The Hyperthermia-enhanced Association between Tropoelastin and Its 67-kDa Chaperone Results in Better Deposition of Elastic Fibers*

Brooke A. Murphy†, Severa Bunda‖, Thomas Mitts§, and Aleksander Hinek‡¶†¶1

From the Cardiovascular Research Program, The Hospital for Sick Children, Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario MSG 1X8, and ‡¶1Human Matrix Sciences, LLC, Visalia, California 93291

The results of our in vitro experiments indicate that exposing cultured human aortic smooth muscle cells and dermal fibroblasts to 39 to 41 °C induces a significant up-regulation in the net deposition of elastic fibers, but not of collagen I or fibronectin, and also decreases the deposition of chondroitin sulfate-containing moieties. We further demonstrate that mild hyperthermia also rectifies the insufficient elastogenesis notable in cultures of fibroblasts derived from the stretch-marked skin of adult patients and in cultures of dermal fibroblasts from children with Costello syndrome, which is characterized by the accumulation of chondroitin 6-sulfate glycosaminoglycans that induce shedding and inactivation of the 67-kDa elastin-binding protein. We have previously established that this protein serves as a reusable chaperone for tropoelastin and that its recycling is essential for the normal deposition of elastic fibers. We now report that hyperthermia not only inhibits deposition of chondroitin 6-sulfate moieties and the consequent preservation of elastin-binding protein molecules but also induces their faster recycling. This, in turn, triggers a more efficient preservation of tropoelastin, enhancement of its secretion and extracellular assembly into elastic fibers. The presented results encourage using mild hyperthermia to restore elastic fiber production in damaged adult skin and to enhance elastogenesis in children with genetic elastinopathies.

Elastic fibers constitute the major fibrotic component of the extracellular matrix (ECM) and are responsible for the resilience of blood vessels, lungs, skin, and the connective tissue framework of internal organs. They are composed of a microfibrillar scaffold made up of several glycoproteins and a core consisting of the unique protein polymer elastin. Elastin is formed after the lysyl oxidase-catalyzed cross-linking of multiple precursor molecules (tropoelastin) that are produced and secreted by fibroblasts, chondrocytes, and vascular SMCs. Elastogenesis is also modulated by the 67-kDa elastin-binding protein (EBP) (6, 7), identified as the catalytically inactive spliced variant of β-galactosidase (S-Gal) that has retained the ability to bind to galactosugars and acquired a unique (frameshift encoding) elastin-binding domain (8, 9). The 67-kDa S-Gal/EBP serves as a molecular chaperone for intracellular tropoelastin, which binds this highly hydrophobic and unglycosylated protein and escorts it through the secretory pathways, protecting it from premature self-aggregation and proteolytic degradation and assuring its orderly assembly into elastic fibers (10). We have described how the coordinated dissociation of tropoelastin from its chaperone and its consecutive assembly into elastic fibers occurs after the binding of S-Gal/EBP to galactosylated components of the microfibrillar scaffold (6, 9–11). We also found that many S-Gal/EBP molecules (40 to 50%) recycle back to the cell interior following delivery of tropoelastin to the cell surface. These chaperone molecules bind again to their new tropoelastin partners in the recycling endosomes and escort them to the cell surface in consecutive rounds (12, 13).

Previous studies have documented that either the primary genetic deficiency of the S-Gal/EBP occurring in patients with GM1-gangliosidosis or Morquio B syndrome (14, 15) or the secondary deficiency of this tropoelastin chaperone, due to the constant shedding induced by its abnormal accumulation of chondroitin 6-sulfate (CS) (16–19) or dermatan sulfate glycosaminoglycans (Hurler disease) (20), consequently prevents the normal assembly of elastic fibers and contributes to the development of the severe clinical phenotypes of these syndromes. Importantly, the genetic manipulations leading to the experimental elimination of proteoglycans rich in chondroitin 6-sulfate and dermatan sulfate (versican 1 and 2 or biglycan) have also been shown to lead to the rescue of S-Gal/EBP and to the restoration of normal elastogenesis in human and animal cells (21–24).

