Effects of Heat Shock on the Expression of Thrombospondin by Endothelial Cells in Culture

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Abstract. Heat-shock proteins from confluent primary cultures of bovine aortic endothelial cells were analyzed by SDS-polyacrylamide gels. In addition to the increased synthesis of the classical heat-shock proteins, there is an increase of a 180,000-mol wt polypeptide in the growth media of heat-shocked cells. Immunoprecipitation with specific antiserum indicates that the 180,000-mol wt polypeptide is thrombospondin. Assay of mRNA levels coding for thrombospondin after brief hyperthermic treatment (45°C, 10 min), followed by a recovery of 2 h at 37°C, results in a twofold increase in mRNA abundance. In contrast, the activation level of the 71,000-mol wt heat-shock protein mRNA occurs at an earlier time than for thrombospondin mRNA. Immunofluorescence microscopy was used to study the intracellular and extracellular distribution of thrombospondin. Thrombospondin is localized to a prominent pattern of granules of intracellular fluorescence in a perinuclear distribution in cells not exposed to heat. Upon heat treatment, the pattern of granules of intracellular fluorescence appears more pronounced, and the fluorescence appears to be clustered more about the nucleus. There are at least three pools of extracellular forms of thrombospondin: (a) the fine fibrillar extracellular matrix thrombospondin; (b) the punctate granular thrombospondin; and (c) the thrombospondin found in the conditioned medium not associated with the extracellular matrix. When bovine aortic endothelial cells are exposed to heat, the extracellular matrix staining of a fibrillar nature is noticeably decreased, with an increase in the number and degree of fluorescence of focal areas where the punctate granule thrombospondin structures are highly localized. No gross morphological changes in extracellular matrix staining of fibronectin was noted. However, the intermediate filament network was very sensitive and collapsed around the nucleus after heat shock. We conclude that the expression of thrombospondin is heat-shock stimulated.

To maintain metabolic homeostasis, both prokaryotic and eukaryotic cells respond to metabolic perturbations by altering their cellular machinery towards the production of so-called “stress” or “heat-shock proteins” (HSPS) (1, 42). Although functionally uncharacterized, these stress proteins appear in all organisms and have been most intensely studied during and after heat treatment (1, 42).

Cells exposed to hyperthermia exhibit altered morphology and reorganization at the levels of transcription and/or translation (9, 22, 39, 43, 49). There are approximately six polypeptides (20,000–30,000 mol wt, 70,000–73,000 mol wt, 80,000–90,000 mol wt, 100,000–110,000 mol wt) which are synthesized at elevated levels in mammalian cells incubated under certain adverse conditions (42). It has been shown that different forms of stress can induce different proteins (42). Some of the agents other than heat shock, which are known to elicit the stress response in prokaryotic or eukaryotic cells include anoxia (12, 39), amino acid analogues (18), sulfhydryl-reacting reagents (29), transition metal ions (29), uncouplers of oxidative phosphorylation (17), viral infections (37), ethanol (30), various antibiotics (14), and certain ionophores (51) and chelators.

Heat-shock proteins have been detected in a variety of cells under normal unstressed conditions. In Drosophila, the low molecular weight heat-shock proteins are transcribed during early development (51). Recently, HSP70 synthesis has been observed during mouse embryogenesis (3) and in embryonal carcinoma cells (2, 33). White et al. (42) have detected basal levels of HSP71 synthesis in adrenals and bladder from unstressed rats in vivo, while Morimoto and Fodor reported high levels of HSP70 synthesis in both embryonic and adult

Abbreviations used in this paper: BAECs, bovine aortic endothelial cells; HSP, heat-shock protein.
Materials and Methods

Endothelial Cells and Cultures

Calf aortic endothelium was isolated according to methods described by Booyse et al. (4) and cultured in HSI-LoSm (Hybridoma Research, Inc., Atlanta, GA) + 5% FCS, penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphoterin (0.25 μg/ml). When passage cells were desired, primary endothelial cells were cloned, passaged, and grown in the same growth media as the parent primary cells. Calf brain capillary endothelium was isolated according to the method of Spatz et al. (44), and rat epididymal endothelium was isolated according to Wagner and Matthews (47). Cells were identified as endothelium morphologically by their cobblestone appearance (34), immunologically by staining with fluorescently labeled anti-Factor VIII (4), and enzymatically by assaying for angiotensin II-converting enzyme. Angiotensin II-converting enzyme activity was assessed as described in the technical bulletin provided by Ventrex Laboratories (Portland, ME), the supplier of the radioactive substrate. In heat-shock experiments, 24-well cluster dishes (2 cm2/well) with confluent monolayers of endothelium were floated in a constant-temperature water bath for the indicated times. 

