Angiotensin II Induces Hypertrophy of Human Airway Smooth Muscle Cells: Expression of Transcription Factors and Transforming Growth Factor-β_1

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Increased smooth muscle mass due to hyperplasia and hypertrophy of airway smooth muscle (ASM) cells is a common feature in asthma. Angiotensin II (Ang II), a potent vasoconstrictor and mitogen for a wide variety of cells, has recently been implicated in bronchoconstriction in asthmatics. However, a possible mitogenic role as well as underlying molecular mechanisms of this octapeptide in human ASM cells are not yet known. We studied the effects of Ang II on ASM cell proliferation and growth and on the expression of three transcription factors, egr-1, c-fos, and c-jun, as well as a cytokine, transforming growth factor-β_1 (TGF-β_1). Human ASM cells were isolated by enzymatic digestion of bronchial smooth muscle obtained from lung resection tissue. Confluent cells were growth-arrested and subsequently incubated with Ang II (100 nM) for different time periods and processed for the measurement of cell growth and gene expression. Ang II significantly induced DNA and protein synthesis in human ASM cells at 8 h, resulting in a net increase in the accumulation of protein over DNA (i.e., cellular hypertrophy) at 16 h of incubation. Cell counts and MTT-reduction assay, however, showed no increase in cell number as a result of Ang II stimulation. Ang II stimulated the expression of egr-1 and c-fos as early as 15 min, reaching maximum levels at 45 min, whereas the expression of c-jun peaked at 2 h of Ang II exposure. Furthermore, steady-state mRNA levels of TGF-β_1 were upregulated by Ang II after 4 h and reached peak levels at 16 h of incubation. Secretion of biologically active TGF-β_1 from human ASM cells was significantly ($P \leqslant 0.02$) enhanced by Ang II incubation after 8 h, which remained elevated until 24 h. Our results suggest that the Ang II-induced transient early expression of transcription factors may regulate autocrine genes like TGF-β_1, of which the subsequent late upregulation could contribute to cellular hypertrophy during, for example, airway remodeling in asthma. **McKay, S., J. C. de Jongste, P. R. Saxena, and H. S. Sharma. 1998. Angiotensin II induces hypertrophy of human airway smooth muscle cells: expression of transcription factors and transforming growth factor-β_1. Am. J. Respir. Cell Mol. Biol. 18:823–833.**

Airway remodeling with inflammatory cell infiltration, epithelial shedding, basement membrane thickening, and increased mass of airway smooth muscle (ASM) is an important determinant of bronchial obstruction and hyperresponsiveness in asthma (1–4). Studies using detailed computer models of human airways indicate that increased ASM mass is by far the most important abnormality responsible for excessive airway narrowing and compliance of the airway wall in asthma (1, 5). In analogy to vascular smooth muscle accumulation in hypertension and atherosclerosis, ASM growth in asthma is a complex phenomenon of which the underlying mechanisms are difficult to investigate *in vivo*. The increased amount of ASM in asthmatics is an indication of abnormal cell proliferation and growth. Both hyperplastic (i.e., increase in cell number) and hypertrophic (i.e., increase in cell size) changes contribute to the increased smooth muscle content of the airway wall (1, 2, 6, 7), but little is known regarding the molecular mechanisms and factors that regulate ASM cell proliferation and growth in asthma.

Recently, a number of growth factors and cytokines derived from inflammatory cells have been implicated in ASM cell division and growth (8–12). The potent circulat-

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Abbreviations: angiotensin II, Ang II; airway smooth muscle, ASM; bovine serum albumin, BSA; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; glyceraldehyde 3-phosphate dehydrogenase, GAPDH; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT; optical density, OD; phosphate-buffered saline, PBS; platelet-derived growth factor, PDGF; transforming growth factor-β_1, TGF-β_1.

ing hormone of the renin–angiotensin system, angiotensin II (Ang II) has been implicated in bronchoconstriction in mild asthmatics (13). Plasma levels of Ang II have been shown to be elevated in patients with acute severe asthma (14). At plasma levels similar to those observed in acute asthma, Ang II was found to enhance methacholine-evoked bronchoconstriction, both in human bronchi *in vitro* and in mild asthmatics *in vivo*, thus suggesting a novel role for Ang II as a putative mediator in asthma (13). Although Ang II has been shown to stimulate proliferation and/or hypertrophy in a wide variety of cells, such as cardiac myocytes, cardiac fibroblasts, and vascular smooth muscle cells (15–17), it remains to be established whether this octapeptide stimulates proliferation and growth of ASM cells.

It is known that growth and differentiation factors stimulate signal transduction pathways in the cell leading to the expression of nuclear proto-oncogenes that include the Fos, Jun, and Egr families (18). Fos and Jun proteins constitute the heterodimer AP-1 (19). The AP-1 and egr-1 transcription factors can regulate a number of target genes during growth factor stimulation and thereby influence cellular growth and differentiation (16, 20, 21). Ang II has been shown to induce a rapid increase in the expression of c-fos and c-jun (members of the leucine zipper family) and egr-1 (a member of the zinc finger family) in vascular smooth muscle cells as well as in cardiac fibroblasts prior to hypertrophy and/or hyperplasia (16, 20, 22). Furthermore, an important cytokine, transforming growth factor-β_1 (TGF-β_1), has synergistically been associated with Ang II. TGF-β_1 is synthesized as a biologically inactive propeptide which must be cleaved to form the active peptide, by proteases such as plasmin (23), before it can exert its function in tissue repair after injury or act as a regulatory peptide in remodeling processes in various tissues, including the heart and the lung (16, 24, 25). Also, TGF-β_1 stimulates the synthesis of extracellular matrix components such as collagens and fibronectin in response to Ang II in vascular smooth muscle cells (17, 25, 26).

To elucidate the potential role of Ang II in ASM growth in asthma, we investigated hyperplasia and hypertrophy in cultured human ASM cells and examined the expression pattern of three proto-oncogenes, namely, egr-1, c-fos, and c-jun. Additionally, we studied the expression of the extracellular matrix regulator TGF-β_1 both at mRNA and protein levels in human ASM cells treated with Ang II.

