Original Article

Endothelin-1 Gene Expression in Endothelial Cells Is Potently Inhibited by a Vasodilator, Dilazep

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Endothelin-1 (ET-1) is considered to be involved in various cardiovascular and renal disorders. The objective of this study was to investigate whether a vasodilator and antiplatelet agent, 1,4-bis[3-(3,4,5-trimethoxybenzoyloxy) propyl]perhydro-1,4-diazepine dihydrochloride monohydrate (dilazep, DZ), has an ET-1-inhibiting effect *in vitro*. Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) pretreated with fetal calf serum were treated with DZ and preproET-1 (PpET-1) transcription was evaluated by Northern blot analysis. ET-1 peptide release in culture medium was evaluated by radioimmunoassay. The effect of DZ on the ET-1 promoter/enhancer apparatus was evaluated in transfection experiments using - 5 kb ET-1 promoter/enhancer constructs. Modest inhibition of PpET-1 gene transcription was detected after 30 min of DZ treatment (0.56 ± 0.19 vs. 1, $p < 0.01$) and more marked inhibition was seen at 24 h (0.04 ± 0.04 vs. 1, $p < 0.0001$). ET-1 peptide release was suppressed strongly after 3 h (382.5 ± 2.9 vs. 673.5 ± 74.5 pg/ml, $p < 0.001$) and 24 h (38.8 ± 9.8 vs. $5,075 \pm 52.0$ pg/ml, $p < 0.0001$). DZ potently inhibited PpET-1 transcription in a concentration-dependent manner (0.42 ± 0.18 vs. 1, $p < 0.001$, at $100 \mu\text{mol/l}$). DZ suppressed PpET-1 transcription in confluent HUVEC at 3 h (0.41 ± 0.11 vs. 1, $p < 0.0001$). DZ strongly inhibited PpET-1 transcription after 1 h of thrombin (TH) treatment (0.30 ± 0.01 vs. 1.51 ± 0.03 , $p < 0.0001$). Transfection experiments using the 5 kb ET-1 promoter-luciferase plasmid revealed that DZ strongly suppressed ET-1 promoter activity by 99% ($p < 0.01$). DZ potently inhibited ET-1 gene expression at the transcription level in serum- or TH-treated endothelial cells. (*Hypertens Res* 2004; 27: 409–415)

Key Words: dilazep, endothelin-1, gene expression, transcription

Introduction

Endothelin-1 (ET-1), which is a potent vasoconstrictor peptide mainly produced in endothelial cells (1), plays an important role in inducing proliferation of vascular smooth muscle cells (2) and cardiac hypertrophy (3) through its specific receptor-mediated signal transduction. Elevated levels of plasma and/or local ET-1 have been reported in patients or animal models of atherosclerosis (4, 5), heart failure (6, 7), pul-

monary hypertension (8) and endotoxic state (9). Various experimental models of hypertension, such as the deoxycorticosterone acetate (DOCA) salt rat, DOCA-salt-treated spontaneously hypertensive rat (SHR), stroke-prone SHR, angiotensin II-infused rat and one-kidney one-clip Goldblatt rat, and some forms of human hypertension, such as moderate- to severe-hypertension and hypertension in African Americans, have been reported to show enhanced ET-1 activity (10).

ET-1 also appears to be involved in the pathogenesis and

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progression of various renal diseases. These changes were recently attributed to ET-1 overproduction in vascular and renal tissues (11). ET-1 induces mesangial cell proliferation as well as extracellular matrix protein accumulation (12, 13), which are associated with vascular remodeling, renal fibrosis and glomerulosclerosis.

Several growth factors, hormones and coagulation factors have been shown to up-regulate ET-1 gene expression (14–16), and these effects are mainly regulated at the transcriptional level. In contrast, some molecules, such as nitric oxide (17) and the long-acting calcium channel antagonist benidipine (18), have been shown to suppress ET-1 production, suggesting that there might be a negative feedback mechanism involved in the regulation of the level of ET-1 production and release in endothelial cells.

1,4-Bis[3-(3,4,5-trimethoxybenzoyloxy)propyl]perhydro-1,4-diazepine dihydrochloride monohydrate (dilazep, DZ) has been used clinically in Europe and Japan as a vasodilator (19), antianginal agent (20), antiplatelet agent (21) and proteinuria-reducing agent (22, 23). Based on reports that DZ relieves proteinuria (22, 23), and that endothelial (1, 14–17, 24) and mesangial cells (24, 25) are important sites of ET-1 production, we hypothesized that DZ could inhibit ET-1 gene expression. In this study, we evaluated the effect of DZ on ET-1 gene expression in cultured endothelial cells.

