The present study characterized the regulation of the genetic expression of the vasoactive peptide endothelin-1 (ET-1) by insulin in bovine aortic endothelial cells. By RNA blot analysis, insulin (1.67 × 10^{-8} M) increased ET-1 mRNA levels by 2.3-fold over the basal within 10 min and attained a maximum (5.3-fold increase) in 2 h. Dose-response studies showed that a maximum effect of insulin was reached at 1.67 × 10^{-8} M although a significant increase can be observed at 1.66 × 10^{-9} M. Radioligand receptor studies indicated that the affinity constant for insulin receptors on endothelial cells correlated closely with the dose response observed for ET-1 mRNA. The ET-1 mRNA half-life was estimated with actinomycin D studies to be 20 min in control cells and was not affected by insulin treatment. Moreover, the effects of phorbol 12-myristate 13-acetate (PMA) and insulin were additive in the induction of ET-1 gene expression. When protein kinase C in the bovine aortic endothelial cells was downregulated by preincubation with 8 × 10^{-7} M PMA for 24 or 48 h, insulin was still able to increase ET-1 mRNA levels whereas PMA was ineffective. Using a chloramphenicol acetyltransferase (CAT) fusion plasmid containing the CAT gene and the 5'-flanking region of the ET-1 gene (Lee, M. E., Bloch, K. D., Clifford, J. A., and Quertermous, T. (1990) J. Biol. Chem. 265, 10446–10450), we observed that 1.67 × 10^{-8} M insulin increased CAT enzyme activity and mRNA levels. The insulin dose-response curve observed for CAT activity correlated with that observed for ET-1 mRNA levels. These results suggest that insulin stimulates expression of the ET-1 gene at the transcriptional level via its own receptors. This effect is mediated mostly through a protein kinase C-independent pathway, suggesting the existence of an insulin-responsive element in the ET-1 gene 5'-flanking sequence.

Insulin resistance and diabetic states have been associated with hypertension, but the biochemical mechanism for their linkage is unclear. Since hyperinsulinemia is often described in both of these clinical states, a direct effect of insulin on the arterial wall has been implicated (1). In vascular cells, insulin receptors have been found on both endothelial and smooth muscle cells (1). Biologically, these cells are also responsive to insulin, with both metabolic and growth effects (1).

In this report we have characterized the effects of insulin on the expression of endothelin-1 (ET-1), a potent vasoconstrictor peptide (2), in aortic endothelial cells. ET-1 was first reported in the supernatant of cultured porcine aortic endothelial cells (2), although recently it has been isolated from other sources (3–5). There are three types of endothelins (ET-1, ET-2, and ET-3) with very similar structure. Although all three endothelins have 21 amino acids with two disulfide intrachain bonds, each has different biological activities and potencies (6). Human ET-1 is synthesized as a 212-amino acid prepropeptide and converted into endothelin after two endopeptidase cleavages (2). The sequence of the human preproendothelin-1 and its chromosomal assignment have been reported (7). Several agents including the platelet-derived polypeptide transforming growth factor-β (8), thrombin (2), and phorbol ester (9) have been reported to regulate the expression of ET-1. In addition, cis-regulatory elements in the 5'-flanking region including the AP-1/c-fos-responsive element (10), the nuclear factor NF-1 binding motif (10), and the TATC (or GATA) motif (11) have been described.

In this study we have focused on the stimulatory effects of insulin on the expression of the ET-1 gene at the mRNA levels and showed that this effect of insulin is on transcription of the ET-1 gene.

MATERIALS AND METHODS

Cell Culture—Bovine aortic endothelial cells (BAE) were isolated as described previously (1) and cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% plasma donor-derived horse serum (PDHS) (GIBCO) in fibronectin-coated 100-mm plates (Nunc, Roskilde, Denmark). Cells were passed every 4–5 days, and confluent dishes of passages 4–12 were used for all the experiments. Two days before the assay, the medium was changed to 2% PDHS-supplemented DMEM, and all serum was removed 3 h before the addition of phorbol 12-myristate 13-acetate (PMA), human insulin.