The results of the present studies involving normal human aortic SMCs and dermal fibroblasts derived from normal human skin, stretch-marked human skin of adult patients, and wrinkled skin of children with CS demonstrate for the first time that exposure to mild hyperthermia (39 to 41 °C) inhibits the deposition of chondroitin sulfate-containing moieties, which are associated with a significant net up-regulation in the deposition of elastic fibers but not collagen I or fibronectin. We then document that, in addition to inhibiting chondroitin 6-sulfate-containing moieties that lead to the preser-
vation of S-Gal/EBP molecules, hyperthermia also induces their faster recycling. This, in turn, triggers a more efficient preservation of newly synthesized tropoelastin, enhancement of its secretion, and extracellular assembly into elastic fibers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture products, including Dulbecco’s modified Eagle’s medium (DMEM), a minimal essential medium, fetal bovine serum (FBS), 0.2% trypsin, 0.02% EDTA and all other products were from Invitrogen. We purchased polyclonal antibody to tropoelastin from Elastin Products (Owensville, MO). The antibody-recognizing AKAAAKA sequence on soluble tropoelastin (25) was a generous gift from Dr. Barry Starcher, University of Texas. The polyclonal anti-S-Gal antibody (8) was used to detect the 67-kDa EBP. Immobilized pepsin and protein A for preparation of anti-S-Gal F(ab)’2 fragments were purchased from Pierce Chemical Company. The Fluor X Fluorescent Dye kit for labeling the anti-S-Gal F(ab)’2 was obtained from Research Organics, Inc. (Cleveland, OH). Monoclonal antibody to total chondroitin sulfate was purchased from Sigma, and monoclonal antibody recognizing chondroitin 6-sulfate was obtained from Seikagaku Corporation (Tokyo, Japan). Monoclonal antibody to fibrillin was purchased from Biomedia Corp. (Foster City, CA). Monoclonal antibody to FKBP65 was purchased from BD Biosciences. Monoclonal antibody to collagen I was purchased from Chemicon International (Temecula, CA). Monoclonal antibody to Hsp47 was purchased from Stressgen Bioreagents (Victoria, BC). Monoclonal antibody to fibronectin was purchased from Sigma. The secondary antibodies fluorescein-conjugated goat anti-rabbit (GAR-FITC) and goat anti-mouse (GAM-FITC) were purchased from Chemicon International. DAPI and propidium iodide were purchased from Sigma. The DNeasy Tissue System for DNA assay and the RNeasy Mini Kit for isolation of total RNA were purchased from Qiagen (Mississauga, ON). The Superscript First Strand Synthesis System for RT-PCR was purchased from Invitrogen. The One-Step RT-PCR Kit was purchased from Qiagen. The Enhanced Chemiluminescence (ECL) Detection Kit and the radiolabeled reagents [3H]valine and [3H]thymidine were purchased from Amer sham Biosciences, and the human GAPDH control was purchased from Clontech (Palo Alto, CA).

**Cell Cultures**—With parental consent and Institutional Ethics Committee approval, SMCs were propagated from small aortic fragments obtained during the autopsy of a 12-year-old male patient who died in a traffic accident. Fibroblasts derived from skin biopsies of three normal boys (4212, a 1-year-old; 4184, a 3-year-old; and 8972, a 6-year-old) and three male patients with CS (OMIM 218040) (7669, a 1-year-old; 4184, a 3-year-old; and 10595, a 2-year-old) were obtained from the cell repository of the Hospital for Sick Children in Toronto. All children who donated cultured cells were previously described (16), and their clinical diagnoses were confirmed by genetic tests. Fibroblasts were also isolated from biopsies of the stretch-marked skin of three female pa-

tients, a 26-year-old (case 1), a 28-year-old (case 2), and a 36-year-old (case 3). All mentioned cells, initially stored at passage 2, were trypsinized and further maintained in a minimal essential medium supplemented with 10% FBS and 1% antibiotics/antimycotics. All experiments were performed on cells from passages 3 to 5. In experiments aimed at assessing ECM production, we initially plated normal aortic SMCs, normal skin fibroblasts, and fibroblasts derived from stretch-marked skin patients (1 × 10^5 cells/dish) and maintained them at 37 °C for 48 h; then, parallel confluent cultures were kept in separate incubators at 37, 39, or 41 °C. Dermal fibroblasts derived from CS patients were plated more densely (2 × 10^5 cells/dish) for immediate confluence; 2 h after plating, parallel cultures were kept at 37, 39, or 41 °C for different periods of time, as indicated in the figure legends. All experiments were performed three times, and quadruplicate cultures from each experiment were used in each assay at the given time.

**Immunostaining**—At the end of the indicated incubation period, parallel cultures maintained at 37, 39, or 41 °C were washed and fixed in cold 100% methanol at −20 °C for 30 min. After a 1-h long blocking with 1% normal goat serum in PBS, cultures were then incubated for 1 h (at room temperature) with antibodies recognizing ECM components (elastin, collagen I, fibronectin, and chondroitin sulfate) or antibodies recognizing the intracellular protein chaperones Hsp47, FKBP65, and S-Gal/EBP. All cultures were washed and then incubated for an additional hour with the appropriate (green) fluorescein-conjugated goat anti-rabbit (GAR-FITC) or goat anti-mouse (GAM-FITC) secondary antibodies. Nuclei were counterstained with (red) propidium iodide. For double immuno- staining, parallel cultures were incubated simultaneously with pairs of monoclonal (anti-FKBP65, anti-Hsp47, or anti-EBP) and polyclonal (anti-tropoelastin) primary antibodies, followed by secondary GAM-FITC and GAR-FITC. The combination of polyclonal anti-S-Gal antibody (detecting EBP) and monoclonal anti-tropoelastin antibodies was also used to visualize the association of these two proteins. The nuclei were counterstained with blue DAPI. All immuno- stained cultures were then mounted in Elvanol and examined with a Nikon Eclipse E1000 microscope equipped with a source of fluorescent light and multiple filters. The images were obtained with a cooled CCD camera (Retiga EX, Qimaging, Surrey, BC). The morphometric analysis was then performed using Image-Pro Plus software from Media Cybernetics (Silver Spring, MD).