Methods

Materials

Dilazep dihydrochloride (Comelian) was donated by Kowa Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Invitrogen Corp. (Carlsbad, USA). Thrombin (TH) was purchased from Sigma Chemicals Co. (St. Louis, USA). Human umbilical vein endothelial cells (HUVEC) and modified MCDB medium were purchased from Cambrex Bioscience Inc. (Walkersville, USA).

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated and cultured as described previously (26) in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. BAEC between the 5th and 8th passage were used for this study.

BAEC were cultured in DMEM containing 10% FCS with 100 µmol/l DZ for 10 or 30 min or for 1, 3, 6, 12 or 24 h. Serum-treated BAEC without DZ treatment were used as controls. Total RNA was extracted after 0, 10, and 30 min and 1, 3, 6, 12 and 24 h of DZ treatment. One ml of culture medium was collected from each culture before extraction of RNA and processed for ET-1 peptide measurement by radioimmunoassay as reported previously (27).

BAEC were cultured in DMEM containing 10% FCS and

1–100 µmol/l DZ. Serum-treated BAEC without DZ treatment were used as controls. Total RNA was extracted after 16 h of DZ treatment.

BAEC were serum-depleted for 16 h, and treated with 100 µmol/l DZ and thrombin (TH) (4 U/ml) at the same time. Total RNA was extracted after 0 (before TH and DZ treatment), 1, 2 and 4 h.

HUVEC were also cultured in modified MCDB medium as reported previously (28) and treated with 10% FCS with or without 100 µmol/l DZ for 3 and 6 h. Serum-treated HUVEC without DZ treatment were used as controls. Total RNA was extracted after 0, 3 and 6 h of DZ treatment.

RNA Isolation and Hybridization

Total RNA was extracted from BAEC cultures at specified times by the acid guanidium thiocyanate phenol chloroform extraction method (26). Northern blotting was performed as described previously (26) using a 1.9 kb EcoR I fragment of bovine prepro-endothelin-1 (b-PpET-1) cDNA (donated by T. Quertermous, Stanford University, Stanford, USA). The degree of hybridization was quantified by densitometric evaluation of the corresponding autoradiogram and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridized bands to correct for possible differences in loading and transfer efficiency.

Transfection and Luciferase Assay

To construct plasmids with firefly luciferase as a reporter gene, directed by the human preproendothelin-1 (h-PpET-1) promoter, an Xba-I/Bgl II digest of the h-PpET-1 genomic fragment, including 5 kb of the 5'-flanking region (donated by T. Quertermous, Stanford University), was subcloned into the pGL2-basic vector (Promega, Madison, USA). BAEC in 100-mm-diameter dishes were transfected with DNAs that had been incubated with 36 µg Lipofectamine reagent (Invitrogen Corp., Carlsbad, USA) as described previously (29).

In brief, BAEC were plated at least 24 h before transfection and were transfected with 5 µg PpET-1 luciferase fusion gene (PpET-1[-5.0LUC]). As an internal standard for transfection efficiency, the vector pRL-SV40 (Promega, Madison, USA) was used. Transfection was performed by incubating cells in the presence of DNA for 4 h. Subsequently, cells were washed and cultured in fresh medium containing 10% FCS. At 24 h after transfection, the cells were re-fed with DMEM/10% FCS, with or without DZ (100 µmol/l). Cell extracts were prepared 48 h after transfection. Cells were washed and lysed with 600 µl lysis buffer (Toyo Ink Co. Ltd., Tokyo, Japan) for 15 min. Cell lysates were then microcentrifuged (15,000 rpm, 15 s), and 20 µl of the clear supernatant was subjected to measurement with a PicaGene-Dual, double luciferase assay system. Luciferase activity was measured in an AutoLumat LB953 Luminometer (Berthold, Gaithersburg, USA) over a 10 s period.

