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# PROSTANOID SYNTHESIS BY VASCULAR SLICES AND CULTURED VASCULAR CELLS OF PIGLET AORTA

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SUMMARY. Biosynthesis of prostanoids was studied in vascular slices of human umbilical arteries, piglet aorta and vena cava as well as in cultured vascular cells of piglet aorta. After pre-incubation with radioactive labeled arachidonic acid, prostanoids in the incubation media of slices or cultured cells were measured by radioimmunoassay or by radioactivity determination of labeled compounds following separation on reserved-phase high performance liquid chromatography. In all vascular slices 6-keto-PGF1α was the main prostanoid found, followed by PGE2. Thromboxane B2 and PGF2α were also formed, but only in trace amounts. In cultured cells taken from the three layers of the vascular wall, the prostanoid profiles differed markedly from those obtained from vascular slices. Each cell strain showed a specific prostanoid pattern. Endothelial cells synthesized predominantly 6-keto-PGF1α and PGF2α. In smooth muscle cells no 6-keto-PGF1α could be detected; here the predominant prostanoid was PGE2. PGF2α was formed in smaller quantities. Fibroblasts synthesized all prostanoids (PGE2, PGF2α, TXB2, 6-keto-PGF1α), PGE2 and PGF2α being the major products. In vascular slices and in cultured endothelial cells, the predominant prostacyclin derivative detected was 6-keto-PGF1α; the enzymatic PGI2-metabolite, 6,15-diketo-PGF1α, could be detected only in piglet vena cava slices in small amounts.

1 - INTRODUCTION. Prostanoids act primarily as local hormones. Their formation in blood vessels may influence platelet vessel wall interaction and may contribute to the regulation of vascular tone. Endothelial cells synthesize prostacyclin ($PGI_2$, 1-4). Its inhibitory effect on platelet function may be related to the anti-adhesive property of normal vascular endothelium for platelets both in vivo and in vitro (5,6). The vasodilatory action of $PGI_2$ formed also in the media of the vascular wall by smooth muscle cells may contribute to the control of vascular tone (7-12). $PGI_2$ was usually measured directly by bioassay or indirectly by determination of its stable hydrolysis product 6-keto-$PGF_{1\alpha}$ using

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thin-layer chromatography or radioimmunoassay. The determination of enzymatically formed $PGI_2$-metabolites such as 6,15-diketo-$PGF_{1\alpha}$ (13) may be useful too for quantifying $PGI_2$. Other prostanoids ($TXA_2$, $PGE_2$, $PGF_{2\alpha}$, $PGD_2$) could as well participate in the regulation of vascular smooth muscle tone or in the interaction between platelets and the vessel wall. In earlier studies, preceding the discovery of $PGI_2$, formation of PGE- and $PGF_\alpha$-like material was reported to occur in vascular slices and in cultured smooth muscle- and endothelial cells (14-17). These observations, however, may have to be reevaluated because the analytical methods used did not allow a sharp differentiation from $PGI_2$ or 6-keto-$PGF_{1\alpha}$ (18).

The aim of the present study was to evaluate the spectrum of prostanoids formed by the vascular wall and to localize their respective formation in the vascular cells. In addition the main derivative of $PGI_2$ should be characterized. Vascular slices of human and porcine origins and cultured porcine aortic vascular cells (endothelial cells, smooth muscle cells, fibroblasts) were incubated with radioactive labeled arachidonic acid, and prostanoids were measured by radioimmunoassay and by radioactivity determination of labeled compounds after separation on reversed-phase high performance liquid chromatography (rp-HPLC).

2 - MATERIALS AND METHODS. The following materials were used : Dulbecco's modified medium containing streptomycin (50 µg/ml), penicillin (50 U/ml), amphothericin B (1 µg/ml) and foetal bovine serum (FBS) from GIBCO Biocult (Glasgow, Scotland). Tissue culture petri dishes from Falcon. Organic solvents from Merck (Darmstadt, West-Germany). Silicic acid (100 mesh) from Mallinkrodt Inc. (St.Louis, Missouri, USA). Instruments and columns for reversed-phase high pressure liquid chromatography from Waters Associates (Milford, Massachusetts, USA). $^3H$-labeled arachidonic acid (specific activity 112 Ci/mM) and $^{14}C$-labeled arachidonic acid (specific activity 250 mCi/mM) from NEN (Boston, Massachusetts, USA). Collagenase (Type I CLS) from the Worthington Biochemical Corporation. Prostanoids were a gift from Dr. John E. Pike (Upjohn Co., Kalamazoo, Michigan, USA). Porcine antifactor VIII serum was kindly provided by Dr. Lavergne (Hôpital Bicêtre, Paris). Antirabbit immunoglobulin, fluoresceine labeled, was purchased from Wellcome (Paris, France).