**Organ Cultures of Explants Derived from Human Skin**—

Fragments of normal and stretch-marked skin (26, 27) collected during plastic surgery procedures from the stretch-marked skin of three female patients, cases 1, 2, and 3, were cut into multiple 4-mm² pieces, placed in culture dishes, and maintained for 5 days at 37 or 41 °C in DMEM containing 5% FBS. At the end of this period, all organ cultures were fixed in 1% buffered formalin, and their transverse serial histological sections were stained with Movat’s pentachrome (28), which shows elastin as black, glycosaminoglycans as green, collagen as yellow, and cell nuclei as dark blue. Previous studies have confirmed that the distribution of black-stained material with
Movat’s method entirely overlaps with immunodetectable elastin (16, 19, 20, 29).

**Monitoring S-Gal/EBP Recycling Kinetics**—Human aortic SMCs were plated on coverslips in 35-mm dishes and grown to subconfluency. Multiple cultures were then preincubated for 1 h with heat-inactivated 3% normal rabbit serum, 3% normal goat serum, and 1% BSA to block the Fc receptors and other possible nonspecific binding sites, then placed on ice at 4 °C (to inhibit intracellular trafficking, including endocytosis). Cultures were then washed with cold PBS and externally labeled for 30 min with 1 μg/ml of F(ab′)2 fragments of anti-S-Gal immunoglobulin directly conjugated to carboxyfluorescein, as previously described (13). At the end of a 30-min labeling period, the cultures were washed again in PBS and incubated at either 37 or 41 °C for different time periods (5, 15, 25, 35, 45, and 55 min). After each chase period, cultures were extensively washed in medium containing 0.2% sodium azide, permeabilized with 100% methanol at −20 °C, and washed again in PBS containing 1% BSA. The nuclei were then counterstained with DAPI.

To confirm that fluorescein-conjugated anti-S-Gal F(ab′)2 binds specifically to the cell surface EBP, parallel culture controls were incubated for 15 min with an excess (5 μg/ml) of nonlabeled anti-S-Gal F(ab′)2 or with a high concentration (1 mg/ml) of κ-elastin, a ligand that binds to the same domain of the EBP as the anti-S-Gal antibody before incubation with labeled immunoglobulin. We also preincubated additional control cultures for 15 min with 0.1 m lactose, which releases EBP from the cell surface (6, 7), before exposure to fluorescein-labeled anti-S-Gal F(ab′)2. We have previously shown that anti-S-Gal does not cross-react with elastin or with the catalytically active form of β-galactosidase (13).

**Metabolic Labeling and Assays of Tropoelastin and Insoluble Elastin**—Aortic SMCs isolated from normal individual and skin fibroblasts derived from normal individuals and WBS and CS patients were initially plated at 1 × 10⁶ cells/35-mm dish and grown to confluence for 2 days in full DMEM with 10% FBS. The quadruplicate cultures were then incubated at 37, 39, or 41 °C for the time periods indicated in the figure legends in the presence of 1 μCi/ml of [³H]valine. The pulse and chase experiments were also performed in cultures of dermal fibroblasts derived from CS patients. At the end of the incubation period, the cultures were assessed for the levels of (metabolically labeled) soluble tropoelastin and insoluble elastin. Briefly, conditioned medium was collected and immunoprecipitated with antibody that recognized the AKAAKAAAKA sequence on soluble tropoelastin (25). The soluble proteins still present in the intracellular compartments were first extracted with 0.1 mol/liter of acetic acid and tropoelastin and were then immunoprecipitated from these extracts. The remaining cell layers containing the newly deposited extracellular matrix were scraped and boiled for 45 min in 0.5 ml of 0.1 N NaOH to solubilize all matrix components except elastin. The resulting pellets containing the insoluble elastin were then solubilized by boiling in 5.7 N HCl. The levels of the [³H]valine-labeled, immunoprecipitable tropoelastin and insoluble elastin were then quantitatively assessed by scintillation counting and normalized per DNA content in each individual culture (16, 17). The levels of metabolically labeled insoluble elastin were also assessed in cultured dermal explants, as previously described (29).

**Assessment of Tropoelastin and S-Gal/EBP Levels on Message and Protein Levels**—Human aortic SMCs and dermal fibroblasts isolated from normal and stretch-marked skin fragments, as well as dermal fibroblasts derived from WBS and CS patients, were incubated at 37 or 41 °C for different time periods indicated in the figure legends. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer’s instructions. Elastin and EBP mRNA levels were examined using Qiagen One-Step RT-PCR, where reverse transcription and PCR were carried out sequentially according to the manufacturer’s instructions with the following primers: human elastin, the sense primer 5′-GGTGCCTGGTTTCCTCAGCCCTGG-3′, the antisense primer 5′-GGGC-CTTGAGATACCCCGTG-3′; the human spliced variant of β-galactosidase (EBP), the sense primer 5′-GTTCCTGGTTGGTGATCCCTCCTCCT-3′, and the antisense primer 5′-GCCAGTGAATGCTGGATGC-3′. In each experiment, we added 0.5 μg of total RNA to each one-step RT-PCR (Qiagen One-Step RT-PCR Kit), and reactions were set up according to the manufacturer’s instructions in a total volume of 25 μl. The amounts of tropoelastin and S-Gal/EBP mRNAs detected in each sample were normalized to the amount of GAPDH mRNA. The levels of tropoelastin and S-Gal/EBP proteins were assessed by Western blots with the respective specific antibodies and standardized to the β-actin levels, as previously described (30).

