Insulin Induces Production of New Elastin in Cultures of Human Aortic Smooth Muscle Cells

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Diabetes mellitus accelerates atherosclerotic progression, peripheral angiopathy development, and arterial hypertension, all of which are associated with elastic fiber disease. However, the potential mechanistic links between insulin deficiency and impaired elastogenesis in diabetes have not been explored. Results of the present study reveal that insulin administered in therapeutically relevant concentrations (0.5 to 10 nmol/L) selectively stimulates formation of new elastic fibers in cultures of human aortic smooth muscle cells. These concentrations of insulin neither upregulate collagen type I and fibronectin deposition nor stimulate cellular proliferation. Further, the elastogenic effect of insulin occurs after insulin receptor activation, which triggers the PI3K downstream signaling pathway and activates elastin gene transcription. In addition, the promoter region of the human elastin gene contains the CAAATAA sequence, consistent with the FoxO-recognized element, and the genomic effects of insulin occur after removal of the FoxO1 transcriptional inhibitor from the FoxO-recognized element in the elastin gene promoter. In addition, insulin signaling facilitates the association of tropoelastin with its specific 67-kDa elastin-binding protein/spliced form of β-galactosidase chaperone, enhancing secretion. These results are crucial to understanding of the molecular and cellular mechanisms of diabetes-associated vascular disease, and, in particular, endorse use of insulin therapy for treatment of atherosclerotic lesions in patients with type 1 diabetes, in which induction of new elastic fibers would mechanically stabilize the developing plaques and prevent arterial occlusions. (Am J Pathol 2012, 180: 715–726; DOI: 10.1016/j.ajpath.2011.10.022)
paired elastogenesis. Consequently, insulin has not been listed among factors that regulate primary elastogenesis in arteries.

Because insulin belongs to the same family of growth factors as a potent elastogenic stimulator, insulin-like growth factor-1 (IGF-1), and the IGF-1 receptor (IGF-1R) shares extensive structural homology with the insulin receptor, we explored whether physiologic and low therapeutic concentrations of this hormone would also up-regulate elastogenesis in primary cultures of SMCs isolated from explants of human aorta. Herein we present data that indicate that insulin may stimulate formation of elastic fibers after interaction with its own receptors and disclosure cellular mechanisms, in which this hormone induces the elastin gene expression and facilitates secretion of newly synthesized tropoelastin.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and other cell culture products were acquired from Gibco-BRL, Life Technologies Corp. (Burlington, ON, Canada). Recombinant human insulin (Humulin R) was purchased from Eli Lilly Canada, Inc. (Toronto, ON, Canada). Recombinant human IGF-1, non-enzymatic cell dissociation solution, proteinase inhibitors, PI3K inhibitor LY294002, glucocorticoid receptor inhibitor RU 486, insulin receptor tyrosine kinase inhibitor AG 1024, transforming growth factor-β inhibitor receptor SB 431542, lysyl oxidase inhibitor β-aminopropionitrile, transcription inhibitor dichlorobenzimidazole riboside, protein translation inhibitor cycloheximide, anti–phospho-T821-Rb antibody, secondary antibody fluorescein-conjugated goat anti-rabbit and fluorescein-conjugated goat anti-mouse, propidium iodide, and DAPI nuclear stains were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Mouse monoclonal antibodies; anti-tropoelastin, anti–α-actin, anti–β-actin, anti-vimentin, anti–pT160-cdk-2, anti–cdk-2, anti–phospho-Tyr (PY-20), and polyclonal antibodies to beta subunits of insulin receptor and IGF-1R were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody to tropoelastin was purchased from Elastin Products Co. Inc. (Owensville, MO). Polyclonal antibody to collagen type I and monoclonal antibody to fibronectin were purchased from Chemicon International, Inc. (Temecula, CA). IGF-IR neutralizing mouse monoclonal antibody and IGF-1R tyrosine kinase inhibitor pircapodophyllin, and cdk-2 inhibitor CVT313 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti–S-GAL/EBP rabbit polyclonal antibody, raised to the elastin/lamin-binding domain of the alternatively spliced variant of β-galactosidase (EBP(S-Gal)), was used to detect the 67-kDa elastin-binding protein. Species- and type-specific secondary antibodies conjugated to horseradish peroxidase, an enhanced chemiluminescence kit, the radiolabeled reagent [3H]-valine and [3H]-thymidine were purchased from Amersham Biosciences Canada, Ltd. (Oakville, ON, Canada). Protein G–bound and M-280 streptavidin–bound Dynabeads were purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada). A DNeasy Tissue system for DNA assay, RNaseasy Mini Kit for isolating total RNA, and One-Step RT-PCR Kit were purchased from Qiagen, Inc. (Mississauga, ON, Canada). Nuclear extracts were prepared from cultured cells using the isolation kit from Active Motif, Inc. (Carlsbad, CA).

