Evidence for a two-step process in prostaglandin secretion

Intracellular accumulation of prostacyclin precedes its release from human endothelial cells in culture

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Cultured endothelial cells (EC) from human umbilical vein were incubated with [U-^{14}C]arachidonic acid (AA) followed by a challenge with thrombin (2 units/ml) or calcium ionophore A23187 (5 μM) for 0.5–10 min at 37°C. In both cases, AA was rapidly liberated from phospholipids and converted into prostaglandin I_2 (PGI_2), as determined by the radioactivity of the stable derivative 6-keto-$PGF_{1\alpha}$. Maximal liberation of AA and synthesis of PGI_2 were achieved within 2 min, but the two compounds first accumulated in EC prior to their release into supernatants. This finding, which was never reported before, raises the question of the mechanism of AA and PG release through the cell membranes and offers a convenient model to investigate this still obscure process.

Endothelial cell *Prostaglandin* *Prostacyclin* *Arachidonic acid* *Secretion* *Thrombin*

1. INTRODUCTION

Among various factors involved in the antithrombotic function of endothelial cells (EC), the secretion of prostacyclin or prostaglandin I_2 (PGI_2), an unstable but powerful PG able to inhibit platelet aggregation and to evoke vasodilation, might play a crucial role [1–3]. As for other oxygenated derivatives of AA, PGI_2 biosynthesis requires a previous liberation from phospholipids through the action of phospholipases [3–5]. In previous studies dealing with this problem, we showed that phosphatidylcholine might represent the major phospholipid involved in AA release upon THR and calcium ionophore A23187 stimulation of EC [6,7]. These results were obtained by a previous labelling of EC lipids with [^{3}H]AA, followed by analysis of supernatant and cell lipids by thin-layer chromatography (TLC) after triggering EC with THR or A23187 for 10 min.

In more recent investigations dealing with the time course of AA liberation using a ^{14}C-labelled precursor, we observed that 6-keto-$PGF_{1\alpha}$, the stable derivative of PGI_2, could be detected in EC soon after THR or A23187 challenge, its appearance in cell supernatants occurring only secondarily. This prompted us to investigate in more detail this sequence of events. The present study describes a two-step process of PGI_2 secretion involving accumulation inside the cells prior to a slow release. So this unexpected finding offers a convenient model to study the mechanism by which PG crosses the membrane of parent cells after biosynthesis.

Abbreviations: EC, endothelial cells; PG, prostaglandin; AA, arachidonic acid; ASA, acetylsalicylic acid (aspirin); THR, thrombin

2. MATERIALS AND METHODS

2.1. *Materials*

All materials and media used for cell culture were purchased from Seromed (Münich, FRG).

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[U-^{14}C]AA was from N.E.N. (Dreieich, FRG). Calcium ionophore A23187 was obtained from Boehringer Mannheim and human thrombin (3000 NIH units/mg protein) was from Sigma (St. Louis, MO). Acetylsalicylic acid (lysin salt) was obtained from Egic laboratory (Amilly, France).

2.2. *Labelling of EC*

EC from human umbilical veins were cultured essentially as in [6] using AB human serum and identified as EC using morphological and immunological criteria [8].

For each experiment, 6 flasks of EC derived from the same cord were used after reaching confluence. After a 20-h incubation at 37°C in a 3% CO_2 atmosphere with 5 ml growth medium containing 0.5 μCi [^{14}C]AA, the supernatant was removed and the cellular monolayer washed twice with 5 ml M199.

2.3. *Stimulation study*

The labelled cells, prepared as above, were incubated with 5 ml M199 containing 5 μM A23187 dissolved in 0.05 ml dimethylsulfoxide or 10 units thrombin in 0.02 ml isotonic NaCl. In control experiments, only the solvent was added. In experiments dealing with the effect of ASA (0.1 mM), the cells were previously incubated for 30 min in the presence of the drug before adding A23187, thrombin or control solvent. Aliquots of the supernatants (0.1 ml) were taken off for radioactivity determination and the whole supernatant was collected, acidified to pH 4 by glacial acetic acid (0.1 ml) and immediately extracted twice with 2 vols ethyl acetate. The remaining cells were scrapped in 5 ml methanol containing 0.5 ml EDTA (0.2 M, pH 7.4). The cell lipids were extracted as in [9] using acidified water (pH 4) instead of buffer.

2.4. *Lipid analysis*

Cell and supernatant lipids were separated by monodimensional TLC on silicagel 60 plates (Merck, Darmstadt) using the upper phase of a mixture of ethyl acetate:acetic acid:isooctane:water (110:20:50:100, v/v). The various spots detected by autoradiography were scrapped into scintillation vials containing 10 ml Instagel (Packard) and radioactivity was determined using an Intertechnique spectrometer.

