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Regulation of endothelin-1 action on the perfused rat liver

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Endothelin-1 (ET-1) was found to be a very potent stimulus for contraction and glycogenolysis in the perfused rat liver. At 1 nM it caused a dramatic increase in portal pressure of 22.1 ± 2.7 cm water and enhanced the glucose output up to 3-fold. Extracellular Ca^{2+} and protein kinase C were involved in the signal transduction of ET-1. ET-1 action does not seem to be mediated by endogenous eicosanoids. The effects of ET-1 were significantly reduced in the presence of 1 μM Iloprost, a prostaglandin I_2 analogue, or by 100 μM sin-1, a nitric oxide donor. In cultured hepatocytes, glycogenolysis was also stimulated by ET-1 although to an extent too small to explain the high glucose output found in the perfused liver.

Calcium ion; Eicosanoid; Nitric oxide; Protein kinase C; Vasoconstriction

1. INTRODUCTION

Endothelin-1 (ET-1) is a very potent vasoconstrictive peptide consisting of 21 amino acids. It was originally identified in the supernatants of porcine aortic endothelial cells [1]. After the cloning of cDNA for ET-1, two other isoforms, ET-2 and ET-3, have been found [2]. While ET-1 is mainly synthesized by endothelial cells, the site of synthesis of the other isoforms is still uncertain [3]. The mechanisms underlying the vasoconstrictive effect of ET-1 on smooth muscle cells have been reported to be due to a mobilization of intracellular Ca^{2+} following phospholipase C activation by inositol Tris-phosphate [4,5] as well as to an influx of extracellular Ca^{2+} through a calcium ion channel [6]. Protein kinase C was also reported to be involved in this process [5,7,8].

In the liver, ET-1 is synthesized by sinusoidal endothelial cells [9]. Binding sites for [^{125}I]ET-1 in the liver have been found in rat liver plasma membranes [10] and in fat-storing cells [11,12]. ET-1 induces Ca^{2+} release and stimulation of glycogen phosphorylase in isolated hepatocytes [10]. In primary cultures of hepatocytes it activates phospholipase C resulting in the generation of inositol phosphates [13]. ET-3 has been reported to induce the increase of portal vein pressure and glycogenolysis in the perfused rat liver [14] and to stimulate Kupffer cells to produce prostaglandin (PG) E_2 and inositol phosphates [15]. Recently we have shown that perisinusoidal stellate (fat-storing) cells that envelope the liver sinusoids [16] and are supposed to regulate the blood flow in the liver sinusoids [17] can contract after ET-1 administration (Kawada et al., submitted). However, it remains to be seen whether all the effects observed with ET-1 in different liver cell systems can be reproduced in the whole organ.

In the present study, we examined the effect of ET-1 on portal pressure and glycogenolysis in the perfused rat liver. Under constant flow rate, portal pressure is dependent on the diameter of vessels and sinusoids; therefore, it is used as a parameter to detect their contraction. Furthermore, the mechanism underlying the change of portal pressure and glycogenolysis induced by ET-1 in the liver as well as the regulation of ET-1 action by local mediators (eicosanoids and nitric oxide) was investigated.

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Abbreviations: ET-1, endothelin-1; NO, nitric oxide; PG, prostaglandin; sin-1, 3-morpholino-sydnoimine-*N*-ethylcarbamide; SNP, sodium nitroprusside.

2. MATERIALS AND METHODS

2.1. *Materials*

Endothelin-1 (ET-1) was purchased from Sigma (Deisenhofen, Germany). Sin-1 (3-morpholino-sydnonimine-*N*-ethylcarbamide) was a gift from Cassella AG (Frankfurt/Main, Germany). Sin-1 was dissolved just before use and was protected from light during the experiment. Collagenase type II (CLS II), fetal-calf serum and newborn-calf serum used for the preparation of hepatocyte cultures were from Biochrom (Berlin, Germany). All other chemicals used were of analytical grade.