Culture of Human Aortic SMCs

Human aortic SMCs (AoSMCs) were propagated from small fragments of aortic media collected during autopsy in three boys (ages, 2 to 10 years) who had died in traffic accidents. Guidelines for the protection of human subjects of the US Department of Health and Human Services and of the Declaration of Helsinki were followed in obtaining tissues for this investigation. Cells migrating from the cultured explants of aortic media were routinely passaged (up to three times) via trypsinization using 0.2% trypsin–0.02% EDTA, and were maintained in DMEM supplemented with 5% FBS and 1% antibiotic-antimycotic mix. The second and third passages of cells that migrated from the original explants of aortic media were routinely probed with antibodies recognizing the SMC-specific α-actin to monitor the preservation of the SMC phenotype. All cells were initially plated at 100,000 cells per dish for immediate confluency so they would begin extracellular matrix production. The cell cultures committed to particular experiments were then transferred to DMEM containing either 2% FBS or to serum-free medium before initiation of particular treatments (see figure legends).

Immunocytochemistry and Morphometry

At the end of treatments, confluent cultures of AoSMCs were fixed in cold 100% methanol at −20°C for 30 minutes, and blocked with 1% normal goat serum for 1 hour. The cultures were then incubated for 1 hour with 10 μg/mL polyclonal antibodies to tropoelastin and/or with 10 μg/mL monoclonal antibody to SMC-specific α-actin. All cultures were then incubated for another hour with the respective fluorescein-conjugated secondary antibodies (fluorescein-conjugated goat anti-rabbit or anti-mouse). Nuclei were counterstained with red-fluorescent propidium iodide or blue-fluorescent DAPI. The cultures were then mounted in elvanol and examined using a microscope (Eclipse E1000; Nikon Instruments, Inc., Melville, NY) attached to a cooled CCD camera (Retiga EX; QImaging Corp., Vancouver, BC, Canada) and a computer-generated morphometric analysis system (Image-Pro Plus software; Media Cybernetics, Inc., Bethesda, MD) as previously described. In each analyzed group, 50 low-power fields from three separate cultures were analyzed, and the areas occupied by immunodetectable elastic fibers were expressed as a percentage of the entire analyzed field.
For assessment of intracellular associations between tropoelastin and the 67-kDa elastin binding protein (EBP), cultures of AoSMCs were incubated in serum-free DMEM for 3 hours before treatment with insulin. Cultures fixed for 30 minutes in cold 100% methanol were then blocked with 1% glycine for 15 minutes, followed by 2% bovine serum albumin with 0.1% Triton X-100 for another hour at room temperature. They were finally exposed for 1 hour to the mixture of 5 μg/mL polyclonal anti–S-Gal/EBP and 5 μg/mL monoclonal anti-tripeniostin antibodies. All cultures were then incubated for another hour with the mixture of fluorescein-conjugated and rhodamine-conjugated secondary antibodies. Nuclei were counterstained with DAPI.

Quantitative Assay of Metabolically Labeled Tropoelastin and Insoluble Elastin

Cells were plated in 35-mm dishes at a density of 100,000 cells per dish and were grown to confluence. Two microcuries [3H]-valine per milliliter fresh medium supplemented with 2% FBS was added 2 hours before the indicated treatments (see figure legends). At the end of each experiment, the conditioned medium was collected and subjected to immunoprecipitation using anti-tripeniostin antibody. The cell layers were washed with PBS and incubated using radioimmunoprecipitation lysis buffer for 10 minutes on ice. After centrifugation, the supernatants were collected and subjected to immunoprecipitation using anti-tripeniostin antibody. The remaining pellets containing extracellular matrix were scraped and boiled in 500 μL 0.1 N NaOH for 30 minutes to solubilize all matrix components except the cross-linked elastin, as previously described.33 Final results (counts per minute) reflecting the total amount of [3H]-valine–labeled insoluble elastin in individual cultures were normalized per their DNA contents (assessed using the Qiagen DNeasy Tissue Kit) and expressed as percentage of control values.