The abbreviations used are: ET-1, endothelin-1; BAE, bovine aortic endothelial; DMEM, Dulbecco’s modified Eagle’s medium; PDHS, plasma donor-derived horse serum; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); PKC, protein kinase C; IRE, insulin-responsive element; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.
sulin, or actinomycin D. In PKC down-regulation experiments, con-
defluent BAE cells were treated with 8 × 10⁶ M PMA for 24 h and then
restimulated with 1.6 × 10⁻⁷ M PMA, 1.67 × 10⁻⁸ M insulin or both
for 30 min.

**Extraction and Analysis of RNA**—Total cellular RNA was extracted
by the guanidinium isothiocyanate and chloroform-phenol method (22) using the commercial
preparation RNAzol (Biotech, Inc., Houston, TX). For cells kept in sus-
pending, medium was aspirated, and cells were washed once with phos-
phate-buffered saline, pH 7.4. RNAzol was added immediately, and
of chloroform, shaken vigorously, and incubated for 15 min at 0 °C
chilled 2-propanol and incubated for 1 h at -20 °C. Precipitated RNA
was collected by centrifugation at 10,000

and resuspended in diethyl pyrocarbonate-treated water. RNA to be
postnuclear supernatant was centrifuged at 100,000

and homogenized with a tight fitting Dounce homogenizer. The

pellet was resuspended in buffer B (buffer A without sucrose) con-

structing 1% Triton X-100, incubated at 4 °C for 30 min, and recentri-
gufed. The soluble fraction was defined as the membranous extract.

**Immuno blotting**—PKC isoform-specific polyclonal antibodies were
developed against synthetic peptides corresponding to residues 310–
334 for PKC-α and 645–673 for PKC-β, in collaboration with Dr. William Heath, Lilly. These antibodies have been characterized pre-
viously (15).

**RESULTS**

**Stimulatory Effect of Insulin on ET-1 Gene Expression**—
Endothelial cells from macro- and microvessels have been shown to be responsive to insulin or contain functional insulin
receptors (1). The presence of a functional insulin receptor in our BAE cell preparation was characterized by insulin recep-
tor binding studies using ¹²⁵I-insulin, which showed 7.9% binding/mg of protein, in keeping with previously published
reports. Insulin at 1.25 × 10⁻⁷ M displaced 50% of the specific
binding, showing high affinity receptors (1, 21).

When BAE cells were incubated in the presence of insulin, time-dependent and dose-response increases in the steady-
state levels of ET-1 mRNA were observed (Figs. 1A and 2A).

**Effect of Insulin and PMA on ET-1 mRNA Half-life**—To
determine whether the effect of insulin is caused by a stabi-
ization of ET-1 mRNA, BAE cells previously treated with
either 1.67 × 10⁻⁶ M insulin or 100 nm PMA were incubated
with 2 μg/ml actinomycin D for 60 min. Total cellular RNA
at five time points was used for Northern blot analysis, and
the densitometric determinations, corrected for the α-tubulin
mRNA signal, are depicted in Fig. 3. The apparent intracel-
ular half-life extrapolated from the curve for control cells
was 20 min, which is similar to the half-life of 15 min of ET-
1 in human umbilical vein endothelial cells as reported pre-
viously by Inoue et al. (10). Insulin-treated BAE cells exhib-
ited higher steady-state levels of ET-1 mRNA at time zero
(2.3-fold increase), and the half-life extrapolated from the
curve was not significantly different from control cells.