Materials and Methods

Materials

Bovine serum albumin (BSA), collagenase, elastase, apotransferrin, ascorbate, insulin, non-enzymatic cell dissociation solution, mouse monoclonal anti-α smooth muscle actin and anti-smooth muscle myosin, antimouse IgG fluorescein isothiocyanate (FITC) conjugate, 4′,6-diamidino-2-phenylindole-2HCl, calf thymus DNA, herring sperm DNA, and trypan blue were purchased from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, MEM nonessential amino acids, gentamicin, penicillin:streptomycin, Fungizone/amphotericin B, and trypsin-ethylenediamenetetraacetic acid (EDTA) were purchased from Life Technologies BV (Breda, The Netherlands). Fetal bovine serum (FBS) was purchased from Bio-Whitaker BV (Verviers, Belgium). [*Methyl*-^{3}H]thymidine, [*methyl*-^{3}H]leucine, (α-P^{32})-dCTP, hybond-N, and redi-prime labeling kit were obtained from Amersham Nederland BV (′s-Hertogenbosch, The Netherlands). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-reduction assay kit was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). The TGF-β_1 E_{max} ImmunoAssay System was procured from Promega Corp. BNL (Leiden, The Netherlands). Anti-cytokeratin and anti-α-CD31 antibodies were obtained from Dako A/S (Glostrup, Denmark). The cDNA probe for human-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from American Type Culture Collection (Rockville, MD). Tissue culture plasticware was obtained from Life Technologies BV. All other chemicals were of molecular biology and/or tissue culture grade and were procured from local suppliers.

Cell Culture

Human airway smooth muscle cells were isolated and cultured according to the methods described previously (10, 27). Briefly, bronchial smooth muscle was dissected from the lobar or main bronchus from lung resection specimens obtained from patients undergoing surgery for lung cancer. After removal of the epithelium, pieces of macroscopically normal smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in HBSS containing 10 mg/ml of BSA (fraction V), collagenase (type XI, 1 mg/ml), and elastase (type IV, 3.3 U/ml) at 37°C in a humidified ASSAB model T154 CO_2 incubator (Clean Air Techniek BV, Woerden, The Netherlands). After enzymatic digestion, the tissue was centrifuged and the resultant pellet was washed in DMEM containing 10% (vol/vol) FBS supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1.5 μg/ml), and subsequently seeded at 2×10^5 cells per 35-mm dish. Fresh medium was replaced every 72 h. After 10–14 d in culture, ASM cells grew to confluence and were then removed from the plastic base of each dish using non-enzymatic cell dissociation buffer and subcultured into tissue culture flasks. At confluency, cells were further passaged by means of trypsinization. Confluent cells in the fifth passage were used for experiments.

Immunocytochemical Characterization of ASM Cells

Monoclonal antibodies against α-smooth muscle actin and smooth muscle cell specific myosin (SM-1 and SM-2) were used as markers to characterize human ASM cells in culture (28). Cells were allowed to attach to multiwell slides for 24 h in FBS-containing medium and were subsequently growth-arrested by incubating for 60–72 h in FBS-free DMEM supplemented with apo-transferrin (5 μg/ml), ascorbate (100 μM), and insulin (1 μM) prior to fixation and staining. Following two washes in ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.6 mM KCl, 1.4 mM

KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2\ H_2O$, pH 7.4), the cells were fixed in ice-cold methanol and permeabilized in PBS containing 0.1% Tween-20. Nonspecific binding was blocked by incubating the cells in 1% BSA in PBS, and the cells were then washed and subsequently incubated with anti-α smooth muscle actin or anti-smooth muscle myosin antibodies. After incubation, the cells were washed twice in PBS and further incubated with affinity purified FITC-conjugated antimouse antibody. Unbound antibody was washed away using distilled water and the sections were dehydrated and mounted in glycerol. Specimens were visualized under a fluorescence microscope (Carl Zeiss BV, Weesp, The Netherlands) and photographed. Human ASM cells stained positive for anti-α smooth muscle actin and anti-smooth muscle myosin. Additionally, the cultures were stained for endothelial and epithelial cell contamination using anti-CD31 and anti-cytokeratin antibodies, respectively. The staining showed that human ASM cell cultures were essentially free (> 95%) of other contaminating cell types. Under the light microscope, the human ASM cells appeared elongated and spindle-shaped with central oval nuclei containing prominent nucleoli. Confluent human ASM cells in culture showed a specific pattern, aligned in parallel so that the broad nuclear region of one cell lies adjacent to the thin cytoplasmic area of another, giving rise to a typical "hill and valley" appearance as described earlier by Twort and van Breeman (27).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from the ASM cells by the guanidinium thiocynate–phenol–chloroform method described previously (16). The RNA concentration was measured by spectrophotometry. For Northern hybridization, samples of total RNA were denatured at 65°C and size-fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide-stained gels were photographed and RNA was transferred to hybond-N membrane by alkaline downward capillary transfer (29). The filters were air-dried and ultraviolet crosslinked in a gene linker (Bio-Rad Labs BV, Veenendaal, The Netherlands). Blots were hybridized at 42°C in a buffer containing 50% deionized formamide, 1.0 M sodium chloride, 1% sodium dodecyl sulfate (SDS), 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 0.2% BSA, 50 mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 2% dextran sulphate, and denatured herring sperm DNA (2 mg/ml). The cDNA probes used for hybridization were: mouse c-fos (2.1-kb fragment), mouse c-jun (2.6-kb fragment), mouse egr-1 (300-bp fragment of zinc finger region), and human TGF-β_1 (1,050-bp fragment). These inserts were labeled with the redi-prime labeling system to a specific activity of approximately 10^9 cpm/μg DNA. Filters were washed under stringent conditions, wrapped in household plastic film, and exposed to Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY) at −80°C for 1–3 d. A GAPDH cDNA probe was used to rehybridize membranes for reference purposes. Hybridization signals were quantified by scanning laser densitometry using the Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden). Densitometric values for each gene were normalized with respective GAPDH mRNA values and expressed as relative optical density (OD) in Ang II-stimulated cells versus control. An optimal concentration of Ang II (100 nM) for the early response of human ASM cells was determined by incubating growth-arrested cells for 1 h in 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M [Sar[1]]-Ang II, and subsequently examining the expression pattern of the proto-oncogene egr-1.

