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The insulinotropic effect of endothelin-1 is mediated by glucagon release from the islet alpha cells

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Abstract

Aims/hypothesis. The circulating concentrations of endothelin-1 (ET-1), a peptide derived from endothelium, are increased in hypertension and diabetes. Endothelin-1 has recently been shown to be an insulinotropic agent. The mechanism of action of endothelin-1 on the endocrine pancreas has not yet been clarified.
Methods. We investigated the action of endothelin-1 on the insulin secretion, the binding of ^{125}I-ET-1 to beta cells as well as its effects on purified beta and non-beta cells from normal rats. The expression of endothelin receptors in alpha- and beta-cell lines and in normal rat islets was also studied.
Results. First, we studied the effects of endothelin-1 on insulin secretion from beta-cell lines (INS-1, βTC3 and MIN6). At all endothelin-1 concentrations applied (1 pmol/l to 1 μmol/l) no change in insulin secretion was found. Ligand-binding experiments on βTC3 cells showed no specific binding of ^{125}I-ET-1. A prominent expression of ET_A-receptor mRNA in an alpha-cell line (αTC1.9) and in normal rat islets was found whereas no expression was found in INS-1 cells. No influence of endothelin-1(1 μmol/l) on insulin secretion stimulated by glucose was detected from purified beta cells. Endothelin-1-(100 nmol/l) increased, however, both insulin and glucagon secretion from a mixture of purified beta and non-beta cells indicating that alpha cells seem to have a key role for the action of ET-1 on insulin secretion.
Conclusion/interpretation. The insulinotropic impact of endothelin-1 is not caused by a direct action on the beta cells but seems to be mediated by a paracrine action, probably secondary to enhanced release of glucagon from the endothelin receptor positive alpha cells. [Diabetologia (1999) 42: 1302–1307]

Keywords Endothelin-1, insulin secretion, glucagon secretion, flow cytometry, alpha cell, beta cell, clonal cell line, endothelin receptor, gene expression.

Endothelin-1 (ET-1) is a peptide derived from endothelium and is part of a family of potent endogenous vasoconstrictor peptides [1]. It has been implicated in the pathogenesis of vascular damage in hypertension and diabetes mellitus. Consequently some studies have shown increased concentrations of circulating ET-1 in both Type I (insulin-dependent) and Type II (non-insulin-dependent) diabetes mellitus [2–4] but others have not been able to show such a relation [5]. Recently, we showed that ET-1 is capable of potentiating insulin secretion, stimulated by glucose, from isolated mouse islets [6] pointing to a direct action on the beta cells. The finding that ET-1 possesses the ability to change glucose metabolism either secondarily to increasing the circulating concentrations of insulin [7, 8] or by modulation of insulin sensitivity [9, 10] underline the importance of clarifying how this peptide influences the endocrine pancreas. The question asked here was whether this peptide potentiates insulin secretion directly by action on the islet beta cells or indirectly by interaction with other islet cell types, e. g. the alpha cells. We therefore stud-

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Abbreviations: ET-1, endothelin-1.

ied whether ET-1 modulates insulin secretion, stimulated by glucose, from beta cells and from purified rat islet cells. To study the possible existence of ET receptors on the beta cells we also did ligand-binding experiments and used reverse transcriptase polymerase chain reaction (RT-PCR) to study the expression of ET-receptor mRNA.

