

## Heat Shock of Vascular Endothelial Cells Induces an Up-regulatory Transcriptional Response of the Thrombomodulin Gene That Is Delayed in Onset and Does Not Attenuate\*

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**Thrombomodulin is a vascular endothelial cell transmembrane protein that forms a 1:1 complex with thrombin, this interaction product forming the basis of a physiologically important natural anticoagulant system. Transcriptional down-regulation of thrombomodulin occurs following exposure of cultured endothelial cells to cytokines, while up-regulation is induced by retinoic acid and dibutyryl cyclic AMP. Thrombomodulin is also regulated developmentally, appearing in the parietal endoderm of 7.5-day-old mouse embryos. We determined that cell surface functional thrombomodulin in cultured human umbilical vein endothelial cells (HUVEC) and A549 cells increased 3.2- and 6.7-fold, respectively, in response to 24 h of continuous 42 °C heat shock stress. Northern analyses of thrombomodulin mRNA accumulation also showed a delayed response that was characterized by an augmentation in mRNA levels that started 12–18 h after the initiation of the stress, and continued to rise, without attenuation, during 48 h of continuous heat shock. Nuclear run-on studies confirmed that the predominant mechanism of augmentation was transcriptional. Furthermore, the heat shock-induced up-regulation of thrombomodulin in HUVEC abrogated the suppressive effect of tumor necrosis factor. Analysis of the 5' region of the thrombomodulin gene revealed six highly conserved tandem copies of the five base pair recognition unit that is the consensus sequence for a heat shock element. We hypothesize that the stress-induced augmentation in thrombomodulin gene transcription is mediated via heat shock factors binding to the heat shock element and that the stress response of thrombomodulin may have a biological role to protect the vascular endothelium during a variety of stresses, including inflammation, infection, and/or development.**

Thrombomodulin is a vascular endothelial cell transmembrane protein that forms a 1:1 stoichiometric complex with thrombin, this interaction product forming the basis of a major natural anticoagulant system. When bound to thrombomodulin, thrombin is able to activate protein C which in turn cleaves

factors Va and VIIIa, thereby down-regulating further thrombin production (1–4). Furthermore, when thrombin is bound to thrombomodulin, the bound thrombin is less available to act on its substrates, particularly platelets and fibrinogen (2, 5). Studies in mice that demonstrate the prevention of thrombin-induced thrombosis by thrombomodulin administration, and its potentiation with anti-thrombomodulin antibodies, lend further evidence as to the *in vivo* importance of thrombomodulin as an anticoagulant (6).

Although thrombomodulin is expressed constitutively on the surface of vascular endothelial cells, its *in vitro* expression may be altered by a variety of modulators and is known to be regulated at several levels (7). Thrombomodulin production may be suppressed by the cytokine tumor necrosis factor (TNF)<sup>1</sup> via both transcriptional and internalization mechanisms (8–10). Thrombin-induced endocytosis of the thrombin-thrombomodulin complex with degradation of thrombin has been invoked as a mechanism for clearing thrombin from the circulation (11–13). Several investigators have reported that agonists including retinoic acid, cAMP, histamine, forskolin, phorbol esters, and thrombin may enhance transcription of thrombomodulin in human umbilical vein endothelial cells (HUVEC), vascular smooth muscle cells, megakaryocyte cell lines, HL60 cells, and F9 embryonal carcinoma cells (7, 14–26). Finally, post-translational modifications, glycosylation, and oxidation of thrombomodulin have also been implicated in the regulation of its functional cell-surface expression (27–29).

Recent studies have begun to identify those 5' regions of the thrombomodulin gene that are important transcriptionally, providing positive and negative regulatory elements for constitutive and modulated expression (30–32). One group (31) determined that the thrombomodulin promoter starting at –51 and including the TATA box is sufficient for response to TNF but only in those cells exhibiting sensitivity to the cytokine. Furthermore, this same group found a major positive acting DNA sequence 30–70 nucleotides upstream of the RNA transcriptional start site as defined by transient thrombomodulin promoter/chloramphenicol acetyltransferase assays and DNase I “footprinting” experiments (31). Our preliminary experiments revealed a heat-sensitive increase of thrombomodulin activity on cultured cells. Subsequent examination of the –80 to –30 region of the promoter revealed multiple tandem copies of a 5-bp recognition unit, nGAAn, and its inverted repeat, that is the consensus sequence referred to as a heat shock element

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; HUVEC, human umbilical vein endothelial cells; HSE, heat shock element; db-cAMP, dibutyryl cyclic AMP; PBS, phosphate-buffered saline; bp, base pair(s); kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSF, heat shock factors; SMC, smooth muscle cells.

(HSE) (33–40). Binding of heat shock factor(s) to the HSE(s) of several genes in yeast and eukaryotic cells is known to enhance transcription rapidly, thereby providing a fundamental mechanism to protect cells from a variety of stresses (36, 41).

In this report we show that transcription of the thrombomodulin gene is highly responsive to heat shock stress in HUVEC and A549 adenocarcinoma cells. This mechanism, which heightens expression of thrombomodulin and interferes with the suppressive effect of TNF on thrombomodulin, may be critical in protecting the vascular endothelium from thrombotic tendencies in pathologic situations and may also be relevant in the developmental regulation of expression of thrombomodulin in mouse embryos and F9 embryonal carcinoma cells (42). The unique nature of the stress-induced response of the thrombomodulin gene may provide a model to further our understanding of the biological role and mechanisms of action of heat shock proteins.

#### EXPERIMENTAL PROCEDURES

**Materials**—Purified bovine protein C was obtained from Enzyme Research Laboratories (South Bend, IN), and the chromogenic substrate, HD-Phe-Pip-Arg-pNA (S2238) was provided by Helena Laboratories (Beaumont, TX). Bovine thrombin and human thrombomodulin from placenta were purified as previously detailed (43–45). Restriction enzymes were from Boehringer Mannheim Canada (Dorval, Quebec), nitrocellulose membranes from Schleicher & Schuell, and  $^{32}$ P-labeled products from ICN Biomedicals (Canada). All-trans retinoic acid and dibutyryl cyclic AMP (db-cAMP) were obtained from Sigma, RNase T1, yeast tRNA, and DNase I were from Life Technologies, Inc./BRL (Burlington, Ontario, Canada), endothelial cell mitogen from Biomedical Technologies Inc. (Stoughton, MA), and NTPs from Pharmacia Biotech. Inc. (Baie d'Urfe, Quebec, Canada). Recombinant human TNF- $\alpha$  purchased from Boehringer Mannheim (Laval, Quebec, Canada) has a specific activity of  $10^8$  units/mg.

