics, Kalamazoo, Michigan.

Results

5-HT stimulated the production of PGI₂ in bovine aortic media explants. This effect was independent from the time at which the experiment was started, since 5-HT was equally effective after a 1-, 8-, or 24-hour culture period following the preparation of the explants (data not shown). The stimulation induced by 5-HT decreased from the thoracic to the abdominal aorta (data not shown). The experiments reported in this paper have been performed with explants dissected from the thoracic portion of the bovine aorta and tested after 24 hours preincubation in the complete culture medium containing 20% FCS. However, the responsiveness to 5-HT was independent from the presence of serum during that culture period (Table 1). The PGI₂ production induced by 5-HT was inhibited by indomethacin (28 μM) (data not shown). It was checked that the immunoreactive material formed in response to 5-HT had the same retention time as authentic 6-K-PGF₁₂ in reversed-phase high-performance liquid chromatography (data not shown).

The maximal stimulation of PGI₂ synthesis was elicited by 5-HT at 10 μM: it was 255±96% of the
challenged for the first time with 5-HT (Figure 2).

amplitude and duration to that obtained in explants

whereas norepinephrine produced a stimulation of

tryptophan, its precursor, nor its products of deg-

radation (5-hydroxytryptophol and 5-hydroxy-

indoleacetic acid) had a detectable effect (Figure 3).

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5-HT, antagonist, at concentration as high as 10 µM, reduced the 5-HT effect on the explants by only ±30%, and these inhibitions were not additive (Table 4).

Several agonists were compared in order to characterize further the action of 5-HT on the bovine aortic media explants: tryptamine; 5-carboxamidotryptamine (5-CT), a 5-HT₁ agonist; and 2-methyl-5-hydroxytryptamine (2-CH₃-5-HT), a 5-HT₃ agonist. As shown in Figure 4, 2-CH₃-5-HT and tryptamine were totally ineffective, whereas 5-CT was as potent and active in increasing PGI₂ production as 5-HT.

In the smooth muscle cells in culture, 2-CH₃-5-HT and tryptamine were also totally ineffective (Figure 5). However, in contrast to the explants, 5-CT was only a partial agonist, less potent and active than 5-HT itself (Figure 5). The ratio of PGI₂ maximal stimulation by 5-CT versus 5-HT was 1.1 ±0.2 (mean±SD of five separate experiments) in the explants but only 0.40±0.21 (mean±SD, three experiments) in the smooth muscle cells in culture.

Discussion

The experimental deendothelialization of the rabbit aorta induces the adhesion of a single layer of platelets on the surface of the lesion and their degranulation. Some of the products released, like platelet factor 4, accumulate locally on the arterial wall for as long as 30 minutes. The adhesion of multiple layers of platelets was reported following pretreatment of the rabbits with high doses of aspirin, suggesting that PGI₂, produced locally by smooth muscle cells, might contribute to protect the vessel against the accumulation of platelet thrombi. The prolonged stimulatory effect of 5-HT on PGI₂ production by the contractile smooth muscle, that we have described, might contribute to this protection. Moreover, it might be one of the reasons why the in vivo production of PGI₂ is enhanced in case of vessel lesions and intravascular activation of platelets. The concentration of 5-HT in serum is about 500 nM, whereas the stimulatory effect that we observed on PGI₂ appeared at concentrations greater than 1 µM. However, as quoted from Berk et al., "one might expect that higher local concentrations would be available at sites of active platelet-vessel wall interactions." The relative insensitivity...
to 5-HT might be a factor that would restrict the stimulation of PGI\textsubscript{2} production to the smooth muscle cells close to the platelets covering the lesion.

The classification of 5-HT receptors is a very complex matter. Recently, Bradley et al\textsuperscript{21} have proposed a functional classification in three categories, 5-HT\textsubscript{1}, 5-HT\textsubscript{2}, 5-HT\textsubscript{3}, based on the action of specific antagonists and agonists. The vascular smooth muscle responses to 5-HT involve distinct receptors. In many vessels like the rabbit aorta\textsuperscript{27} the rat aorta\textsuperscript{28} and the rat caudal artery\textsuperscript{20} the contraction induced by 5-HT is mediated by 5-HT\textsubscript{2} receptors and is inhibited competitively by the specific antagonists ketanserin,\textsuperscript{20} and LY 538572\textsuperscript{9} at nanomolar concentrations. However, the contraction of the dog saphenous vein\textsuperscript{27} and the rabbit basilar artery\textsuperscript{20} in response to 5-HT is not inhibited by ketanserin and is mimicked by the 5-HT\textsubscript{1} agonist 5-CT. In some vessels, 5-HT has a vasodilatory effect, reproduced by 5-CT, suggesting that it is linked to a 5-HT\textsubscript{1} receptor.\textsuperscript{21} In coronary arteries, 5-HT induces an endothelium-dependent vasodilation, apparently mediated by 5-HT\textsubscript{1} receptors, as it is inhibited by methiothepin.\textsuperscript{31-33} Finally, other vascular effects seem to be mediated by receptors different from the 5-HT\textsubscript{1}, 5-HT\textsubscript{2}, and 5-HT\textsubscript{3} receptors. According to Cohen and Colbert,\textsuperscript{34} the 5-HT receptor responsible for canine basilar artery contraction is neither 5-HT\textsubscript{2} nor 5-HT\textsubscript{3}: It would be similar to the receptor common to tryptamine and 5-HT described in the rat stomach fundus.\textsuperscript{35} Cohen\textsuperscript{36} observed a direct relaxing effect of 5-HT (1-10 \mu M)