Assessment of Cell Proliferation

Cells were plated in 35-mm culture dishes (100,000 cells per dish) containing DMEM supplemented with 5% FBS. The cultures were grown to 70% to 80% confluency and serum-starved overnight to synchronize the cell cycles. The cultures were then transferred to DMEM containing 2% FBS and 1 μCi [3H]-thymidine/mL, and the quadruplicate cultures were incubated with or without indicated doses of insulin for 48 hours. The total amount of [3H]-thymidine–labeled DNA in individual cultures was then assessed via scintillation counting as previously described.31–33 The parallel control and insulin-treated cultures were then resolved on SDS-PAGE gel and subjected to Western blotting using indicated antibodies.

Immunoprecipitation

At the end of indicated treatments (see Figure 4 legend), the cultured cells were lysed as described, and 300-μL aliquots of cell extracts containing 300 μg proteins were incubated at 4°C for 3 hours with the aliquots of protein G–bound Dynabeads (Invitrogen Corp.) that were conjugated with either rabbit polyclonal anti-tripeniostin, goat polyclonal anti-Glut10, or rabbit polyclonal anti–S-Gal antibodies, according to the manufacturer’s instructions. The beads carrying the final immunoprecipitation products were then washed with PBS, resuspended in sample buffer, and boiled for 5 minutes. The released proteins were then resolved on SDS-PAGE gel and subjected to Western blotting using indicated antibodies.

Elastin Gene Expression

At the end of the treatments, total RNA was extracted from quadruplicate cultures in each experimental group using the RNeasy Mini Kit per the manufacturer’s instructions. One microgram total RNA from each sample was added to one-step RT-PCR (One-Step RT-PCR Kit; Qiagen, Inc.). The reactions were set up in a total volume of 25 μL per the manufacturer’s instructions. The reverse transcription step was performed for elastin and 18S ribosomal RNA reactions at 50°C for 30 minutes, followed by 15 minutes at 95°C. The elastin PCR reaction using sense primer 5′-GGTGCGGTTGTTCTTCAGCTG-3′ and antisense primer 5′-GGCCCTGAGATACCCCA-GTG-3′ was performed as previously described35 The products were analyzed using ethidium bromide staining and densitometry-based image analysis using an optical system (Gel Doc 1000; Bio-Rad Laboratories, Inc., Hercules, CA). The amount of tropoelastin mRNA was standardized relative to the amount of 18S ribosomal RNA and expressed as percentage of control values.

Western Blotting

At the end of all treatments, cells from quadruplicate cultures in each experimental group were lysed using NP40 cell lysis buffer following the manufacturer’s instructions (Invitrogen Corp.). Thirty micrograms of protein extracts from each sample was suspended in standard sample buffer, resolved on 4% to 12% gradient SDS-PAGE gels, transferred to nitrocellulose membranes, and subjected to Western blotting using antibodies as previously described31–33

Determination of Interactions Between FoxO1 and Elastin Gene Promoter

Confluent cultures of human AoSMCs were maintained for 16 hours in DMEM with 2% FBS and then treated in the presence or absence of 10 nmol/L insulin and 10 μmol/L PI3K inhibitor LY294200 for 30-minute periods. Cells
were then scraped, and their nuclear extracts were prepared using an isolation kit (Active Motif, Inc.) according to the manufacturer’s instructions. To explore whether the human elastin gene promoter may bind FoxO1 transcription regulating factor and whether such an interaction could be modified by insulin, the DNA oligonucleotides 5′-GCACCCTCCAAATAAACCACACCGTA-3′ and 5′-TA-

CGGTGTGTTATTTGGGGTGC-3′, reflecting a putative Fox-O binding domain located within the human elastin gene promoter, were synthesized. The equal molar amounts of both single-stranded DNA oligonucleotides were annealed in Tris-EDTA buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)] to form the double-stranded DNA probe. Then, 20-μL aliquots of M-280 streptavidin containing Dynabeads conjugated with 40 pmol of our biotinylated DNA probe were mixed with samples of nuclear extracts (containing 100 μg protein) derived from cells that were maintained for 30 minutes in the presence or absence of 30 nmol/L insulin and/or 10 μmol/L LY294002.

All preparations were subsequently incubated for 2 hours in 200 μL binding buffer [50 mmol/L KCl, 10% glycerol, 20 mmol/L HEPES (pH 7.9), 1 mmol/L MgCl₂, 1 mmol/L DTT, 0.1 μg poly(DI-DC) (polydeoxyinosinic-deoxyctydyllic), and proteinase inhibitors]. After binding, the beads were washed four times in the same binding buffer, without poly(DI-DC). The bound proteins were resolved using 8% SDS-PAGE and probed using Western blotting with the anti-FoxO1 monoclonal antibody.