**Cell Culture**—HUVEC were harvested and cultured as previously reported (46, 47). Experiments were performed on cells at passages 2–5. A549 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cell cultures were incubated at 37 °C (or exposed to the specified "heat shock" temperature) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were exposed to retinoic acid and/or db-cAMP at final concentrations of 1  $\mu$ M and 0.5 mM, respectively, at 37 °C for the noted periods of time and washed with PBS prior to further processing. The measurements of functional cell surface thrombomodulin, RNA isolations, and nuclear extractions for run-on assays were conducted after cells were grown to 2–3 days post-confluence. Media was changed 24 h prior to all experiments.

**Protein C Activation**—Cells were grown in 24-well dishes, exposed to heat shock for varying periods of time, and the levels of functional cell surface thrombomodulin were evaluated using the synthetic amidolytic substrate, S2238, by methods previously reported (47). Cells were counted following the reaction, and the rate of change in absorbance at 405 nm/viable cell was determined. Viability of cells was evaluated by trypan blue exclusion.

**Preparation of cDNA Probes**—The plasmid Bluescript (pBS) containing a 6.4-kb genomic DNA fragment spanning all of the transcribed human thrombomodulin gene was prepared as previously outlined (8). The DNA probe used to detect thrombomodulin mRNA was a *XhoI/XbaI* 3.1-kb fragment from the thrombomodulin gene that spans the entire coding region for the protein. The cDNA probe for rat brain  $\beta$ -tubulin (RBT3) was a gift from Dr. Matt Fenton, Cambridge, MA (48). The plasmid pGAD28 containing the cDNA for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was provided by Dr. Robert J. Schwartz, Houston, TX (49). cDNA encoding human tissue factor was a gift of Dr. William H. Konigsberg (Yale University School of Medicine, New Haven, CT). The *Sau3A-HindIII* fragment of this cDNA spanning nucleotides 90–1364 (50) was used for tissue factor mRNA detection. DNA encoding the human heat shock protein HSP70 was provided by Dr. Jack Hensold (Case Western Reserve University, Cleveland, OH). All DNA inserts were subcloned into pBS for the purposes of nuclear run-on experiments. Plasmids were purified by alkaline lysis and CsCl gradient centrifugation (51). The cDNA probes were purified on a low melting point agarose gel and radiolabeled with [ $\alpha$ - $^{32}$ P]dCTP using the random primer synthesis method (52). The specific activity of the probes was approximately  $2 \times 10^6$  counts/min/ng of DNA.

**RNA Isolation and Northern Analysis**—Total RNA was isolated from the cells by the single step method of Chomczynski *et al.* (53). Northern analyses were performed as described previously (47, 51).

**Isolation of Nuclei and Measurement of *in Vitro* Gene Transcription**—Following heat shock, approximately  $5 \times 10^7$  cells/sample in monolayers were washed with ice-cold  $1 \times$  PBS. The cells were incubated on ice for 5 min with cold lysis buffer containing 0.15 M NaCl, 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.6% Nonidet P-40, and scraped into prechilled centrifuge tubes in which the lysates were spun for 5 min at  $500 \times g$ . The resulting nuclear pellets were gently resuspended in  $1 \times$  storage buffer (100 mM Tris-HCl, pH 7.9, 4 mM MgCl<sub>2</sub>, 4 mM MnCl<sub>2</sub>, 200 mM NaCl, 0.4 mM EDTA, 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 40% glycerol, 0.1 mM phenylmethylsulfonyl fluoride) and frozen in liquid nitrogen.

Nuclear run-on assays were performed by incubating the nuclei for 30 min at 27 °C with 150  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (3000 Ci/mmol) in a reaction buffer containing 100 mM Tris-HCl, pH 7.9, 4 mM MgCl<sub>2</sub>, 4 mM MnCl<sub>2</sub>, 0.2 M NaCl, 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM each of ATP, CTP, GTP, and dithiothreitol, 10 mM creatine phosphate, and 40 units of RNasin (8, 54, 55). The reactions were stopped by addition of 125  $\mu$ g of tRNA and 24  $\mu$ g of DNase I at 37 °C for 10 min. Further DNase I was added as necessary until the solutions were no longer viscous. To each 200- $\mu$ l sample was added 100  $\mu$ l of a buffer containing 30 mM Tris-HCl, pH 7.9, 30 mM EDTA, 1.5% SDS, and proteinase K to a final concentration of 300  $\mu$ g/ml. This mixture was incubated for 30 min at 42 °C, after which total RNA was extracted as detailed above.

Each preparation of nuclei yielded  $5\text{--}10 \times 10^6$  counts/min of labeled nuclear RNA. Equal numbers of counts of the labeled RNA were hybridized at 42 °C for 2–3 days in the Northern blot hybridization buffer with 5  $\mu$ g of each of the plasmid cDNA that were denatured and immobilized by dot-blotting onto nitrocellulose filters. The filters were washed sequentially at 55 °C in  $1 \times$  SSC, 1% SDS, and  $0.2 \times$  SSC, 0.2% SDS for 15 min each. This was followed by a wash at 37 °C with  $2 \times$  SSC containing RNase A 10  $\mu$ g/ml and RNase T1 5 units/ml for 15 min, prior to final washes in  $2 \times$  SSC. The  $^{32}$ P-labeled RNA bound specifically to the filters was visualized by autoradiography at  $-70$  °C using intensifying screens. Nonspecific hybridization was determined to be negligible, as assessed by using filters containing the plasmid Bluescript.