Fig. 1. Effect of DZ on ET-1 gene expression of serum-treated BAEC. A: A typical Northern blot after hybridization with [α^{32} P]dCTP-radiolabeled b-PpET-1 probe (upper panel) ($n = 4$). The same filter was stripped and re-hybridized with murine GAPDH probe as an internal control (lower panel). ET-1, endothelin-1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CON, control; DZ, dilazep. B: Time-dependent effect of DZ on ET-1 gene expression in serum (10% FCS)-treated BAEC. Data represent the mean $\pm$ SD of the relative density of normalized ET-1, which is the ratio of the densitometric value of the hybridized ET-1 band to the GAPDH band for their respective lanes with the control arbitrarily given a value of 1 ($n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$). C: Time-dependent suppression of ET-1 peptide release in subconfluent BAEC culture ($n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

Statistical Analysis

Data are expressed as the mean $\pm$ SD. ANOVA and unpaired Student's *t*-test, or paired *t*-test when appropriate, were performed, and statistical significance was set at $p < 0.05$.

Results

Time-Dependent Effect of DZ on PpET-1 Transcription in Serum-Treated BAEC

Figure 1A shows a representative result of Northern blot analysis demonstrating the time-dependent inhibition of PpET-1 transcription by DZ in BAEC treated with 10% FCS under a subconfluent condition. In comparison to the PpET-1 transcription in serum-treated BAEC (control = 1), treatment with 100 μ Mol/l DZ significantly inhibited the transcription at as early as 30 min (0.56 ± 0.19 vs. 1, $p < 0.01$), and more potently thereafter (0.14 ± 0.04 , $p < 0.0001$ at 12 h; and 0.04 ± 0.04 , $p < 0.0001$ at 24 h) (Fig. 1B). DZ also strongly suppressed ET-1 peptide release in culture medium after 3 h (382.5 ± 2.9 vs. 673.5 ± 74.5 pg/ml, $p < 0.001$) and 24 h (38.8 ± 9.8 vs. $5,075 \pm 52.0$ pg/ml, $p < 0.0001$) of incubation of BAEC in 10% FCS-treated medium, as shown in Fig. 1C.

The effect of DZ in BAEC culture under a confluent con-

dition was also investigated. A strong inhibition of ET-1 was observed in serum-treated BAEC under a confluent condition after 12 h of incubation with DZ (0.47 ± 0.31 vs. 1, $p < 0.05$ at 12 h; 0.52 ± 0.4 vs. 1, $p = 0.05$, at 24 h). ET-1 peptide release was significantly inhibited at these time intervals (control: $3,950 \pm 842.9$ pg/ml; DZ-treated BAEC: 955.5 ± 21.4 , $p < 0.001$ at 12 h; 983.5 ± 88.3 , $p < 0.001$, at 24 h).

Concentration-Dependent Effect of DZ on PpET-1 Transcription in Serum-Treated BAEC

As shown in Fig. 2, DZ at 10–100 μ Mol/l inhibited PpET-1 transcription in a concentration-dependent manner (0.69 ± 0.18 vs. 1 at 10 μ Mol/l, $p < 0.05$; 0.58 ± 0.20 vs. 1, $p < 0.01$ at 50 μ Mol/l; 0.42 ± 0.18 vs. 1, $p < 0.001$ at 100 μ Mol/l DZ).

Effect of DZ on PpET-1 Transcription in Serum-Treated HUVEC

In serum-treated HUVEC under a confluent condition, DZ strongly suppressed PpET-1 transcription at 3 h (0.41 ± 0.11 vs. 1, $p < 0.0001$) and 6 h (0.42 ± 0.12 vs. 1, $p < 0.0001$) of incubation, as shown in Fig. 3. ET-1 peptide release was also strongly inhibited by DZ (control: $1,390 \pm 46.2$ pg/ml; DZ-treated HUVEC: 299.0 ± 13.9 pg/ml at 3 h, $p < 0.0001$; $357 \pm$

Fig. 2. Concentration-dependent effect of DZ on ET-1 gene expression in serum (10% FCS)-treated BAEC. Data represent the mean $\pm$ SD of the relative density of normalized ET-1 after Northern blot hybridization with [α^{32} P]dCTP-radio-labeled b-PpET-1 probe. The same filter was stripped and hybridized with murine GAPDH probe as an internal control. Normalized values were calculated as the ratio of the densitometric value of the hybridized ET-1 band to the GAPDH band for their respective lanes with the control arbitrarily given a value of 1 ($n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

12.7 pg/ml at 6 h, $p < 0.0001$).