**Data Analysis**

In all biochemical studies, quadruplicate samples in each experimental group were assayed in three separate experiments. Means, standard errors, and standard deviations were calculated for each experimental group. Statistical analysis was performed using one-way or two-way analysis of variance followed by Bonferroni’s test or Student t-test as appropriate (when only two sets of data were compared). P ≤ 0.05 was considered significant.

**Results**

**Insulin Stimulates Deposition of Elastic Fibers in Cultures of Human AoSMCs**

Results of the first series of experiments revealed that 24-hour treatment of confluent cultures of the aortic media–derived cells with 0.5 to 100 nmol/L insulin induced a dose-dependent increase in their production of the immunodetected elastic fibers and up-regulated levels of metabolically labeled insoluble elastin (Figure 1A). We also documented that 95% ± 3% of cells present in the first, second, and third passages of cells derived from the original aortic media explants demonstrated abundant expression of the SMC-specific α-actin. However, results of additional tests revealed that in addition to a potent elastogenic effect on these aortic media–derived SMCs, 1 nmol/L insulin also stimulated deposition of new elastic fibers in the parallel cultures derived from aortic adventitia, which contained primarily fibroblasts and less than 10% of the α-actin–positive SMCs (Figure 1B). In contrast, the insulin-treated cultures of both cell types did not reveal any up-regulation in deposition of immunodetectable fibronectin or collagen I (data not shown). Results of the quantitative assay of [³H]-thymidine incorporation and immunodetection of Ki-67 proliferative antigen in 48-hour cultures of AoSMCs demonstrated that treatment with 0.5 to 100 nmol/L insulin did not induce any up-regulation in their basic proliferative rate (Figure 1C).

**Insulin Stimulates Elastogenesis Through Activation of Insulin Receptor and Triggers a Downstream Signaling Pathway that Includes PI3K**

Because IGF-1 is a potent stimulator of elastogenesis, and both insulin and IGF-1 can cross-activate their highly homologous receptors when applied in micromolar concentrations, we also tested whether the observed up-regulation in net deposition of elastin would also be due to the cross-activation of IGF-1R by insulin. Results of experiments in which parallel immunoprecipitation using antibodies to beta subunits of insulin receptors and IGF-IR were followed by Western blotting using anti–phospho-Tyr antibody demonstrated that nanomolar concentrations of insulin induced dose-dependent phosphorylation of the insulin receptor, observed 30 minutes after addition of insulin, but did not affect phosphorylation of IGF-IR, which was phosphorylated only in cultures treated with IGF-1 (Figure 2A).