### Table 3. Inability of 5-Hydroxytryptamine to Stimulate PGI\textsubscript{2} Production in Endothelial Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Bovine aorta (ng/ml)</th>
<th>Human umbilical vein (ng/ml)</th>
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<tbody>
<tr>
<td>ADP 10 \mu M</td>
<td>19.5±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>ADP 100 \mu M</td>
<td>26.8±0.8</td>
<td>ND</td>
</tr>
<tr>
<td>Bovine thrombin (5U/ml)</td>
<td>4.6±0.2</td>
<td>14.0±2.0</td>
</tr>
<tr>
<td>5-HT 10 \mu M</td>
<td>4.0±0.8</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>5-HT 100 \mu M</td>
<td>4.8±0.6</td>
<td>0.8±0.1</td>
</tr>
</tbody>
</table>

Endothelial cells cultured from either bovine aorta or human umbilical vein were incubated for 30 minutes in the presence of the tested agents. The results are expressed as mean±SD of triplicate determinations in one representative experiment out of four for the bovine aorta and two for the umbilical vein. ND, not determined.

### Table 4. Effect of 5-Hydroxytryptamine Antagonists on the 5-Hydroxytryptamine-Stimulated Release of PGI\textsubscript{2} From Bovine Aortic Media Explants

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Ketanserin (3 \mu M)</td>
<td>18±13 (n=5)</td>
</tr>
<tr>
<td>Ketanserin (10 \mu M)</td>
<td>33±12 (n=7)</td>
</tr>
<tr>
<td>Cyproheptadine (10 \mu M)</td>
<td>47±9 (n=5)</td>
</tr>
<tr>
<td>Methiothepin (10 \mu M)</td>
<td>36±12 (n=3)</td>
</tr>
<tr>
<td>Ketanserin (10 \mu M)+methiothepin (10 \mu M)</td>
<td>34±20 (n=2)</td>
</tr>
</tbody>
</table>

Explants were incubated for 60 minutes in the presence of 5-hydroxytryptamine (5-HT) (10 \mu M); the various antagonists were added 10 minutes before. Results represent the percent inhibition of the stimulatory effect of 5-HT (mean±SD, n=number of separate experiments in which each condition was tested in triplicate).
on canine coronary arteries, which was independent from the endothelium and which might be mediated by another yet unidentified receptor. Finally, some 5-HT effects can be mediated by α-adrenergic receptors. Coughlin et al concluded that the stimulation by 5-HT of PG12 production in bovine aortic smooth muscle cells is mediated by 5-HT2 receptors, on the basis of a potent competitive inhibition by ketanserin. In agreement with them, we have shown that 5-CT stimulates PG12 release in these cells with a lower potency and maximal effect than 5-HT itself. According to Feniuk et al, 5-CT is indeed a partial 5-HT2 agonist. In the rabbit aorta, it is 15 times less potent than 5-HT, with only half its efficacy. However, in bovine aortic media explants, the stimulation of PG12 release by 5-HT was only partially inhibited by ketanserin, which excludes a major contribution of 5-HT2 receptors. The specificity of this 5-HT action has been established by a comparison with its precursor, its degradation products and other amines. In particular, the 5-HT action is not mediated by α-receptors since noradrenaline produces a much smaller stimulation than 5-HT itself. The total absence of a tryptamine effect excludes the involvement of tryptamine receptors, described in other tissues. Recently, Hirafuji et al reported similar observations suggesting that 5-HT stimulates the release of PG12 from the rat aorta via receptors distinct from the 5-HT2 subtype. The characterization of these receptors will require further studies and the development of selective ligands for the multiple 5-HT receptor subtypes.

The fact that smooth muscle cells may exist in different phenotypes is well known, although the profound differences between smooth muscle cells in culture (synthetic state) and the majority of the smooth muscle cells present in adult arteries (contractile state) have not been underscored enough in many studies. Recently, we have shown that phorbol 12-myristate, 13-acetate, a selective activa-
tor of protein kinase C, as well as ionophore A23187, were potent stimuli of PGI₂ production in the cultured smooth muscle cells, whereas they were both totally ineffective on bovine aortic media explants.⁴⁰ Although other factors, like the interaction with elastin⁴¹ or collagen fibers, might explain the differences between explants and cultured cells, our study suggests that the phenotypic modulation from the contractile to the synthetic state might be accompanied also by a fundamental change in the mechanisms which control PGI₂ production.

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Key Words • serotonin • smooth muscle • aorta • prostacyclin
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