Effect of DZ on PpET-1 Transcription in Serum-Depleted and TH-Stimulated BAEC

TH stimulation resulted in a significant increase in PpET-1 transcription (1.51 ± 0.03 vs. 1, $p < 0.01$ at 1 h; 1.24 ± 0.02 vs. 1, $p < 0.05$ at 2 h; Fig. 4A); however, treatment of DZ resulted in a marked reduction in PpET-1 mRNA from 1 h of DZ treatment (0.30 ± 0.01 vs. 1.51 ± 0.03 , $p < 0.0001$, at 1 h; 0.08 ± 0.02 vs. 1.24 ± 0.02 , $p < 0.0001$, at 2 h; Fig. 4B). These PpET-1 mRNA expression levels were significantly lower in comparison to the basal PpET-1 transcription (0.30 ± 0.01 vs. 1.0 ± 0 , $p < 0.0001$, at 1 h; 0.08 ± 0.02 vs. 1.0 ± 0 , $p < 0.0001$, at 2 h).

Effect of DZ on ET-1 Promoter Activity

To determine whether the decrease in ET-1 gene expression induced by DZ treatment was mediated by promoter/enhancer activity of the ET-1 gene, transfection experiments were carried out using the ET-1 promoter-luciferase reporter gene construct, containing 5 kb of the h-PpET-1 promoter. As shown in Fig. 5, ET-1 promoter activity was strongly suppressed by 99% ($p < 0.01$) after 24 h of DZ treatment.

Discussion

We demonstrated that DZ strongly inhibited PpET-1 tran-

scription and ET-1 peptide release in serum-treated BAEC under a subconfluent condition (Fig. 1). DZ also inhibited transcription of PpET-1 mRNA and ET-1 peptide release in a confluent BAEC culture. Transfection experiments using reporter plasmids linked to the 5' flanking region of the ET-1 gene demonstrated that DZ strongly suppressed ET-1 promoter activity, which resulted in down-regulation of ET-1 gene transcription (Fig. 5). This strong inhibition of PpET-1 transcription in the presence of potent ET-1 stimulation with 10% FCS has important clinical implications.

We have also demonstrated that DZ potently inhibited ET-1 gene expression in confluent HUVEC in the presence of 10% FCS. The pharmacological effects of DZ, such as vasodilation (19), amelioration of cardiomegaly (30) and proteinuria reduction (22, 23), which are likely to be ET-1-related effects, may be due to ET-1 inhibiting effect of DZ. However, further experiments will be necessary to clarify whether these effects are indeed mediated by ET-1.

Serum contains several growth factors, and it is quite difficult to evaluate their individual contributions. We therefore investigated whether a similar enhancement of PpET-1 occurred in TH-treated BAEC. In agreement with previous studies, TH stimulation significantly increased PpET-1 transcription under serum depletion. We also demonstrated strong inhibition of ET-1 gene expression by DZ (Fig. 4) in TH-stimulated BAEC. These PpET-1 mRNA expression levels were significantly lower in comparison to the basal PpET-1 transcription under serum depletion. This strong effect of DZ could partly explain its apparent in countering hemostasis and thrombogenesis. It has been previously reported that DZ inhibits tissue factor expression in HUVEC and monocytes (28), indicating its possible use as an anti-thrombotic agent (28, 31). With its additional pharmacological effect of antiplatelet activity (22, 23), DZ may be clinically useful for managing various thrombosis- and coagulation-related vascular disorders.

Several clinical studies using ET-1 receptor antagonists are currently underway to investigate their potential amelioration of the harmful effects of ET-1, such as vascular hypertrophy, atherosclerosis and renal dysfunction. From the results of our study, DZ is expected to be effective for the clinical management of various cardiovascular and renal disorders in which the ET-1 system is up-regulated.