Results of quantitative RT-PCR assays, monitoring of the elastogenic response of AoSMCs to insulin in the time course, revealed that 10 nmol/L insulin caused significant up-regulation of the tropoelastin mRNA level in 4 hours, and the peak of this effect was observed in cultures treated for 8 hours. Inasmuch as pretreatment of parallel cultures with transcription inhibitor (50 µmol/L dichloro-benzimidazole riboside) completely abolished the insulin-induced up-regulation of tropoelastin mRNA levels (data not shown), we concluded that insulin causes initiation of elastin gene transcription and does not promote tropoelastin mRNA stability. Then, we documented that elastogenic effects of 10 nmol/L insulin, observed at the mRNA level, were abrogated in cultures pretreated and subsequently maintained using AG1024, which inhibits phosphorylation of the insulin receptor, or with a highly selective inhibitor of PI3K (LY294002). In contrast, pretreatment and incubation of parallel cultures with the specific inhibitor of IGF-1R tyrosine kinase (pircpodophyllin) did not inhibit the elastogenic effects induced by 10 nmol/L insulin (Figure 2B). Results of quantitative immunocytochemistry using anti-tropoelastin antibody (Figure 2C) and assessment of [³H]-valine–labeled insoluble elastin further demonstrated that 10 nmol/L insulin induces the ultimate deposition of elastin through activation of its own receptors and triggers a signal that involves PI3K (Figure 2D, left panel). In contrast, we also demonstrated that inhibition of PI3K (with LY294002) also abolished the elastogenic effects induced by IGF-1 (Figure...
Figure 1. Low concentrations of insulin induce production of elastic fibers in cultures of cells derived from human aorta. A: Representative micrographs depict immunodetected elastic fibers (green) in 24-hour cultures (second passage) of SMCs derived from human aortic media. Morphometric evaluation (lower left panel) and quantitative assay of the metabolically labeled insoluble elastin (lower right panel) demonstrate that low concentrations (0.5 to 100 nmol/L) of insulin induce new elastogenesis in a dose-dependent manner. Nuclei were counterstained with propidium iodide (red). *P < 0.01, **P < 0.001. B: Representative micrographs demonstrate that the second passage of cells derived from human aortic media, exhibiting SMC-specific α-actin, and the parallel cultures derived from aortic adventitia, containing primarily fibroblasts and only single α-actin–positive SMCs, deposit multiple immunodetected elastic fibers in response to 48-hour treatment with 10 nmol/L insulin. Nuclei were counterstained with DAPI (blue). C: Quantitative assay (right) measuring incorporation of [3H]-thymidine and immunodetection of Ki-67 proliferative antigen in 48-hour cultures of AoSMCs (left) demonstrate that treatment with 0.5 to 100 nmol/L insulin did not induce any up-regulation of their basic proliferative rate. Nuclei were counterstained with hematoxylin (blue). All cells were maintained in medium with 2% FBS. Scale bars = 15 μm. All results are derived from three separate experiments in which quadruplicate cultures from each experimental group were assessed.
Figure 2. Insulin stimulates elastogenesis through exclusive activation of the insulin receptors and the downstream signaling pathway that includes PI3K. **A:** Results of immunoprecipitations with antibodies recognizing insulin receptor (anti-IR beta subunit) and IGF-IR (anti-IGF-1 beta subunit) followed by immunoblotting with anti-phospho-Tyr antibody demonstrate that 30-minute treatment of cultured AoSMCs (second passage, maintained in serum-free medium) with 0.5 to 10 nmol/L insulin induced dose-dependent phosphorylation of the insulin receptor but did not affect phosphorylation of the IGF receptor, which phosphorylation could only be induced using 10 nmol/L IGF-1. **B:** Results of RT-PCR assays reveal that 8-hour treatment of AoSMCs (maintained in serum-free medium) with 10 nmol/L insulin induced significant up-regulation in levels of tropoelastin mRNA. This effect of 10 nmol/L insulin was not observed in cultures pretreated for 30 minutes and subsequently maintained in the presence of insulin receptor kinase inhibitor (Ag1024) or PI3K inhibitor (LY294002). In contrast, pretreatment and consecutive incubation of parallel cultures using the specific inhibitor of IGF-1R tyrosine kinase (picropodophyllin) did not inhibit the elastogenic effects induced by 10 nmol/L insulin. Representative photomicrographs of 24-hour cultures of human AoSMCs (maintained in medium with 2% FBS) immunostained with anti-tropoelastin antibody (green) (scale bars = 15 μm) (C) and results of their morphometric evaluation, as well as assessment of [3H]-valine–labeled insoluble elastin (D) confirmed that inhibition of insulin receptor kinase and PI3K (left) but not IGF-1R kinase (right) eliminated the elastogenic effects of 10 nmol/L insulin. The elastogenic effects of 10 nmol/L IGF-1 observed in parallel cultures were also eliminated after inhibition of IGF-1R kinase or PI3K. All results are derived from three separate experiments in which quadruplicate cultures from each experimental group were assessed. *P < 0.001.
2D, right panel). Thus, the present data suggested that both elastogenic signaling pathways, induced by either insulin or IGF-1, require activation of PI3K. However, the results of the following series of experiments clearly indicated that the consecutive steps of these two pathways, downstream of PI3K, may diverge and ultimately initiate the elastin gene transcription in different ways.

It has been suggested that the final steps of the IGF-1–induced elastogenic signaling pathway leads to activation of a regulatory element on tropoelastin gene promoter (retinoblastoma control element). In addition, we have recently established that the IGF-1–initiated elastogenic signal includes activation of the cyclin E/cdk-2 complex that causes site-specific phosphorylation of retinoblastoma on threonine 821, a prerequisite step that consequentially enables the binding and delivery of the Sp1 transcription activation factor to the retinoblastoma control element and initiation of elastin gene transcription. Now we demonstrate that in contrast to IGF-1–treated AoSMCs, in which specific inhibition of cdk-2 with CVT313 abolished the final elastogenic effect of this growth factor, the insulin-treated cells still exhibited heightened deposition of elastic fibers when cultured in the presence of the same cdk-2 inhibitor (Figure 3A). In addition, we established that, in contrast to IGF-1, insulin did not induce phosphorylation of cdk-2 on tyrosine 160, required for activation of this kinase, or promote the site-specific phosphorylation of retinoblastoma on threonine 821 (Figure 3B). These data strongly suggested that insulin would induce activation of the elastin gene through the action of a different control element located within the elastin gene promoter than the IGF-1. Thus, we attempted identification of such a putative insulin-dependent control element within the elastin gene.