2.2. *Animals*

Male Wistar rats weighing between 200 and 250 g fed ad libitum with Altromin were used throughout. They were from Interfauna (Tuttlingen, Germany).

2.3. *Liver perfusion*

Rat livers were perfused in a non-recirculating manner [18] using Krebs–Henseleit buffer containing 118 mM NaCl, 4.74 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.87 mM $NaHCO_3$, and 2.54 mM

$CaCl_2$, pH 7.4, equilibrated with 95% (v/v) O_2 and 5% CO_2. Perfusion pressure was monitored continuously in an open glas capillary tube (6 mm) connected to the inflow. A change of 10 cm water corresponds to 7.35 mmHg or 980.6 Pascal.

2.4. *Preparation of hepatocytes in primary culture*

Hepatocytes were prepared under aseptic conditions as described previously [19], plated on collagen-coated dishes (2.5×10^6 cells, 60 mm diameter) and kept in primary culture at 37°C. During the first 3 h, cells were incubated with 3 ml RPMI 1640 medium supplemented with 10% fetal-calf serum, penicillin 100 U/ml and streptomycin 50 μg/ml, thereafter in the same medium containing 5% newborn-calf serum. After 24 h, they were kept for 4 h in 25.5 mM glucose and 1 μM insulin to enhance the synthesis of glycogen. Cells were washed free of glucose and stimulated with ET-1 in glucose-free Hank's medium, pH 7.4. At the times indicated, glucose concentration in the medium was determined according to Kunst [20]. Protein content in primary cultures of hepatocytes was measured according to Bradford [21].

3. RESULTS AND DISCUSSION

3.1. *Endothelin-1 action on the perfused rat liver*

ET-1 administered to the isolated perfused rat liver induced an increase of the portal vein pressure and stimulated glucose output (Fig. 1, A and B). The portal pressure started to increase immediately after addition of ET-1 and reached its maximum (22.4 ± 2.7 cm water) between 20 and 25 min (Fig. 1A). After removal of ET-1 the portal pressure remained at the plateau level for 2 min and then decreased gradually. The pressure did not return to the initial level 30 min after removal of ET-1; a small but significantly elevated portal pressure (7.5 ± 1.2 cm water) was still present. The kinetics of glucose output appeared to differ from the kinetics of the change of portal pressure. Within 5 min after addition of ET-1 the glucose output was increased 3-fold. The glucose output decreased slightly during the following 20 min, rose again at 25 min and decreased gradually thereafter (Fig. 1B). The increase in glucose output and portal pressure was dose-dependent (Fig. 1, A and B). 0.1 nM ET-1 induced a maximal increase in portal pressure of 6.1 ± 1.8 cm water at 30 min and stimulated glucose output to 160% of the control value at 10 min. 10 nM ET-1 induced the same maximum of portal pressure and the biphasic glucose output as 1 nM ET-1. The effects of these two concentrations differed from each other in their kinetics only. The maximal increase of portal pressure appeared already at 12 min after stimulation with 10 nM ET-1.

3.2. *Effect of extracellular Ca^{2+} on endothelin-1 action*

ET-1 was reported to induce the influx of extracellular Ca^{2+} into smooth muscle cells [1,6]. To test whether extracellular Ca^{2+} was also required for the increase of portal pressure and glycogenolysis, the liver was stimulated by 1 nM ET-1 in the absence of Ca^{2+}. Unspecifically bound Ca^{2+} was removed before stimulation with ET-1 by a 5 min perfusion in Ca^{2+}-free medium containing 2 mM EGTA. Under these conditions, ET-1 induced just a small increase of portal pressure (maximal level, 2.5 ± 0.8 cm water) (Fig. 1C). The functional integrity of the liver after treatment with Ca^{2+}-free medium was checked by stimulating the liver with 1 nM ET-1 after re-addition of Ca^{2+}. As shown in Fig. 1C, the liver pretreated with Ca^{2+}-free medium was fully responsive to ET-1 when the perfusion buffer was supplemented with Ca^{2+}; the maximal increase in portal pressure was 18.6±3.8 cm water. Extracellular Ca^{2+} was also necessary for the increased glucose output induced by ET-1; in the absence of Ca^{2+} the ET-1-elicited glucose output was suppressed (Fig. 1D, diamonds). Addition of Ca^{2+} to the medium restored the increase of glucose output. Thus, extracellular Ca^{2+} is necessary for the action of ET-1.