The addition of 160 nm PMA for 30 min appeared to increase the
half-life of ET-1 mRNA although no conclusion can be drawn from our
plot.
Effect of Insulin and PMA on ET-1 Gene Expression in Control and PKC-down-regulated BAE Cells—It has been proposed that activation of PKC may be involved in the regulation of the ET-1 gene expression in endothelial cells since there is an AP-1 consensus motif in the promoter region. In addition, phorbol esters have been shown to have a stimulatory effect on mRNA levels (9, 10). Thus, we decided to investigate whether PKC has a role mediating the effect of insulin on the expression of the ET-1 gene. PKC activity was down-regulated in BAE cells after 24 h of incubation with 800 nM PMA (Fig. 4 and Table I). Table I shows that PKC activity after PMA treatment was decreased by 83% in the membrane-associated activity. No increase in PKC activities was observed when stimulation was repeated with 160 nM PMA. Western blot from both control and down-regulated BAE cells was performed. Among PKC isoforms α, β1, β2, and γ, only α and β2 were identified (Fig. 5). In down-regulated BAE cells, both PKC isoforms were dramatically decreased by immunoblot, corresponding to the decrease in activity levels. When control cells were incubated in the presence of 100 ng/ml insulin (1.67 × 10⁻⁸ M) or 160 nM PMA for 30 min, increases of 2.1 ± 0.1 and 3.3 ± 0.9 fold, respectively, of ET-1 mRNA levels were observed. When both agents were combined, an additive effect was detected (5.5 ± 1.5-fold increase). In PKC down-regulated cells, only insulin was able to increase ET-1 mRNA expression although the effect was smaller than in control cells, and no additive effect with PMA was observed (2.2- and 2.4-fold increase in insulin and insulin plus PMA, respectively). Similar findings were observed after 48 h of down-regulation with PMA. PKC activities were decreased by a magnitude comparable to what they were after 24 h of PMA treatment, and BAE cells were still able to respond to insulin but not to PMA (results not shown).
Effect of Insulin on ET-1 Expression

Insulin Regulation of ET-1-CAT Fusion Gene in Transiently Transfected BAE Cells—Since insulin appeared not to affect the half-life of ET-1 mRNA, we decided to determine whether insulin can affect the transcriptional rate of ET-1 by using transient transfection experiments of plasmids containing a 4.4 kb of the 5'-flanking region of the ET-1 gene ligated to the gene for CAT. This 5'-flanking region of ET-1 gene has been reported previously to contain the promoter elements for this gene (16). To optimize conditions for the transfection into BAE cells, the experiments were performed in BAE cells that were harvested 48 and 72 h after the addition of the ET-1 CAT construct plasmid DNA. Cells were treated with 1 mg/ml (1.67 x 10^-7 M) of insulin for 3 h before harvesting. The results in Fig. 6 show that insulin caused a significant increase in the normalized CAT activity at either time after transfection (3.6- and 2.3-fold after 48 and 72 h of transfection, respectively).

The dose-response effect of insulin on CAT activity normalized for β-galactosidase was shown in Fig. 7. Similar to the dose-response curve of insulin for ET-1 mRNA levels shown in Fig. 2, the maximum was reached with 1.67 x 10^-7 M insulin (3.7-fold increase), and the response decreased at higher concentrations (1.67 x 10^-7 M). The effect of insulin on expression of CAT mRNA was also determined by solution hybridization studies. As shown in Fig. 8 insulin at 1.66 x 10^-7 M was able to increase CAT mRNA by 2.5-fold. Therefore, these results suggest that insulin was increasing the expression of the ET-1 gene by modification of the transcriptional rate.

DISCUSSION

Vascular endothelial cells play an important role in the control of the local vascular tonus through the release to the extracellular medium of vasoactive substances such as endothelial cell-derived relaxing factor or endothelin, which may act on adjacent smooth muscle cells (2, 22). Hormonal regu-

![Fig. 4. Effect of insulin and PMA on ET-1 mRNA levels in control and PKC-down-regulated cells. A confluent monolayer of BAE cells was fed 48 h with DMEM, 2% PDHS and serum-deprived for 3 h before any treatment. Half of the cells were treated with 800 nM PMA for 24 h before the experiment to deplete the PKC and were stimulated with 1.67 x 10^-6 M insulin (INS) or 160 nM PMA for 30 min. *, p < 0.025 versus control; **, p < 0.001 versus control. Northern blot was performed as in previous figures and the values represent the mean of three or four experiments (mean ± S.E.).](image)

![Table I. Protein kinase C activity in normal and down-regulated BAE cells](table)

| Cytosol | Membrane |
|--------|----------|
| % of control | |
| Control | 100 | 100 |
| Control + PMA* | 52 | 225 |
| Down-regulated | 38 | 55 |
| Down-regulated + PMA* | 34 | 38 |

* Restimulated with 0.16 μM PMA for 30 min.