Protein Labeling

Primary cultures of endothelial cells were grown in 24-well cluster dishes to a density of 5 × 10³ cells/cm². The cell monolayers were washed with growth medium and replaced with 200 μl of fresh medium containing 80 Ci [³⁵S]methionine (SA 1.066 Ci/mmol; New England Nuclear, Boston, MA). In addition, the monolayers were labeled with 2.3 × 10⁶ Ci/mmol (New England Nuclear, Boston, MA) according to the method of Mumbly et al. (35). Labeling times were as indicated. The cells were then washed in Hanks’ balanced salt solution buffered with Heps (15 mM), pH 7.4 (buffered HBSS), and lysed in 200 μl of 5% SDS, 50% sucrose, 40 mM diithiothreitol (DTT), and 312 mM Tris-HCl (pH 6.8). Proteins released into the media were analyzed by first removing the growth medium from the cell monolayers and diluting fourfold with buffered HBSS containing protease inhibitors (1 mM diisopropylfluorophosphate [DFP]; 0.005% phenylmethylsulfonyl fluoride [PMSF]; 1 μg/ml leupeptin, 4 μg/ml P-toluene sulfonyl-l-arginine methyl ester [TAME]; 10 mM N-ethylmaleimide [NEM]). 20 μl of this suspension was then solubilized in sample buffer to give a final concentration of 2.5% (wt/vol) SDS, 25% sucrose, 20 mM DTT, and 156 mM Tris-HCl (pH 6.8).

Gel Electrophoresis and Autoradiography

The amount of [³⁵S]methionine incorporated into individual proteins in the cell or released into the medium was assessed by uniform-concentration polyacrylamide gels as described by Laemmli (24). In addition, two-dimensional isoelectric focusing gels prepared according to O'Farrell (38) were used to examine the protein profiles. The isoelectric focusing gels (3% polyacrylamide) were prepared with LKB ampholines (LKB Instruments, Inc., Gaithersburg, MD) to form linear gradients in the range of pH 4–8. The gradient was measured by slicing the isoelectric focusing gel into 5-mm sections, which were placed in 2 ml of degassed distilled water. The samples were shaken and the pH determined. The gels were stained with Coomassie Brilliant Blue R-250 (0.1%) in 10% isopropanol and 10% acetic acid at room temperature overnight and destained in 10% isopropanol, 10% acetic acid for 10% isopropanol, 10% acetic acid. 

Immunohistochemistry

Radioimmune precipitation was performed by harvesting radiolabeled growth medium proteins into PBS, pH 7.2, containing 0.1% SDS and 0.5% Triton X-100. The monoclonal antibody against platelet thrombospondin was obtained from Dr. Deane F. Mosher. Its characterization has been described elsewhere (16). The immunoprecipitation of bovine aortic endothelial cell-derived thrombospondin was carried out essentially as described by Mumbly et al. (35).

Immunofluorescence Microscopy

Immunofluorescence localization of thrombospondin in bovine aortic endothelial cell cultures was performed according to the technique of Ruagi et al. (41), using anti-thrombospondin immunoglobulin obtained from Dr. Deane F. Mosher. A mouse monoclonal antibody against human fibronectin was purchased from Calbiochem-Behring, Corp. (San Diego, CA; model No. 341647), and a mouse monoclonal anti-vimentin antibody was purchased from ICN Immunobiocemicals (Costa Mesa, CA; model No. 69-706). Rhodamine-labeled goat anti-rabbit IgG was from Cochrane Medical (Malvern, PA; model No. 2211-0081).

Immunoblotting

Proteins were solubilized in sample buffer to give a final concentration of 2.5 (wt/vol) SDS, 25% sucrose, 20 mM diithiothreitol, and 156 mM Tris-HCl (pH 6.8). The samples were subjected to electrophoresis on SDSPAGE gels, electroblotted onto nitrocellulose paper, and incubated with antibodies followed by anti-mouse IgG alkaline phosphatase conjugate (Pro- megabiotec, Madison, WI). The bands were visualized based on a method reported by Huyhn et al. (15).

