Volume 263, number 1, 117–120 FEBS 08304 April 1990

Protein kinase C-regulated production of prostacyclin by rat endothelium is increased in the presence of lipoxin A4

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Received 13 February 1990

Prostacyclin is generated by cultured rat endothelial cells. Compound blocking activity of protein kinase C and cyclic nucleotide-dependent protein kinases (H7) and compound blocking interaction between Ca^{2+} and calmodulin (W7) diminish generation of prostacyclin in rat endothelial cells. These compounds give a synergistic effect when they are introduced to the endothelial cell cultures simultaneously. Compound HA1004, an inhibitor of cAMP- and cGMP-dependent protein kinases has no effect on prostacyclin generation. Lipoxin A4, a potent direct stimulator of protein kinase C, rapidly induces prostacyclin generation in rat endothelium in a dose- and time-dependent fashion. Lipoxin A4-induced generation of prostacyclin can be inhibited by H7 and W7 but not by HA1004. Lipoxin B4 has no significant effect on prostacyclin generation in rat endothelium. In conclusion, our results demonstrate that generation of prostacyclin by rat endothelial cells is regulated via a pathway involving protein kinase C and Ca^{2+}.

Endothelium; Prostacyclin generation; Lipoxin; Protein kinase C

1. INTRODUCTION

One of the important functions of endothelial cells (EC) is production of prostacyclin (PGI_2), a potent vasodilator and an agent preventing platelet aggregation. Generation of PGI_2 is regulated by several protein and lipid mediators [1–3]. In some cells, protein kinase C (PKC) has been shown to participate in the transduction of the signal leading to the generation of PGI_2 [4–6].

We examined the generation of PGI_2 by non-stimulated or lipoxin (LX)-treated rat heart EC. To examine the role of protein kinases in the generation of PGI_2, we employed 3 inhibitors of protein kinase activities: H7, a blocker of PKC and cAMP- and cGMP-dependent protein kinase (cAMP-PK and cGMP-PK, respectively) activities [7], W7, a blocker of the interaction between calmodulin and Ca^{2+} [8], and HA1004, a blocker of the cAMP-PK and cGMP-PK activities [9].

2. MATERIALS AND METHODS

2.1. *Isolation and culture of rat heart endothelial cells*

EC were isolated from 7–10-day-old DA rat hearts according to the modified method of Kasten [10], as described in detail elsewhere [11,12]. Endothelial origin of the cells had been confirmed on the basis of their growth pattern and antigen expression [11–13]. EC were seeded at the density of 10^4 cells/microwell (96 wells/plate) in 250 μl of culture medium (D-Val MEM + 10% FCS + gentamycin). Twenty-four hours after seeding, the cells were used for the experiments.

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2.2. *Chemicals*

Lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Protein kinase inhibitors H7, W7 and HA1004 were obtained from Seikagaku America (St. Petersburg, FL, USA). Radioimmunoassay (RIA) kits for the measurement of 6-keto-prostaglandin F1 alpha (6-keto-PGF1a) were from Amersham (England).

2.3. *Prostacyclin measurements*

EC were induced to generate PGI_2 by changing the culture medium in the microwells to fresh medium. In the experiments where the effects of different chemicals on the generation of PGI_2 were examined, new medium was supplemented with LX and/or with inhibitors of protein kinase activities. Levels of PGI_2 were assessed by radioimmunoassay of 6-keto-PGF1a after different periods of incubation at 37°C. Results presented in the figures are pg 6-keto-PGF1a/100 μl sample of culture medium obtained from microwells containing 10^4 EC in 250 μl of culture medium. Results are mean values of 3–5 determinations. Statistical significances were counted using the Student's *t*-test.

2.4. *PKC activity measurements*

Activity of PKC, in the presence of compounds H7, W7 and HA1004, was measured in crude extracts obtained from EC cultures. The enzyme activity measurement and procedure of obtaining the crude extract containing PKC was described in detail by Kikkawa et al. [14].

3. RESULTS

EC constitutively generate PGI_2. Removal of PGI_2 from the culture medium of EC, by changing the culture medium, stimulates generation of this compound (measured as the level of 6-keto-PGF1a) until the basal level of the culture medium is reestablished.

