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# NITRIC OXIDE BUT NOT PROSTACYCLIN IS AN AUTOCRINE ENDOTHELIAL MEDIATOR

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**Abstract**—Using porcine aortic endothelial cells, the present study investigates whether stimulation of prostacyclin ($PGI_2$) and nitric oxide also causes elevation of the respective second messengers cAMP and cGMP in the endothelial generator cells. The calcium ionophore A23187 at 0.3–3 $\mu$M increased endothelial cGMP levels up to 27-fold in an L-arginine-dependent manner as assessed through complete inhibition by $N^G$-monomethyl-L-arginine (100 $\mu$M). The 36-fold $PGI_2$ stimulation by 3 $\mu$M A23187 was not accompanied by an intracellular increase in cAMP or an enhanced cAMP efflux. Correspondingly, the $PGI_2$ mimetic iloprost (10 pM–100 $\mu$M) did not change endothelial cAMP levels. However, forskolin (1–100 $\mu$M) and prostaglandin $E_2$ ($PGE_2$) (0.1–10 $\mu$M) produced concentration-dependent increases in cAMP with a 9-fold and 8-fold stimulation at 100 $\mu$M forskolin and 10 $\mu$M $PGE_2$, respectively. These results demonstrate that in contrast to NO, $PGI_2$ acts as a strictly paracrine hormone without affecting the respective second messenger cAMP in the endothelial generator cells.

Prostacyclin ($PGI_2$†) and nitric oxide are vasoactive endothelium-derived autacoids and play an important role in the regulation of vascular tone and blood cell activity [1]. The biological effects of $PGI_2$ and NO are brought about by their respective second messengers cAMP and cGMP [2, 3]. Thus, it has recently been demonstrated that endothelial cells inhibit platelet aggregation by two separate but synergistic mechanisms involving $PGI_2$-induced cAMP stimulation and NO-induced cGMP stimulation [4]. Whereas the role of $PGI_2$ and NO in paracrine signaling between endothelial and other vascular cells is widely accepted [1], conflicting results have been published concerning a possible autocrine function of the two autacoids. In previous studies, NO formation in endothelial cells was reported to have no effect on endothelial cGMP [5–7] whereas other investigators found substantial increases in cyclic GMP using the same stimuli [8–10]. Similarly, a $PGI_2$-induced elevation of endothelial cAMP has been reported [11, 12] although, on the other hand, no $PGI_2$ receptors were found in vascular endothelium [13]. It has recently been suggested that cyclic nucleotides regulate autacoid release from endothelial cells [14, 15]. Thus, the question as to whether $PGI_2$ and NO affect their respective second messenger system in the endothelial generator cell has become even more relevant since such an autocrine signal transduction pathway may serve as a negative feed-back loop in autacoid synthesis. The aim of the present study was to compare $PGI_2$ and NO in terms of their possible autocrine effects by measuring the levels of cAMP and cGMP in endothelial cells in response to simultaneous stimulation of $PGI_2$ and NO by the calcium ionophore A23187.

## MATERIALS AND METHODS

*Materials.* LLC-$PK_1$ cells (ATCC CL101) were purchased from the American Type Culture Collection, Rockville, MD, U.S.A. Fetal calf serum, Dulbecco's modified Eagle medium and penicillin–streptomycin were obtained from Gibco, Eggenstein, F.R.G. L-NMMA was purchased from Bachem (Bubendorf, Switzerland). Iloprost was from Schering (Berlin, F.R.G.) and $PGE_2$ was purchased from Cayman (Ann Arbor, MI, U.S.A.). Acetylated low density lipoprotein labelled with Dil-Ac-LDL was from Paesel and Lorei (Frankfurt, F.R.G.). Calcium ionophore A23187 and all other reagents were from the Sigma Chemical Co. (Deisenhofen, F.R.G.).

*Cell culture.* Porcine aortic endothelial cells were isolated according to Gryglewski *et al.* [16] and cultured in Dulbecco's modified Eagle medium, supplemented with 15% fetal calf serum, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. The cells were grown in a humidified incubator at 37° and 5% $CO_2$. Only endothelial cells at passage 1 were used for experiments. The cells exhibited the characteristic cobblestone morphology and were further identified as endothelial cells by their increased uptake of Dil-Ac-LDL [17]. In randomly selected cultures, more than 98% of the cells were found to fluoresce after incubation with Dil-Ac-LDL. LLC-$PK_1$ pig kidney epithelial cells (passage 201–203) were maintained under the same conditions as the endothelial cells.