Cell Integrity

Cell integrity was assessed by trypan blue exclusion, lactate dehydrogenase

894
Effect of continuous heat treatment on the protein profiles of the growth media of BAECs. BAECs were labeled with \(^{35}\text{S}\)-methionine for times (minutes) indicated after transfer from 37\(^\circ\)C to 41.5\(^\circ\)C (B) or 43\(^\circ\)C (C) or at 37\(^\circ\)C without heat-shock treatment before labeling (A). The protein profiles were analyzed by SDS–PAGE (9% uniform gel) and fluorography. Equal numbers of cell equivalents per lane were added. The molecular weights of the proteins which respond to heat stress are indicated by \(*\). The position of human fibronection (FN), thrombospondin (TS), and bovine serum albumin (BSA) in the gel are indicated. Note BSA in serum binds \(^{35}\text{S}\)-methionine. The band labeled in the gel at position indicated “BSg’ is not newly synthesized material but rather \(^{35}\text{S}\)-methionine associated with serum BSA.

(LDH) and \(^{56}\text{Cr}\)-release from endothelial monolayers. Lactate dehydrogenase determinations were carried out as described by Wroblewski and La Due (50) who adapted the classical assay of Kubowitz and Ott (23). The amount of LDH present in a sample was expressed as the specific activity of the enzyme (units of enzyme activity/mg of total protein).

Endothelial monolayers were also assessed for viability using a \(^{56}\text{Cr}\)-release assay. The monolayers growing in a 24-well cluster dish were first washed with growth medium; then 1 ml of \(^{56}\text{Cr}\)-containing medium (5–6 × 10\(^5\) cpm/ml) was added to each well (10\(^6\) cells/well) and incubated at 37\(^\circ\)C for 3 h. After this incubation period, the medium was removed, and the cells were washed twice with growth medium. The cultures were then placed under desired experimental conditions. At the end of the experiment, both the growth medium and the monolayer were examined for \(^{56}\text{Cr}\) content. The monolayers were lysed with 1 N NH\(_4\)OH and the samples counted in a liquid scintillation counter. Based on the total number of cpm in the cells before heat treatment, the percentage of \(^{56}\text{Cr}\) released was determined.

RNA Isolation

Confluent monolayers of endothelial cells were washed with buffered HBSS and detached with trypsin-EDTA. The cells were collected and resuspended while gently vortexing in a lysis buffer containing 1% diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO), 100 mM NaCl, 10 mM CaCl\(_2\), and 30 mM Tris-HCl, pH 7.4. Triton X-100 was added to a final concentration of 0.5%, and the suspension was immediately vortexed. After spinning out the nuclei (4,000 g for 5 min), the supernatants were transferred to new Eppendorf tubes. 5 vol of cold 7.5 M guanidinium-HCl (Bethesda Research Laboratories, Bethesda, MD), 25 mM citrate pH 7.0, and 0.5% N-laurylsarcosine were added, while the suspension was mixed, followed by the addition of 0.025 vol of 1 M acetic acid. Nucleic acids were precipitated overnight at -20\(^\circ\)C in the presence of 0.5 vol of 95% ethanol. The pellet was collected at 4,000 g for 5 min and the supernatant carefully discarded. The precipitation of nucleic acids was repeated as described above using one-half the original volume of guanidinium-HCl solution without N-laurylsarcosine. The RNA pellets were recovered by differential centrifugation and 0.2 m NaCl, and 2 vol of 95% ethanol were added. The nucleic acids were precipitated again overnight at -20\(^\circ\)C. The final RNA pellet was collected by centrifugation, resuspended in a small volume of sterile distilled water, and stored at -70\(^\circ\)C until used.

Northern Gel Hybridization and Slot Blot Analysis

Total RNA was then subjected to electrophoresis on denaturing formaldehyde gels, blotted onto nitrocellulose, and hybridized to nick-translated probes. After hybridization at 60\(^\circ\)C, the filters were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) - 0.1% SDS at 55\(^\circ\)C, dried, and exposed to X-ray film (Kodak, XAR-2). The plasmid containing the human HSP71 probe was a gift of Dr. Lee Weber (13).

The quantity of thrombospondin mRNA was determined by slot blotting by standard procedures suggested by the supplier (Schleicher and Schuell, Keene, NH) hybridization probe was prepared from the MI insert by Eco RI digestion and preparative agarose gel electrophoresis followed by nick-translation to a specific activity of 10\(^8\) cpm/\(\mu\)g. The hybridization and subsequent washes were done at high stringency.

Results

Expression of a 180,000-mol wt Polypeptide during Hyperthermia

BAECs exhibit properties attributable to stressed cells when incubated at high temperatures (19, 20, and data to be published elsewhere). When primary cultures of BAECs are ex-
posed for 2 h at 41.5°C, there is an induction of HSP71 (the most inducible of the HSP70 family and which is clearly distinguishable on two-dimensional gels from HSP73, the cognate polypeptide) (19, 20). If these cells are stressed at 43°C for 2 h, as many as five major heat-shock proteins (HSP71, 73, 80, 90, and 100) are evident (19, 20, and data to be published elsewhere) and if they are exposed to a brief hyperthermic challenge (45°C, 10 min) followed by a return to 37°C for 2 h, the induction of large amounts of HSP71 and small increases in HSP73 are noted (19, 20). Thus, heat-shock proteins are differentially expressed in confluent primary cultures of BAECs under conditions of brief hyperthermic treatment or continuous heat treatment (19, 20).