3.3. *Effect of a protein kinase C inhibitor on endothelin action*

In vascular smooth muscle cells, ET-1 has been reported to activate phospholipase C producing inositol phosphates and diacylglycerol [4,5]. Diacylglycerol activates protein kinase C leading to the phosphorylation of several proteins including filamentous proteins of the cytoskeleton and glycogen phosphorylase kinase [22]. Recent reports suggest that the protein kinase C-dependent phosphorylation of filamentous proteins is responsible for the long-lasting contraction of vessels that is characteristic for ET-1 action [8,23]. In stellate cells ET-1 strongly activates phospholipase C (Kawada et al., submitted). To test whether protein kinase C is involved in the increase of portal pressure and glucose

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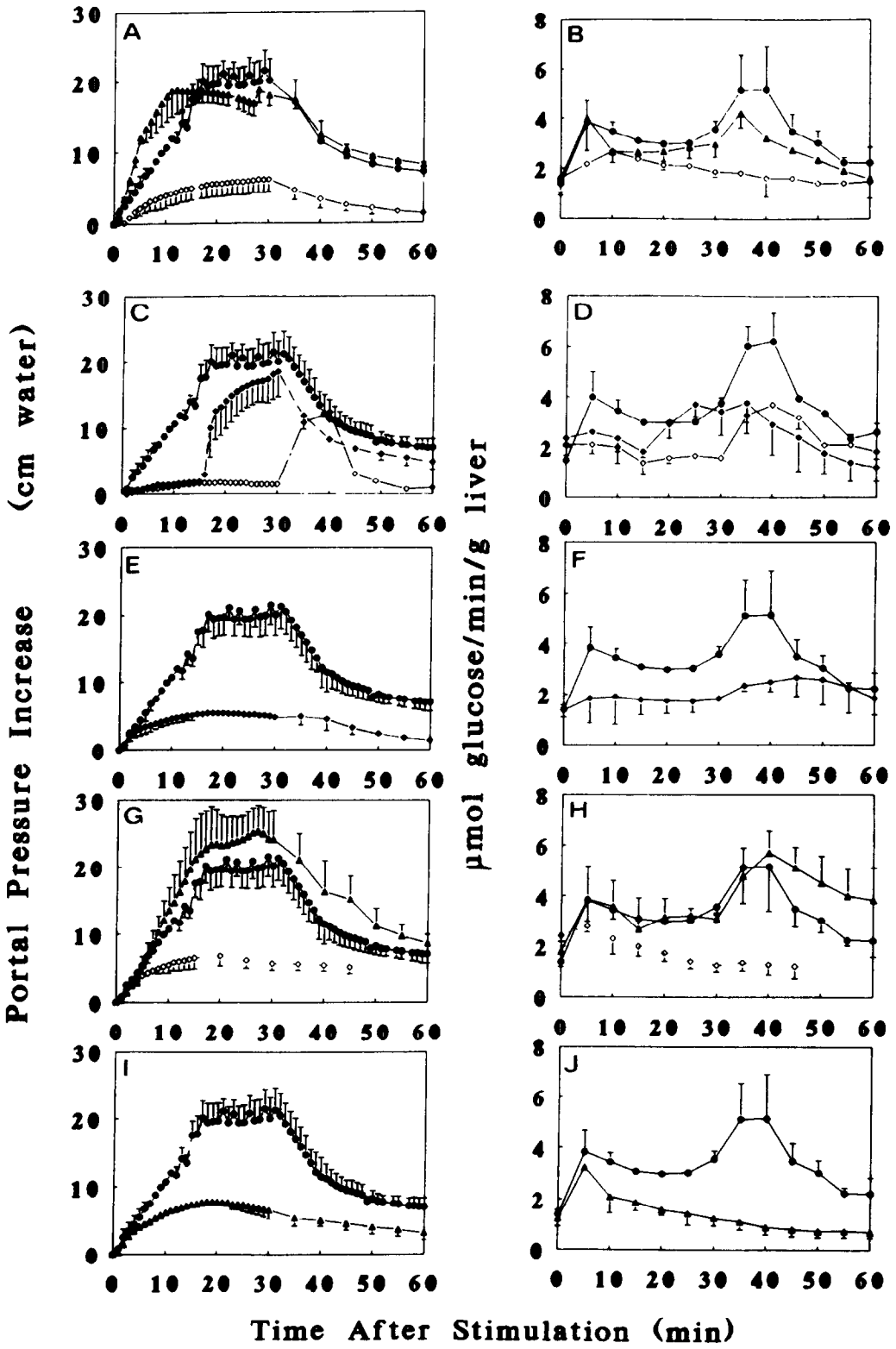
Fig. 1. Increase of portal pressure and glucose output after stimulation with ET-1 in the presence of different effectors. The livers were preperfused for 30 min before addition of ET-1. After the indicated times of exposure to ET-1, perfusions were continued for 30 min without any effector. Portal pressure and glucose output were determined as described in Materials and Methods. The change of portal pressure was determined in A, C, E, G and I. Glucose output was determined in B, D, F, H and J. The rate of glucose output by the untreated perfused liver was constant with a coefficient