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# ENDOTHELIN-1 INDUCES PROSTACYCLIN RELEASE FROM BOVINE AORTIC ENDOTHELIAL CELLS

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**SUMMARY**: The effects of endothelin-1 (ET-1) on the release of prostacyclin from cultured bovine aortic endothelial cells were studied. ET-1 induced a time- and dose-dependent release of 6-keto $PGF_{1\alpha}$, the stable metabolite of prostacyclin, with an apparent $EC_{50}$ value of 3.0 ±0.9 nM (n=6). ET-1 up to a concentration of 500 nM did not affect cellular integrity. Preincubation of the cells for 30 min with 10 $\mu$M indomethacin inhibited ET-1 (100 nM) - induced prostacyclin release by 90 % . These findings indicate that ET-1 can directly stimulate prostacyclin release from endothelial cells probably through a receptor mediated mechanism. 

Endothelin-1 (ET-1) first isolated from conditioned medium of cultured vascular endothelial cells (1) is now well characterized as a potent constrictor of vascular and non-vascular smooth muscles both in vivo and in vitro (c.f. 2). In addition to its contractile action, ET-1 has been shown to inhibit ex vivo aggregation of rabbit platelets (3) as well as in vivo platelet aggregation in the dog (4) and rabbit (5), whereas it has no effect on in vitro platelet aggregation (3,4). Since indomethacin and aspirin can block the antiaggregatory action of ET-1, the involvement of an antiaggregatory cyclooxygenase product, presumably prostacyclin, has been suggested (3,4). This view is further supported by the findings that the in vivo anti aggregatory action of ET-1 in the dog was accompanied by enhanced plasma levels of 6-keto $PGF_{1\alpha}$, the hydrolytic metabolite of prostacyclin (4). The present experiments were designed to study the effects of ET-1 on prostacyclin release from monolayer

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of cultured endothelial cells and to compare the effects of ET-1 to those of thrombin, a potent stimulus of prostacyclin synthesis (6).

## MATERIALS AND METHODS

**Drugs and chemicals**. Synthetic endothelin-1 (ET-1, Peninsula Labs, Belmont, CA) was dissolved in distilled water and further diluted in 0.15 M NaCl. Thrombin (Thrombostat) was purchased from Parke Davis, Scarborough, Ont. Indomethacin (Merck Frosst, Montreal, Canada) was dissolved in ethanol and diluted with 0.15 M NaCl. The final concentration of ethanol in the culture medium was 0.5 %. Medium 199 and lactate dehydrogenase kit were purchased from Sigma Chemical Co., St. Louis, MO; fetal calf serum from Gibco, Grand Island, N.Y. and 6-keto $PGF_{1\alpha}$ antiserum from Cayman Labs, Ann Arbor, MI.

**Endothelial cell culture**. Bovine aortic endothelial cells were obtained from collagenase-treated (300 U/ml for 15 min at 37°C) bovine aortae (9), seeded in T 75 culture flasks (Falcon) and grown in Medium 199 supplemented with 15 % fetal calf serum (heat inactivated at 56°C for 30 min), L-glutamine 2 mM, penicillin 100 U/ml, streptomycin 0.1 mg/ml and fungizone 2.5 $\mu$g/ml. After achieving confluence, the cells were detached by using EDTA/trypsin treatment (0.4 mM / 0.04 % in HBSS without $Ca^{2+}$ and $Mg^{2+}$) for 3 min at 37°C, and were subcultured for up to four times. The cells were finally seeded in 6-well plates (Falcon) with the same medium. The identity of endothelial cells grown in this fashion was verified by morphology and by demonstration of angiotensin-converting enzyme activity. The viability of cells was greater than 95 % as assessed by trypan blue exclusion.

**Experimental protocols**. When the cells reached confluence, the culture medium was replaced 8 h before the experiments. Cells were incubated for the indicated time period with ET-1 (0.1-500 nM), thrombin (0.01-0.1 U/ml) or their vehicle. ET-1 concentrations higher than 500 nM were not studied because of the limited availability of the peptide. In the experiments with indomethacin, the endothelial cells were preincubated for 30 min with 10 $\mu$M indomethacin before challenge with 100 nM ET-1. Only one concentration of agonists was added to each well. At the end of the incubation period, aliquots of the medium were removed to determine concentrations of 6-keto $PGF_{1\alpha}$, the stable metabolite of prostacyclin and lactate dehydrogenase activity, respectively. The samples for 6-keto $PGF_{1\alpha}$ were stored at - 20°C until assayed. Lactate dehydrogenase activity was measured on the day of the experiments using a commercial kit.