**Metabolic Labeling with [³⁵S]Sulfate and Assessment of Sulfated Glycosaminoglycan Production**—Skin fibroblasts derived from three normal individuals and three CS patients were initially plated at 50,000 cells/dish in normal medium in 35-mm culture dishes immediately after trypsinization. Two hours later, the medium was changed to sulfate-free DMEM containing 2 μCi/ml of [³⁵S]sulfate, and the parallel quadruplicate cultures were incubated at 37 and 41 °C for 24 h. At the end of the incubation period, the cultures were extensively washed in PBS and then digested at 60 °C for 2 h with proteinase K (250 μg/ml). The cultures were then rinsed with ammonium acetate and incubated for another 20 min at 100 °C to inactivate the proteinase K. This step was followed by precipitation with 100% ethanol at −20 °C. The precipitates were finally lyophilized and dissolved in water, and the content of the newly produced sulfated glycosaminoglycans were immunoprecipitated with antibodies recognizing total chondroitin sulfate and chondroitin 6-sulfate. The obtained products were then quantitatively assessed by scintillation counting and normalized per DNA content in each individual culture.

**Data Analysis**—In all studies, quadruplicate samples in each experimental group were assayed in at least three separate experiments. We calculated the mean ± S.D. for each group and carried out statistical analyses. Unpaired Student’s t tests were performed, and the statistical significance shown. p values less than 0.05 (p < 0.05) were considered significant.
RESULTS

Cultured Cells Exposed to Hyperthermia Produce More Elastic Fibers Than Cultures Maintained at 37 °C—The results of initial experiments examining the effect of hyperthermia on the production of elastic fibers demonstrated that cultures of normal human skin fibroblasts and normal human aortic SMCs maintained for 5 days at 41 °C produced more immunodetected elastic fibers than cultures kept at 37 °C. Remarkably, all cultures exposed to 41 °C demonstrated a significant decrease in the level of chondroitin sulfate-containing moieties (Fig. 1A). Morphometric analysis of multiple parallel cultures immunostained with specified antibodies further
indicated that hyperthermia exclusively up-regulated the deposition of elastic fibers by an average of 94 to 112% and down-regulated the deposition of chondroitin sulfate-containing moieties by an average of 82 to 88%. Meaningfully, exposure to hyperthermia did not affect the levels of other fibrous ECM components, such as collagen type I or fibronectin (Fig. 1B). The significant and selective increase in the deposition of immunodetectable elastic fibers in hyperthermia-exposed cultures was further validated by the results of the quantitative biochemical assay of (cross-linked) insoluble elastin (Fig. 1B). The time course observations indicated that hyperthermia-induced enhancement in the deposition of radioactive elastin was evident as early as day 1 and steadily increased until day 7 (Fig. 1C). Moreover, we found that 7-day-long exposure of cultured cells to only 39 °C also induced a significant up-regulation in the net deposition of [3H]valine-labeled insoluble elastin. The observed values were only insignificantly lower than the values noted in cultures kept at 41 °C. Of practical importance, additional results showed that the cultures of both cell types exposed to 41 or 39 °C for only 3 h/day also demonstrated a significant increase in the net deposition of insoluble elastin (assessed in 7-day-old cultures), as compared with control cultures kept at 37 °C (Fig. 1D). Using the same techniques, we also documented that 7 days of exposure to hyperthermia induced production of new elastic fibers in monolayer cultures of dermal fibroblasts isolated from biopsies of the stretch-marked skin of three female patients (cases 1, 2, and 3 (Fig. 2, A and B)). We then demonstrated that hyperthermia also induced production of long elastic fibers in organ cultures of skin explants derived from these patients (Fig. 2, C and D). In contrast, the parallel explants (derived from the same initial biopsies) maintained at 37 °C revealed only thin and short elastic fibers. It is noteworthy that histological sections of skin explants exposed to 41 °C did not reveal any accumulation of elasticotic material as has been reported in skin exposed to 43 °C (31–33). The heightened number of elastic fibers in explants maintained for 7 days at 41 °C (morphometric evaluation revealed a 2–3-fold increase) were additionally validated by proportionally higher values of metabolically labeled insoluble elastin detected in parallel cultures. Jointly, both methods indicated that dermal explants of stretch-marked skin produced significantly more new elastic fibers when cultured at 41 °C. Because the number and size of elastic fibers observed in sections of explants maintained for 7 days at 37 °C also exceeded values observed in the initial biopsies evaluated at time 0 (Fig. 2D), we excluded the possibility of elastolysis in explants cultured at 37 °C.