3. RESULTS

Fig.1 represents an autoradiogram of lipid extracts from both EC and supernatants after TLC separation. Besides phospholipids, which were most abundant in cell extracts, several spots appeared upon cell stimulation with THR. Among these, phosphatidic acid and diglycerides were only detected in EC extracts, whereas 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 (cyclo-oxygenase products), hydroxy-eicosanoic acids (lipoxygenase metabolites) [10] and free AA were seen in both EC and supernatant extracts. As observed in [6,7], the 5 latter compounds displayed a maximal increase in 10-min supernatants, 6-keto-$PGF_{1\alpha}$ and free AA being the major compounds. However, their

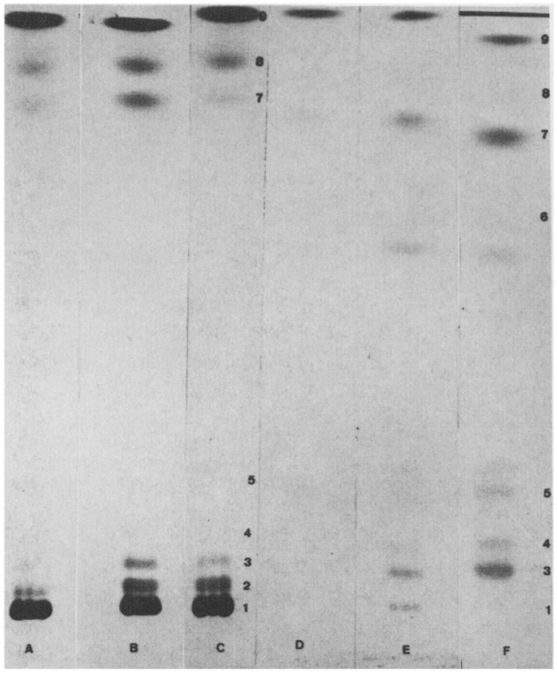

Fig.1. Thin-layer chromatography of radioactive compounds formed after thrombin stimulation of [^{14}C]AA-labelled EC. Cell lipids: lane A, control (unstimulated cells); lanes B,C, thrombin-stimulated cells after 2 min (B) and 10 min (C). Supernatant lipids: lane D, control (unstimulated cells); lanes E,F, thrombin-stimulated cells after 2 min (E) and 10 min (F). Numbers refer to the following compounds: 1, phospholipids; 2, phosphatidic acid; 3, 6-keto-$PGF_{1\alpha}$; 4, $PGF_{2\alpha}$; 5, PGE_2; 6, hydroxy-eicosanoic acids; 7, AA; 8, diglycerides; 9, other neutral lipids.

presence in EC, especially after short incubation times, indicated that PGI_2 and liberated AA might accumulate in EC before releasing into the medium.

To have further insight into this process, radioactive free AA and 6-keto-$PGF_{1\alpha}$ were quantified in lipid extracts from both EC and supernatants after stimulation for different times with THR or A23187. As shown in fig.2A, THR stimulation evoked a rapid synthesis of PGI_2, which became maximal already after 2 min. But until 1-2 min, 6-keto-$PGF_{1\alpha}$ remained confined to the cell extract, whereas a slow release process took place afterwards, leading to a 2-times higher amount of PGI_2 in the medium after 10 min stimulation. When considering free AA, a similar pattern was observed (fig.2B), although some amounts of it were already present in EC before THR stimulation, in agreement with our previous data [6,7]. In this case, it must also be noted that the total amount of AA decreased slightly between 5 and 10 min, probably indicating some reacylation into phospholipids.

Almost identical results were also obtained for both compounds with A23187 (fig.3A,B), the only difference being a larger mobilization of AA by comparison with THR.

Fig.4 and 5 illustrate the effect of acetylsalicylic acid (ASA) on PGI_2 and AA accumulation in the two compartments (cells and supernatants). Confirming data of fig.2, 6-keto-$PGF_{1\alpha}$ and AA were more abundant in EC than in supernatants after 1 min stimulation, whereas the reverse was observed after 10 min. In all cases, pretreatment of EC with ASA suppressed completely the PGI_2 appearance