**Insulin Induces Dissociation of FoxO1 Transcription Inhibitor from the FoxO-Recognized Element, Identified Within the Elastin Gene Promoter**

Insulin initiates transcription of numerous genes via the mechanism causing detachment of the transcription inhibitors [belonging to the forkhead box O (FoxO) superfamily] from their unique domains, FoxO-recognized elements (FREs) located in the cis-regulatory regions of their promoters. We identified that the CAAATAA sequence located on position −1948 in the human elastin gene promoter is highly homologous to the FRE consensus. We have previously established that hydrophobic and unglycosylated tropoelastin must be chaperoned through the secretory pathways by the 67-kDa EBP, identified as the catalytically inactive spliced variant of S-Gal. Therefore, we explored whether insulin could also modulate interactions between tropoelastin and its EBP/S-Gal chaperone. Results of experiments in which confluent cultures of AoSMCs were incubated for only 20 minutes in the presence or absence of 10 nmol/L insulin demonstrated that the cell extract of insulin-treated cultures did not demonstrate any increase in basic levels of intracellular S-Gal/EBP or tropoelastin (assessed using Western blot analysis) (Figure 4). However, at the same time, significantly more S-Gal/EBP could be co-immunoprecipitated using anti-tropoelastin antibody from the extracts of insulin-treated cells than from untreated cultures. This effect of insulin could not be induced in parallel cultures of cells pretreated for 30 minutes with PI3K inhibitor (Figure 4). Further analysis of parallel cultures of...
AoSMCs subjected to the double immunostaining with anti-S-Gal and anti-tropoelastin antibodies (Figure 4E) demonstrated that in untreated cells, the tropoelastin can be immunodetected primarily in endosomal reticulum (green fluorescence), apart from the peripheral secretory vesicles containing the immunodetected S-Gal/EBP (red fluorescence). In contrast, in cells exposed for 20 minutes to 10 nmol/L insulin, the tropoelastin co-localizes
with S-Gal/EBP in numerous peripheral vesicles (yellow fluorescence). Inasmuch as the insulin treatment could not induce such tropoelastin/S-Gal/EBP co-localization in cells preincubated using LY294002 (data not shown), we concluded that insulin-activated pathways that involve PI3K also facilitate transportation of tropoelastin into the secretory endosomes, where it can meet the S-Gal/EBP chaperone.

Discussion

In adult human tissues, activation of the tropoelastin gene and production of new elastic fibers can be only transiently resumed under the influence of several factors released during inflammation, repair, and remodeling of the injured tissues.1–6,52–55 While both primary insulin deficiency and decreased cellular sensitivity to insulin have been implicated in the pathogenesis of the impaired healing processes, atherosclerosis, and hypertension, all frequently observed in patients with either type 1 or type 2 diabetes,56–62 the possibility of a direct contribution of insulin to the cellular and molecular mechanisms controlling deposition of elastic fibers has not yet been explored.

Previous studies with the objective of elucidation of the elastogenic effects of other humoral factors have revealed the existence of several regulatory elements within the elastin gene promoter that could interact with various transcription factors and, consequently, stimulate

Figure 4. Independent of its genomic elastogenic effect, insulin enhances tropoelastin secretion and facilitates association between tropoelastin and its S-Gal/EBP chaperone. Results of two quantitative assays measuring the levels of immunoprecipitated tropoelastin in confluent cultures of AoSMCs that were first maintained for 2 hours in serum-free medium containing [3H]valine and the lysyl oxidase inhibitor (BAPN) and then incubated for 1 hour in the presence or absence of 10 nmol/L insulin. Assays demonstrated that extracts of AoSMCs incubated in the presence of 10 nmol/L insulin contain significantly less metabolically labeled soluble tropoelastin immunoprecipitated (A) or Western blotted (B) using anti-tropoelastin antibody than do their untreated counterparts. Conversely, their conditioned media contain significantly more immunodetected soluble tropoelastin when compared with untreated cultures. These insulin-induced changes in levels of newly synthesized tropoelastin could not be observed in parallel cultures, in which activity of PI3K was inhibited by 1 hour preincubation with LY294002. C: Results of experiments in which confluent cultures of AoSMCs (second passage) were incubated in serum-free medium for only 20 minutes in the presence or absence of 10 nmol/L insulin demonstrate that the cell extract of insulin-treated cultures do not demonstrate any increase in basic levels of intracellular S-Gal/Elastic fiber or tropoelastin detected using Western blot analysis. D: At the same time, the insulin-treated cells contain significantly more S-Gal/Elastic fiber, co-immunoprecipitated using anti-tropoelastin antibody, when compared with their untreated counterparts. In contrast, insulin could not induce the same effect in parallel cultures pretreated for 30 minutes using PI3K inhibitor. All results were derived from three separate experiments in which quadruplicate cultures from each experimental group were assessed.*P < 0.001. E: Representative micrographs depict cultures of AoSMCs (third passage) subjected to double immunostaining with anti–S-Gal/Elastic fiber antibodies (green fluorescence) and anti-tropoelastin antibodies (red fluorescence). Results show that in untreated cells, most tropoelastin (green) is localized to the central endoplasmic reticulum and is separate from peripheral endosomes (red) containing S-Gal/Elastic fiber. In contrast, cells treated for 20 minutes with 10 nmol/L insulin reveal co-localization of tropoelastin and its S-Gal/Elastic fiber chaperone in the peripheral secretory vesicles (yellow fluorescence). Nuclei were counterstained with DAPI (blue). Scale bars = 5 μm.
or inhibit elastin gene transcription.1,63 For example, IGF-1 can induce a specific signaling pathway leading to activation of the retinoblastoma control element on the elastin gene promoter, thereby triggering tropoelastin gene expression.25–27 In contrast, glucocorticoids, after binding to their unique response elements located in another region of the elastin gene promoter, can also induce up-regulation of tropoelastin gene expression.64 In addition, transforming growth factor-β-induced signaling has been implicated in activation of yet another control element within the elastin gene promoter and the consequent up-regulation in levels of tropoelastin-encoding mRNA.65