In addition to the induction of the classical heat-shock proteins in BAECs exposed to hyperthermia, there is an increase of a 180,000 mol wt polypeptide in the growth medium of these cells. The amount of the 180,000-mol wt band increases gradually with an increase in the length of hyperthermic treatment. In addition to the 180,000-mol wt polypeptide, BAECs, which are responding to heat-stress, release two polypeptides with molecular masses of 45,000 and 18,400 D into the growth medium (Fig. 1). Fig. 1 demonstrates the polypeptide profile in one-dimensional polyacrylamide gels of the growth media from cultures labeled with [35S]methionine for times indicated after transferring from 37°C to 41.5°C (Fig. 1 B) or 43°C (Fig. 1 C) or at 37°C without any heat-shock treatment before labeling (Fig. 1 A).

Fig. 2 shows the protein profile in one-dimensional SDS–polyacrylamide gels of growth media from cultures labeled with [35S]methionine for times indicated after brief hyperthermic treatment (45°C, 10 min) followed by a return to normal culture conditions (Fig. 2 B) or at control temperature without heat-shock treatment prior to labeling (Fig. 2 A). As under conditions of continuous heat treatment (Fig. 1), the amount of the 180,000-mol wt and 45,000-mol wt polypeptide increases with time (Fig. 2 B). In addition the 220,000-mol wt polypeptide increases. A change in the 220,000-mol wt polypeptide was not readily apparent under conditions of continuous heat treatment (from 41°C to 43°C). The increases in these polypeptides in the growth media of BAECs exposed to continuous heat treatment (41°C to 43°C) or brief hyperthermic treatment (45°C, 10 min) was not due to cell

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**Figure 2.** Effect of brief hyperthermic treatment on the protein profiles of the growth media of BAECs. The BAECs were labeled with [35S]methionine for times (minutes) indicated. (A) Represents cells at 37°C with no heat-shock treatment and (B) cells transferred from 37°C to 45°C for 10 min and returned to 37°C. The protein profiles were analyzed by SDS–PAGE (9% uniform gels). Each lane contained the same number of cell equivalent. The molecular weights of the proteins which respond to heat stress are indicated [*]. The position of human fibronectin (FN), thrombospondin (TS), and bovine serum albumin (BSA) in the gel are shown.
Figure 3. Effect of continuous heat treatment on the protein profile of the growth media of BAECs. BAECs were labeled with [3H]-proline for times (hours) indicated after transfer from 37°C to 41.5°C (B) or at 37°C without heat-shock treatment prior to labeling (A). D represents the protein profile of the growth medium of cells transferred from 37°C to 41.5°C for 4 h and returned to 37°C for time indicated or (C) the protein profile of the growth medium of cells at 37°C without heat shock. The protein profiles were analyzed by 9% SDS-PAGE and fluorography. Each lane contained the same number of cell equivalents. The 220,000- and the 180,000-mol wt polypeptides are indicated.

Note that, in control cultures (37°C), the relative intensity of individual bands changed somewhat from cell batch to cell batch. These differences were not related in any way to small changes in cell density or length of time in culture.

Examination of one-dimensional gels of 35S- or 3H-labeled proteins in the growth media of cells exposed to continuous heat treatment at 41.5°C for 4 h reveals many polypeptides which label with [35S]methionine (Fig. 1 B) and only two major ones, a 220,000-mol wt and a 180,000-mol wt polypeptide, which label with 3H-proline (Fig. 3, A and B). In growth media, the 180,000-mol wt polypeptide makes up ~1% of total [35S]methionine-labeled proteins secreted by heat-treated cells and 20% of the total [3H]proline-labeled proteins. When the cells incubated at 41.5°C for 4 h are returned to 37°C for 24 h, the protein profile of the growth media and monolayers are the same as those of controls (Fig. 3, C and D).

Two-dimensional gel analysis of [3H]proline-labeled growth media demonstrates the presence of the 180,000-mol wt polypeptide in the growth media of cells incubated at 37°C for 4 h and an increased amount of the polypeptide in the growth media of cells incubated at 41.5°C for the same time period (data not shown). The 180,000 mol wt polypeptide found in the growth media has pI of 4.5 as determined by isoelectric focusing.