[^{3}H]Thymidine and [^{3}H]Leucine Incorporation Assays

Effects of Ang II on DNA and protein biosynthesis were assessed by incorporation of [*methyl*-^{3}H]thymidine and [*methyl*-^{3}H]leucine, respectively. An optimum concentration of Ang II (100 nM) was determined by incubating growth-arrested ASM cells for 24 h in 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M [Sar[1]]-Ang II, and subsequently examining [^{3}H]thymidine and [^{3}H]leucine incorporation. Confluent cells were washed in PBS, detached by trypsinization, and transferred into 24-well plates at a seeding density of 2×10^4 cells/well. After 5 d in culture the cells were growth-arrested as described above. Cells were incubated with [*methyl*-^{3}H]thymidine or [*methyl*-^{3}H]leucine (1 μCi/well) in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum), or DMEM containing 100 nM [Sar[1]]-Ang II for 4, 8, 16, 24, or 48 h. After stimulation, the cells were washed in PBS, fixed with ice-cold methanol, and exposed to ice-cold trichloroacetic acid (5% wt/vol). The acid-insoluble fraction was lysed in 0.3 M NaOH and the incorporated radioactivity was determined in a Packard 1500 Tricarb liquid scintillation analyzer (Packard-Becker BV, Delft, The Netherlands) (9).

Assessment of Cellular Hypertrophy and Hyperplasia

Protein/DNA ratio. ASM cell hypertrophy in relation to Ang II was assessed by calculating the ratio of total protein to DNA content. Cells were plated at a seeding density of 2×10^4 cells/well in 24-well plates. After 5 d the cells were growth-arrested as described above, and then incubated in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum), or DMEM containing 100 nM [Sar[1]]-Ang II for 4, 8, 16, 24, or 48 h. After stimulation, the cells were washed in ice-cold PBS and lysed in 0.3 M NaOH. The total DNA content was determined fluorimetrically using the method described by Kapuscinski and Skoczylas (30). The total protein content was estimated colorimetrically using the method described by Bradford (31). Serial concentrations of calf thymus DNA and BSA were used for the calibration curves.

MTT assay. In order to assess cellular hyperplasia in response to Ang II, MTT assays were performed. Cells in the fifth–sixth passage were transferred to 24-well plates at a seeding density of 2×10^4 cells/well. After 24 h, the cells were growth-arrested as described above and subsequently incubated in either fresh FBS-free DMEM or DMEM containing 100 nM [Sar[1]]-Ang II for 4, 8, 16, 24, or 48 h. Proliferation was assessed using the MTT dye technique, which relies on the specific metabolic reduction of the tetrazolium salt MTT by living cells as described earlier (32). Following incubations, the cells were washed twice in PBS, 200 μl of MTT in DMEM (final concentration 0.5 μg/ml) was added to each well, and the cells were incubated for 5 h at 37°C. After incubation, the blue formazan product was solubilized by the addition of 200 μl of solubilization solution (10% SDS in 0.01 M HCl) to each well and incu-