of variation of 11.5% during the experimental period. The closed circles refer to stimulation with 1 nM ET-1. Bars represent the S.D. ($n = 3$). (A and B) Livers were perfused with 10 nM ET-1 (closed triangles), or 0.1 nM ET-1 (open diamonds) for 30 min. (C and D) The livers were stimulated either with ET-1 for 30 min in Ca^{2+}-free medium and thereafter in buffer containing Ca^{2+} (open diamonds), or for 15 min in Ca^{2+}-free medium, then stimulated a second time with 1 nM ET-1 in the presence of Ca^{2+} (closed diamonds). To remove unspecifically bound Ca^{2+}, livers were perfused for 5 min with 2 mM EGTA before stimulation in Ca^{2+}-free medium. (E and F) Livers were stimulated with 1 nM ET-1 and 20 nM staurosporin (closed diamonds). (G and H) Stimulation was performed for 30 min in the presence of 1 nM ET-1 and 50 μM indomethacin (closed triangles) or for 15 min in the presence of 1 nM ET-1 and 1 μM Iloprost (open diamonds). (I and J) Livers were perfused with 1nM ET-1 and 100 μM sin-1 (closed triangles).

output, stimulation of the perfused liver with ET-1 was carried out in the presence of 20 nM staurosporin, an inhibitor of protein kinase C (Fig. 1E and F). The increase of portal pressure and glucose output was almost totally inhibited in the presence of staurosporin. These results indicated that both extracellular Ca^{2+} and pro-



tein kinase C activity were required for the reactions induced by ET-1 in the perfused liver.