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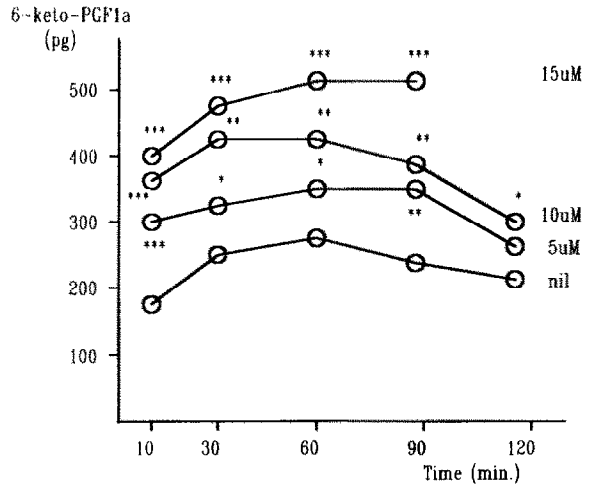

Fig. 1. Generation of PGI_2, by rat heart EC, induced by change of the culture medium to fresh medium (nil) or to fresh medium supplemented with different amounts (5, 10 or 15 μM) of LXA_4. Values are means of 5 separate experiments. Statistical significances in Student's t-test: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

For non-stimulated rat heart EC, seeded at a density of 10^4 cells/microwell in a volume of 250 μl of culture medium, the basal level of PGI_2 is 180–250 pg/100 μl of the culture medium and is generated within 30 min. However, the highest rate of PGI_2 generation occurs within the first 10 min (Fig. 1, nil treatment curve).

It was possible to modulate the generation of PGI_2 by rat heart EC in culture, which is induced by a change of the culture medium, with inhibitors of the activities of several protein kinases. H7 inhibits the activities of 3 protein kinases: PKC (K_i = 6.0 μM), cAMP-PK (K_i = 3.0 μM), and cGMP-PK (K_i = 5.8 μM). At a concentration of 10 mM, it had a very weak effect on the generation of PGI_2, but at a concentration of 30 mM, it diminished the level of PGI_2 in the culture medium (Fig. 2A). HA1004, at low concentrations, is an inhibitor of cAMP-PK and cGMP-PK (K_i = 2.3 and 1.3 μM, respectively), whereas higher concentrations affect also the activity of PKC (K_i = 40.0 μM). In our experiments, the presence of a 2 mM concentration of HA1004 in the culture medium led to increased generation of PGI_2 exceeding the basal level. HA1004, at a concentration of 10 mM still had a potentiating effect on the generation of PGI_2, but the effect was lower than with the 2 mM concentration of this inhibitor (Fig. 2B). W7 is a compound known to block calmodulin-Ca^{2+} interactions (IC_{50} = 31 μM). It had a strong inhibitory effect on the generation of PGI_2 at concentrations ranging from 20 to 50 mM (Fig. 2C). However, at a concentration of 20 mM, W7 had only a short-lasting inhibitory effect (Fig. 2C). Treatment of EC with a combination of H7 and W7, at low concentrations of 10 and 20 mM, respectively, produced a synergistic inhibitory effect (Fig. 2D).