Materials and methods

Preparation and incubation of purified islet cells. Islets were isolated from male Lewis rats weighing 170–180 g (M&B, Denmark) using the collagenase digestion technique. They were incubated overnight at 4 °C, 95 % air/5 % CO_2 in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 10 % fetal calf serum (Life Technologies, Md., USA). After treatment with trypsin (0.02 mg/ml; Boehringer Mannheim, Mannheim, Germany) at 4 °C the cells were sorted by a fluorescence-activated cell sorting system (FACStar Plus, Becton Dickinson, Mountain View, Calif., USA) to give purified beta and non-beta cells [11]. For incubation experiments with ET-1 the purified beta cells were seeded at a density of 0.1 million cells/vial in 96-well plates (NUNC, Roskilde, Denmark). In experiments with mixtures of beta and non-beta cells the density was 62 000 beta /38 000 non-beta cells per vial. After overnight culture at 37 °C, 95 % air/ 5 % CO_2 in RPMI 1640 supplemented with 10 % fetal calf serum the cells were washed with a modified Krebs-Ringer Buffer (KRB) and used for incubation studies. The modified KRB contained 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.28 mmol/l $CaCl_2$, 25 mmol/l HEPES and human serum albumin (Boehringer Mannheim), pH 7.4. Pre-incubations were carried out for 60 min at 37 °C, 95 % air/ 5 % CO_2 in 200 µl of the modified KRB supplemented with 3.3 mmol/l glucose followed by incubations for 60 min at 37 °C in 95 % air/ 5 % CO_2 in 200 µl of the modified KRB supplemented with glucose and ET-1 (Sigma Chemical, St. Louis, Mo., USA). After this 150 µl of the incubation medium was removed for analyses of insulin and glucagon.

Incubation studies on cell lines. The INS-1 cells [12] were grown in monolayer in a modified RPMI 1640 medium (Gibco) supplemented with 10 % (v/v) fetal calf serum 10 mmol/l HEPES, 100 IU/ml penicillin, 100 mg/ml streptomycin (all Life Technologies), 1 mmol/l sodium pyruvate and 50 mmol/l β-mercaptoethanol in 24-well plates (NUNC). The MIN6-cells [13] were grown in RPMI 1640 and βTC3-cells [14] and αTC1.9 cells [15] in DMEM (Gibco), both supplemented with 10 % fetal calf serum. The cells were seeded at a density of 200 000 cells 2 days before the incubation experiments. The incubation experiments were carried out as described above for purified islet cells.

Ligand binding. We used βTC3 cells for competition studies with ^{125}I-ET-1 and cold ET-1. The cells were seeded onto 6-well plates 2 days before the experiments at a density of 1 000 000 cells per well. The wells were rinsed with a binding buffer containing 130 mmol/l NaCl, 4.7 mmol/l KCl, 1.4 mmol/l $MgSO_4$, 2.5 mmol/l NaH_2PO_4, 10 mmol/l HEPES, 1 mmol/l EGTA and 3.0 mmol/l *D*-glucose before the binding experiments. The experiments were carried out for 120 min at 4 °C in the above mentioned binding buffer supplemented with human serum albumin (Boehringer) and 50 000 cpm/ml of ^{125}I-ET-1 or ^{125}I-glucagon (both with a specific activity of 74 TBq/mmol; Amersham International, Bucks, UK). After rinsing the cells with ice-cold binding buffer, 1.0 ml NaOH was added and the plates were left for 30 min. The solution was then used for γ-counting and subsequent protein analysis. The binding of ^{125}I-glucagon and its displacement by cold glucagon (Sigma) was used as control.

RT-PCR assay for detection of ET_A-receptor mRNA. We isolated RNA from rat islets, the beta-cell line INS-1 and from an alpha-cell line αTC1.9 using TriZol reagent (Gibco). The ET_A-receptor primers used in the polymerase chain reaction (PCR) spanned a product of 310 base pairs. The sense ET-primer (5'GCCATTGAAATCGTCTCCATC) corrresponds to mouse ET-receptor cDNA 16 to 36 and the antisense (5'TTTGCCACTTCTCGACGCTG) corresponds to base-pair 307 to 326 (GeneBank Accession no. L20340). The reverse transcriptase was done using random hexamer primers. The cDNA product was amplified by addition of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Brandsburg, N.J., USA). Each cycle of amplification consisted of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension for 2 min (74 °C). We ran 36 cycles and the final cycle was terminated by a 6-min extension period (74 °C). Finally, 8 µl of the PCR product was loaded on 2 % agarose gel, stained with ethidiumbromide and photographed. A similar protocol was used for negative controls except that the reverse transcriptase was omitted.