**Cyclic AMP Assay**—Following incubation of cell monolayers at 42 °C for the noted times, the cells were placed on ice, washed three times with cold PBS, scraped, and suspended gently in 1 ml of PBS. Cells were counted and assessed for viability. Ice-cold ethanol was added to a final concentration of 65% (v/v), the precipitated proteins were pelleted at  $2000 \times g$  for 15 min at 4 °C, and washed once with ice-cold 65% ethanol. The supernatants were pooled, cleared again by centrifugation of any precipitate, and dried by speed-vac lyophilization. The dried extracts were suspended in a suitable volume of assay buffer prior to analysis using the Biotran enzyme-immunoassay kit according to the manufacturer's instructions (Amersham, Montreal, Quebec, Canada).

**Analysis of Data**—Statistical analyses of data were conducted by standard techniques (56) with the aid of StatView computer program for the Macintosh (Abacus Concepts Inc., CA). The means are provided with associated standard errors (S.E.) or standard deviations (S.D.).

#### RESULTS

The 5' ends of both the human and murine thrombomodulin genes contain a highly conserved sequence just upstream of the TATA box that consists of six inverted repeats of a five bp consensus element (nGAAn), referred to as the HSE (Fig. 1). The two pairs of central pentads are perfect repeats separated by an interval nucleotide. Flanking each side of these four, is an additional imperfect pentad with correct nucleotide spacing. When in arrays of two or more inverted repeats, the HSE has been shown to provide a site of interaction with heat shock factors (HSF), thereby conferring on the target gene a means of rapid transcriptional up-regulation when exposed to a variety of stresses, including exposure to heat (36, 57).

We first examined the effect of heat shock on the cell-surface expression of functional thrombomodulin in HUVEC and A549 cells, both of which are known to express thrombomodulin constitutively. Functional levels of thrombomodulin were quantitated by chromogenically measuring the time-dependent conversion of protein C to its active form in the presence of exogenously added thrombin. During continuous exposure of A549 cells to 42 °C heat shock, cell surface functional throm-



Human	5'	GCAATCCGAGTATGCGGCATCAGCCCTTCCC	-80
Mouse	5'	GTAATCCGAGAACGCAGCTTCAGCCCTTCCC	-88
Human		AC CAGGC <u>ACTTC CTTC</u> T <u>TTTCC</u> CGAAC GTCCA	-47
Mouse		AC CAGGC <u>ACTTC CTTC</u> T <u>TTTCC</u> CGAAC GTGCA	-55
HSE		NAAGN NCTTC NTTCN NTTCN NGAAN NTTCN	
Human		GGGAGGGAGGGCCGGGCATTATAAAC	-20
Mouse		AAGAGGGAGGGCCGGGCATTATAAAC	-28
HSE			

FIG. 1. 5' regions of human and mouse thrombomodulin genes. The consensus sequence for the classic heat shock element (HSE) is compared with the highly conserved putative HSE in the thrombomodulin gene. Nucleotides in **bold** indicate imperfect repeats that flank the central four perfect repeats (**bold and underlined**). Numbering of human and mouse thrombomodulin genes is from Yu *et al.* (31) and Niforas *et al.* (32), respectively.

bomodulin decreased by approximately 50% at 3 and 6 h, but by 24 h had increased 6.7-fold as compared with parallel control cells maintained at 37 °C (Fig. 2A). A similar early transient decrease in functional cell-surface thrombomodulin was not detected in HUVEC, although by 24 h, thrombomodulin had increased 3.2-fold (Fig. 2B). Longer exposures of the HUVEC to 42 °C led to loss of cell adherence to the culture plate, preventing reliable quantitative studies at later time points. Consequently, A549 cells, which maintained both viability and adherence, were studied at periods of continuous heat shock of up to 48 h, and exhibited a persistent and continuous augmentation in thrombomodulin cell-surface functional expression (Fig. 2A) with no evidence of attenuation.

Most classic heat shock up-regulatory transcriptional responses occur within minutes (57), whereas both the transcription of previously active non-heat shock genes and the translation of pre-existing mRNAs are usually repressed (58). In the preceding experiments, functional cell surface thrombomodulin expression was transiently suppressed in A549 cells during 42 °C heat shock exposures of less than 6 h. However, in contrast to other non-heat shock proteins, continued heat shock resulted in an eventual increase in expression of the functional cell surface thrombomodulin, with persistent augmentation without attenuation. This complex response to heat shock might be explained partially by alterations in protein and/or mRNA stability that may be dependent on the degree of thermal stress. Alternatively, rapid changes in transcription during the course of the heat shock stress, partly as a result of HSF(s) binding to the consensus HSE, could result in the apparent biphasic observed response.

In order to clarify the above, we determined the effect of heat shock on the levels of HUVEC and A549 cell thrombomodulin steady-state mRNA by Northern blot analyses. As seen in Fig. 3, the specific 3.8-kb thrombomodulin mRNA was readily detectable in cells maintained at 37 °C. Within 2 h of transferring the cells to 42 °C, thrombomodulin mRNA decreased markedly and remained suppressed to almost undetectable levels for over 6 h, the kinetics of which were similar to those seen with A549 cell surface functional thrombomodulin expression. This was subsequently followed by a continuous and dramatic accumulation of specific thrombomodulin mRNA, the upward trend continuing during the entire 48 h of heat shock stress applied (Fig. 4). The magnitude of the mRNA response exceeded that seen for cell-surface functional protein expression, although the kinetics of the response in the A549 cells were again similar (Fig. 5). In parallel Northern blots, heat shock elicited no change in the mRNA levels of GAPDH (Figs. 3–5), thereby confirming the specific nature of the response. In contrast to the response seen with thrombomodulin, HSP70 mRNA responded "classically," increasing rapidly and dramatically from