Hyperthermia Does Not Affect the Steady-state Level of Tropoelastin mRNA but Improves the Durability of the Newly Translated Elastin—In an attempt to determine the mechanism by which hyperthermia stimulates an increase in net elastin deposition in cultures of dermal fibroblasts and aortic SMCs, we first compared the steady-state levels of tropoelastin mRNA in cultures maintained at 37 and 41 °C for 24 h. The results of quantitative RT-PCR analysis showed that exposure to hyperthermia did not affect elastin mRNA levels (Fig. 3A). On the other hand, Western blot analysis using an antibody recognizing the AKAAKAAAAAAK sequence (exposed only in soluble tropoelastin and hidden in polymerized elastin) detected more full-length (72 kDa) tropoelastin and less lower molecular mass tropoelastin degradation products in extracts of dermal fibroblasts and aortic SMCs exposed to 41 °C for only 3 h, as compared with their counterparts kept at 37 °C (Fig. 3B). Because RT-PCR analysis demonstrated no change in tropoelastin mRNA levels during a 3-h exposure to 41 °C (data not shown), these results suggest that hyperthermia improves the durability of newly translated tropoelastin. Furthermore, we found that dermal fibroblasts and aortic SMCs (pulsed for 3 h with [3H]valine) that were exposed to 41 °C secreted significantly more 3H-labeled tropoelastin that was immunoprecipitated with anti-AKAAKAAAAAKA antibody from the conditioned medium following a 3-h chase than cultures kept at 37 °C (Fig. 3C). Jointly, the presented results indicate that, in addition to preserving newly produced tropoelastin better, hyperthermia also stimulates secretion of this precursor protein.

Hyperthermia-exposed Cells Demonstrate Heightened Levels of Intracellular S-Gal/EBP—Although cells exposed to hyperthermia did not demonstrate any increase in the steady-state levels of S-Gal/EBP mRNA (Fig. 3D), they revealed increased levels of this tropoelastin chaperone detected by Western blotting (Fig. 3E). This suggested that the heightened levels of S-Gal/EBP detected in hyperthermia-exposed cells did not result from the increased synthesis of this protein but likely reflected its intracellular retention. Moreover, a double immunostaining using antibodies against tropoelastin and S-Gal/EBP clearly documented a peculiar pattern of association between intracellular tropoelastin and its chaperone in hyperthermia-exposed cells. We found that, in aortic SMCs maintained at 37 °C, the majority of tropoelastin (green fluorescence) was detected in the endoplasmic reticulum apart from the S-Gal/EBP (red fluorescence) and that colocalization of these two proteins (yellow fluorescence) occurred only in the peripheral vesicular compartment. In contrast, cells maintained at 41 °C demonstrated that the bulk of the intracellular tropoelastin colocalized with S-Gal/EBP (yellow fluorescence) in a large endosomal compartment localized in the perinuclear region and in numerous peripheral vesicles (Fig. 3F).

Interestingly, we also investigated the possible involvement of two other molecular chaperones in the hyperthermia-induced preservation of intracellular elastin; these experiments produced negative results. We found that neither Hsp47, a known molecular chaperone for such proline-rich proteins as collagen (34, 35) nor FKBP65, previously shown to colocalize with tropoelastin in the distended endoplasmic reticulum of brefeldin-treated cells (36), would interact with tropoelastin. Western blot analysis and immunostaining indicated that, whereas hyperthermia up-regulated the levels of Hsp47 in both tested cell types, it did not influence basal levels of immunodetectable FKBP65 (data not shown). The double immunostaining of cultured dermal fibroblasts and aortic SMCs maintained at 37 or 41 °C also indicated that neither Hsp47 (data not shown) nor FKBP65 (Fig. 3G) colocalized with intracellular or extracellular elastin.
We have previously established that S-Gal/EBP acts as a recyclable chaperone that delivers multiple tropoelastin molecules to the cell surface in several consecutive rounds (13). We therefore examined whether hyperthermia might affect the intracellular trafficking of S-Gal/EBP and whether the kinetics of its recycling would potentially improve the net secretion of tropoelastin in aortic SMCs. To trace EBP trafficking, multi-
ple subconfluent cultures of aortic SMCs were cooled to 4 °C (to inhibit intracellular trafficking, including endocytosis), externally labeled with anti-S-Gal antibody for 15 min, and then transferred to incubate at either 37 or 41 °C for different periods (5, 15, 25, 35, 45, and 55 min). We previously demonstrated, using both regular fluorescence microscopy and con-