2.1. Vascular slices. Umbilical cords were obtained immediately after delivery from healthy women who had not taken aspirin-like drugs during the last two weeks of pregnancy. Aorta and vena cava from young piglets (1-2 weeks old) were dissected immediately after killing. Umbilical arteries and piglet vessels were rinsed with cold NaCl (0.9%) and cut into rings of 1-2 mn width. The rings (0.2-0.5 g tissue wet weight) were incubated with $^{14}C$-

labeled arachidonic acid (1 μCi/g of tissue) for 2 hrs at 37°C in 2 ml 0.1M Tris HCl-buffer, pH7.4. After this period, the supernatants were removed for prostanoid analysis, the vascular segments were washed with 0.9% NaCl solution in order to remove all free radioactivity and were reincubated at 37°C in Tris-buffer for an additional 2 hour incubation period.

2.2. Vascular cell cultures. Cultured cells of the three layers of the vascular wall were obtained from piglet aorta. Endothelial cells were isolated by treatment with 0.1% collagenase solution (200 U/mg) for 2 minutes at 20°C (19). Smooth muscle cells from the aortic wall were grown from explants (20). Fibroblasts were obtained by preparing explants from the adventitia of the aorta. All cells were cultured in 35 mm petri dishes in Dulbecco's modified medium. For growing cells, foetal bovine serum (FBS) was added at a final concentration of 20% for endothelial cells and 10% for smooth muscle cells and fibroblasts. At confluence all cultured cells were kept in a maintenance medium containing 2% FBS. Cultured endothelial cells were characterized by the presence of factor VIII antigen (21). Cultured vascular cells were incubated with labeled arachidonic acid just before they reached confluence. The medium was removed and replaced by FBS-free medium containing $^{3}H$- or $^{14}C$-labeled arachidonic acid (1-2 μCi/ml medium) for 1 hour. FBS was subsequently added to a final concentration of 2%. After 24 hours the medium was removed and analyzed (day 1). New medium was added and removed after an additional 24 hour incubation period (day 2).

2.3. Determination of prostanoids. For analysis of prostanoids the incubation media of vascular slices or cultured cells were acidified with 1 N-HCl to pH 3.5 and extracted twice with 3 volumes of chloroform/methanol (2:1). Extracts were purified on an open silicic acid column chromatography (22). The first eluate (5 ml benzene/ethyl-acetate, 60:40) containing arachidonic acid and its unpolar metabolites was discarded, the second eluate (10 ml benzene/ethyl-acetate/methanol, 60:40:20) containing the prostanoids was evaporated at 37°C under a stream of nitrogen. The eluates were stored in 1 ml ethyl-acetate at -20°C. The recovery at this stage averaged 75% for each prostanoid. For separation on reversed-phase high performance liquid chromatography (rp-HPLC), the samples were dissolved in 200 μl of the elution solvent and after centrifugation (15 minutes at 4000 x g and 4°C) applied to the column. The conditions of rp-HPLC were the following : reversed-phase column with radial compression (radial PAK $A^{R}$, 8 x 100 mm); mobile phase : water/acetonitrile/acetic acid 70:30:0.1; flow rate : 1 ml/min., isocratic elution. The column pressure was continously recorded (Omniscribe$^{R}$ recorder, Houston Instruments). Fractions were collected every minute (Retriver 3, ISCO, Lincoln, Nebraska, USA) in plastic microtubes, frozen at -80°C and lyophilized. The dry residues were dissolved in RIA-buffer (22). Prostanoids were determined by two methods : either by liquid scintillation counting of the radioactive prostanoids formed from labeled arachidonic acid or by radioimmunoassay. The separation of tritium-labeled prostanoid standard substances on rp-HPLC is shown in figure 1. $^{3}H$-labeled prostanoids had a slightly more polar behaviour than the unlabeled and $^{14}C$-labeled prostanoids. The following prostanoids were measured by RIA : 6-keto-PGF1α, 6,15-diketo-PGF1α, TXB2, PGF2α, PGE2