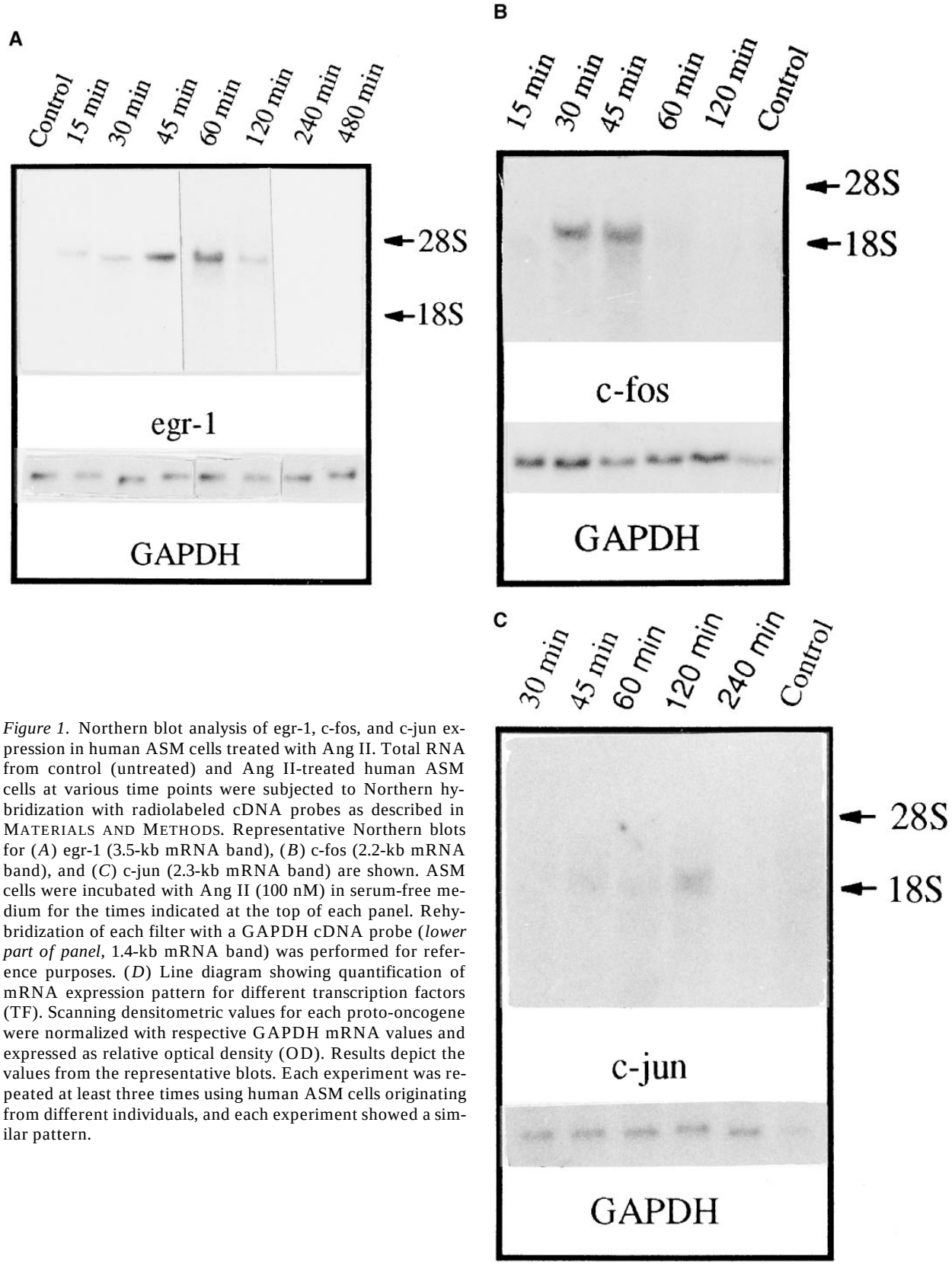


Figure 1. Northern blot analysis of egr-1, c-fos, and c-jun expression in human ASM cells treated with Ang II. Total RNA from control (untreated) and Ang II-treated human ASM cells at various time points were subjected to Northern hybridization with radiolabeled cDNA probes as described in MATERIALS AND METHODS. Representative Northern blots for (*A*) egr-1 (3.5-kb mRNA band), (*B*) c-fos (2.2-kb mRNA band), and (*C*) c-jun (2.3-kb mRNA band) are shown. ASM cells were incubated with Ang II (100 nM) in serum-free medium for the times indicated at the top of each panel. Rehybridization of each filter with a GAPDH cDNA probe (*lower part of panel*, 1.4-kb mRNA band) was performed for reference purposes. (*D*) Line diagram showing quantification of mRNA expression pattern for different transcription factors (TF). Scanning densitometric values for each proto-oncogene were normalized with respective GAPDH mRNA values and expressed as relative optical density (OD). Results depict the values from the representative blots. Each experiment was repeated at least three times using human ASM cells originating from different individuals, and each experiment showed a similar pattern.

bating for a further 16 h at 37°C. An aliquot of 200 μl from each duplicate well was then transferred to a 96-well microtiter plate, and the OD was determined by automated dual wavelength spectrophotometry (Bio-Rad Labs) at a test wavelength of 595 nm and a reference of 690 nm. The change in optical density (Δ_{OD}), which correlates directly with change in cell numbers, was plotted against time as described previously by Hirst and colleagues (10).

Hemocytometry. Cells were seeded into 24-well plates and treated as described above. Following stimulation, the medium was removed and the cell monolayers were resuspended in 100 μl trypsin-EDTA at room temperature for 10 min. An equal volume of trypan blue was added to the cell suspension before counting in a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany). At least 120 cells were counted from each well to minimize counting error.

Detection of TGF-β_1 Protein in Conditioned Media

Confluent cells in the fifth passage were growth-arrested as described above, and then incubated in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum), or DMEM containing 100 nM [Sar^1]-Ang II for 4, 8, 16, or 24 h. Media were removed and stored at −20°C until assay. Biologically active TGF-β_1 protein was quantified by enzyme-linked immunosorbent assay (ELISA) using a TGF-β_1 E_{max} ImmunoAssay System (Promega Corp. BNL, Leiden, The Netherlands) following the suppliers' instructions.

Statistical Analysis

Data are given as means ± SEM. Statistical analysis was performed by using the Student's *t* test. Significance was accepted at $P \leq 0.05$.

Results

Expression of Transcription Factors in Relation to Ang II

Representative Northern blots showing the expression pattern of three proto-oncogenes, following Ang II stimulation of human ASM cells, are shown in Figures 1A–1C. Ang II induced the expression of mRNAs encoding egr-1 (*panel A*) and c-fos (*panel B*) as early as 15 min, while c-jun expression was increased after 45 min of Ang II stimulation (*panel C*). Figure 1D shows the quantitative analysis, by scanning laser densitometry, of the Northern blots for each proto-oncogene. Densitometric analysis revealed that the induction of c-fos and egr-1 was transient and reached a maximum at 45 min followed by an abrupt decline; whereas the expression of c-jun was also transient but reached a maximum at 120 min and gradually returned to basal levels after 4 h. The relative OD values for the levels of mRNA encoding egr-1, c-fos, and c-jun increased