*Incubation procedure and determination of cyclic nucleotides and 6-oxo-$PGF_{1\alpha}$.* Cells grown to confluence in 35-mm culture dishes were washed

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† Abbreviations: $PGI_2$, prostacyclin; L-NMMA, $N^G$-monomethyl-L-arginine; $PGE_2$, prostaglandin $E_2$; Dil-Ac-LDL, 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine-perchlorate.

twice with 2 mL of a balanced salt solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH, buffered to pH 7.3. Cells were exposed for 10 min at 37° to the L-arginine antagonist L-NMMA [18] and/or the arginine enantiomers in the balanced solution containing 0.5 mM isobutylmethylxanthine. A23187, iloprost, $PGE_2$ or forskolin was added and the incubation was continued for another 10 min at 37°. The final assay volume was 1 mL. The supernatant was aspirated and aliquots were radioimmunoassayed for 6-oxo-$PGF_{1\alpha}$, an index metabolite of $PGI_2$ [19], or for cAMP. Intracellular cyclic nucleotide levels were determined by radioimmunoassay after terminating the reaction by addition of ethanol and subsequent evaporation as described previously [20, 21].

*Statistical analysis.* The data are means ± SEM of N = 6 observations. Differences were analysed by Student's two-tailed *t*-test. P values of $< 0.05$ were considered to be significant.

## RESULTS

### *Effect of L-NMMA on A23187-induced endothelial cGMP stimulation*

A23187 (0.3–3 μM) induced a concentration-dependent increase in endothelial cGMP with a 6-fold elevation at 0.3 μM and a maximal 27-fold stimulation over basal levels (5.4 ± 0.4 pmol cGMP/$10^6$ cells) at 3 μM. In the absence of isobutylmethylxanthine, basal levels were only 1.5 pmol cGMP/$10^6$ cells. Under these conditions, only at the highest concentration of A23187 used (3 μM) was a significant 3-fold increase in cGMP observed. The L-arginine antagonist L-NMMA (0.3–100 μM) attenuated A23187-induced cGMP stimulation concentration-dependently (Fig. 1A). At 0.3 and 100 μM L-NMMA, a 30 and 94% decrease in cGMP stimulation by A23187 was observed, respectively. The inhibition by L-NMMA (100 μM) was abolished completely by 2 mM L-arginine but not by 2 mM D-arginine (Fig. 1B). Basal cGMP levels remained unaltered by L-NMMA or the arginine enantiomers.

### *Effect of A23187 on endothelial $PGI_2$ and cAMP*

A23187 (0.3–10 μM) increased endothelial $PGI_2$ formation in a concentration-dependent manner. The increase was 9-fold at 0.3 μM and 37-fold at 10 μM (Fig. 2). cAMP levels remained unaffected at all concentrations of A23187 investigated (0.3–10 μM, Fig. 2).

### *Effect of forskolin, $PGE_2$ and iloprost on endothelial cAMP*

The receptor-independent activator of adenylate cyclase forskolin caused concentration-dependent increases in endothelial cAMP (Fig. 3). At 1 and 100 μM forskolin a 5- and 12-fold stimulation of intracellular cAMP was detected, respectively. $PGE_2$ (0.1–10 μM) was also found to augment intracellular cAMP synthesis with a 3-fold stimulation at 0.1 μM and an 8-fold elevation at 10 μM (Fig. 3). In contrast, the stable $PGI_2$ mimetic iloprost was without effect on intracellular cAMP levels at all the concentrations used (10 pM–100 μM Fig. 3).

### *Effect of A23187, iloprost and forskolin on cAMP efflux in endothelial cells*

In order to investigate whether the lack of effect on intracellular cAMP by $PGI_2$ was due to a rapid cAMP efflux into the extracellular space, cAMP levels in the cell supernatant were measured (Table 1). After stimulation with 10 μM A23187 and 10 μM iloprost no increase in intracellular or extracellular cAMP was detected. The marked intracellular stimulation of cAMP by 100 μM forskolin was accompanied by only a slight and non-significant increase in extracellular cAMP levels (Table 1).