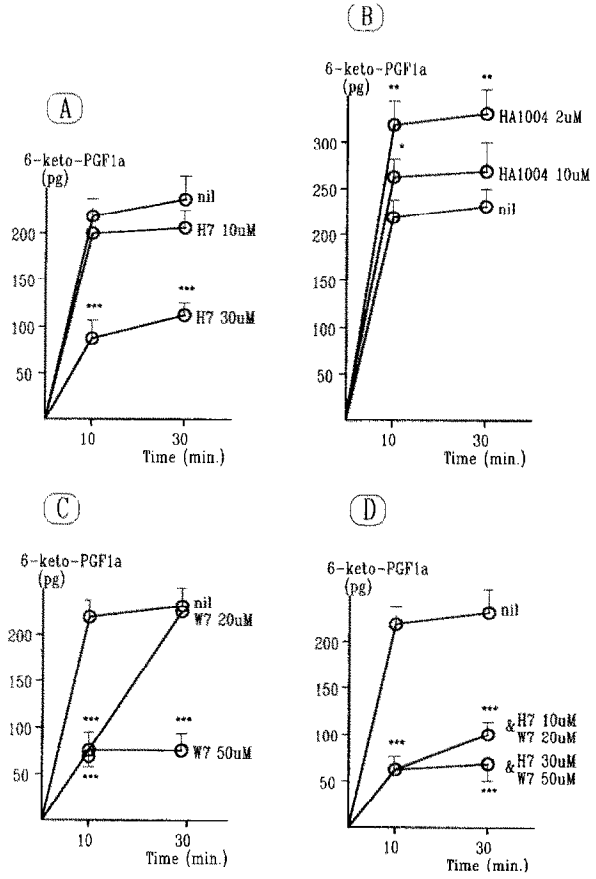

Fig. 2. Effects of protein kinase inhibitors: H7 (A), HA1004 (B), W7 (C) and H7 + W7 (D) on the generation of PGI_2 by rat EC after change of culture medium. Values are means ± SD of 3 separate experiments. Statistical significances in Student's t-test: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

Because the above results suggested involvement of the PKC pathway in the generation of PGI_2, experiments were done to examine the effect of H7, W7 and HA1004 on the PKC activity. As seen in Table I, all tested compounds had an inhibitory effect on PKC activity. However, inhibition of PKC activity varied between inhibitors and was dependent on the inhibitor

Table I

The effect of compounds H7, W7 and HA1004 on the activity of protein kinase C

Compound	Concentration (μM)	Inhibition of PKC activity (%)
H7	10	48 ± 5
	30	95 ± 5
W7	20	10 ± 7
	50	69 ± 8
HA1004	2	4 ± 3
	10	14 ± 5

Inhibition values are means ± SD of 3 separate experiments

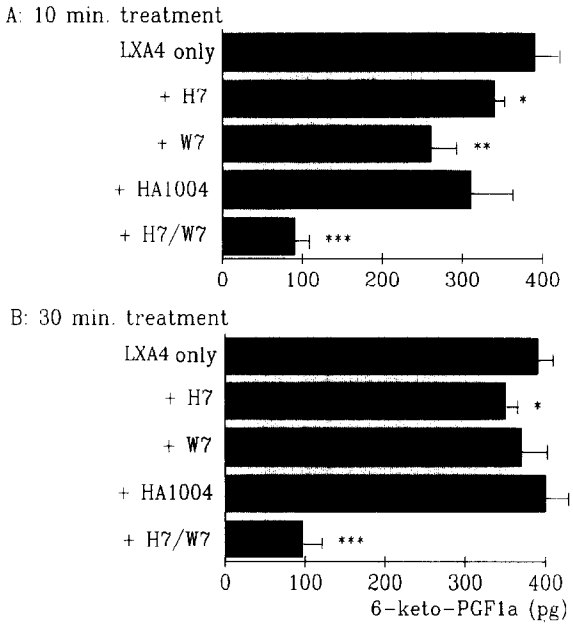

Fig. 3. Effect of the inhibitors of protein kinase activity, H7, W7 and HA1004 added to the cultures of rat EC 10 min prior to addition of LXA_4. The concentration of 6-keto-PGF1a was measured 10 min (A) and 30 min (B) after introduction of LXA_4 to the cultures of EC. Values are means ± SD of 3 separate experiments. Statistical significances in Student's *t*-test: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

concentration used. These results are in agreement with results published by others [15,16].

LX have been demonstrated to induce generation of PGI_2 by cultured human umbilical cord EC [2]. Moreover, it has been suggested that, similarly to other arachidonic acid cascade compounds, LX activate PKC directly [17]. Therefore, we examined the effect of LXA_4 and LXB_4 on the generation of PGI_2 by rat EC as well as the effect of protein kinase inhibitors on the LX-induced generation of PGI_2. LX used in experiments demonstrated different potentials to induce PGI_2. LXA_4 had a strong dose- and time-dependent enhancing effect (Fig. 1), whereas LXB_4 failed to increase significantly the generation of PGI_2 (not shown). When both LXs were combined for treatment of EC, no synergistic or antagonistic effect of LXB_4 was observed on the LXA_4-induced generation of PGI_2 (not shown).