Insulin and glucagon assays. Insulin was measured by radioimmunoassay with a guinea-pig, anti-porcine insulin antibody and mono-^{125}I-(Tyr A14)-labelled human insulin as tracer and rat insulin as standard. Free and bound radioactivity was separated using ethanol. Glucagon was measured by radioimmunoassay (Linco Res., Mo., USA). Endothelin-1 in the concentrations studied did not interfere with the assays.

Protein analysis. The cells were lysed in 0.1 mol/l NaOH and total protein was measured by Bratfords method using Bio-Rad Protein assay Dye reagent (Bio-Rad Laboratories, Hercules, Calif., USA).

Statistics. To avoid variation from day to day in the insulin output from the purified islet cells, the results were related to the mean output on that particular day. The data hereby obtained were analysed using Students unpaired *t* test. Insulin secretion from beta-cell lines was analysed by ANOVA and Newman-Keuls test. The level of significance was set at $p < 0.05$. Results are expressed as means ± SEM.

Results

Effects of ET-1 on insulin secretion from clonal beta-cell lines. The effect of ET-1 on insulin secretion from INS-1 cells was studied in the presence of 16.7 mmol/l glucose (Fig. 1). Glucose (16.7 mmol/l) stimulated insulin secretion by approximately 120 % compared with 3.3 mmol/l glucose ($p < 0.01$). When applied at 16.7 mmol/l glucose ET-1 (1 pmol/l to 1 µmol/l) did not change the insulin secretion. In contrast, as expected, carbacholin (100 µmol/l) potentiated insulin secretion stimulated by glucose ($p < 0.05$). In addition, we studied the effects of ET-1 on insulin secretion stimulated by glucose from two other beta-

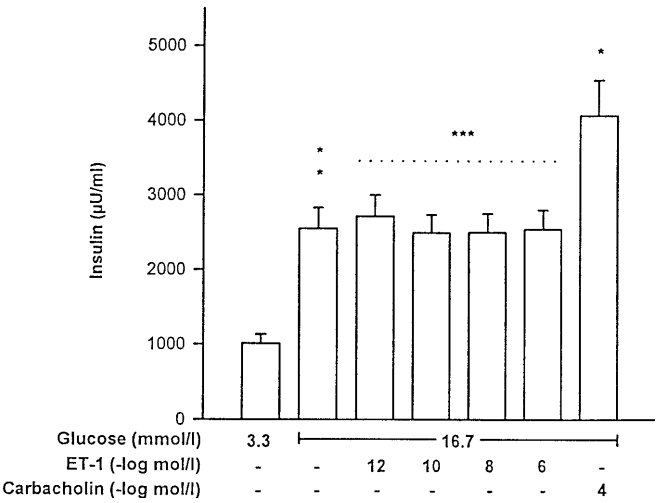

Fig. 1. Effects of ET-1 (1 pmol/l to 1 μmol/l) and carbacholine (100 μmol/l) on insulin secretion from INS-1 cells incubated for 60 min at 3.3 or 16.7 mmol/l glucose. The columns show means ± SEM from four separate experiments each containing eight single incubations. *$p < 0.05$ compared with 16.7 mmol/l glucose itself, **$p < 0.01$ compared with 3.3 mmol/l glucose itself, ***not significantly different from 16.7 mmol/l glucose itself

cell lines, βTC3 and MIN6 with identical observations, i.e. there was no change in insulin secretion (Table 1).

Studies on insulin secretion from purified beta cells. When expressed in arbitrary units (per cent change from mean of the entire data set, (see Methods section), an increase in glucose from 3.3 to 16.7 mmol/l stimulated insulin secretion two-fold (0.57 ± 0.05 vs. 61.19 ± 0.13; $p < 0.05$) from the purified normal rat beta cells. When ET-1 (100 nmol/l) was added in the presence of 16.7 mmol/l glucose no change in insulin release could be detected (1.19 ± 0.13 vs. 1.21 ± 0.97). The number of single incubations were six to nine done in six separate experiments.

Studies on insulin and glucagon secretion from mixed, purified, beta and non-beta cells. When purified beta and non-beta cells were mixed at a ratio of 5:3 at the prevailing glucose concentration of 16.7 mmol/l, ET-1 (100 nmol/l) potentiated both insulin (in arbitrary units, see above) from 1.00 ± 0.05 to 1.28 ± 0.09 ($p < 0.05$) and glucagon secretion from 0.84 ± 0.03 to 1.06 ± 0.09 ($p < 0.05$). The number of single incubations were 12–13 done in eight separate experiments.