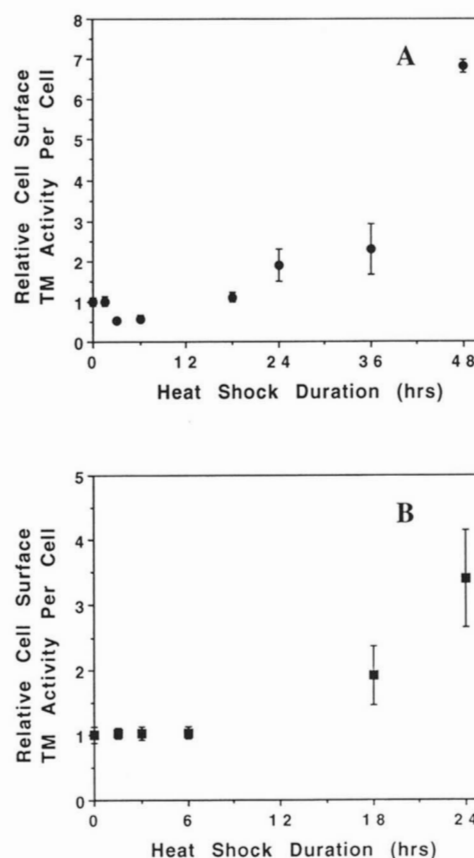


FIG. 2. Cell surface expression of functional thrombomodulin in response to heat shock. A549 cells (A) and HUVEC (B) were grown to post-confluence at 37 °C and exposed to 42 °C for varying periods of time. Cell-surface thrombomodulin activity was determined as detailed under "Experimental Procedures." The results reflect the mean of an experiment done in triplicate with associated standard error bars and are representative of three independent studies.

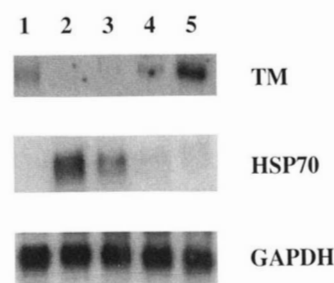


FIG. 3. Northern blot analysis of HUVEC in response to heat shock. Post-confluent HUVEC were exposed to continuous 42 °C heat shock for 0, 2, 4.5, 7, and 20 h (lanes 1–5, respectively). 10 µg of total RNA from each sample were separated on a denaturing formaldehyde-agarose gel as detailed under "Experimental Procedures." Accumulation of specific mRNA for thrombomodulin, HSP70, and GAPDH were evaluated using the appropriate radiolabeled cDNA probes.

initially undetectable levels within 1 h of the stress and subsequently attenuating to a less elevated base line. When the cells were exposed to a transient heat shock of 90 min followed by incubation at 37 °C, the mRNA for HSP70 rose rapidly and again declined abruptly to a lower plateau when the heat stress was withdrawn. Under these conditions of transient stress to the cells, there was no subsequent increment in thrombomodulin mRNA level in HUVEC or A549 cells (data not shown).

Abravaya *et al.* (59) has reported a differential effect of heat shock responses in HeLa cells that depends on the magnitude of the difference between growth and heat shock temperatures.



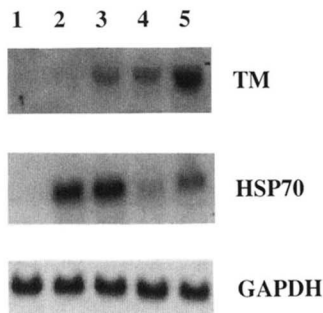


FIG. 4. Northern blot analysis of HUVEC in response to heat shock. HUVEC were exposed to 42 °C continuous heat shock for 0, 18, 24, 36, and 48 h (lanes 1–5, respectively) and evaluated as in Fig. 3.

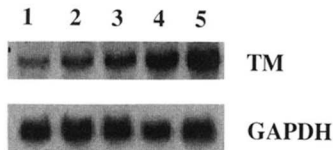


FIG. 5. Northern blot analysis of A549 cells in response to heat shock. Post-confluent A549 cells were exposed to 42 °C continuous heat shock for 0, 18, 24, 36, and 48 h (lanes 1–5, respectively) and evaluated as in Fig. 3.

We therefore considered the possibility that altering the degree of stress might elicit a more classical thrombomodulin heat shock response. However, although the kinetics of thrombomodulin mRNA accumulation varied somewhat, the pattern of response as a result of exposing the cells to 41 or 43 °C was not different as compared with the response following the 42 °C stress (not shown).

Our results indicated that heat shock leads to a complex time-dependent variation in the expression of thrombomodulin mRNA in HUVEC and A549 cells, but direct support for the hypothesis that this effect was due to alterations in transcription was required. Using nuclear “run-on” experiments, we evaluated *in vitro* transcription in both control and heat shock-treated HUVEC. As depicted in Fig. 6, HSP70 gene transcription was undetectable in the control unstressed cells. Following continuous 42 °C heat shock, HSP70 mRNA transcription was increased dramatically by 90 min, followed by a diminished but easily detectable level as the stress continued past 6 h. In contrast, and similar to the Northern analyses, thrombomodulin gene transcription was dramatically suppressed within 1.5 h of the onset of heat shock. The diminished transcription persisted for over 18 h, after which time it began to increase gradually and persistently for the 48 h examined. We could not entirely exclude the possibility that heat shock also altered the stability of the mRNA thereby further contributing to the pattern of accumulation of thrombomodulin mRNA and protein levels. However, the similar time frame of changes in thrombomodulin gene transcription, mRNA accumulation, and functional cell surface expression of the receptor suggested that transcription was likely the predominant mechanism. Transcription of tubulin, GAPDH, and human tissue factor genes remained unaltered during the course of the heat shock stress, indicating the relatively specific nature of the thrombomodulin response (data not shown).

We have previously reported that a rapid decline in thrombomodulin gene transcription in HUVEC is a major mechanism by which the cytokine, TNF, suppresses cell surface functional expression of the receptor (8). In contrast, TNF causes tissue factor expression to be markedly up-regulated in vascular endothelial cells via transcriptional mechanisms (60). These two cell-surface proteins therefore, by their action, enhance the procoagulant properties of the vascular endothelial cell surface

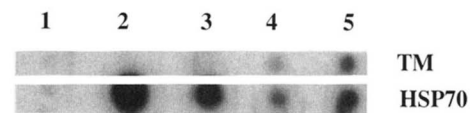


FIG. 6. Nuclear run-on studies of thrombomodulin and HSP70 genes in HUVEC exposed to heat shock. Post-confluent HUVEC were exposed to 42 °C continuous heat shock for 0, 1.5, 18, 24, and 48 h (lanes 1–5, respectively). *In vitro* transcription was evaluated as detailed under “Experimental Procedures.”

when modulated by TNF. If the observed early heat shock-induced suppression of thrombomodulin was mediated by TNF, we would therefore anticipate that tissue factor mRNA levels would be augmented during that same period of time. Our Northern analyses and nuclear run-ons indicated that heat shock does not alter tissue factor expression (data not shown), largely excluding the possibility that TNF is mediating the down-regulation of thrombomodulin gene transcription during heat shock. Further evidence to support our conclusion is the fact that the heat shock response of A549 cells, a cell line that has been shown to be largely resistant to TNF-induced suppression of thrombomodulin (31), is similar to that of HUVEC with respect to an early transient decrease in thrombomodulin mRNA levels.