Here, for the first time, we demonstrate that transcription of the elastin gene also can be up-regulated via the insulin-triggered signaling pathway. We first documented that insulin (0.5 to 10 nmol/L) induced dose-dependent phosphorylation of the insulin receptor but did not affect phosphorylation of the IGF-1 receptor. The specificity of this newly discovered phenomenon has been further confirmed in that pretreatment of AoSMC cultures with AG1024 caused inhibition of insulin receptor phosphorylation and, consequently, halted insulin-stimulated elastogenesis. We also demonstrated that the elastogenic effects of low dosages of insulin were still observed in cultures in which the IGF-1R kinase was specifically inhibited with picropodophyllin. It must also be mentioned that pretreatment of parallel cultures of AoSMCs using either glucocorticoid receptor inhibitor (RU486) or transforming growth factor-β receptor inhibitor (SB431542) did not diminish the insulin-induced elastogenesis (data not shown). This evidence additionally eliminated the possibility that low dosages of insulin would induce cross-activation of IGF-1, glucocorticoid, or transforming growth factor-β receptors.

While the results of the present study (Figure 2D) indicated the potential overlap of the downstream signaling pathways (up to activation of PI3K) induced by IGF-1 and insulin, it was also demonstrated that, in contrast to IGF-1, insulin did not stimulate the cyclinE/cdk-2–dependent site-specific phosphorylation of threonine 821 on retinoblastoma protein that enables binding of the Sp1 transcription factor and its consecutive delivery to the cytoplasmic translocation of blocking transcription factors belonging to the FoxO superfamily, bound to the specific DNA sequences containing consensus (G/C)(T/A)AAAC(T/AA)A, named an FRE element.42–46,66 However, until now, no study has searched for the alleged FRE within the elastin gene promoter. Therefore, results of our analysis of the elastin gene promoter, which yielded identification of the CAAATAA DNA probe sequence (−1948) consistent with a FRE consensus within the elastin gene promoter (Figure 3C), provided the new perspective. Most importantly, we observed that the synthetic DNA probe with this putative FRE sequence bound and sequestered the protein (recognized using anti-FoxO1 antibody) from the nuclear extracts of human AoSMCs cultured in the absence of insulin. Inasmuch as such association between the CAAATAA DNA probe and FoxO1 could not be detected in cells treated for 30 minutes with insulin, we concluded that insulin induces a certain modification of FoxO1 that likely causes its dissociation from the CAAATAA domain located within the elastin gene promoter. Further observation that insulin could not induce a similar effect in cells in which activity of PI3K has been inhibited (by LY294002) enabled us to conclude that PI3K-dependent phosphorylation of FoxO1 is prerequisite for the final execution of the insulin-triggered elastogenic signal. This conclusion is consistent with previously published data66,67 that demonstrated that activation of the PI3K-Akt/SGK pathway by insulin triggers phosphorylation of nuclear FoxO proteins that consequently enable their dissociation from genomic DNA and binding to chaperone protein 14-3-3, which facilitates their translocation to the cytoplasm, where phosphorylated FoxOs are degraded.