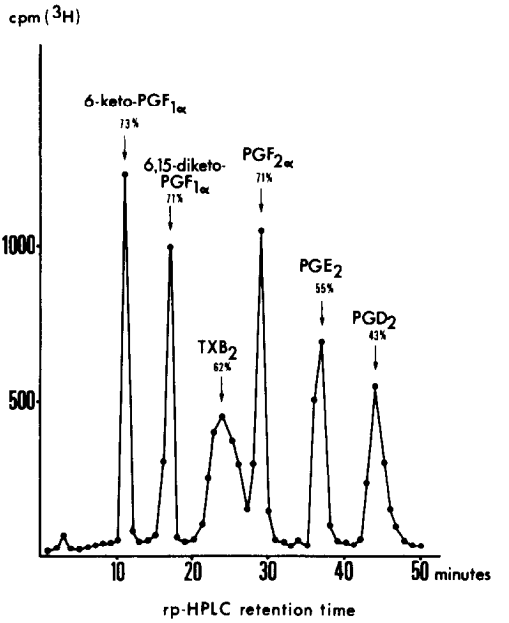


Fig. 1. Separation of 3H-labeled PG-standards on reversed-phase high performance liquid chromatography (rp-HPLC). Recovery after rp-HPLC is shown as percent of injected 3H-PG's. For separation conditions see methods.

and PGD2. For all RIA's of prostanoids except for PGF2α iodinated tracers were used (23). The technics for production of the specific antibodies, the methodology and the respective sensitivities of the RIA's are described elsewhere (24).

3 - RESULTS. In all vascular slices studied the major prostanoid formed was prostacyclin determined by its stable hydrolysis product 6-keto-PGF$_{1\alpha}$. The radioactive profile of prostanoids formed from incubation of $^{14}$C-labeled arachidonic acid with piglet aortic slices is shown in figure 2a. Similar radioactive profiles were obtained from the other vascular segments derived from human umbilical arteries or piglet vena cava. The results of the radioactive profiles were confirmed by radioimmunological determination (Table 1). 6-keto-PGF$_{1\alpha}$ was the major prostanoid, followed by PGE$_2$. The latter was formed at a relative high rate (28%) in slices of piglet vena cava when compared to piglet aorta (7%). Thromboxane B$_2$ and PGF$_{2\alpha}$ were also synthesized, but only in trace amounts. Washing of slices markedly decreased the total prostanoid synthesis, but did not change the relative distribution of the prostanoids formed.