3.4. *Endothelin-1 and eicosanoids*

Stimulated non-parenchymal liver cells are able to produce eicosanoids (for a review, see [24]) that induce vasocontraction and glycogenolysis in the perfused liver, e.g. thromboxane A_2 [25]. Since ET-3, an isoform of ET-1, has been reported recently to induce PGE_2 production in Kupffer cells [14], one can assume that the effect of ET-1 is mediated by eicosanoids. For this reason, the effects of ET-1 were examined in the presence of 50 μM indomethacin, an inhibitor of cyclooxygenase (Fig. 1G and H). However, neither the increase in portal pressure nor the stimulation of glycogenolysis induced by ET-1 were affected by indomethacin. On the contrary, the ET-1-mediated elevation of portal pressure was consistently, though not in a highly significant manner, enhanced in the presence of indomethacin suggesting that some endogenous eicosanoids might reduce the contraction induced by ET-1. As Iloprost, a PGI_2 analogue, led to relaxation of precontracted stellate cells in culture [26], we tested whether it counteracted the elevation of portal pressure caused by ET-1 (Fig. 1G). One μM Iloprost suppressed the maximal increase of portal pressure induced by ET-1 from 22.4 ± 2.7 to 6.8 ± 1.4 cm water. Similarly the stimulation of glucose output was strongly reduced in the presence of Iloprost (Fig. 1H). Therefore, we assume that endogenous PGI_2 antagonizes the effect of ET-1 in the liver. However, further experiments may be required to show whether ET-1 stimulates the formation of endogenous PGI_2 in the perfused liver.

3.5. *Endothelin-1 and nitric oxide*

Nitric oxide (NO) is a novel vasodilatory factor produced by endothelial cells [27,28], platelets, activated macrophages and neutrophiles (for a review, see [29]). When NO is released from endothelial cells, it easily diffuses into smooth muscle cells underlying endothelial cells and leads to cell relaxation by increasing cGMP via the activation of soluble guanylate cyclase [30]. In the liver, hepatocytes from rats pretreated with *Corynebacterium parvum* were shown to produce nitrite and nitrate, the oxidation products of NO [31]. Stimulated Kupffer cells also synthesized NO [32,33]. On the other hand, SNP and sin-1, two substances which liberate NO, can react with precontracted stellate cells to induce relaxation (Kawada et al., submitted). For this reason, we examined whether NO can affect the increase of portal pressure and glycogenolysis induced by ET-1. 100 μM sin-1 markedly reduced the maximal increase of portal pressure caused by ET-1 from 22.4 ± 2.7 to 7.8 ± 0.5 cm water (Fig. 1I). The increased glucose output by ET-1 was only slightly reduced at an initial stage, but afterwards it gradually decreased to the basal level (Fig. 1J). The results indicate that contraction induced by ET-1 in the perfused liver can be inhibited by the action of a NO donor. It is not known whether ET-1 can induce the production of NO to counterbalance the contraction elicited in this system.

3.6. *Endothelin-1 and bile flow*

ET-1 also affected bile flow during stimulation with ET-1 (Table I). The extent of bile flow seems to be correlated with portal pressure. In the presence of vasodilatory agents like sin-1 and Iloprost, the biliary flow rate was restored to normal values. One can assume that in vivo ET-1 not only leads to vasocontraction but also, perhaps as a secondary phenomenon, to glycogenolysis and a reduced bile flow.

3.7. *Effect of endothelin-1 on hepatocytes in primary culture*

One or 10 nM ET-1 stimulated the rate of glucose output of hepatocytes only to a small extent (Fig. 2A); at 5 min to 123% and 125%, and at 30 min to 117% and 114% of the control value, respectively. Even with higher concentration (50 nM), the degree of stimulation was the same as that for 1 or 10 nM ET-1. These values were much lower than those elicited by 2 μM glucagon (320% of control, Fig. 2B). Obviously, the strong increase of glucose output by the liver is mediated only in part by a direct effect of ET-1 on hepatocytes; the first glucose peak that appeared 5 min after ET-1 addition mirrors ET-1 action. At that time, the increase of the portal pressure was 25% (5.5 ± 1.2 cm H_2O, $n = 3$) of the maximum. Furthermore, the maximal increase of glycogen phosphorylase activity has been found 5 min after ET-1 [10]. The second peak of glucose output starting 25 min after ET-1 addition seems to be due to other mechanisms that are related to the change of portal pressure, e.g. hypoxia in some sinuoids.