It has previously been demonstrated that insulin promotes the trafficking and the cell surface translocation of small cytoplasmic vesicles. Therefore, we tested whether it would also facilitate secretion of the newly produced tropoelastin. By tracing the fate of the [3H]-valine–labeled tropoelastin, we demonstrated that conditioned media of AoSMC cultures treated with insulin for only 1 hour contained a significantly higher level of the newly synthesized (immunoprecipitable) tropoelastin than did their untreated counterparts. Consistently, the cell extracts of these cultures demonstrated a lower level of intracellular tropoelastin than did control cultures. The parallel cultures pretreated with PI3K inhibitor did not exhibit any changes in extracellular or intracellular levels of metabolically labeled tropoelastin in response to insulin. These data strongly suggested that insulin, independent of its genomic effect, also facilitates secretion of the newly synthesized tropoelastin.

Because tropoelastin is not subjected to posttranslational modifications, it must be escorted through the secretory pathways. Although a transient association between tropoelastin and two proteins residing in the endoplasmic reticulum (BIP and FKBP65) has been suggested,68 until now, only one 67-kDa EBP, identified as S-Gal, has been characterized as a functional chaperone associated with tropoelastin in the peripheral endosome and secretory vesicles, as well as facilitating its orderly assembly into elastic fibers on the cell surface.30,47–51 We have previously established that after delivery of tropoelastin to the cell surface, 40% to 50% of S-Gal/EBP molecules can recycle back to the cell interior of AoSMCs to bind again to their new tropoelastin partners in the recycling endosomes.50 We also described that certain conditions such as raising temperature or elimination of galactosugars can accelerate the speed and enhance the efficiency of tropoelastin secretion.52,53 Inasmuch as insulin induces fast rearrangement of actin filaments that likely facilitates translocation of small vesicles that deliver glucose transporters to the cell surface and also induces...
recycling of these vesicles, we anticipated that insulin would also affect the intracellular trafficking of vesicles containing S-Gal/EBP, thereby facilitating the trafficking of tropoelastin-EBP complexes through the secretory pathways. Our results revealed that even 20-minute treatment of cultured AoSMCs with 10 nmol/L insulin caused a marked increase in the intracellular association between these two proteins, documented by co-immunoprecipitation followed by Western blotting (Figure 4D). In addition, the double immunostaining clearly demonstrated that 20-minute treatment with insulin caused translocation of tropoelastin from the central endoplasmic reticulum to the peripheral secretory endosomes, where it could be co-localized with S-Gal/EBP (Figure 4E). While these results illustrated that insulin facilitates the final (S-Gal/EBP-dependent) steps of tropoelastin secretion, it is not clear whether insulin would also affect translocation of the newly synthesized tropoelastin from the endoplasmic reticulum to the distal Golgi apparatus and secretory endosomes. Thus, additional studies are needed to clarify this. It must be mentioned, however, that our preliminary results, that is, immuno–co-localization of tropoelastin to the small vesicles containing glucose transporter Glut10 and co-immunoprecipitation of these two molecules from the AoSMC extracts (data not shown), may spark the attractive hypothesis that insulin-dependent Glut10 might also participate in the early steps of intracellular trafficking of tropoelastin. This hypothesis could be also endorsed because other investigators have recently localized Glut10 in the endoplasmic reticulum and in the Golgi complex, and patients bearing mutated SLC2A10 gene encoding Glut10 develop the arterial tortuosity syndrome associated with the extracellular elastosis.

In summary, our results demonstrate that low dosages of insulin induce the elastogenic effect that is solely induced through the interaction with insulin receptor and involves the downstream activation of PI3K. We further propose that the ultimate up-regulation of elastic fiber deposition by insulin is executed through the initiation of two parallel mechanisms, initiation of the elastin gene expression and enhancement of tropoelastin secretion. Herein, we provide experimental data that suggest that the insulin-dependent initiation of the elastin gene transcription occurs after dissociation of the FoxO1 transcription factor from the specific domain (FRE) identified within the elastin gene promoter. Furthermore, we demonstrate that insulin may also facilitate the association of tropoelastin with S-Gal/EBP chaperone and ultimately contribute to its efficient secretion. We believe that our discovery of elastogenic action of insulin enables better understanding of the pathologic mechanisms by which the initial lack of insulin in type 1 diabetes or the insulin resistance in type 2 diabetes contribute to development of hypertension and the rapid progression of atherosclerosis. In particular, our data endorse insulin therapy of atherosclerotic lesions in patients with type 1 diabetes, in which induction of new elastic fibers would mechanically stabilize the developing plaques and prevent the imminent arterial occlusions.

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