Endothelial cells from different origins respond to continuous heat treatment (42°C, 1 h) also by increasing the amount of a 180,000-mol wt polypeptide present in their growth media. Primary cultures of BAECs (Fig. 4, lane 1), passaged cultures of BAECs (passage 12) (Fig. 4, lane 2) and bovine brain capillary endothelial cells (passage 9) (Fig. 4, lane 4) show an increased amount of a 180,000-mol wt polypeptide upon heat stress. Rat epididymal endothelial cells (passage 8) (Fig. 4, lane 3) show an increase in a 190,000-mol wt polypeptide. The 180,000-mol wt polypeptides comigrate on one-dimensional gels with purified human platelet trom-
Effect of heat treatment on the protein profile of the growth media of endothelial cells from different origins. Endothelial cells from primary (lane 1) and passage 12 (lane 2) cultures of bovine aortic endothelium, passage cultures of rat epididymal capillaries (passage 8) (lane 3), and passage cultures of bovine brain capillaries (passage 9) (lane 4) were labeled with \(^{35}\text{S}\)methionine for 1 h at 37\(^\circ\)C (A) or 42\(^\circ\)C (B). The protein profiles were analyzed in a linear 6–15% concentration gradient. Each lane contained the same number of cell equivalents. The position of human fibronectin (FN), thrombospondin (TS), and bovine serum albumin (BSA) are indicated.

The induced 180,000-mol wt polypeptide is immunoprecipitated by a monoclonal antibody to human platelet thrombospondin (Fig. 5). Preincubation of the antibody with excess human platelet thrombospondin completely prevents the immunoprecipitation of this band (data not shown). The 220,000-mol wt polypeptide is fibronectin as determined by immunoprecipitation experiments (data not shown).

To determine whether the appearance of the 180,000-mol wt polypeptide in the growth media requires new transcription (as does HSP71; data not shown) and translation, cells were incubated for 10 min at 37\(^\circ\)C with either actinomycin D (2 \(\mu\)g/ml) or cycloheximide (5 \(\mu\)g/ml) before temperature elevation and labeling. This concentration of cycloheximide blocks the overall general translation of cellular proteins by more than 80% in these cells. Actinomycin D at 1 \(\mu\)g/ml inhibits transcription by 100% in the cells. The appearance of the 180,000-mol wt polypeptide into the growth media after heat treatment is completely blocked by cycloheximide and partially blocked by actinomycin D (Fig. 6). As such, the expression of this polypeptide appears to require continued translation.

Transcriptional Regulation of HSP71 and Thrombospondin in BAECs

To determine the level of control in BAECs, transcription of HSP71 gene was measured at 37\(^\circ\)C (Fig. 7, lane A), 41.5\(^\circ\)C by heat shock. The protein band of 180,000-mol wt is identified as bovine thrombospondin by quantitative removal of the polypeptides by immunoprecipitation with anti-human platelet thrombospondin immunoglobulin. Lane 1, polypeptides secreted by control BAECs labeled with \(^{35}\text{S}\)methionine 2 h; lane 2, polypeptide secreted by a 2-h treatment at 41.5\(^\circ\)C; lane 3, isolation of polypeptides from culture media of heat-shocked BAECs by immunoprecipitation; lane 4, supernatant after removal of the polypeptides with antibodies against human platelet thrombospondin.
Figure 6. Effect of cycloheximide and actinomycin D on the protein profiles of the growth media of BAECs. Cells were preincubated for 10 min at 37°C with cycloheximide (5 μg/ml) or actinomycin D (2 μg/ml). The cells were then labeled with 3H-proline and returned to 37°C (A and C) or to 41.5°C (B and D) for 4 h. The labeled proteins were analyzed by SDS–PAGE and fluorography. (A) is the profile of the growth media of control and (B) of heat-shocked cells after Coomassie Brilliant Blue staining showing exogenous proteins in the serum of cultured medium. C and D are the corresponding fluorographs. Arrows indicate the proteins whose relative synthesis is inhibited by treatment. (a) Untreated cells; (b) cells exposed to cycloheximide; and (c) cells exposed to actinomycin D.

Figure 7. The accumulation of HSP71 RNA in confluent primary cultures of BAECs exposed to heat stress. Equal amounts of RNA for 2 h (Fig. 7, lane B), 45°C for 10 min (Fig. 7, lane C), and 45°C for 10 min followed by a return to 37°C for 2 h. The expression of the HSP71 gene is most abundant when cells are exposed to brief hyperthermic treatment followed by a return to control temperatures. The pattern of abundance of the mRNA for the HSP71 gene at each temperature tested reflects the amount of HSP71 polypeptide expressed in the cell (19, 20).

