Thrombin Regulaton of Endothelin-1 Gene in Isolated Human Pulmonary Endothelial Cells*

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Endothelin-1 (ET-1) is a potent vasoconstrictor elicited from endothelial cells in response to a variety of stimuli, and mounting evidence implicates ET-1 as an important mediator for a number of vascular diseases including pulmonary vascular disease. The mature 21 amino acid ET-1 peptide is derived by proteolytic processing from preproendothelin-1, a 212 amino acid precursor. Previous studies have used functional assays to provide a fairly detailed picture of the basal regulation of ET-1 in bovine endothelial cells, and in COS cells. In addition, a limited analysis of stimuli-induced ET-1 expression, in functional studies, has been conducted. However, these studies have been conducted in a heterologous system whereby regulation of the human ET1 gene was assessed in bovine or porcine aortic endothelial cells, COS cells, HeLa cells, or NIH3T3 cells, and in some cases, produced variable results.

In this work we provide a detailed analysis of the expression and regulation of the human ET1 gene in human pulmonary endothelial cells. The aim of these studies was to investigate the effect of thrombin, a physiologic mediator potentially involved in the pathogenesis of pulmonary vascular disease, on the molecular regulation of ET1.

**MATERIALS AND METHODS**

Human pulmonary artery (HPA) endothelial cells were isolated from pulmonary arteries of heart transplant donors by collagenase treatment and scraping of the endothelium as described previously. Human pulmonary microvascular (HPV) endothelial cells were obtained commercially (Clonetec Corp; San Diego, Calif). ET-1 mRNA levels were evaluated by Northern blot analysis under both basal- and thrombin (10 U/mL)-stimulated conditions (0.5 to 4 h) and in response to actinomycin D (4 μM), an RNA synthesis inhibitor using human ET-1 or rat cathepsin B cDNAs.

To determine chromatin structure of the ET1 gene, DNase I hypersensitive sites were defined using lysoloeic permeabilized cells and increasing concentrations of DNase I in both basal- and thrombin-stimulated cells. Digested DNA was evaluated by Southern blot analysis using indirect end labeling to map the positions of the hypersensitive sites. Both low and high resolution chromatin structures were evaluated in standard 1% agarose gel and an agarose gel system (MetaPhor; FMC Bioproducts; Rockland, Maine, respectively).

To evaluate further for promoter activity, portions of the 5' flanking region cloned into the promoterless human growth hormone (hGH) reporter vector, pOOGH were transfected into human pulmonary endothelial cells and evaluated for growth hormone release (reporter assay) under basal- and thrombin-stimulated conditions. A number of constructs were generated from a human ET-1 genomic P1 clone we obtained and several from Dr. David Wilson. The ET-1/hGH constructs include -8.0 kb, -5.2 kb, -2.4 kb, -1.38 kb, -0.952 bp, -0.628 bp, -0.184 bp, -0.375 bp, -0.141 bp, and -0.088 bp. The cells were transfected with diethylaminoethyl-dextran, chloroquine, and dimethyl sulfoxide method, and 1 day after transfection, the cells were equally distributed into control and treated plates. Statistical comparisons were made for each vector individually to detect differences under stimulus vs control conditions. Values were calculated in a paired two-tailed t test.

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RESULTS

Thrombin exposure induced ET-1 mRNA levels after 30 min with a peak induction of two- to threefold after 1 h by a transcriptionally dependent process in both HPA and HPV endothelial cells. Analysis of chromatin structure identified a number of sites that reside in the 5′ flanking region and within the ET1 gene. The approximate positions, relative to start of transcription, of all the hypersensitive sites are as follows: −6.7 kb, −3.2 kb, −1.3 kb, −700 bp, −350 bp, −170 bp, −150 bp, +40 bp, +300 bp, +700 bp, +5.6 kb, and +5.8 kb. We observed no change in these sites between basal and thrombin-stimulated cells, which suggests that the ET1 gene promoter is a preset promoter.

Promoter deletion analysis in both HPA and HPV endothelial cells demonstrated that −141 to −373 bp is essential for basal-dependent and −373 to −484 is necessary for thrombin-dependent expression. However, full expression for both conditions required an element within −952 bp. The relative fold induction of the reporter gene in response to thrombin was two- to threefold, which is similar to ET-1 mRNA induction and suggests that the thrombin-dependent regulatory regions are contained within −952 bp of the 5′ flanking region of the ET1 gene and is dependent solely on a transcriptional event.

DISCUSSION

A composite of the estimated regions of the DNase I hypersensitive sites in the ET1 gene and 5′ flanking region deletion analysis is shown in the Figure 1. Only those constructs that contain regions of DNase I hypersensitivity with an increase in construct size are shown. The putative cis-regulatory regions defined by DNase I correlate with those regions shown to be important for basal- and thrombin-stimulated expression of ET-1 in transient transfection assays.

Our studies have provided an analysis of the transcriptional activity of the ET1 gene promoter. These data are the first such analysis of the function of the human ET1 gene promoter in a homologous system with a tissue type relevant to human disease and specifically pulmonary disease. It is also the first detailed analysis of stimuluss-induced ET1 gene promoter function. At present, the transcriptional regulation of the ET1 gene appears to be a complex system with the possibility of a number of interacting regulatory sites involved in full basal- and thrombin-dependent expression. This may not be surprising because ET-1 is an extremely potent vasoconstrictor, and its expression must be highly regulated to ensure the maintenance of appropriate vascular tone.

REFERENCES


Endothelial Cell Hypoxic Stress Proteins*

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Among mammalian cells, vascular endothelial cells (EC) are often exposed to changes in their local environment, particularly decreases in ambient oxygen tension. Thus, tolerance to acute hypoxia and adaptation to chronic

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