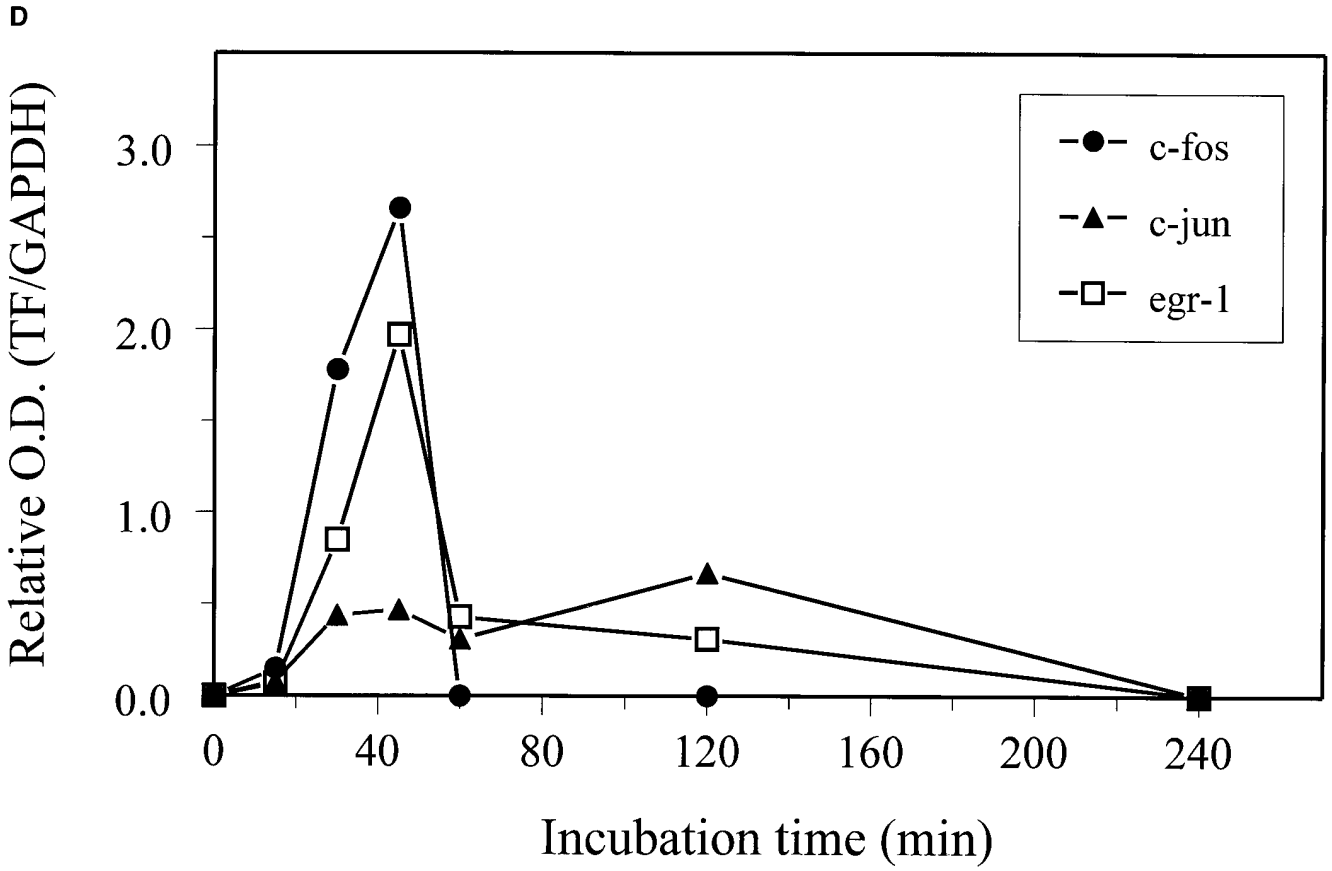


Figure 1. Continued.

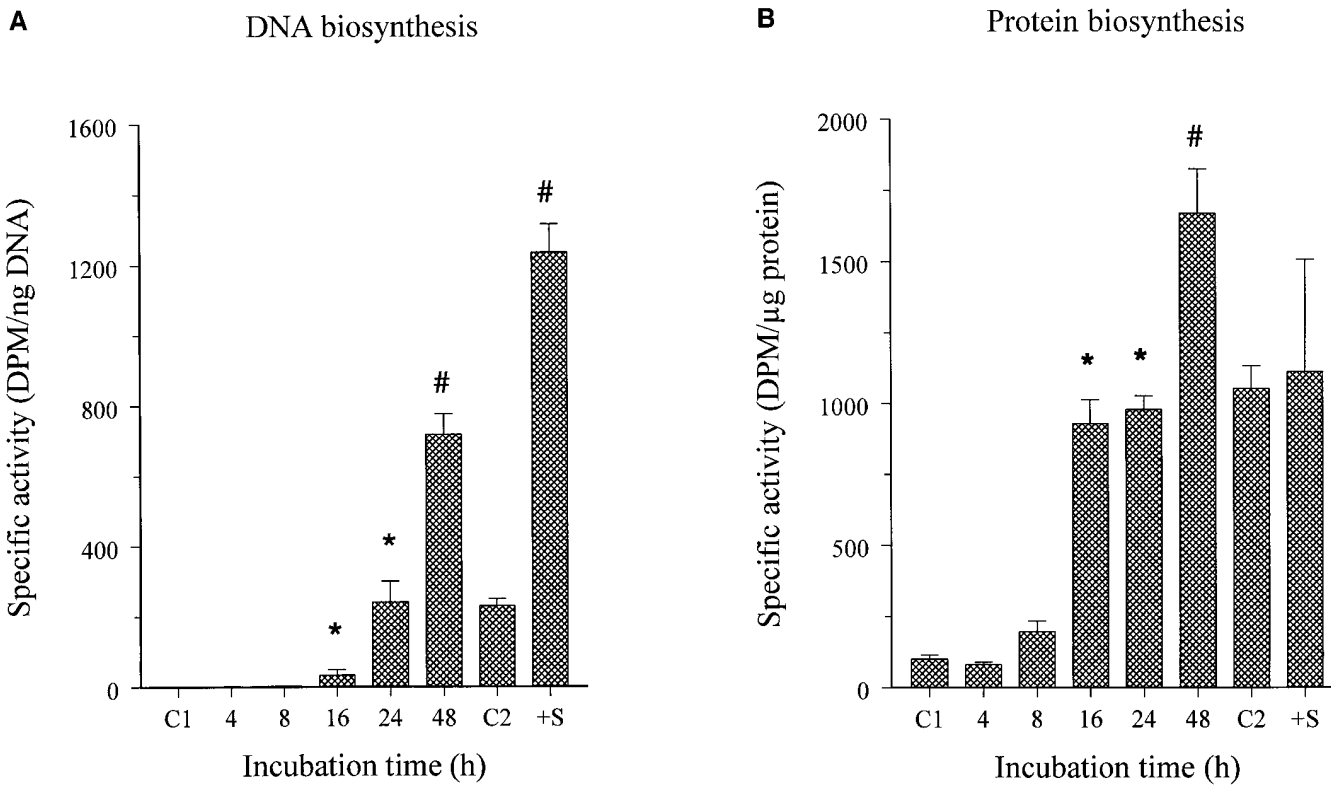


Figure 2. Ang II-induced DNA (*A*) and protein (*B*) biosynthesis in human ASM cells. [^{3}H]Thymidine and [^{3}H]leucine incorporation were measured in confluent, growth-arrested ASM cells that were stimulated with 100 nM Ang II and compared with untreated cells (C1 = 4 h; C2 = 48 h) or cells treated with medium containing 10% FBS (+S). Values are calculated from three independent experiments of four determinations each and expressed as specific activity (dpm/ng DNA or dpm/μg protein). $^*P \leq 0.05$ compared with C1; $^{\#}P \leq 0.05$ compared with C2.