Several investigators have previously reported that thrombomodulin may be up-regulated by retinoic acid and/or db-cAMP in a variety of cultured cells, including HUVEC, Dami cells, and F9 embryonal carcinoma cells (16, 17, 19, 20, 23). The time course of elevation of thrombomodulin mRNA and cell-surface expression induced by heat shock was reminiscent of the effect of these modulators on the expression of the receptor. This prompted us to consider whether 1) heat shock might increase intracellular cAMP levels, thereby increasing thrombomodulin gene transcription, and 2) modulation of the cells with retinoic acid and/or db-cAMP might enhance thrombomodulin gene transcription via a heat shock element-dependent mechanism. To examine the first possibility, A549 cells and HUVEC were exposed to varying periods of heat shock and were subsequently extracted for quantitation of intracellular cAMP levels. In parallel, cell surface thrombin-dependent protein C activation was determined during the time course and found to be similar to that seen in Fig. 2. In a representative experiment performed in triplicate following 48 h of exposure of A549 cells to 37 or 42 °C, cAMP levels were 864 fmol/10<sup>6</sup> cells (S.D. = 224) and 800 fmol/10<sup>6</sup> cells (S.D. = 267), respectively, while they were 1072 fmol/10<sup>6</sup> cells (S.D. = 237) and 828 fmol/10<sup>6</sup> cells (S.D. = 261), respectively, in HUVEC. In spite of a marked increase in thrombomodulin mRNA and cell-surface functional levels, there was no significant difference ( $p > 0.5$ ) in cAMP levels in HUVEC or A549 cells at all time points up to 48 h of exposure to 37 versus 42 °C.

The second possibility, *i.e.* that following exposure of the cells to retinoic acid and/or db-cAMP heat shock factor binding to the HSE might be enhanced thereby augmenting thrombomodulin gene transcription, was not evaluated directly. However, this scenario was not felt to be likely, since it has previously been reported that F9 cells constitutively express high levels of HSP70 that gradually diminish following differentiation with retinoic acid and db-cAMP (61). Our findings indicated that there was no detectable change in HSP70 mRNA in HUVEC after 3 and 5 days of treatment with retinoic acid and db-cAMP, during which time thrombomodulin mRNA increased several-fold (not shown). We recognize, however, that this does not exclude the possibility that other heat shock factors may play a critical role in up-regulating thrombomodulin during exposure to these “differentiating” agents.

The heat shock response is ubiquitous to essentially all cells, and we therefore considered the possibility that non-thrombo-

modulin-producing cells might be induced to transcribe thrombomodulin mRNA under this stress. MRC5 human embryonal lung fibroblasts (Connaught Laboratories, Toronto, Canada) were consequently exposed to continuous heat shock of 43 °C following culture at 37 °C. Thrombomodulin mRNA remained undetectable during the entire 48 h of continuous heat shock (data not shown), suggesting that stronger negative regulatory forces were active in preventing thrombomodulin expression in these cells, or alternatively, that the accessibility of the necessary HSF(s) to bind to the HSE of thrombomodulin was restricted within the chromatin in these normally non-thrombomodulin producing cells (62).

Finally, we examined the effect of heat shock on the regulation of thrombomodulin by TNF by exposing HUVEC to either 37 or 42 °C for a total of 24 h, in the presence or absence of TNF (100 units/ml) for the final 20 h. In a representative experiment performed in triplicate at 37 °C, TNF suppressed thrombomodulin cell surface functional activity to  $56.6 \pm 7.9\%$  (S.D.) as compared with control cells. Following heat shock alone, thrombomodulin activity increased to  $222 \pm 41\%$  (S.D.). Administration of TNF while the cells were exposed to 42 °C resulted in thrombomodulin activity levels of  $205 \pm 53\%$  (S.D.), not significantly different from heat shocked cells alone ( $p > 0.5$ ). These experiments support our hypothesis that heat shock may protect thrombomodulin from the down-regulatory forces of TNF in HUVEC.

#### DISCUSSION

As part of an ongoing investigation into mechanisms for the up-regulation of thrombomodulin, we had observed that heat shock caused a progressive increase in cell-surface functional thrombomodulin as evaluated by thrombin-dependent protein C activation. Subsequent analysis of the DNA sequence of the thrombomodulin promoter revealed the presence of a putative HSE spanning the 5' region of the human thrombomodulin gene from -77 to -47 (CAGGCACTTCCTTCCTTTTCCCGAACGTCCA), that is entirely conserved in the human and mouse, except for a single nucleotide difference. Furthermore, Yu *et al.* (31) have shown that the approximately 70 bp upstream of the transcriptional start site have positive functional activity and that specific interaction products essential for thrombomodulin gene transcription protect the DNA sequence from -65 to -43 on the coding strand. The elucidation of the mechanism by which this region exerts a positive regulatory function has hitherto not been reported. We hypothesized that the putative HSE in the thrombomodulin gene may confer heat stress inducibility in those cells that express thrombomodulin constitutively and that heat shock would up-regulate expression via transcriptional mechanisms. This was confirmed in HUVEC and A549 cells by analyses of cell-surface functional expression of thrombomodulin, mRNA levels and *in vitro* nuclear run-on assays. The observed augmentation in thrombomodulin transcription was particularly noteworthy because of 1) the strikingly delayed response, and 2) the absence of any recovery or attenuation during 48 h of continuous heat shock stress.