Displacement studies. Displacement of ^{125}I–ET–with cold ET-1 on βTC3–cells was also carried out. There was a lack of displacement of ^{125}I–ET–with ET-1 at the concentrations used (10 pmol/l-1 μmol/l) (Fig. 2A). The relatively low total binding and the lack of displacement show that there is no specific binding of ^{125}I–ET–to the βTC3 cells. As a positive control we found a clear displacement of ^{125}I–glucagon by cold glucagon (1 pmol/l to 1 μmol/l) (Fig. 2B).

RT-PCR for ET_A-receptor mRNA. A prominent signal appeared at the appropriate size in the two lanes where cDNA from αTC1.9-cells was loaded (lanes 3 and 4) (Fig. 3). No signal was seen in INS-1 cells (lanes 5 and 6) whereas normal rat islets also showed a clear signal for ET_A-receptor mRNA (lanes 7 and 8). No signal was seen in the lane where the reverse transcriptase was omitted (lane 2).

Discussion

Endothelin-1, an ubiquitous peptide, has been proposed to be involved in many different regulatory functions both in the central nervous system as a neurotransmitter [16] and in the periphery as a potent vasoactive substance derived from endothelium [1]. This peptide can modulate the release of several hormones such as growth hormone [17], parathyroid hormone [18] and testosterone [19, 20]. Regarding the potential impact of ET-1 on the endocrine pancreas, recent work has shown the capability of ET-1 to increase insulin concentrations in vivo in the rat [7, 8] and dog [21]. This peptide possibly also changes glucose metabolism immediately by lowering insulin sensitivity as shown in healthy humans [10] and rats [9]. The insulin sensitivity in humans has, however, also been reported unchanged in response to ET-1 infusion [22]. We have recently shown that ET-1 potentiates insulin release, stimulated by glucose, from the islets [6]. A direct effect of ET-1 on

Table 1. Effects of ET-1 (1 pmol/l to 1 μmol/l) on insulin secretion from the beta-cell lines MIN6 and βTC3 incubated at 3.3 or 16.7 mmol/l glucose

ET-1	3.3 mmol/l glucose	16.7 mmol/l glucose				
(-log mol/l)	–	–	12	10	8	6
MIN6	36 ± 6	113 ± 25^{a}	106 ± 21^{NS}	108 ± 23^{NS}	101 ± 20^{NS}	102 ± 29^{NS}
βTC3	79 ± 9	132 ± 9^{a}	115 ± 8^{NS}	133 ± 7^{NS}	127 ± 8^{NS}	142 ± 10^{NS}

Insulin given in μU/ml, means ± SEM from a total of 8–12 single incubations of clonal cells done in two experiments.
[a] $p < 0.01$ (different from 3.3 mmol/l glucose)
[NS] not different from 16.7 mmol/l glucose per s