FIGURE 3. Hyperthermia does not affect the steady-state level of tropoelastin and S-Gal/EBP mRNAs, but transiently enhances the intracellular levels of these two proteins and then stimulates their intracellular association and joint secretion. A, results of quantitative RT-PCR analysis demonstrating that a 24-h long exposure of dermal fibroblasts and aortic SMCs to hyperthermia did not affect elastin mRNA levels. B, Western blots with an antibody recognizing only soluble tropoelastin detected more full-length (72 kDa) tropoelastin and less lower molecular weight degradation products in cells exposed to 41 °C for only 3 h, as compared with their counterparts kept at 37 °C. C, cells exposed to 41 °C (pulsed for 3 h with [3H]valine) secreted significantly more [3H]valine-labeled tropoelastin (immunoprecipitated from the conditioned medium) following a 3-h chase than cultures kept at 37 °C. D, cells exposed to hyperthermia did not demonstrate any increase in the steady-state levels of S-Gal/EBP mRNA. E, Western blotting revealed increased protein levels of this tropoelastin chaperone. F, representative micrographs showing a double immunostaining with antibodies against S-Gal/EBP (red fluorescence) and tropoelastin (green fluorescence) confirmed that hyperthermia enhanced the intracellular levels of these two proteins and their association (yellow fluorescence) in the perinuclear endosomal compartment and peripheral secretory vesicles. G, representative micrographs depicting parallel cultured aortic SMCs immunostained with anti-FKB65 (red fluorescence) and with anti-tropoelastin (green fluorescence) show that FKB65 does not colocalize with intracellular or extracellular elastin. Scale bars = 5 μm.
focal microscopy, that exposing aortic SMCs to anti-S-Gal antibody at 4 °C for 15 min resulted in the exclusive labeling of cell surface EBP molecules. The present experiments, which monitored aortic SMCs by fluorescence microscopy, demonstrated that 5 min after the transfer of cultures to 37 °C, the anti-S-Gal-EBP complexes were still detected on the cell surface, but, during the next 10 min, they began an internalization that could be clearly detected in the endocytic vesicles and in the endosomal cisternas localized in the cell center 15 min after the cell transfer to 37 °C. Approximately 10 min later, the anti-S-Gal-labeled EBP molecules were concentrated mostly in the perinuclear endosomal compartments, and following the next 3 10-min intervals, the anti-S-Gal-EBP complexes were present in the peripheral endosomal cisternas, in exocytic vesicles, and again at the cell surface (Fig. 4, left panels). Importantly, we demonstrate that the overall S-Gal/EBP recycling process occurred significantly faster in cells maintained at 41 °C, resulting in a second round of its recycling 55 min after the transfer of cells to 41 °C (Fig. 4, right panels).

Hyperthermia Induces Inhibition of Chondroitin 6-Sulfate Accumulation in CS-derived Fibroblasts, thereby Restoring Normal Elastogenesis—Because normal dermal fibroblasts exposed to 41 °C produce fewer chondroitin sulfate moieties (Fig. 1A) and contain more S-Gal/EBP than counterparts maintained at 37 °C (Fig. 2, E and G), we speculated that hyperthermia might also inhibit the synthesis of glycosaminoglycans containing chondroitin 6-sulfate in CS-derived fibroblasts, which were characterized by the selective accumulation of this glycosaminoglycan, which triggers the shedding of S-Gal/EBP (16, 19).

Indeed, we were able to demonstrate that CS fibroblasts exposed to hyperthermia (just 2 h after plating into the secondary cultures) demonstrated heightened levels of intracellular tropoelastin, higher rates of its secretion, and much higher ultimate deposition of elastic fibers than their counterparts maintained at 37 °C (Fig. 5, A–C). Using immunostaining with anti-chondroitin sulfate and anti-chondroitin 6-sulfate antibodies, we also documented that exposure to hyperthermia rectified the peculiar accumulation of these glycosaminoglycans that has been consistently observed in parallel cultures of CS-derived dermal fibroblasts maintained at 37 °C (Fig. 5D). The results of further experiments clearly indicated that hyperthermia significantly inhibited the new synthesis of [35S]sulfate-labeled glycosaminoglycans that could be immunoprecipitated from extracts of normal fibroblasts and CS-derived fibroblasts using antibodies that recognize total chondroitin sulfate and chondroitin 6-sulfate (Fig. 5E). Importantly, we also demonstrated that the hyperthermia-dependent inhibition of the deposition of chondroitin 6-sulfate by cultured CS fibroblasts coincided with a marked increase in cell-associated S-Gal/EBP, as immunodetected by Western blotting (Fig. 5F). Moreover, double immunostaining (Fig. 5G) further demonstrated that hyperthermia-exposed CS fibroblasts contained more secretory vesicles (yellow fluorescence), in which the immunodetected EBP (red fluorescence) colocalized with the immunodetected tropoelastin (green fluorescence). It is noteworthy that we also observed a similar range in the inhibition of the deposition of immunodetectable and immunoprecipitable [35S]sulfate-labeled chondroitin sulfates in all tested cell types after exposure to 39 °C (data not shown).

DISCUSSION

It has been well established that exposing mammalian cells to extreme hyperthermia (42 to 43 °C) initiates multiple signaling pathways that can lead either to cell death or extensive aberrations in cell function (37–40). It has also been reported that exposing human skin to extreme hyperthermia (43 °C) results in accumulation of amorphous elastin aggregates resembling those observed in photo-aged skin (31–33). In contrast, the results of our study indicate for the first time that exposure to 39 or 41 °C induces significant enhancement in the deposition of elastic fibers in cultures of normal aortic SMCs, as well as in cultures of dermal fibroblasts derived from normal human skin and from patients affected by an acquired idiopathic elastinopathy that has caused stretch marks (26, 27, 41, 42) or the secondary elastinopathy observed in genetic CS. Because such a mild hyperthermia did not induce up-regulation in the deposition of fibronectin or collagen type I (Fig. 1), we concluded that the observed enhancement of elastogenesis results from a distinctive temperature-induced mechanism and does not reflect the general stimulation of ECM synthesis and/or secretion.