In addition, the level of expression of the thrombospondin gene was determined (Fig. 8) on the same RNA samples used in Fig. 7. At 41.5°C (Fig. 8 B), the amount of transcript presented is the same as at 37°C (Fig. 8 A). However, when cells are exposed for 10 min to 45°C (Fig. 8 C), the abundance of mRNA declines by ~30% relative to control. Furthermore, brief hyperthermic treatment (45°C, 10 min) followed by a recovery of 2 h at 37°C (Fig. 8 C) results in a twofold increase in messenger for thrombospondin. The expression of the thrombospondin gene occurs at a later time than that of the heat-shock mRNA.

**Immunofluorescence of Extracellular Matrix Proteins of Cells Exposed to Hyperthermia**

To morphologically localize the thrombospondin in the cell (10 μg) were fractionated into agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a 32P-labeled cDNA clone. (A) RNA from unstressed BAECs; (B) heat-shock RNA from BAECs exposed to continuous heat treatment at 41.5°C for 2 h; (C) heat-shock RNA from BAECs exposed for 10 min to 45°C; and (D) heat-shock RNA from BAECs exposed for 10 min to 45°C followed by a return to 37°C for 2 h.
Figure 8. Slot-blot showing the accumulation of thrombospondin RNA in confluent primary cultures of BAEC exposed to heat stress. The slot-blot was probed with the M1 clone of human endothelial cell thrombospondin. (A) RNA from unstressed BAECs; (B) heat-shock RNA from BAEC exposed for 2 h at 41.5°C; (C) heat-shock RNA from BAEC exposed for 10 min to 45°C; and (D) heat-shock RNA from BAEC exposed for 10 min to 45°C followed by a return to 37°C for 2 h.

RNA from BAECs exposed to continuous heat treatment at 41.5°C for 2 h; (C) heat-shock RNA from BAEC exposed for 10 min to 45°C; and (D) heat-shock RNA from BAEC exposed for 10 min to 45°C followed by a return to 37°C for 2 h.

Figure 9. Immunolocalization of thrombospondin in BAEC cultures. BAECs were grown on 16-mm glass coverslips and allowed to reach confluence. Fixed cells were stained for thrombospondin. (a) BAECs at 37°C; (b) after exposure to 41.5°C for 4 h. a and b are corresponding phase-contrast micrographs. Note that, after exposure to heat, there is a decrease in the fibrillar thrombospondin network and an increase in the number of local areas of fluorescence. Fixed and permeabilized cells were also stained for thrombospondin. (c) BAECs at 37°C and (d) after exposure to 41.5°C for 4 h. Note that, after exposure to heat, there is a decrease in the fibrillar thrombospondin network. The perinuclear staining remains; however, it appears somewhat more clustered about the nucleus. In addition, fixed and permeabilized cells were stained for fibronectin. (e) BAECs at 37°C; (f) after exposure to 41.5°C for 4 h; (e') and (f') are corresponding phase-contrast micrographs. Note that, after exposure to heat, fibrillar fibronectin network remains basically unaltered; however, the perinuclear staining appears somewhat more clustered about the nucleus (arrow). Finally, fixed and permeabilized cells were stained for vimentin. (g) BAECs at 37°C and (h) after exposure to 41.5°C for 4 h. (g') and (h') are corresponding phase-contrast micrographs. Note that, upon mild heat stress, there is partial collapse of the intermediate filament network.
Figure 10. Effect of heparin on the protein profile of the growth media of endothelial cells from different origins. Endothelial cells were labeled with [3H]proline for 4 h in the presence or absence of heparin (100 μg/ml). (1) Primary cultures of BAEC; (2) passage cultures of BAECs; (3) passage cultures of rat epididymal capillaries; and (4) passage cultures of bovine brain capillaries. The protein profiles were analyzed by 9% SDS-PAGE and fluorography. Each lane contained equal numbers of cell equivalents. The position of human fibronectin (FN), thrombospondin (TS), and bovine serum albumin (BSA) in the gel are indicated. (A) Short and (B) long exposure of the same gel bearing radioactive polypeptides to X-ray film. Note the change in ratio in the 220,000- and 180,000-mol wt bands in the presence of heparin.

is noticeably decreased, with increase in the number and degree of fluorescence of the focal areas indicated by arrows (Fig. 9 b). When confluent cultures are fixed, permeabilized, and stained with specific antibody to thrombospondin, a prominent pattern of granules of intracellular fluorescence in a perinuclear distribution is evident (Fig. 9 c). However, when these cells are exposed to heat stress for 4 h at 41.5°C, the pattern of granules of intracellular fluorescence appears more pronounced, and the fluorescence appears to be clustered more about the nucleus than in control cells (Fig. 9 d). Furthermore, the extracellular matrix staining of a fibrillar nature is noticeably decreased. Raugi et al. (41), have reported the identical pattern of extracellular matrix staining for bovine aortic endothelial cells. They demonstrated that the extracellular fluorescence was not due to trapping or adsorption of thrombospondin from serum contained in culture medium.