### *Effect of iloprost on cAMP in LLC-$PK_1$ cells*

To preclude a down-regulation of $PGI_2$ binding sites under the culture conditions used, iloprost-induced cAMP stimulation was measured in LLC-$PK_1$ cells which were grown under the same conditions as the endothelial cells. In this cell type, iloprost (0.01–1 μM) increased intracellular cAMP concentration-dependently with a 3-fold elevation at 0.1 μM and a maximal 20-fold stimulation at 1 μM (Fig. 4).

## DISCUSSION

The present data demonstrate that endothelial cGMP stimulation by the calcium ionophore A23187 is strictly L-arginine-dependent and can thus be attributed to autocrine activation of the L-arginine/NO pathway. Our data are in contrast to previous reports by other authors who were unable to demonstrate NO-dependent endothelial cGMP elevation [5–7]. There are several possible reasons for the apparent discrepancy between their and our findings. In repeatedly passaged cells, as used in some of the above mentioned studies [5, 7], the responsiveness of soluble guanylate cyclase to NO-induced activation may be lost, a phenomenon that has been demonstrated in both endothelial and non-endothelial cells [22, 23]. Moreover, the detection of a substantial NO-dependent cGMP stimulation in cultured cells seems to require the presence of a phosphodiesterase inhibitor, as shown in the present and in a previous study [24]. Thus, Loeb *et al.* [6] may have missed an increase in endothelial cGMP levels since they conducted their experiments in the absence of a phosphodiesterase inhibitor. Furthermore, our findings are in line with a recent report demonstrating the existence of NO-sensitive soluble guanylate cyclase in endothelial cells [25]. As far as the function of endothelial cGMP is concerned, evidence has been raised that NO-induced cGMP stimulation in endothelial cells serves as a negative feedback mechanism or switch-off signal for NO formation [26, 14].

In contrast to NO, $PGI_2$ in our study failed to activate its respective second messenger system in endothelial cells. The marked increase in $PGI_2$ formation by A23187 in endothelial cells was not followed by a stimulation of endothelial cAMP synthesis. Similarly, endothelial cAMP levels remained unaltered in the presence of the stable $PGI_2$ mimetic iloprost. However, the receptor-independent activator of adenylate cyclase forskolin