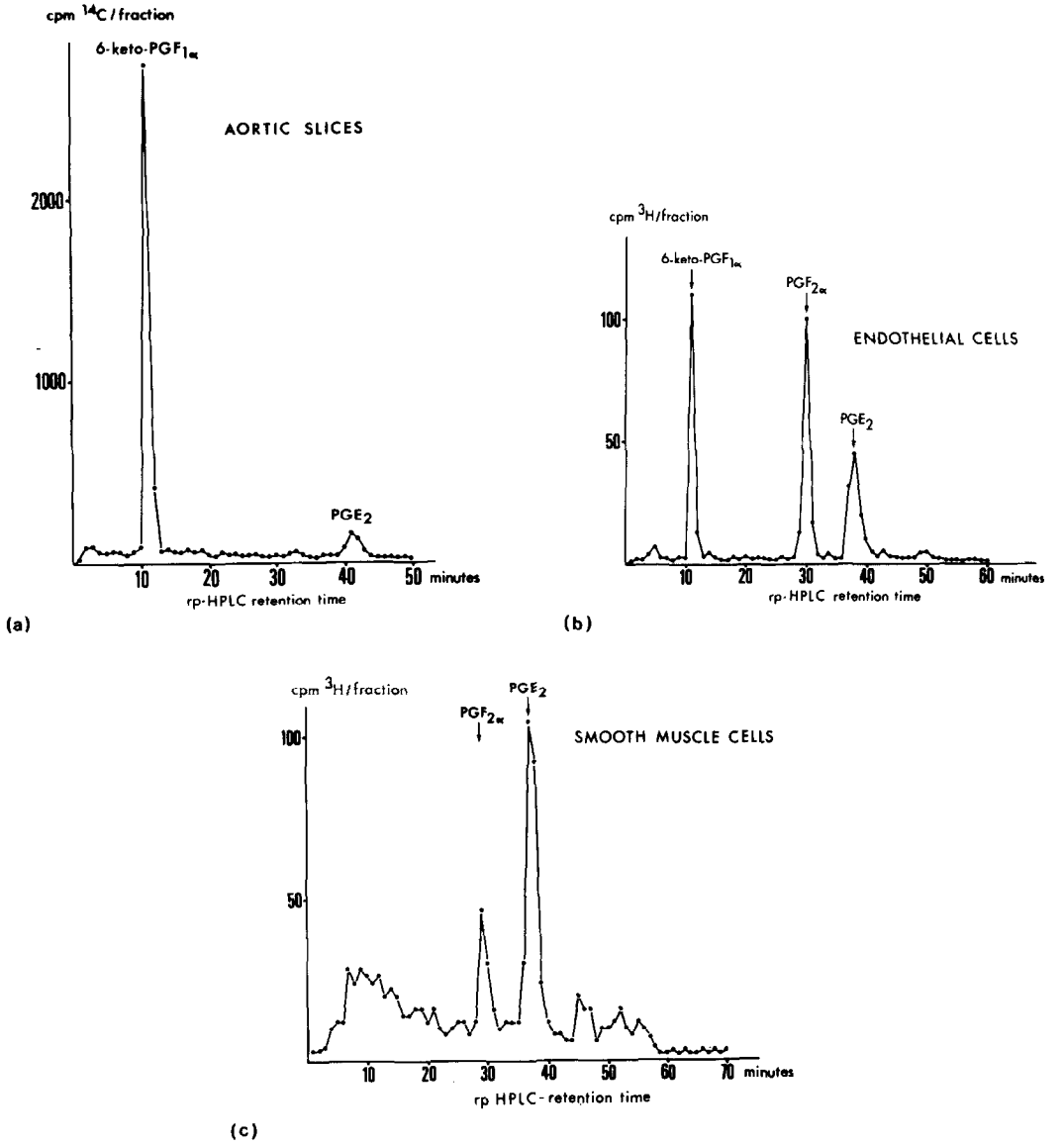


Fig. 2a-c. Prostanoid synthesis following incubation of labeled arachidonic acid with slices (a), cultured endothelial cells (b) or smooth muscle cells (c) derived from piglet aorta. Labeled prostanoids were separated on rp-HPLC (see Fig.1.).

The prostanoid spectra found in cultured endothelial cells, smooth muscle cells and fibroblasts derived from piglet aorta were quite different. Each cell type showed a specific prosta-

Table 1. Prostanoid (PG) formation by vascular slices following incubation with ($^{14}C$) arachidonic acid. PG's were determined by RIA after separation on rp-HPLC. Values are given as ng/g wet weight of tissue and hour.

| | 6-keto-$PGF_{1\alpha}$ | 6,15-diketo-$PGF_{1\alpha}$ | $TXB_2$ | $PGF_{2\alpha}$ | $PGE_2$ | $PGD_2$ |
|---|---|---|---|---|---|---|
| PIGLET AORTA | | | | | | |
| -1st incubation | 225 | <0.1 | 4.8 | 4.2 | 17.0 | <0.1 |
| -2nd incubation after washing of slices | 58 | <0.1 | 2.0 | 0.8 | 4.8 | <0.1 |
| PIGLET VENA CAVA | | | | | | |
| -1st incubation | 390 | 2.3 | 10.5 | 13.4 | 162.0 | <0.1 |
| -2nd incubation after washing of slices | 107 | 0.8 | 4.4 | 2.2 | 43.0 | <0.1 |
| HUMAN UMBILICAL ARTERIES | 72 | <0.1 | 0.9 | 0.2 | 3.0 | <0.1 |

noid pattern (Fig. 2b,c, Table 2). The ratio of $PGI_2$ to the total prostanoid formation was the highest in endothelial cells. In smooth muscle cells, the predominant PG was $PGE_2$, no 6-keto-$PGF_{1\alpha}$ could be detected. In both cell lines, $PGF_{2\alpha}$ was formed in high amounts whereas $TXB_2$ could not be found. Fibroblasts synthesized $PGE_2$ and $PGF_{2\alpha}$ and, in addition, $TXB_2$ and 6-keto-$PGF_{1\alpha}$. The total amount of prostanoids formed in the absence of exogenous arachidonic acid decreased on the second incubation day (data not shown), but the relative proportion of the formed

Table 2. Prostanoid (PG) formation by cultured piglet aortic cells after incubation with ($^{3}H$) arachidonic acid. PG's were determined by RIA after separation on rp-HPLC Values (mean of two experiments) are given in % of total PG-formation*

| | | 6-keto-$PGF_{1\alpha}$ | 6,15-diketo-$PGF_{1\alpha}$ | $TXB_2$ | $PGF_{2\alpha}$ | $PGE_2$ | $PGD_2$ |
|---|---|---|---|---|---|---|---|
| Endothelial cells | day 1 | 58 | 0 | 0 | 32 | 10 | 0 |
| | day 2 | 44 | 0 | 0 | 33 | 23 | 0 |
| Smooth muscle cells | day 1 | 0 | - | 2 | 21 | 77 | 0 |
| | day 2 | 0 | - | 0 | 10 | 90 | 0 |
| Fibroblasts | day 1 | 4 | - | 15 | 17 | 64 | 0 |
| | day 2 | 2 | - | 10 | 20 | 68 | 0 |

*Culture medium containing FBS was analysed for prostanoids which were substracted from the results obtained with cultured cells.

prostanoids remained unchanged. Neither in vascular slices nor in cultured vascular cells $PGD_2$ could be detected. In all cases the predominant $PGI_2$ derivative was 6-keto-$PGF_{1\alpha}$; the enzymatically formed $PGI_2$ metabolite, 6,15-diketo-$PGF_{1\alpha}$, could be detected as immunoreactive compound in small amounts only in slices of piglet vena cava.