In addition to staining for thrombospondin, confluent cultures were fixed, permeabilized, and stained with a specific antibody to fibronectin. These cells show a prominent pattern of extracellular fibrillar staining (Fig. 9 e). When the cells are exposed for 4 h to 41.5°C, the extracellular fibrillar pattern is not noticeably altered; however, a pattern of intracellular fluorescence appears more pronounced (Fig. 9 f), and the fluorescence appears to be clustered more about the nucleus than in control cells (Fig. 9 e).

The vimentin cytoskeleton is known to collapse in cells exposed to heat stress (II). Therefore, fixed and permeabilized BAECs from normal growth conditions and after exposure to heat were stained with a vimentin antibody (Fig. 9 g). At 37°C, the cells show a network of wispy, well-spread filamentous structures. However, cells exposed to 41.5°C for 4 h show partial reorganization or collapse of the intermediate filament structure about the nucleus (Fig. 9 h). The collapse of the vimentin cytoskeleton becomes more severe with the degree and duration of heat stress (data not shown).
**Pattern of Protein Synthesis in Endothelial Cells from Different Origins Exposed to Heparin**

It has been shown that addition of heparin, a glycosaminoglycan, to culture media of endothelial cells enhances their proliferation and increases their life span (45). Recently, Majack et al. (31) reported that heparin regulates thrombospondin synthesis and matrix deposition by cultured rat aortic smooth muscle cells. Because of these two facts and our interest in the role of thrombospondin in the growth and proliferation of endothelial cells as it may relate to a "stress" or injured state, the pattern of protein synthesis in endothelial cells from different origins exposed to heparin was compared to the protein profile generated by endothelial cells after exposure to heat stress.

Fig. 10 shows the protein profile in one-dimensional SDS–polyacrylamide gels of growth media from cultures labelled with [3H]proline for 4 h in the presence of heparin (100 µg/ml). As with heat stress, the amount of thrombospondin in the media of BAECs appear to increase in the presence of heparin. In addition, with all four endothelial cell types examined, the thrombospondin/fibronectin ratio in growth media is altered, i.e., is increased relative to control. It is important to note that no HSP71 induction was observed in endothelial monolayers exposed to heparin for 4 h. The protein profile of cells incubated in the presence of heparin appeared identical to that of cells at 37°C (data not shown).

**Discussion**

This is the first example in the literature in which increased amounts of a 180,000 mol-wt polypeptide have been shown to appear in the growth media of cells exposed to hyperthermia. We report here that, as the HSP71 band intensifies with time of exposure to continuous heat treatment, there is a concomitant increase in a 180,000-mol wt band in the growth media of endothelial cells. The appearance of this 180,000-mol wt polypeptide in the growth media has been extensively characterized. Its appearance is blocked by preincubation with cycloheximide, suggesting that the expression of this polypeptide may be translationally regulated. When the protein profiles of the growth media of control and heat-shocked cells are examined, the 180,000–, 45,000–, and 18,400-mol wt bands appear to be altered. In addition, a change in the 220,000-mol wt polypeptide was noted (Fig. 2). However, this change was not apparent under conditions of continuous heat treatment. It is clear that the appearance of the 180,000-mol wt polypeptide is not due to cell lysis.

The 180,000-mol wt polypeptide, generated in the growth media of BAECs exposed to hyperthermia, appears by several criteria to be bovine thrombospondin; they are immunoprecipitation with specific antiserum, immunoblotting with thrombospondin antibodies (data not shown) and examination of the growth media of cells exposed to heat stress under non-reducing conditions (data not shown). Data in this paper suggest several possibilities for the increase in the 180,000-mol wt polypeptide. During continuous heat stress, it could be derived from a translational alteration, a posttranslational modification, or a change in the turnover of the thrombospondin pools. Consistent with posttranslational regulation of thrombospondin is our observation that the Golgi region is rearranged in heat-stressed BAECs as determined by electron microscopy (Ketis and Karnovsky, unpublished data). However, cells exposed to brief hyperthermic challenge followed by a recovery period at 37°C show an increase in transcription of the thrombospondin gene, and Welch and Suhan (49) have shown that heat-stressed cells retain a normal morphology during later times of recovery. Thus, the expression of thrombospondin by endothelial cells in response to heat stress is regulated by mechanisms which act at several levels of gene expression and/or protein processing.