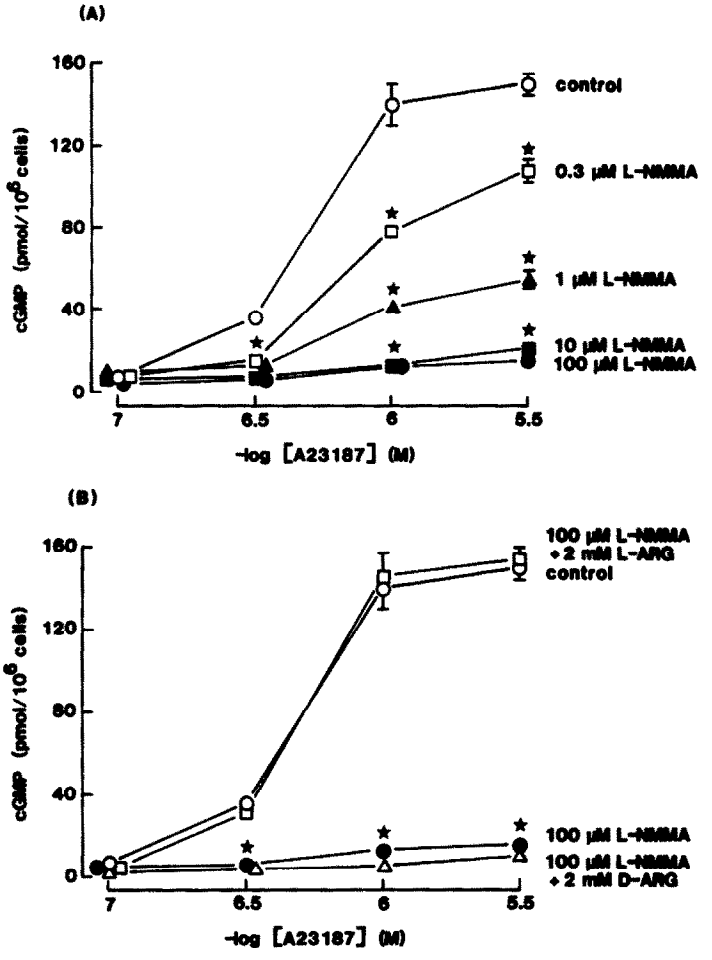


Fig. 1. Effect of L-NMMA on cGMP stimulation by A23187 in porcine aortic endothelial cells (A); reversal of the L-NMMA-induced inhibition by L-arginine but not by D-arginine (B). Incubations were carried out as described in Materials and Methods. Basal cGMP level was $5.4 \pm 0.4$ pmol/$10^6$ cells. * $P < 0.05$; treatment vs control. The data are means ± SEM of N = 6 observations.

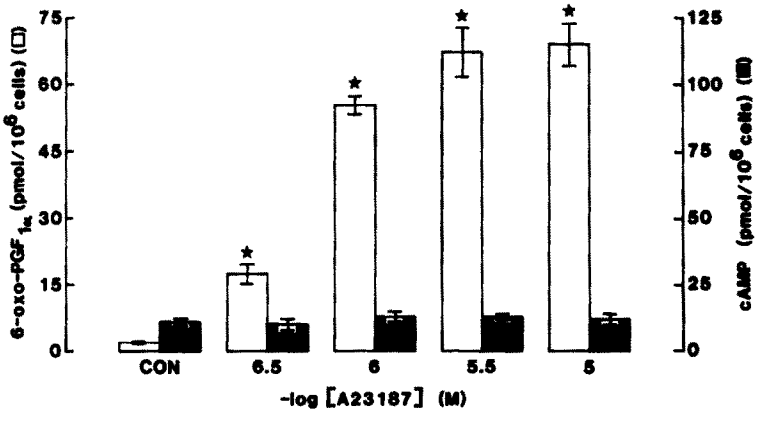


Fig. 2. Effect of A23187 on $PGI_2$ formation and cAMP levels in porcine aortic endothelial cells. Incubations were carried out as described in Materials and Methods. * $P < 0.05$; treatment vs control (CON). The data are means ± SEM of N = 6 observations.

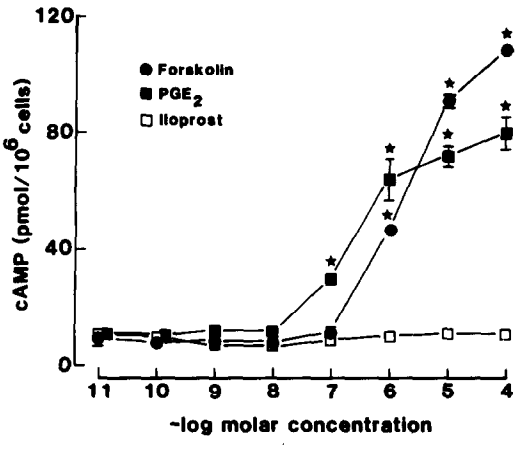


Fig. 3. Effect of forskolin, $PGE_2$ and iloprost on cAMP levels in porcine aortic endothelial cells. Incubations were carried out as described in Materials and Methods. * $P < 0.05$; treatment vs basal cAMP level (8.9 ± 0.9 pmol/$10^6$ cells). The data are means ± SEM of N = 6 observations.