The 5- and 7-day-old cultures of all tested cell types exposed to 41 °C did not show any increase in the number of apoptotic cells, but they demonstrated a 40–55% decrease in their proliferation rate (data not shown). We have previously reported that the addition of exogenous insoluble elastin to cultures of fibroblasts derived from CS (16) and Hurler syndrome (20) sharply inhibited their abnormally high proliferation rate. This phenomenon was attributed to the fact that hydrophobic elastin sequestrates mitogenic growth factor (e.g. PDGF) from the FBS-containing medium. We now conclude that the observed decrease in proliferation rate of cells maintained at 41 °C did not reflect a toxic effect of hyperthermia, but was induced by the heightened deposition of insoluble elastin sequestrating the FBS-derived mitogens.

The beneficial biological effects observed in diverse cell types, tissues, and organs exposed to mild heating have been linked mostly to the heightened expression of heat shock proteins (43–46) acting as intracellular chaperones of numerous structural and ECM proteins, ensuring their proper folding and protection from premature degradation, as well as transportation to their proper destination. However, the results of our study failed to demonstrate colocalization between tropoelastin and the representative heat shock protein Hsp47, a molecular chaperone of proline-rich collagen (34, 35). This eliminated the possibility that Hsp47 would also protect tropoelastin, which shares high levels of proline residues and a certain structural similarity with collagen precursors. Results from additional experiments also eliminated the involvement of a putative elastin-associating protein FKBP65 (peptidyl-prolyl cis-trans isomerase), which normally facilitates the proper folding of other proline-rich proteins (47). Importantly, the presented data shows that the hyperthermia-in-
duced up-regulation of elastic fiber deposition can be mecha-

nistically linked to a more efficient association between the
newly produced tropoelastin and its well established chaper-
one, S-Gal/EBP.

It has been previously reported that the binding of galacto-
sugar-containing moieties to the lectin site of the S-Gal/EBP
induces a change in the three-dimensional folding of this mol-
ecule, resulting in the hiding of its elastin-binding and neura-

FIGURE 4. Hyperthermia induces faster recycling of the S-Gal/EBP. To trace S-Gal/EBP trafficking, multiple subconfluent cultures of aortic SMCs were cooled to 4 °C and externally labeled with anti-S-Gal antibody for 15 min and then transferred either to 37 or 41 °C for the indicated periods of time. Representative micro-
graphs obtained by fluorescence microscopy show that 5 min after the transfer of cells to 37 °C, the anti-S-Gal-labeled EBP molecules were still detected on the cell surface. They began internalization during the next 10 min and could be clearly detected in the endocytic vesicles and endosomal cisterns localized in the cell center 15 min after transferring the cell to 37 °C. The anti-S-Gal/EBP complexes were concentrated mostly in the perinuclear endosomal compartments (25 min after transferring the cell to 37 °C); following the next 3 10-min intervals, the anti-S-Gal/EBP complexes were present in the peripheral endosomal cisterns, in exo-
cytic vesicles, and, again, at the cell surface (left panels). The overall EBP recycling process occurred significantly faster in cells maintained at 41 °C, resulting in a sec-
ond round of S-Gal/EBP recycling 55 min following the transfer of cells to 41 °C (right panels). Scale bar = 5 μm.
Hyperthermia induces the inhibition of chondroitin 6-sulfate deposition and the consecutive recovery of normal elastogenesis in cultures of CS fibroblasts. A, CS fibroblasts maintained for 3 h at 41 °C displayed four times more intracellular \[^{3}H\]valine-labeled tropoelastin and eventually secreted three times more of this metabolically labeled protein than their counterparts kept at 37 °C. B, representative micrographs depicting 5-day-old cultures of CS fibroblasts immunostained with anti-elastin antibody show the recovery of normal elastogenesis in hyperthermia-exposed cultures of CS fibroblasts. Scale bar = 15 μm. C, results of quantitative assays of metabolically labeled insoluble elastin in 5-day-old cultures of CS fibroblasts confirmed these results and additionally showed that CS fibroblasts heated for only 3 h/day also demonstrated significantly higher levels of newly produced insoluble elastin than their counterparts maintained at 37 °C. D, representative micrographs depicting 5-day-old cultures of CS fibroblasts immunostained with anti-chondroitin sulfate and anti-chondroitin 6-sulfate antibodies. CS-derived fibroblasts exposed to 41 °C demonstrated a strikingly lower content of both immunodetected chondroitin sulfates than cells maintained at 37 °C. E, Western blot analysis further showed that the heat-exposed CS fibroblasts demonstrated a marked increase in cell-associated S-Gal/EBP and tropoelastin, which occurred without any increase in their respective mRNA levels. G, representative micrographs showing 24-h cultures of CS-derived fibroblasts (double immunostained with anti-EBP and anti-tropoelastin antibodies) demonstrate that the hyperthermia-exposed cells contain more secretory vesicles (yellow fluorescence), in which the immunodetected EBP (red fluorescence) colocalizes with the immunodetected tropoelastin (green fluorescence). Scale bar = 5 μm.
minidase-1-binding domains, as well as consecutive dissociation from the cell membrane (48). It has also been demonstrated that the pericellular accumulation of galactosugar-bearing moieties causes an extensive shedding of S-Gal/EBP molecules from the cell surface, prevents their recycling to the cell interior, and interferes with the normal assembly of tropoelastin into elastic fibers (12–16). The particular accumulation of chondroitin 6-sulfate-rich proteoglycans, biglycan, and versicans 1 and 2, which cause the functional elimination of the S-Gal/EBP and a consequent lack of normal elastogenesis, has been implicated in the formation of the occlusive neointima of atherosclerotic and injured arteries, as well as in the development of chronic obstructive pulmonary disease (20, 49–52). Most importantly, previous work has also demonstrated that the experimental elimination of biglycan and versican 1 deposition restored normal levels of functional S-Gal/EBP and reversed impaired elastogenesis (21–24).