In eukaryotic cells, stress-induced up-regulation in transcription is mediated by HSF that in unstressed cells are present in the nucleus and cytoplasm in a non-DNA binding state. Under stress, the HSF oligomerizes, accumulates inside the nucleus, binds to the HSE in the 5'-flanking sequence of the responsive gene, and leads to a rapid increase in transcription, as exemplified by the response of HSP70 in our studies using HUVEC and A549 cells (33, 34). This is generally followed by a "recovery" phase in which gene transcription decreases upon return to pre-heat shock temperatures. As elucidated by Sorger (33), however, this classical response is not the only response.

Exposure of budding yeast cells to elevated temperatures also leads to sustained and persistent changes in HSF activity. Furthermore, COOH terminus deletion mutants have revealed that separate domains of the HSF are required for transient *versus* sustained heat shock transcriptional activation (63), and each may respond to different stimuli. In eukaryotic cells, this dual mechanism is not clearly evident, although Abravaya *et al.* (59) have demonstrated that HeLa cells that were heat shocked at 42 °C showed transient HSF activation, while at 43 °C, HSF activity could be maintained during several hours of continuous heat shock.

To our knowledge, apart from thrombomodulin, there are no other examples in mammalian cells in which continuous heat shock is manifest by a transient decrease, followed by a gradual increase in gene transcription and sustained elevation. There are several possible explanations for thrombomodulin's uncharacteristic response to heat shock. 1) Although the HSE confers stress inducibility, multiple cis-regulatory elements confer a range of constitutive and inducible transcriptional responses, and consequently, the kinetics and magnitude of DNA binding activity of HSF to the HSE may be alternatively regulated (57). 2) The specific HSF(s) mediating the increment in thrombomodulin transcription may bind particularly slowly to the HSE either due to intrinsic properties of the HSF, or due to the presence of negative transcription factor(s). The latter may either interfere with binding or variably regulate the otherwise generally rapid effect of HSF on increasing transcription. 3) Diversity in the sequence of the HSE may also play a part in altering the binding and dissociation rates of HSFs (59). In this respect, the promoter region of the thrombomodulin gene has two pairs of consensus repeats separated by a single thymidine, the effect of which has not been examined, but may play a role in the unusual response of this gene to heat shock. 4) Lack of release of bound HSF from the HSE of thrombomodulin at the temperatures and times studied may explain the extended response that has similarly been observed for HSP70 in HeLa cells when exposed to 43 °C (59).

Although its natural anticoagulant properties have been carefully characterized, the multidomain structure of thrombomodulin and its presence in a variety of non-vascular tissues suggests that it may have other functions. Thrombomodulin appears early in the organogenesis of vascular tissue in parietal endoderm of 7.5-day-old mouse embryos (64), but at 10.5 days is also present in other nonvascular structures (42, 64). Treatment of F9 mouse teratocarcinoma cells with retinoic acid followed by db-cAMP leads to differentiation into primitive parietal endoderm accompanied by expression of thrombomodulin (also referred to as fetomodulin) (23, 42, 64). "Classic" heat shock protein expression in mammals is also developmentally regulated, and its constitutive expression may be critical for normal development (for review, see Refs. 65, 66). A role for heat shock gene expression has also been implicated in the differentiation of several cells, including muscle cells, myelomonocytic cells (67), and in the human cell line K652 (68). In spite of this, we could not demonstrate a definite relationship between HSP70 synthesis and the expression of thrombomodulin in F9 cells. The *in vitro* differentiation of F9 cells by treatment with retinoic acid and db-cAMP is characterized by a steady decline in HSP70 mRNA expression (61) from otherwise spontaneously elevated levels. This is in contrast to thrombomodulin, which is absent in undifferentiated F9 cells and is markedly increased during differentiation by transcriptional mechanisms (23, 42, 64). An indirect mechanism linking heat shock proteins and thrombomodulin synthesis is possible, but evidence for such a connection is lacking.



Since the kinetics and intensity of the increment in thrombomodulin in response to either retinoic acid/db-cAMP or heat shock were similar, we postulated that retinoic acid might act indirectly on the promoter by augmenting HSF binding to the HSE of thrombomodulin. Our experiments determined that HSP70 mRNA was not responsive to treatment with retinoic acid/db-cAMP. This, however, does not exclude the possibility of a role of HSF in augmenting transcription of thrombomodulin following exposure to retinoic acid/db-cAMP. More direct evidence for such a role will require *in vivo* genomic footprinting analyses and/or gel mobility-shift assays (59, 69).

Heat shock proteins and their response to stress comprises the most highly conserved system in evolution. This fact underlines the importance of their function in the protection of cells and organisms from the harmful effects of a variety of stresses and supports the contention that the heat shock response plays a critical role in several biological systems and disease states, including development, ischemia, inflammation, tissue damage, cancer, and aging (for reviews, see Refs. 35, 36). In keeping with the functional significance of the heat shock response, it is intriguing to note that with few exceptions heat shock genes are intronless. This is otherwise a rare finding in higher eukaryotes that has been postulated to ensure rapid and accurate protein expression, without the need for intron processing. The fact that the thrombomodulin gene is also lacking in introns (70) further supports the physiologic importance of thrombomodulin.

The heat shock-induced response of thrombomodulin may be important in situations more generally labeled as high stress. For example, studies by Fink *et al.* (71) have demonstrated that thrombomodulin function and antigen levels decrease immediately following balloon injury to rabbit aortas, which, however, recover by 7 days. This "renewal" of thrombomodulin following tissue injury probably is due to induced thrombomodulin activity on normally thrombomodulin-negative smooth muscle cells (SMC). This observation is consistent with *in vitro* data presented by Soff *et al.* (21) who determined that *in situ* in the arterial wall, thrombomodulin is normally absent from the SMC layer, whereas in SMC cultures, functional thrombomodulin and its mRNA are readily detectable. Fink *et al.* (71) postulated that the SMC up-regulation of thrombomodulin following injury might be related to enhanced access to varied nutrients, growth factors or cytokines (71). We believe that as a means of protecting the vasculature from thrombotic potential, the "heat shock"-like stress response may be the underlying mechanism.