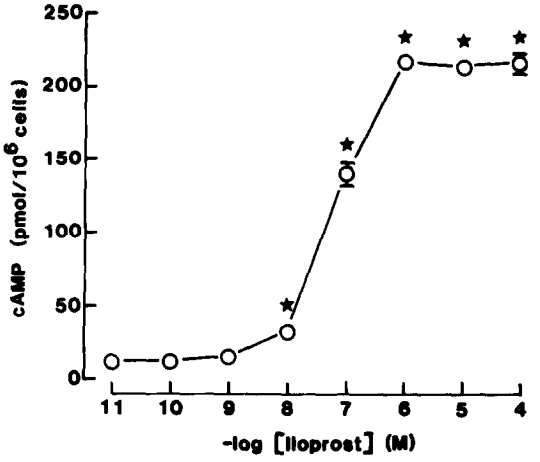


Fig. 4. Effect of iloprost on cAMP levels in LLC-$PK_1$ pig kidney epithelial cells. Incubations were carried out as described in Materials and Methods. * $P < 0.05$; treatment vs basal cAMP level (11.0 ± 0.9 pmol/$10^6$ cells). The data are means ± SEM of N = 6 observations.

Table 1. Effect of A23187, iloprost and forskolin on intracellular and extracellular levels of cAMP in porcine aortic endothelial cells

| Addition | cAMP (pmol/$10^6$ cells) | |
|---|---|---|
| | Intracellular | Extracellular |
| None (control) | 9.6 ± 1.0 | 4.9 ± 0.7 |
| A23187 (10 μM) | 8.4 ± 0.8 | 4.3 ± 0.5 |
| Iloprost (10 μM) | 10.0 ± 0.9 | 4.8 ± 0.6 |
| Forskolin (100 μM) | 111.4 ± 6.4* | 7.0 ± 1.5 |

Incubations were carried out as described in Materials and Methods. * Denotes a significant change ($P < 0.05$) in cAMP level as compared to control value. The data are means ± SEM of N = 6 observations.

produced a substantial increase in endothelial cAMP synthesis. This finding demonstrates the existence of active adenylate cyclase in endothelial cells and precludes desensitization of this enzyme on the post-receptor level as an explanation for the observed unresponsiveness to $PGI_2$. Moreover, an increased cAMP efflux, which could have masked the stimulation of intracellular cAMP, did not occur. Another possible explanation for the observed lack of effect of $PGI_2$ on endothelial cAMP is the loss or down-regulation of $PGI_2$ receptors on endothelial cells in culture as has been shown in the case of muscarinic receptors [9]. However, in LLC-$PK_1$ pig kidney epithelial cells, which were maintained under the same conditions as the endothelial cells, $PGI_2$ elevated cAMP in a concentration-dependent and saturable manner, arguing against an artificial loss of $PGI_2$ binding sites under our cell culture protocol. Moreover, the receptor-dependent agonist $PGE_2$ was found to stimulate cAMP in endothelial cells demonstrating that endothelial prostaglandin receptors are probably well preserved in culture.

Our findings are in contrast to previous investigations reporting endothelial cAMP stimulation by $PGI_2$ [11, 12]. However, these authors were unable to demonstrate a $PGI_2$-dependent activation of adenylate cyclase in endothelial membrane preparations which is postulated for a receptor-dependent activation of the adenylate cyclase/cAMP system and has been shown in other cell types such as platelets [2, 27]. Thus, it seems questionable as to whether the increase in endothelial cAMP observed in the above mentioned studies is actually due to stimulation of $PGI_2$ receptors. Although little is known about the distribution and possible species differences of $PGI_2$ receptors in the vasculature, recent findings suggest the absence of $PGI_2$ receptors in vascular endothelial membranes [13]. These observations are in line with the lack of $PGI_2$-induced cAMP stimulation in endothelial cells reported here.

Although cAMP has been shown to attenuate autacoid release from endothelial cells [15], our results suggest that the release of the autacoid $PGI_2$ is not controlled in a negative feedback loop. Whereas for NO, being cytotoxic and a promoter of severe hypotension during septic shock [28, 29], a fast acting switch-off mechanism seems to be useful, cytoprotective $PGI_2$ [30] may not require a similar regulatory pathway via endothelial cAMP. This may be particularly important for the pharmacological use of $PGI_2$-stimulating drugs since, according to our results, attenuation of their vasodilatory and platelet inhibitory effects via endothelial cAMP stimulation will not occur.

In summary, our results demonstrate a fundamental difference in the action of the two autacoids NO and $PGI_2$. Whereas NO leads to autocrine stimulation of endothelial cGMP, $PGI_2$ acts as a strictly paracrine hormone without affecting the respective second messenger cAMP in the endothelial generator cell.

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