Our observation that the hyperthermia-exposed cultures of human SMCs and fibroblasts demonstrated a significant down-regulation in the deposition of immunodetectable chondroitin sulfate-bearing moieties (Fig. 1, A and B), agrees with the previously published data of Wegrowski (53). Because the peculiar inhibition of chondroitin sulfate deposition in hyperthermia-exposed cells consistently coincided with heightened elastogenesis, we further explored the mechanistic background of this phenomenon. We specifically used dermal fibroblasts derived from CS patients, in which impaired elastogenesis is linked to up-regulation in the deposition of chondroitin 6-sulfate (four times higher levels than in normal skin fibroblasts) and a consequent secondary S-Gal/EBP deficiency (16, 19). Importantly, we established that exposing these cells to 41 °C induced a significant inhibition in the production of chondroitin 6-sulfate-bearing moieties (Fig. 5, D and E) that coincided with a significant increase in the levels of intracellular S-Gal/EBP and tropoelastin, as well as with a remarkable enhancement of intracellular association between tropoelastin and its chaperone, S-Gal/EBP (Fig. 5, F and G). Thus, we propose that the heat-induced inhibition of chondroitin 6-sulfate synthesis consequently eliminates the accelerated shedding of functional S-Gal/EBP reported in CS. Therefore, this hyperthermia-induced phenomenon that then initiates a more efficient return of S-Gal/EBP molecules to the recycling endosomes, likely constitutes a prerequisite step for the rectification of the secondary deficiency in this reusable tropoelastin chaperone observed in CS-derived cells maintained at 37 °C. Because we also observed a similar hyperthermia-induced inhibition in the production of immunodetectable and immunoprecipitable chondroitin sulfates in cultures of normal skin fibroblasts and normal aortic SMCs, we conclude that the above documented mechanism is not restricted to CS cells and that it likely contributes to the rescue of functional S-Gal/EBP in other cell types that are exposed to hyperthermia for a longer time.

Because the exposure of all tested cell types to 41 °C did not stimulate any increase in the basal levels of tropoelastin mRNA, but did enhance the levels of intact (72 kDa) intracellular tropoelastin, we propose that the hyperthermia-induced up-regulation in the net deposition of elastic fibers is preceded by the enhanced protection of newly synthesized tropoelastin by rescued S-Gal/EBP molecules. Importantly, the results of pulse and chase experiments (tracing the fate of the [3H]valine-labeled tropoelastin) and a double immunostaining confirmed that hyperthermia induced a more efficient secretion of the tropoelastin-S-Gal/EBP complexes in all tested cell types.

We also documented a significantly faster recycling of S-Gal/EBP in cells maintained at 41 °C (Fig. 4). Hence, we suggest that this hyperthermia-induced mechanism further contributes to the acceleration of tropoelastin secretion and to the ultimate up-regulation of elastic fiber production.

The results of our in vitro experiments, particularly those showing better net elastogenesis in cultures exposed to 39 °C for only 3 h/day, encourage future studies aimed at the development of novel therapeutic strategies for early diagnosed CS patients, in whom the initially lower than normal deposition of elastin has been mechanistically connected to a heightened proliferation of SMCs (16) and hypertrophic cardiomyopathy (19). Because the most active synthesis of an elastin present in adult tissues occurs during the last trimester of fetal life and in the early neonatal period (54), we specifically propose that a hyperthermia-based therapy could be most beneficial during this period. The feasibility of such a therapy is also supported by the published data (55) indicating that exposing infants to a sauna did not affect their cardiovascular performance. Because the exposure to 41 °C does not induce any pathological elastosis, but did up-regulate the deposition of normal elastic fibers in primary cultures of dermal fibroblasts and dermal explants derived from patients with stretch-marked skin (Fig. 2), we also suggest that the application of mild local heating should be considered in therapies aimed at stimulating elastogenesis in stretched-mark and wrinkled skin.

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