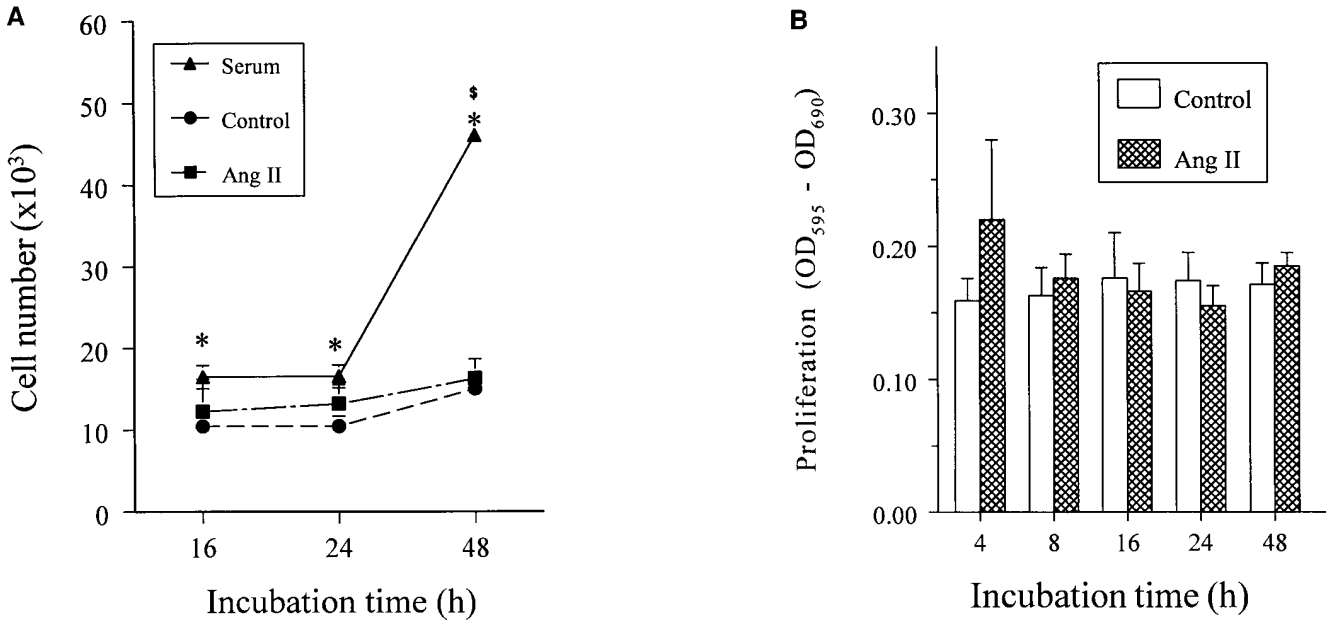


Figure 3. Proliferative response of human ASM cells exposed to Ang II. Proliferation was assessed by hemocytometry (*A*) and by using the MTT dye technique as described in MATERIALS AND METHODS (*B*). Values are represented as means ± SEM of four measurements from three separate experiments. $^*P \leq 0.05$ compared with control; $^{\$}P \leq 0.05$ compared with Ang II-treated cells.