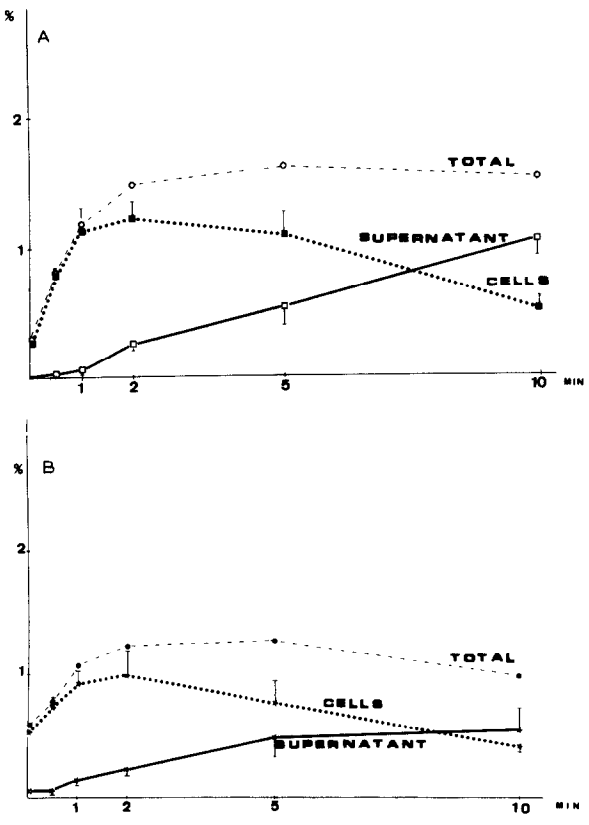

Fig.2. (A) Synthesis of PGI_2 upon thrombin stimulation (2 units/ml) of human EC. Values correspond to means ± SE (4 experiments). (B) Liberation of free AA upon thrombin stimulation (2 units/ml) of human EC. Values correspond to means ± SE (4 experiments).

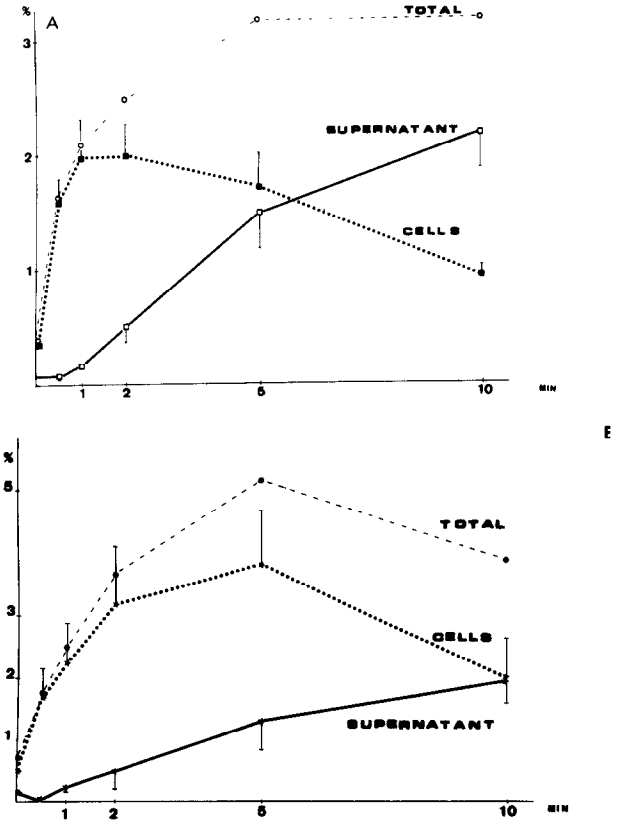

Fig.3. (A) Synthesis of PGI_2 upon A23187 stimulation (5 μM) of human EC. Values correspond to means ± SE (4 experiments). (B) Liberation of free AA upon A23187 stimulation (5 μM) of human EC. Values correspond to means ± SE (4 experiments).

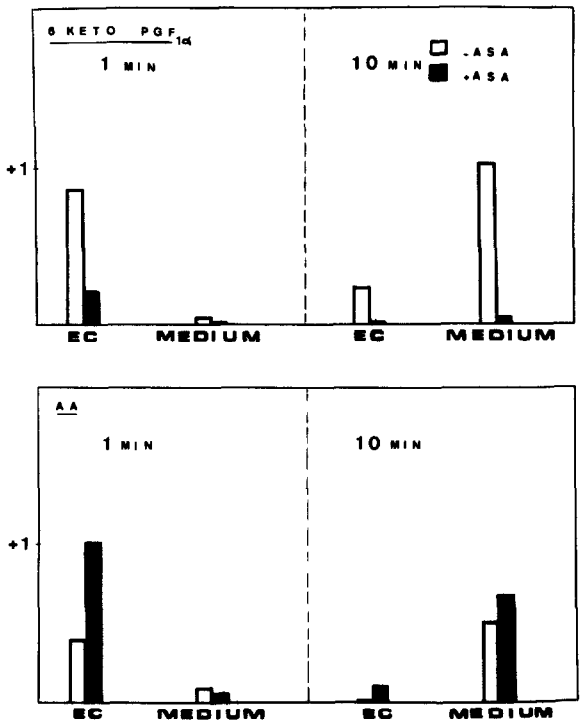

Fig.4. Radioactivity changes of 6-keto-$PGF_{1\alpha}$ and free AA cell associated or secreted upon thrombin stimulation without (□) or with ASA (0.1 mM) (■). Results are expressed as percentages of total radioactivity present in cells + supernatants, compared with unstimulated control cultures.