![Fig. 5. Immunoblot of protein kinase C isoforms in down regulated BAE cells. Proteins from cytosolic (CYTO) and membrane fractions (MEM) were separated on 7.5% SDS-polyacrylamide gel electrophoresis (10 μg/lane) and transferred to Schleicher & Schuell nitrocellulose. Nitrocellulose was blocked with 3% bovine serum albumin in rinse buffer at 4 °C for 16 h. The membrane was incubated with 2 μg/ml affinity-purified antibody against α or β, at 4 °C for 16 h. Blots were washed then incubated with protein A (Amersham) at 4 °C overnight and washed four times with rinse buffer containing 1.0 M NaCl. Results were visualized by autoradiography. CC, control cells; CP, cells with PMA; DC, down-regulated cells; DP, down-regulated with PMA.](image)

![Fig. 6. Insulin regulation of ET-1 CAT fusion gene in transiently transfected BAE cells. The 4.4-kb ET-1 CAT construct (20 μg) was co-transfected with the β-galactosidase construct (10 μg) into BAE cells by using a calcium phosphate batch transfection protocol (16). Freshly trypsinized BAE cells were treated with 1 ml of the calcium phosphate precipitate containing the plasmid and were fed with DMEM containing 10% PDHS (GIBCO) in 100-mm dishes. The next day the cells were washed four times with phosphate-buffered saline and grown to confluence in 2% PDHS/DMEM (24 h). The medium was changed to 0.2% bovine serum albumin for 3 h, and insulin was added at 1.67 x 10^-7 M for 3 h. 11°CChloramphenicol acetylated was calculated and normalized for the expression of β-galactosidase in the same cell extract. The fold effect of insulin was determined, and the results from three different experiments were averaged (mean ± S.E.). *, p < 0.005; **, p < 0.01.](image)
under the same conditions stated for Fig. 6. Insulin was added at concentrations of $1.67 \times 10^{-5}$, $1.67 \times 10^{-6}$, and $1.67 \times 10^{-7}$ M for 3 h. The results are the mean ± S.E. of three separate transfections in duplicate.

**FIG. 7.** Dose response to insulin ET-1 CAT fusion gene in transiently transfected BAE cells. The experiment was performed under the same conditions stated for Fig. 6. Insulin was added at concentrations of $1.67 \times 10^{-5}$, $1.67 \times 10^{-6}$, and $1.67 \times 10^{-7}$ M for 3 h. The results are the mean ± S.E. of three separate transfections in duplicate.

**FIG. 8.** Effect of insulin on CAT mRNA expression using the ET-1 promoter as measured by S1 analysis. BAE cells were transfected as in Figs. 6 and 7. Total cellular RNA was harvested 48 h after transfection, and CAT mRNA was measured by S1 nuclease protection assay. BAE cells were exposed to $1.67 \times 10^{-5}$ M insulin or vehicle for 3 h. The probe was generated using an insert from the adenovirus EZ promoter linked to the CAT gene within M13mp18 (20). The probe was produced by liberation from M13 by EcoRI digestion is 367 nucleotides in length, 150 of which would be protected by CAT mRNA (indicated by the arrow). The results shown are representative of two independent S1 protection assays.

The concentration range observed in the dose-dependent increase in ET-1 transcription correlated closely with the affinity constant of the insulin receptor, suggesting that this action of insulin is mediated by binding to its own receptor. It is very unlikely that insulin is exerting this effect via the insulin-like growth factor-1 receptor, in view of the low affinity of the insulin-like growth factor-1 receptor for insulin (21). Furthermore, studies using specific antibodies to the insulin receptor were also able to enhance ET-1 gene expression (data not shown). Because of our previous finding that endothelial cells from capillaries are more responsive to insulin than those from macrovessels (1), we would predict that insulin should also be able to regulate ET-1 expression in microvessel endothelial cells, which have been reported to produce ET-1 (23).