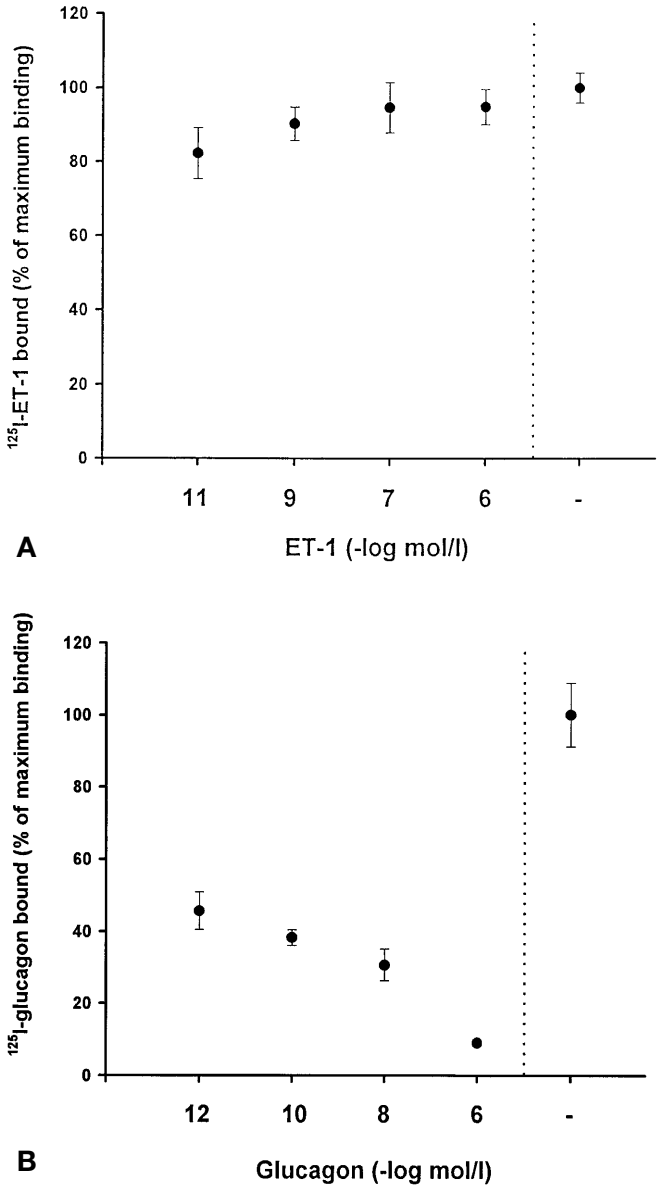

Fig. 2 A, B. Displacement from βTC3 cells of (**A**) ^{125}I-ET-1 by cold ET-1 (10 pmol/l to 1 μmol/l) or (**B**) ^{125}I-glucagon by cold glucagon (1 pmol/l to 1 μmol/l). Each point on the curves represents means ± SEM from triplicates in three separate experiments

the islet beta cells would seem to be a probable explanation underlying the immediate insulinotropic effect. Note that this study shows, however, that ET-1 is not able to change insulin release from three different beta cell lines, i.e. INS-1, MIN6 and βTC3 cells. Being aware of the risk of artefactual findings in clonal cell lines, we included studies in fluorescence-activated cell sorting purified islet cells from normal rats. Using the normal, purified beta cells, we obtained similar results, i.e. ET-1 did not change insulin secretion from beta cells. Furthermore, our receptor-binding experiments using ^{125}I-ET-1 showed a low total binding of ^{125}I-ET-1 to βTC3 cells and no displacement by cold ET-1 supporting ET-1 receptors not being present on beta cells. As a control we showed that ^{125}I-glucagon was displaced by cold glucagon. Glucagon receptors have been shown on beta-cell lines [23].

To gain further insight, we supplemented the ligand-binding experiments with studies on the expression of ET_A-receptor mRNA. Using RT-PCR we found that ET_A-receptor mRNA exists in the alpha-cell line, αTC1.9, but not in the beta–cell line, INS-1. We also showed that mRNA for the ET_A receptor is expressed in normal rat islets. Similar results were obtained using islets from normal mice (unpublished results). It is well known that ET receptors are present on endothelial cells [20] apparently, however, a significant part of the specific mRNA detected in islets originates from alpha cells.

Taken together, our results seem to rule out that this peptide has any direct action on the islet beta cells. Instead our findings point to the possibility that another islet cell type might be involved in the insulin secretion induced by ET-1 from intact islets. This is rendered probable by the finding that ET-1 was capable of stimulating insulin secretion when purified rat beta and non-beta cells were mixed. The purified non-beta cells consisted mainly (approximately 90 %) of alpha cells [11] and it is therefore likely that the concomitant presence of alpha and beta cells is a prerequisite for insulin secretion induced by ET-1. In line with this we showed a slight, but statistically significant, increase in glucagon secretion from mixed beta and non-beta cells in response to stimulation with ET-1 at high glucose. This finding seems at variance with experiments in vivo in rats where continuous ET-1 infusion immediately suppresses plasma pancreatic glucagon concomitant with decreased blood glucose and delayed increments in plasma insulin [7]. Those authors found that ET-1 enhanced insulin concentrations despite a preceding suppression of glucagon and blood glucose concentrations. The complexity of in vivo experimental conditions and the relatively high glucose concentration applied in our present study could explain this discrepancy between effects of ET-1 on glucagon secretion in vitro and in vivo.