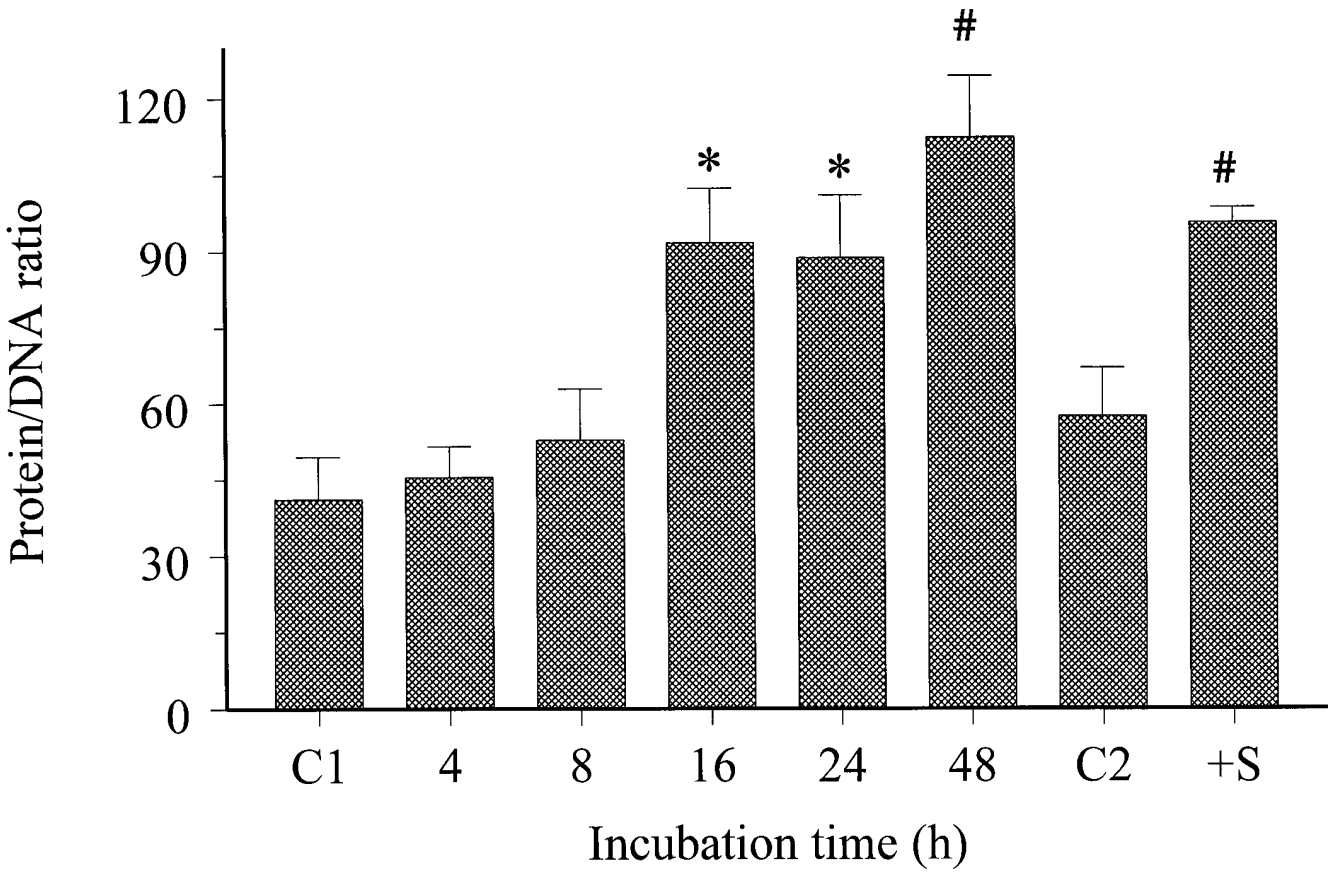


Figure 4. Ang II-induced cellular hypertrophy in human ASM cells. Total DNA and protein contents of ASM cells in the presence and absence of Ang II (100 nM) were measured. DNA and protein values were calculated from three independent experiments of four determinations each, and the protein/DNA ratio was calculated. Values are expressed as means ± SEM. C1 = control (untreated) cells at 4 h; C2 = control cells at 48 h; +S = cells incubated with medium containing 10% FBS. $^{*}P \leq 0.05$ compared with C1; $^{\#}P \leq 0.05$ compared with C2.

from 0 to their maximum values of 1.97, 2.66, and 0.67, respectively, after Ang II stimulation of human ASM cells.

Effects of Ang II on ASM Proliferation and Growth

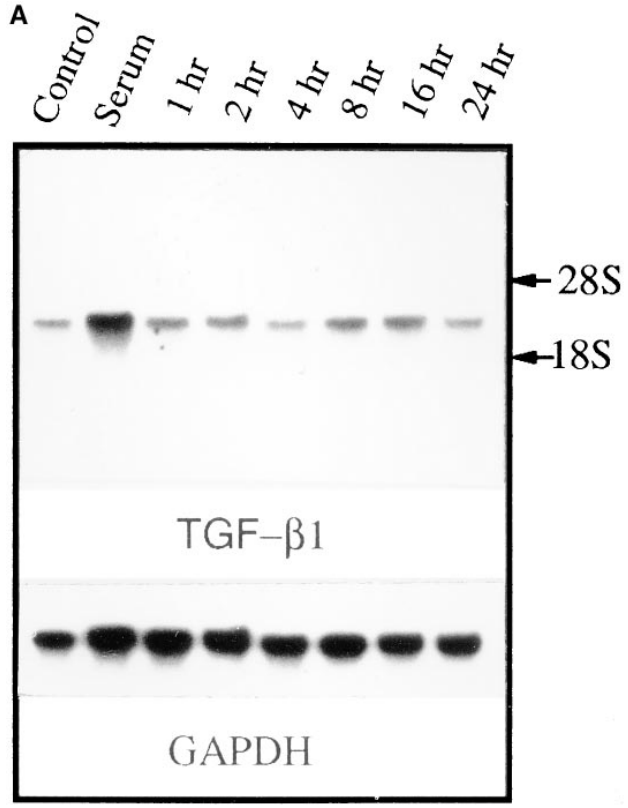
The initial aim of this study was to determine whether Ang II induces an increase in DNA and protein synthesis in ASM cells made quiescent in defined serum-free medium. Biosynthesis of DNA and protein from their respective labeled precursors (thymidine and leucine) was calculated as specific activity and represented as dpm/ng DNA (Figure 2A) or dpm/μg protein (Figure 2B). Data show that the biosynthesis of both DNA and protein was minimal until 8 h of Ang II incubation. However, a significant increase was observed in both DNA and protein biosynthesis at 16 h of Ang II stimulation and these levels remained elevated until 48 h as compared with controls.

In order to assess whether the DNA biosynthesis in human ASM cells, treated with Ang II, was accompanied with an increase in cell number, we performed hemocytometry and MTT assays. Data on hemocytometry showed that Ang II incubation did not significantly increase the cell number with time as compared with respective controls. However, serum already induced a significant ($P \leq 0.05$) increase in cell numbers at 16 h as compared with controls, and at 48 h cell numbers were considerably higher ($P \leq 0.001$) as compared with respective control and Ang II-treated cells (Figure 3A). MTT-reduction assay data showed no significant change in OD values at 4, 8, 16, 24, or 48 h of Ang II incubation as compared with respective controls, indicating that Ang II did not cause ASM cell proliferation (Figure 3B).

In a separate set of experiments, the total DNA and protein contents of the ASM cells were determined in relation to Ang II stimulation with time. Figure 4 shows the effect of Ang II on the protein/DNA ratio in human ASM cells. In order to assess Ang II-induced hypertrophy in human ASM cells, we calculated the protein-to-DNA ratio at different time points. After 16 h of Ang II incubation, the protein/DNA ratio of the ASM cells increased significantly ($P \leq 0.001$) compared with controls, indicating that the cells were synthesizing more protein than DNA, suggesting cellular hypertrophy.

Effects of Ang II on TGF-β_1 Expression

To evaluate the effects of Ang II on TGF-β_1 expression, we analyzed total cellular RNA from serum-fed ASM cells and cells stimulated with 100 nM Ang II for various time intervals and compared the expression pattern with re-