Heat shock stress induction of thrombomodulin may also be relevant with respect to recent *in vivo* septic shock experiments by Drake and co-workers (72). Following injection of baboons with lethal doses of *Escherichia coli*, vascular endothelial cell surface thrombomodulin antigenic expression remains unchanged from normal in spite of markedly elevated TNF levels. Cell culture *in vitro* data would have predicted a down-regulation in expression of thrombomodulin in response to these elevated TNF levels (8, 73). However, our *in vitro* experimental findings support the hypothesis that the forces driving to down-regulate thrombomodulin expression (*e.g.* TNF) in this case are offset by the inflammatory reaction, creating a heat shock response that augments thrombomodulin synthesis, and thereby serves to protect the vasculature from further thrombotic damage. Furthermore, this is consistent with recent *in vivo* studies in which the lethal effects of sepsis in mice are abrogated by hyperthermia (74). These investigators (74) have found that the protective effect of heat shock correlates with the appearance of HSP72 and that this is maximal at least 12 h following heat shock initiation. Based on our data, this is sufficient time

for augmentation of thrombomodulin synthesis to have occurred, consequently providing a means of protecting the vasculature from thrombotic damage.

There have been few other studies on the response of endothelial cell molecules to heat shock stress. One group, however, (75) has demonstrated that thrombospondin, a glycoprotein synthesized by platelets and endothelial cells, and suggested to play a role in the growth and proliferation of endothelial cells, increases in response to heat stress. The time course of thrombospondin's increment following administration of the stress is later than that of HSP70, although detailed studies to evaluate for eventual attenuation of the response were not performed. We speculate that the heat shock stress response might play a key role in "turning on" responsive genes such as thrombomodulin and thrombospondin, the purpose of which may be to maintain a thromboresistant surface under a variety of pathological conditions.

Finally, Udelsman and co-workers (76), using a unique *in vivo* model, have determined that vascular HSP70 levels rise dramatically in response to mild restraint stress in spite of a lack of apparent tissue or cellular damage. Furthermore, they have also reported that the HSP70 response diminishes with aging. We hypothesize that the expression of thrombomodulin may be similarly exquisitely sensitive to minimal stress *in vivo* and therefore may play a fundamental role in maintaining homeostasis. For example, a dampened stress response of thrombomodulin in the aging animal would be consistent with the observed age-associated increased risk of thrombosis and vascular disease in humans. Further studies to explore these hypotheses and the up-regulation of thrombomodulin are underway, the results of which may provide new insights into the prevention and/or treatment of vascular thrombotic disease and atherogenesis.

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## REFERENCES

- Esmon, C. T. (1987) *Science* **235**, 1348–1352
- Esmon, C. T. (1992) *Arterioscler. Thromb.* **12**, 135–145
- Fulcher, C. A., Gardiner, J. E., Griffin, J. H., and Zimmerman, T. S. (1984) *Blood* **63**, 486–489
- Suzuki, K., Stenflo, J., Dahlback, B., and Teodorsson, B. (1983) *J. Biol. Chem.* **258**, 1914–1920
- Parkinson, J., Bang, N., and Garcia, J. (1993) *Arterioscler. Thromb.* **13**, 1119–1123
- Gomi, K., Zushi, M., Honda, G., Kawahara, S., Matsuzaki, O., Kanabayashi, T., Yamamoto, S., Maruyama, I., and Suzuki, K. (1990) *Blood* **75**, 1396–1399
- Hirokawa, K., and Aoki, N. (1991) *J. Cell. Physiol.* **147**, 157–165
- Conway, E. M., and Rosenberg, R. D. (1988) *Mol. Cell. Biol.* **8**, 5588–5592
- Moore, K. L., Esmon, C. T., and Esmon, N. L. (1989) *Blood* **73**, 159–165
- Lentz, S. R., Tsiang, M., and Sadler, J. E. (1991) *Blood* **77**, 542–550
- Maruyama, I., and Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 15432–15438
- Conway, E., Boffa, M., Nowakowski, B., and Steiner-Mosonyi, M. (1992) *J. Cell. Physiol.* **151**, 604–612
- Horvat, R., and Palade, G. (1993) *Eur. J. Cell Biol.* **61**, 299–313
- Hirokawa, K., and Aoki, N. (1990) *J. Biochem. (Tokyo)* **108**, 839–845
- Hirokawa, K., and Aoki, N. (1991) *Biochem. J.* **276**, 739–743
- Ishii, H., Kizaki, K., Uchiyama, H., Horie, S., and Kazama, M. (1990) *Thromb. Res.* **59**, 841–850
- Ito, T., Ogura, M., Morishita, Y., Takamatsu, J., Maruyama, I., Yamamoto, S., Ogawa, K., and Saito, H. (1990) *Thromb. Res.* **58**, 615–624
- Iwashima, Y., Sato, T., Watanabe, K., Ooshima, E., Hiraishi, S., Ishii, H., Kazama, M., and Makino, I. (1990) *Diabetes* **39**, 983–988
- Maruyama, I., and Soejima, Y. (1990) *Ann. N. Y. Acad. Sci.* **598**, 538–539
- Maruyama, I., Soejima, Y., Osame, M., Ito, T., Ogawa, K., Yamamoto, S., Dittman, W. A., and Saito, H. (1991) *Thromb. Res.* **61**, 301–310
- Soff, G. A., Jackman, R. W., and Rosenberg, R. D. (1991) *Blood* **77**, 515–518
- Tanaka, A., Ishii, H., Hiraishi, S., Kazama, M., and Maezawa, H. (1991) *Clin. Chem.* **37**, 269–272
- Weiler, G. H., Yu, K., Soff, G., Gudas, L. J., and Rosenberg, R. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2155–2159
- Kizaki, K., Naito, S., Horie, S., Ishii, H., and Kazama, M. (1993) *Biochem. Biophys. Res. Commun.* **193**, 175–181
- Archipoff, G., Beretz, A., Bartha, K., Brisson, C., de la Salle, C., Froget-Leon, C., Kiklein-Soyer, C., and Cazenave, J. (1993) *Br. J. Pharmacol.* **109**, 18–28
- Bartha, K., Archipoff, G., de la Salle, C., Lanza, F., Cazenave, J., and Beretz, A. (1993) *J. Biol. Chem.* **268**, 421–429
- Ye, J., Esmon, C., and Johnson, A. (1993) *J. Biol. Chem.* **268**, 2373–2379