**Measurement of 6-keto $PGF_{1\alpha}$**. Concentrations of 6-keto $PGF_{1\alpha}$ were measured by a direct enzyme immunoassay according to the method of Pradelles et al.(8) . The detection limit of the assay was 0.3 pg/tube. Intrassay and interassay coefficients of variation were 5.6 % (n=5) and 9.4 % (n=4), respectively. The 6-keto $PGF_{1\alpha}$ antiserum cross-reacted with $PGF_{2\alpha}$ 12 %, 6,15-diketo $PGF_{1\alpha}$ 8 % and $TXB_2$ 0.2 %. Prostacyclin production was calculated as the difference between the amount of 6-keto $PGF_{1\alpha}$ released by cells challenged with the agonist minus the amount released by identically treated but unchallenged cells.

**Statistical analysis**. Values are reported as means ± SEM. Statistical analysis of the data was performed by Dunn multiple contrast hypothesis test (9) or by Mann-Whitney U test. A $p < 0.05$ level was considered significant for all tests.

## RESULTS

### Endothelin-1-induced prostacyclin release

Initial studies demonstrated that 100 nM ET-1 is capable of releasing prostacyclin from cultured bovine endothelial cells in a time-dependent manner (Fig.1). Enhanced prostacyclin production was observed at 1 min of incubation, and the maximal effect was reached within 15 min (Fig.1). Similar time course of changes in prostacyclin release was observed when cells were stimulated with thrombin (Fig.1). The stimulatory effect of 100 nM ET-1 was calculated to be equivalent to that of 0.03-0.04 U/ml of thrombin.

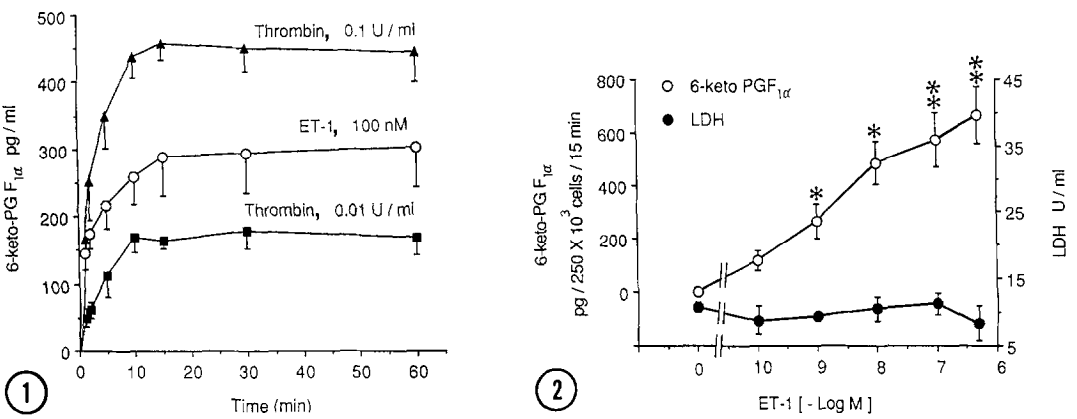


**Fig. 1**. Time course of 6-keto $PGF_{1\alpha}$ release from cultured bovine aortic endothelial cells by endothelin-1 (ET-1) and thrombin. Cells were seeded in 6-well plates, incubated in 1.9 ml of incubation medium and were stimulated with ET-1 (100 nM) or thrombin (0.01 and 0.1 U/ml). At the indicated times an aliquot was removed for determination of 6-keto $PGF_{1\alpha}$ concentration. Values were calculated as the difference between 6-keto $PGF_{1\alpha}$ levels in the medium of stimulated cells minus that of identically treated but unstimulated cells. Values are means ± SEM of 3-6 separate experiments in triplicate.