3.8. *Concluding remarks*

ET-1 is a very potent agonist for vasoconstriction and glycogenolysis in the liver acting at nanomolar concentrations. The strong increase in portal pressure caused by ET-1 in the perfused liver could be due to the con-

Table I

Influence of vasocontraction on bile flow

Effectors	Bile flow (μl/min)	Increase of portal pressure (cm water)
None	9.8 ± 1.0 (n = 11)	± 0 (n = 16)
1 nM ET-1	5.6 ± 1.4 (n = 3)	22.4 ± 2.7 (n = 3)
1 nM ET-1 + 1 μM Iloprost	8.0; 9.0	6.8 ± 1.4 (n = 3)
1 nM ET-1 + 100 μM Sin-1	9.7; 11.3	7.8 ± 0.5 (n = 3)

The livers were perfused under the conditions described in the legend of Fig 1 The values represent means ± S.D.; in two instances (n = 2), bile flow data are given individually.

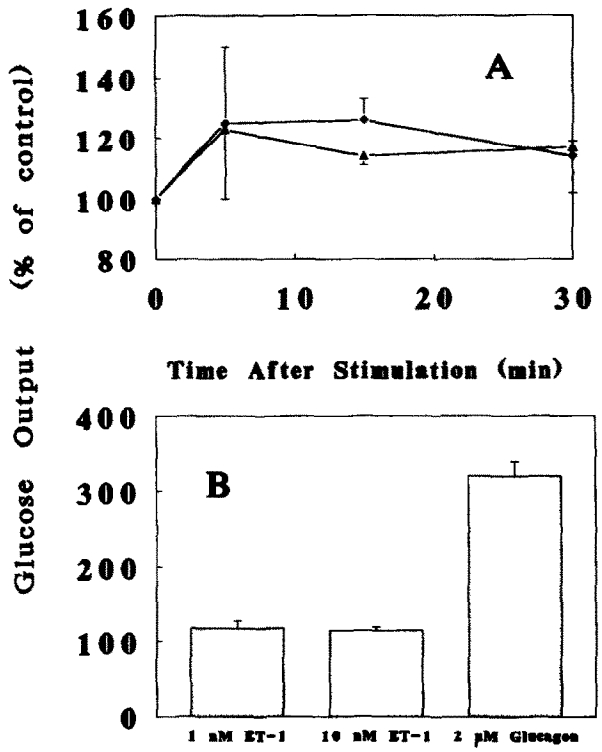


Fig. 2. Effect of ET-1 and glucagon on glycogenolysis in hepatocytes in primary cultures. Culture conditions were described in Materials and Methods. (A) Time course of glycogenolysis with different ET-1 concentrations. Closed diamond, 10 nM ET-1; closed triangle, 1 nM ET-1. 100% referred to the glucose output in non-stimulated cells. Basal cumulative glucose output was 11 ± 1, 16 ± 6, 24 ± 9 and 43 ± 14 nmol/mg protein at 0, 5, 15, and 30 min, respectively. Mean values from 3 independent cell batches ± S.D. are given. Note that the rate of glucose output was almost constant during the experimental period. (B) Comparison of glycogenolysis induced by ET-1 and glucagon. Glucose was measured after 30 min incubation with effectors.

traction of stellate cells that envelope the liver sinusoids. However, one can not exclude the possibility that ET-1 also reacts with smooth muscle cells of hepatic blood vessels. Extracellular Ca^{2+} and protein kinase C were required for vasocontraction and glycogenolysis. The effects of ET-1 could be reduced by sin-1, a NO donor, and Iloprost, a PGI_2 analogue. Since both, NO and PGI_2, can be synthesized in the liver, it is tempting to assume that they are formed simultaneously *in vivo* to counterbalance the action of ET-1.

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