28. Lentz, S. R., and Sadler, J. E. (1991) *J. Clin. Invest.* **88**, 1906–1914
29. Glaser, C., Morser, J., Clarke, J., Blasko, E., McLean, K., Kuhn, I., Chang, R.-J., Lin, J.-H., Vilander, L., Andrews, W., and Light, D. (1992) *J. Clin. Invest.* **90**, 2565–2573
30. Tazawa, T., Hirose, S., Suzuki, K., Hirokawa, K., and Aoki, N. (1993) *J. Biochem. (Tokyo)* **113**, 600–606
31. Yu, K., Morioka, H., Fritze, L., Beeler, D., Jackman, R., and Rosenberg, R. (1992) *J. Biol. Chem.* **267**, 23237–23247
32. Niforas, P., Sanderson, G., Bird, C., and Bird, P. (1993) *Biochim. Biophys. Acta* **1173**, 179–188
33. Sorger, P. (1991) *Cell* **65**, 363–366
34. Jakobsen, B., and Pelham, H. (1991) *EMBO J.* **10**, 369–375
35. Welch, W. (1993) *Phil. Trans. R. Soc. Lond.* **339**, 327–333
36. Morimoto, R. (1993) *Science* **259**, 1409–1410
37. Pelham, H. (1982) *Cell* **30**, 517–528
38. Mirault, M., Southgate, R., and Delwart, E. (1982) *EMBO J.* **1**, 1279–1285
39. Perisic, O., Xiao, H., and Lis, J. (1989) *Cell* **59**, 797–806
40. Xiao, H., Perisic, O., and Lis, J. (1991) *Cell* **64**, 585–593
41. Sarge, K., and Morimoto, R. (1991) *Gene Exp.* **1**, 169–173
42. Imada, S., Yamaguchi, H., Nagumo, M., Katayanagi, S., Iwasaki, H., and Imada, M. (1990) *Dev. Biol.* **140**, 113–122
43. Conway, E. M., Lau, H. K., Bauer, K. A., and Rosenberg, R. D. (1987) *J. Lab. Clin. Med.* **110**, 567–575
44. Salem, H. H., Maruyama, I., Ishii, H., and Majerus, P. W. (1984) *J. Biol. Chem.* **259**, 12246–12251
45. Kurosawa, S., and Aoki, N. (1985) *Thromb. Res.* **37**, 353–364
46. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2751
47. Conway, E., Nowakowski, B., and Steiner-Mosonyi, M. (1992) *Blood* **80**, 1254–1263
48. Bond, J., Robinson, G., and Farmer, S. (1984) *Mol. Cell. Biol.* **4**, 1313–1319
49. Dugaiczky, A., Haron, J., Stone, E., Dennison, O., Rothblum, K., and Schwartz, R. (1983) *Biochemistry* **22**, 1605–1613
50. Spicer, E., Horton, R., Bloem, L., Bach, R., Williams, K., Guha, A., Kraus, J., Lin, T., Nemerson, Y., and Konigsberg, W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 5148–5152
51. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY
52. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
53. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
54. McKnight, G., and Palmiter, R. (1979) *J. Biol. Chem.* **254**, 9050–9058
55. Greenberg, M., and Ziff, E. (1984) *Nature* **311**, 433–438
56. Zar, J., (1974) *Biostatistical Analysis* (Cliffs, E., ed), Prentice Hall, Inc., New York
57. Morimoto, R., Sarge, K., and Abravaya, K. (1992) *J. Biol. Chem.* **267**, 21987–21990
58. Lindquist, S. (1986) *Annu. Rev. Biochem.* **55**, 1151–1191
59. Abravaya, K., Phillips, B., and Morimoto, R. (1991) *Genes & Dev.* **5**, 2117–2127
60. Conway, E. M., Bach, R., Rosenberg, R. D., and Konigsberg, W. H. (1989) *Thromb. Res.* **53**, 231–241
61. Bensaude, O., and Moragne, M. (1983) *EMBO J.* **2**, 173–177
62. Lee, M., and Garrard, W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9166–9170
63. Sorger, P. (1990) *Cell* **62**, 793–805
64. Imada, M., Imada, S., Iwasaki, H., Kume, A., Yamaguchi, H., and Moore, E. (1987) *Dev. Biol.* **122**, 483–491
65. Heikkila, J. (1993) *Dev. Genet.* **14**, 1–5
66. Nakai, A., and Morimoto, R. (1993) *Mol. Cell. Biol.* **13**, 1983–1997
67. Fincato, G., Polentarutti, N., Sica, A., Mantovani, M., and Colotta, F. (1991) *Blood* **77**, 579–586
68. Phillips, B., and Morimoto, R. (1991) *Heat Shock and Development* (Hightower, N. L., ed) pp. 167–187, Springer-Verlag, Berlin
69. Abravaya, K., Phillips, B., and Morimoto, R. (1991) *Mol. Cell. Biol.* **11**, 586–592
70. Jackman, R. W., Beeler, D. L., Fritze, L., Soff, G., and Rosenberg, R. D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6425–6429
71. Fink, L., Eidt, J., Johnson, K., Cook, J., Cook, C., Morser, J., Marlar, R., Collins, C., Schaefer, R., Xie, S., Hsu, S., Hsu, P. (1993) *Int. J. Dev. Biol.* **37**, 221–226
72. Drake, T., Cheng, J., Chang, A., and Taylor, F. (1993) *Am. J. Pathol.* **142**, 1458–1470
73. Nawroth, P., and Stern, D. (1986) *J. Exp. Med.* **163**, 740–745
74. Hotchkiss, R., Nunnally, I., Lindquist, S., Taulien, J., Perdizet, G., and Karl, I. (1993) *Am. J. Physiol.* **265**, R1447–1457
75. Ketis, N., Lawler, J., Hoover, R., and Karnovsky, M. (1988) *J. Cell Biol.* **106**, 893–904
76. Udelsman, R., Blake, M., Stagg, C., Li, D., Putney, D., and Holbrook, N. (1993) *J. Clin. Invest.* **91**, 465–473

**Heat shock of vascular endothelial cells induces an up-regulatory transcriptional response of the thrombomodulin gene that is delayed in onset and does not attenuate.**

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