Only scanty information exists on the mechanism of action of ET-1 on insulin release. A role for nitric oxide in insulin secretion induced by ET-1 in vivo has been proposed because the nitric oxide inhibitor *N*-monomethyl-L-arginine (NMLA) abolishes insulin secretion induced by ET-1 [8]. It is known that ET-1 is capable of stimulating nitric oxide production in perfused arterial vessels [24]. In our initial study, using isolated islets, it could be speculated that the endothelium still present in the islets play a part in the insulin release induced by ET-1. The present study clearly points to an important role for the alpha cells. The combined action of glucagon from

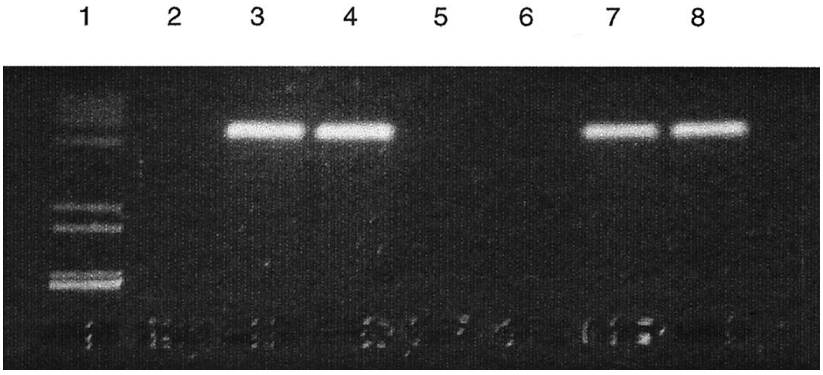

Fig. 3. RT–PCR analysis of ET_A-receptor mRNA. Samples of the PCR reactions were fractionated in a 2% agarose gel. The gel stained with ethidiumbromide is shown. Lane: 1, size markers; 2, no reverse transcriptase added (negative control); 3–4, αTC1.9 cells; 5–6, INS-1 cells; 7–8, rat islets

the alpha cells and a substance derived from endothelium e.g. nitric oxide could, however, also be operating.

It is noteworthy that increased ET-1 immunoreactivity has been shown in chronic pancreatitis and, in particular, that ET-1 immunoreactivity is colocalized with insulin and glucagon [25]. Pancreatic ET can be released by intragastric alcohol as shown in the anaesthetized cat [26]. It is also known that ET can reduce pancreatic blood flow as shown in the dog [27]. In a recent study ET_B receptor-like immunoreactivity on the rat islet beta and delta cells was reported [28]. Our present study clearly shows that no functional ET receptors exist on beta cells.

Note, our conclusions are not based on a single finding but rather on a number of different experiments all pointing in the same direction. Thus, it is hypothesized that ET-1 stimulates islet alpha cells to release glucagon which subsequently increases insulin secretion. It is proposed that a paracrine mechanism is responsible for insulin secretion induced by ET-1. The physiological and pathophysiological role of ET-1 on the endocrine pancreatic function is still to be clarified. It is clear, however, that this peptide is capable of changing islet secretion on a short-term basis. Whether a constant increased ET-1 concentration, presumably being present in pathological conditions such as hypertension and diabetes, might affect long-term islet hormone secretion in a deleterious direction is not known. The circulating ET concentrations in humans of 1–5 pmol/l are several orders of magnitude lower than the concentrations we applied in our islet cell experiments. It might well be, however, that an ET concentration in nanomolar range is achieved in the islets due to local release from the endothelium, e.g. in atherosclerosis vascular ET increases remarkably in the face of only a slight increase in circulating ET [29].

In conclusion, it is suggested that the insulinotropic effect of ET-1 is mediated by a paracrine action secondary to stimulation of glucagon release from ET-receptor mRNA positive alpha cells.

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