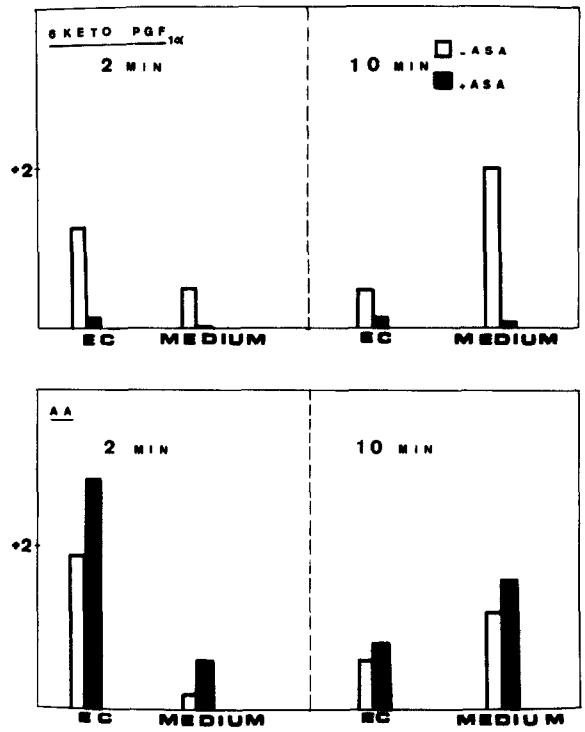

Fig.5. Radioactivity changes of 6-keto-$PGF_{1\alpha}$ and free AA cell associated or secreted upon A23187 stimulation without (□) or with ASA (0.1 mM) (■). Results are expressed as percentages of total radioactivity present in cells + supernatants, compared with unstimulated control cultures.

in both cells and medium. In contrast, the amount of AA increased, especially in the cell extracts after short incubation periods. The lower effect of ASA on the amount of AA after 10 min might be explained by a reacylation of the excess of AA not converted into PGI_2. These results give further support to the identification of 6-keto-$PGF_{1\alpha}$ in both supernatants and cell extracts.

4. DISCUSSION

PG and other oxygenated derivatives of AA are lipidic mediators able to transfer information between a parent cell and a target cell. For instance, the biological system used here involves EC, which can inhibit platelet function and evoke vasodilation through the release of the powerful PGI_2 [1–3]. AA liberation from phospholipids is thought to occur at the inside of the producing cell. Such a view is supported by the fact that AA availability to cyclo-oxygenase and lipoxygenases is mostly regulated by phospholipase A_2. This calcium-dependent enzyme becomes activated through calcium mobilization to the cytoplasm upon specific cell stimulation by various agonists or calcium ionophores [3–7,11]. This hypothesis also fits our previous observation that membrane phospholipid asymmetry retains around 95% of total AA at the inside of human platelets [12].

However, nothing is known about the mechanism allowing PG release from stimulated cells. Many studies dealing with PG synthesis were based on PG determination in the medium using, for instance, radioimmunoassays [13], whereas metabolic investigations often involved lipid extraction of the total medium, including cells and extracellular space [11,14–16]. To our knowledge, this work brings the first observation that a clear dissociation exists between the process of intracellular PG biosynthesis and the process of

secretion. Indeed, 6-keto-$PGF_{1\alpha}$, the stable derivative of the major PG synthesized by EC, was identified in EC as an early product on the basis of its chromatographic behaviour as well as on the fact that its synthesis was completely suppressed by ASA treatment. Also, its release from cells was quite simultaneous with its appearance into supernatants.

This finding might have three significant consequences: (i) from a practical point of view, it implies that PG determination in cell supernatants does not reflect the time course of PG synthesis, since the compounds could appear only secondarily in the cell medium; (ii) from a basic point of view, it raises the general question of the mechanism allowing lipid mediators to cross the cell membrane. As for free fatty acids, the question is still open as to whether a single diffusion/partition or a specific carrier-mediated process is involved in such a transport [17,18]. In this respect, EC could offer a convenient model for studies dealing with this transport mechanism. Comparison with other cell systems might also be very helpful in investigating this problem (iii) from a pharmacological point of view, it implies that besides a modulation of the enzymes involved in the biosynthesis of PG and other parent compounds, drugs able to modify the release process might also prove useful in the field of inflammation or thrombosis.

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