McKeown-Lango et al. (32) have reported that exogenously added [125I]-labeled platelet thrombospondin bound in two fractions to cultured fibroblasts, and the fractions were distinguished by their relative rates of turnover. One fraction of thrombospondin was degraded within minutes by a saturable, endocytic process. The remainder was localized to extracellular matrix and appeared to be more slowly degraded over several hours. The amount of thrombospondin degraded after 4 h at 37°C represented 12% of the added radioactivity. The rate of degradation of thrombospondin by BAECs at 37°C is presently unknown.

Thrombospondin has been detected previously in matrices of BAECs (41). BAECs at 37°C exhibit both intracellular and extracellular fluorescence when probed for thrombospondin (Fig. 5). Staining is observed in a perinuclear pattern (presumably representing thrombospondin in Golgi vesicles and secretory granules) as well as in a fibrillar extracellular pattern. After heat stress, a notable amount of the extracellular stained material is decreased. These observations with the biochemical data suggest that thrombospondin turnover may be altered. It is important to note that the addition of leupeptin, a calcium protease inhibitor, had no effect on the expression of thrombospondin by heat-stressed cells (data not shown).

Recently, Majack et al. (31) have shown that heparin regulates thrombospondin synthesis and matrix deposition by cultured rat aortic smooth muscle cells, and Thornton et al. (45) have shown that addition of heparin to culture media of endothelial cells enhances their proliferation and increases their life span. Because of these two facts and our interest in the role of thrombospondin in the growth and proliferation of endothelial cells as it may relate to a "stress" or injured state, the pattern of protein synthesis in endothelial cells from different origins exposed to heparin was compared with the protein profile obtained from endothelial cells exposed to heat stress. As with heat stress, the amount of thrombospondin in the growth media of BAECs appears to increase in the presence of heparin. However, no HSP71 induction was observed in endothelial monolayers exposed to heparin for 4 h. The protein profile of control cells and monolayers treated with heparin appeared to be the same.

Ketis and his colleagues (Ketis, N. V., J. Lawler, and M. J. Karovsky, manuscript in preparation) have observed that the growth of endothelial cells in culture is altered after exposure to hyperthermia. Growth is initially retarded followed by exponential growth (data to be presented elsewhere). During the recovery period, the amount of thrombospondin in the growth media increases (Fig. 2) as does the transcription of the thrombospondin gene (Fig. 8). We speculate that thrombospondin may aid in the recovery of cells from heat shock. Thrombospondin, a trimeric glycoprotein composed of apparently identical subunits of 180,000-mol wt, is thought to have a role in platelet aggregation (10, 40). It has been
shown to have a binding domain for heparin (8, 26, 27), fibronectin (25), fibrogen (28), collagen (25, 36), and possibly growth factors (31). In addition, Lahav et al. (25) have provided evidence for the interaction of thrombospondin with fibronectin during platelet adhesion. Recently, it has been suggested (31) that thrombospondin may function as an extracellular “integrator” of growth stimulatory and inhibitory signals, since the amount of thrombospondin incorporated into the extracellular matrix of smooth muscle cells is regulated by both PDGF- and heparin-like glycosaminoglycans. The amount of thrombospondin in the smooth muscle cell environment, for any vascular injury, may determine the extent of smooth muscle cell thrombospondin interaction and the extent of smooth muscle cell response. We are currently examining the role of thrombospondin in the growth and proliferation of endothelial cells as it may relate to a “stress” or injured state and how endothelial injury or stress may affect the surrounding vascular milieu, specifically smooth muscle cells.

In summary, we present several novel observations pertaining to the patterns of protein synthesis in cultured bovine aortic endothelial cells subjected to hyperthermia; namely, (a) the appearance in the growth media of a 180,000-mol wt polypeptide; (b) that the 180,000-mol wt polypeptide induced by heat stress is thrombospondin as determined by several criteria; (c) that brief hyperthermic treatment followed by a recovery of 2 h at 37°C results in a twofold increase in messenger for thrombospondin; (d) that in addition to thrombospondin, the expression of at least two other polypeptides in the growth media of endothelial cells is altered; and (e) that heparin also increases the amount of thrombospondin present in the growth media of BAECs. We speculate that thrombospondin may be involved in the recovery of endothelial cells from a stressed or injured state.

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Ketis et al. Heat-shock Protein Synthesis in Endothelium 903
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