Inhibitors of protein kinase activities, such as H7, W7 or HA1004, when added to the EC cultures simultaneously with LXA_4, did not have significant effects on the generation of PGI_2 (not shown). However, when they were added to the EC cultures 10 min prior to adding LXA_4, some of them had a significant effect on LXA_4-induced generation of PGI_2. H7 and W7 inhibited LXA_4-induced generation of PGI_2 but their effect was short lasting, and the level of PGI_2 equal to this generated by LXA_4 alone, was reached by EC within 30 min. HA1004, at a concentration of 10 mM, had an inhibitory effect on LXA_4-induced generation of PGI_2, and similarly to H7 and W7. The level of PGI_2 equal to this, induced by LXA_4 alone, was established within 60 min after introduction of inhibitor to the cell cultures. When H7 and W7, at concentrations of 30 mM and 50 mM, respectively, were added together to the EC cultures, a strong synergistic inhibitory effect on the generation of PGI_2 was obtained (Fig. 3).

4. DISCUSSION

Generation of PGI_2 is one of the major functions of EC [18–20] and in human EC this process has been shown to be regulated by PKC [4]. Our results suggest that also in rat heart EC PGI_2 generation is regulated via a pathway involving PKC. Generation of PGI_2 was inhibited by compound H7 blocking activity of PKC, cAMP-PK, cGMP-PK, and compound W7, whereas compound HA1004, at a concentration inhibiting the activities of cAMP-PK and cGMP-PK only, did not inhibit PGI_2 generation but even increased it. It suggests that PKC and Ca^{2+} are involved in the generation of PGI_2 by rat EC. This finding is also supported by the observed inhibitory effects of all compounds on the PKC activity itself. The observation that HA1004 increases PGI_2 production by EC correlates with the finding by Kroll et al. [21] who demonstrated that cyclic AMP, a stimulator of cAMP-PK, inhibits the production of PGI_2. This may suggest that PGI_2 production by rat EC might be positively regulated by PKC and negatively regulated by cAMP-PK. Therefore, since PGI_2, similarly to other prostaglandins, exerts its actions on the cells by increasing the cytosolic levels of cAMP, it suggests the existence of a negative feed-back self-regulatory system in the generation of PGI_2 by EC.

Brezinski et al. [2] have demonstrated that LXA_4 is able to increase the production of PGI_2 by human EC. LXA_4, as well as LXB_4, have been demonstrated to induce direct activation of PKC [17]. Our results are in agreement with these findings. First, LXA_4, but not LXB_4, induces the generation of PGI_2 by rat EC in a time- and dose-dependent manner. It was possible to diminish the effect of LXA_4 on the generation of PGI_2 with H7 and W7 but not with HA1004. However, the inhibitors exerted their effect on PGI_2 generation only when they were added to the EC cultures at least 10 min prior to addition of LXA_4. This may suggest that the penetration rate of these inhibitors is slower than that of LXA_4 [15]. Another observation of ours, that the effects of H7 and W7 on the non-treated EC lasts longer than effects of these inhibitors on EC treated with LXA_4, suggests that either LXA_4-activated PKC do not respond significantly to the inhibitors used, or that the presence of LXA_4 hastens the process of recovery of the ability of the PKC to bind ATP [15]. Longer preincubation periods, up to 60 min, did not cause significant effects as compared to 10 min preincubation times

(not shown). Synergism of inhibitory effects of H7 and W7 on the generation of PGI_2 by EC stimulated with LXA_4 supports the suggestion that protein kinase C is involved in the LXA_4-dependent PGI_2 generation (similarly to native generation of PGI_2) [16].

The biological significance of the finding that LXA_4 increases the generation of PGI_2 by EC is still unknown. As a main source of LX, eosinophils have been named. Therefore, it is possible to speculate that in pathological conditions in sites where eosinophilia occurs (e.g. the often-observed blood eosinophilia preceding transplant rejection, [22]), the locally produced LX might serve as triggers of the self-protecting mechanism leading to the protection of the EC from the damage via increased generation of PGI_2.

Acknowledgements: We thank Dr Pekka Häyry for critically reading this manuscript and Ms Leena Saraste for editorial help. The research was supported by grants from the Finnish Academy, the Sigrid Juselius Foundation, the Finnish Kidney Foundation, Helsinki, and from the Research and Science Foundation of Farmos, Turku, Finland.

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