DZ has been reported to potentiate the negative inotropic effect of adenosine on guinea-pig atria by preventing both its accumulation by atrial tissue and degradation by deaminase (32). It has also been reported that blockade of adenosine receptors resulted in the attenuation of the effect of DZ on vasodilation (33) and tissue factor expression (28). However, our preliminary data using the non-selective adenosine receptor antagonist 1,3-dipropyl-8-*p*-sulphophenylxanthine (DPSPX, 10^{-8} mol/l) indicated that adenosine receptor blockade did not modify the ET-1-inhibiting effect of DZ in BAEC (data not shown). Therefore, the effect of DZ on ET-1 production may not be mediated through adenosine recep-

Fig. 3. Effect of DZ on the transcription of PpET-1 mRNA in a confluent HUVEC culture under serum (10% FCS) treatment. A: A typical Northern blot after hybridization with [α^{32} P]dCTP-radiolabeled h-PpET-1 probe (upper panel) (n = 4). Human GAPDH was used as an internal control (lower panel). ET-1, endothelin-1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CON, control; DZ-3, dilazep treatment for 3 h; DZ-6, dilazep treatment for 6 h. B: Relative density of normalized ET-1, which is the ratio of the densitometric value of the hybridized ET-1 band to the GAPDH band for their respective lanes with the control arbitrarily given a value of 1. Data represent the mean $\pm$ SD. The white bar represents serum- and the black bar DZ-treated HUVEC (n = 4). *** p<0.0001. CON, control; DZ-3, dilazep treatment for 3 h; DZ-6, dilazep treatment for 6 h.

Fig. 4. Effect of DZ on ET-1 gene expression of TH-treated BAEC. A: A typical Northern blot after hybridization with [α^{32} P]dCTP-radiolabeled b-PpET-1 probe (upper panel) (n = 4). The same filter was stripped and re-hybridized with murine GAPDH probe as an internal control (lower panel). TH, thrombin; DZ, dilazep; ET-1, endothelin-1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B: Effect of DZ on ET-1 gene expression in serum-depleted and TH-treated BAEC. Data represent the mean $\pm$ SD of the relative density of normalized ET-1, which is the ratio of the densitometric value of the hybridized ET-1 band to the GAPDH band for their respective lanes with the control value arbitrarily given a value of 1 (n = 4, * p<0.05, ** p<0.01, *** p<0.0001).

Fig. 5. Effect of DZ on relative luciferase activity as an index of ET-1 promoter activity in 10% FCS-treated BAEC after transfection (4 h) with a h-PpET-1 luciferase fusion gene (PpET-1[-5.0LUC]) genomic fragment including 5 kb of the 5'-flanking region in serum-treated BAEC (10% FCS for 24 h) and further culture for 24 h with or without 100 μ mol/l DZ. Vector pRL-SV40 was used as an internal standard for analysis of transfection efficiency (n = 9, ** p<0.01).

tors. Further studies on the extra- and intra-cellular signal transduction system, including studies on the role of adenosine in ET-1 production, will be necessary to elaborate the mechanism of action of DZ.

Study Limitations

The ET-1-inhibiting effect of DZ was observed at concentrations of 10–100 μ mol/l. A relatively high concentration of DZ (100 μ mol/l) was used in these experiments because the strongest inhibition of PpET-1 transcription was observed at this concentration. In comparison to the serum DZ concentration used clinically in humans (approximately 1 μ mol/l, personal communication), the range of 10–100 μ mol/l is quite high.

In vitro cell culture with 10% FCS is a very strong stimulatory condition for ET-1 expression, and actual clinical situations resulting in ET-1 stimulation may be milder in intensi-

ty. The ET-1 inhibiting effect by lower concentrations of DZ may be anticipated *in vivo* or in real clinical situations. In some ischemia-reperfusion models, lower concentrations of DZ (5–10 $\mu\text{mol/l}$) have been reported to be effective in suppressing the ischemia-reperfusion injury (20, 34, 35). In actual clinical practice, a typical pharmacological dose regime of DZ has been shown to exhibit signs of a cardioprotective effect in patients with renal failure under hemodialysis (36).

The present study showed that DZ suppressed ET-1 promoter activity. However, it is still not clear how DZ can access transcription factors in the nucleus to modulate the ET-1 promoter. Transcriptional regulation of the ET-1 gene has been thoroughly studied in previous studies (37, 38), and we have demonstrated the role of transcription factors such as *c-Jun*, *c-Fos* and GATA2 protein in binding to their cognate-binding sites in the promoter region of the ET-1 gene (37). Further studies will be needed to clarify the effect of DZ in the transcription apparatus of the ET-1 promoter and elaborate the signal transduction pathway.

In conclusion, DZ inhibited ET-1 gene expression in cultured endothelial cells at the transcriptional level. These findings could have important clinical implications for the treatment of cardiovascular and renal diseases in which the ET-1 system is up-regulated.

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