**Fig. 2**. Effect of endothelin-1 (ET-1) on 6-keto $PGF_{1\alpha}$ production and lactate dehydrogenase (LDH) release by cultured bovine aortic endothelial cells. The culture and experimental conditions were identical to those described in the legend to Fig.1. Cells were stimulated with various concentrations of ET-1 for 15 min, then aliquots of the medium were removed for determination of 6-keto $PGF_{1\alpha}$ concentration and LDH activity. The 6-keto $PGF_{1\alpha}$ production was calculated as the difference between the amount released by stimulated cells minus that of identically treated but unstimulated cells. Values are means ± SEM of 6 separate experiments in triplicate. x, $p < 0.05$; xx, $p < 0.01$ (compared to control by Dunn multiple contrast hypothesis test).

When cells were exposed for 15 min to increasing concentrations of ET-1, a dose-dependent increase in prostacyclin release was observed (Fig.2). At a concentration as low as 1 nM, ET-1 induced significant increase in 6-keto $PGF_{1\alpha}$ levels (Fig.2). The apparent $EC_{50}$ value for ET-1 was 3.0 ±0.9 nM (n=6). ET-1 up to a concentration of 500 nM did not affect lactate dehydrogenase release from the cells (Fig.2).

**Inhibition of ET-1-induced prostacyclin release by indomethacin**

In another set of experiments, the effect of 100 nM ET-1 on prostacyclin release was compared in the absence and presence of indomethacin. Preincubation of the cells for 30 min with 10 $\mu$M indomethacin inhibited ET-1 - induced prostacyclin formation on average by 90 % (520 ± 82 and 49 ± 20 pg 6-keto $PGF_{1\alpha}$ released by 250 000 cells per 15 min in the absence and presence of indomethacin, respectively, n=4, $p < 0.05$). Neither indomethacin nor its vehicle affected lactate dehydrogenase release (data not shown).

## DISCUSSION

The present study demonstrates that ET-1 can induce time-, and dose-dependent release of prostacyclin, as measured by its major stable metabolite, 6-keto $PGF_{1\alpha}$, from cultured bovine aortic endothelial cells. Significant prostacyclin release was detected at ET-1 concentrations as low as 1-10 nM. These concentrations are comparable to those reported to inhibit in vivo platelet aggregation (3-5) or to induce prostacyclin release (4). The time course of prostacyclin release by ET-1 was similar to that of thrombin. Similarly to thrombin (6), ET-1 stimulated prostacyclin production without addition of exogenous arachidonic acid, indicating that the substrate for prostacyclin synthesis must have been endogenous arachidonic acid. The findings that ET-1 did not affect cellular viability, as evidenced by unchanged lactate dehydrogenase release, coupled with the observation that indomethacin nearly completely blocked ET-1 - induced prostacyclin release indicate that the enhanced prostacyclin formation cannot be attributed to a non-specific lytic action of ET-1 on endothelial cells, rather it was a consequence of the interaction between ET-1 and its putative membrane receptors. Although specific ET-1 receptor antagonists presently are not available, recent reports on the specific binding of ET-1 to brain capillary endothelial cells (10) lend further support to this view.

The present experiments give little information on the mechanisms by which ET-1 can stimulate prostacyclin synthesis. Indirect evidence that ET-1 can activate phospholipase $A_2$ came from experiments reporting a sustained prostacyclin release following ET-1 administration in vitro (11,12) and in vivo (4). Stimulation of phospholipase $A_2$ in cultured smooth muscle (13,14) and glomerular mesangial cells (15) has been subsequently demonstrated by adding ET-1 to cells labeled with [$^3$H]-arachidonic acid. Presently it is not known whether ET-1 activates phospholipase $A_2$ directly via a G protein or indirectly by increasing intracellular $Ca^{2+}$ concentration (16). It is also possible that ET-1 might activate phospholipase $A_2$ and C in endothelial cells by parallel but independent mechanisms as it was reported for vascular smooth muscle cells (14).

In summary, the present findings provide direct evidence that ET-1 is capable of releasing prostacyclin from endothelial cells. Thus, one may assume that ET-1 released either by endothelial or other blood-borne cells (17) may act on the same cells and can induce prostacyclin release, which in turn, may mediate and/or modulate its biological actions, including inhibition of platelet aggregation.

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