4 - DISCUSSION. The synthesis of prostanoids was comparable in vascular slices of human and porcine origin : the major PG found was 6-keto-$PGF_{1\alpha}$ followed by $PGE_2$; $PGI_2$ is considered to be the major vascular arachidonate metabolite mainly produced in the inner layer of the vascular wall (25,7), but only in a few studies the vascular formation of $PGI_2$ was estimated in relation to the synthesis of other prostanoids.

Bovine vessels from different vascular regions formed mainly 6-keto-$PGF_{1\alpha}$; only in the bovine umbilical artery, $PGE_2$ was the major product (26). We found that the piglet vena cava showed a higher ratio of $PGE_2$ to $PGI_2$ when compared to the aorta. The relationship between $PGE_2$ and $PGI_2$ may be influenced by differences in cofactor concentrations, as has been shown for microsomes of bovine seminal vesicles (27). The prostanoids $PGF_{2\alpha}$ and $TXA_2$ which generally cause vadoconstriction were also formed in the vascular slices studied, but only in trace amounts. Whether in vivo traumatisation also induces increased synthesis of the vasodilatory prostanoids $PGI_2$ and $PGE_2$ cannot be decided from these in vitro studies.

In order to evaluate the synthesis of these prostanoids by different vascular cells, PG-biosynthesis was measured in cultured endothelial cells, smooth muscle cells and in fibroblasts, derived from the three layers of piglet aorta. Each cell type was found to have a specific prostanoid spectrum. Endothelial cells synthesized the highest rate (52%) of $PGI_2$ as compared to the other cell types. Synthesis of $PGI_2$ has been demonstrated in endothelial cells from different species and origin (1-4,28). In our study, we found also a high rate of $PGF_{2\alpha}$ formation (32%) in endothelial cells. In smooth muscle cells, $PGE_2$ was the predominant prostanoid followed by $PGF_{2\alpha}$; 6-keto-$PGF_{1\alpha}$ was not detectable. The latter finding differs from results reported by other laboratories in different species (2,8-10), and may be specific for the pig. Thus, synthesis of $PGI_2$ has been shown in smooth

muscle cells derived from human arteries (2), rat (8,9), and rabbit (10) aorta, but was not demonstrable in smooth muscle cells from pig aorta (29). Similarly to the smooth muscle cells, fibroblasts, cultured from the adventitia of piglet aorta, synthesized $PGE_2$ and $PGF_{2\alpha}$ in high amounts, but, in addition, produced also $TXB_2$ and 6-keto-$PGF_{1\alpha}$. The resulting spectrum of prostanoids accords well with that found in fibroblasts from human foreskin (28). Synthesis of $PGI_2$ was reported also by other laboratories to occur in 3T3 fibroblasts from mouse embryo (23,31) and in human skin fibroblasts (2). The heterogenous prostanoid profile found in fibroblasts may be related to the pluripotent biological property of this relatively undifferentiated cell type.

The prostanoid pattern obtained in slices or in cultured cells derived from the same vascular segment (piglet aorta) was quite different. In vascular slices, prostanoid synthesis may be stimulated when compared to the cultured cells. A differential oxygenation and metabolic activity of the three layers of the vascular wall when incubated as slices could result in a predominant stimulation of $PGI_2$ by the endothelial layer. $PGI_2$ found in piglet aortic slices could also be formed by the non-endothelial layers of the vascular wall,what is supported by the demonstration of $PGI_2$-formation in de-endothelialized vascular tissue (7,32). $PGE_2$ found in piglet aortic slices may derive from smooth muscle cells and fibroblasts. The relative high formation rate of $PGF_{2\alpha}$ in all cultured cell types may be due to the culture conditions : the dispersion of cells and their growth on unphysiological surfaces in an artifical medium could lead to an alteration in the distribution and activity of the enzymes which transform PG-endoperoxides into prostanoids.

In summary, prostanoid formation in vascular slices and cultured vascular cells was found to differ markedly which may be partly due to the different experimental conditions. The specific prostanoid profile of the vascular cells could be related to their different functions.

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