Our data showed that the addition of insulin did not alter the ET-1 mRNA half-life indicating that changes in mRNA levels may be a result of an increase in transcription rate. This possibility is firmly supported by the results of transient transfection studies in endothelial cells using constructs which utilized the ET-1 promoter linked to the CAT reporter gene. Insulin increased CAT expression to a similar extent and in a similar dose-response manner as found by Northern blot analysis for ET-1 mRNA. These findings strongly suggest that the insulin agonist effect is mediated at the transcription level and not by prolonging mRNA half-life. This effect of insulin is not unusual since this hormone has been reported to regulate the transcription rate of genes such as glucokinase, pyruvate kinase, amylase, glyceroldehyde-3-phosphate dehydrogenase phosphoenolpyruvate carboxykinase, c-fos, and c-myc (24–32). In some cases, such as amylase and c-fos activation, the effect of insulin was reported to occur in less than 30 min (28, 29), similar to the rapid effect seen here with ET-1. The existence of cis-acting regulatory sequences mediating the effect of insulin on the expression of several genes have been shown previously. The best characterized mechanism of insulin regulation of gene expression is that associated with the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (30–32). Recently, a 5′-specific insulin-responsive element (IRE) has been recognized to direct this hormonal effect on phosphoenolpyruvate carboxykinase gene expression, and a specific DNA-binding protein has been implicated (31). A different IRE has been characterized in association with the glyceroldehyde-3-phosphate dehydrogenase gene (33). Based on an analysis of nucleotide sequence critical for formation of an insulin-sensitive protein-DNA complex, the authors proposed a minimal structural IRE. They found that 8 consecutive bases of the motif are perfectly conserved in the promoter region of other insulin-responsive genes. In the 5′-flanking region of the ET-1 gene at base pairs –2988 to 2979 we have found a sequence, CCCGCAATTCG, that matches 9 bases of the proposed IRE (the underlined Δ is a C in the GADPH IRE). The ET-1 gene contains a second sequence at base pairs –136 to –145 (GGCCCTGGGCT) which has been found in association with other genes regulated by insulin (27, 30, 36). The functional meaning of this second motif is unknown. It is possible that one of these sequences functions as a cis-acting element which mediates the insulin responsiveness of the ET-1 gene. This hypothesis is currently under study in our laboratory.

The action of insulin appears to differ from the effect of PMA in several aspects. As reported previously, we have confirmed that PMA, an activator of protein kinase C, will also increase ET-1 mRNA levels in steady state (9, 10). Our actinomycin D experiments suggest that the effect of PMA may be to increase the stability of the mRNA although more studies are needed to support this speculation. However, it is also possible that PMA could be increasing transcription rate as well since previous reports describing the promoter region for ET-1 have identified an 8-base pair AP-1 consensus sequence. Such sequences have been suggested to mediate responsiveness to phorbol esters, probably by activating PKC (34, 35). This mechanism, nevertheless, is probably not the main pathway by which insulin activates the expression of ET-1 gene, since reducing the activities of PKC in the endo-
Effect of Insulin on ET-1 Expression

Clinically, the finding that insulin at physiological levels can enhance ET-1 expression could be important for the understanding of the relationship between hyperinsulinemia and vascular disorders such as hypertension and acceleration of atherosclerosis (37). Recently, Yamaguchi et al. (23) reported that endothelial cells exposed to elevated glucose produced a greater amount of ET-1. The mechanism of this effect of glucose was not identified, but it could be mediated by PKC. Activation of PKC has been shown previously to occur in endothelial cells exposed to high glucose levels (15). Thus, it is tempting to speculate that a combination of hyperinsulinemia and hyperglycemia will increase ET-1 expression in a paracrine fashion in the diabetic state. The resultant increase in ET-1 expression may have a role in causing some of the vascular abnormalities found in diabetic patients.

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