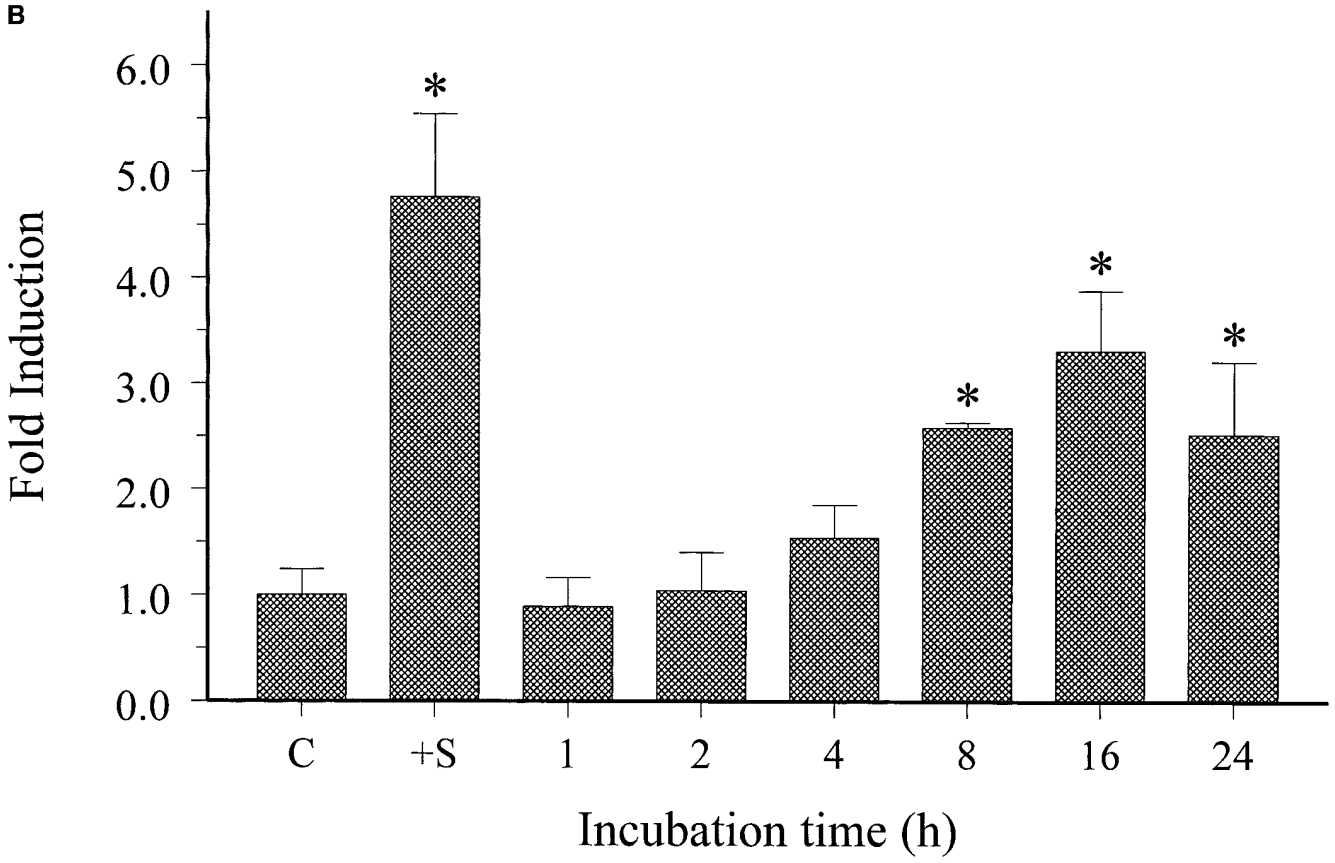


Figure 5. TGF-β_1 mRNA expression in human ASM cells in relation to Ang II. (*A*) A representative Northern blot showing the major 2.5-kb mRNA band encoding TGF-β_1. RNA from control and Ang II-treated human ASM cells was hybridized with a radiolabeled human TGF-β_1 probe as described in MATERIALS AND METHODS. Human ASM cells were incubated with Ang II (100 nM) in serum-free medium for the times indicated at the top of the panel. Filters were reprobed with a radiolabeled GAPDH cDNA for reference purposes (*lower part of panel A*). (*B*) Bar graph showing quantitative analysis of the Ang II-induced TGF-β_1 expression. Scanning densitometric values for TGF-β_1 were normalized with respective GAPDH mRNA values. Values are means of the normalized signal ± SEM ($n = 4$) and expressed as fold induction versus control (control value set at 1.0). $^{\#}P \leq 0.05$ versus control (control cells at 24 h).

spective controls. Using a human-specific cDNA probe encoding TGF-β_1, we detected an mRNA species of 2.4 kb in human ASM cells (Figure 5A). TGF-β_1 expression was drastically increased ($P \leq 0.001$) in serum-fed cells as compared with the control cells. Scanning laser densitometric analysis of TGF-β_1 expression showed that Ang II significantly induced ($P \leq 0.001$) the steady-state mRNA levels which became apparent after 8 h and reached a maximum level at 16 h ($P \leq 0.001$) as compared with controls (Figure 5B). To evaluate whether the increase in mRNA levels for TGF-β_1 were accompanied with an increase in secreted protein, biologically active TGF-β_1 was measured in the conditioned medium after stimulation of the ASM cells with Ang II for various time intervals. Indeed, a significant increase in TGF-β_1 protein was observed after 8 h, which remained elevated at 24 h (Table 1).

Discussion

This is the first study showing that Ang II, in the absence of serum, induces hypertrophy in human airway smooth muscle cells *in vitro*. In an attempt to discern whether Ang II could induce cell hypertrophy, cell hyperplasia, or both, we used several biochemical markers. On the basis of [^{3}H]leucine incorporation, protein over DNA accumulation in the cell (33), MTT-reduction assays, and cell number determinations, our results clearly indicate a hypertrophic response to Ang II in human ASM cells. Our data also show that Ang II induced an increase in [^{3}H]thymidine incorporation into DNA without significantly altering the cell number in human ASM cells. Furthermore, the magnitude of increase in [^{3}H]thymidine incorporation was much lower (55%) than that seen with FBS, suggesting that Ang II may act as a weak mitogen for human ASM cells.

Studying vascular smooth muscle cells *in vitro*, several groups have demonstrated cell proliferation (34) whereas others have shown only cellular hypertrophy after Ang II stimulation (15, 17, 35). Ang II-induced hypertrophy in vascular smooth muscle cells has been shown to be associated with an increase in expression and secretion of the autocrine growth factor platelet-derived growth factor-AA (PDGF-AA) (15, 36). PDGF is a potent mitogen for vascular smooth muscle cells and it is quite possible that in analogy to these cells, human ASM cells express and secrete PDGF in response to Ang II, resulting in an increase in DNA synthesis. Moreover, Ang II induces the synthesis and release of other autocrine–paracrine mitogenic factors such as endothelin-1 and basic fibroblastic growth factor (8, 15). Therefore, in this study, Ang II-induced DNA synthesis may be attributed to the autocrine induction of such growth factors, although it does not appear that the cells are capable of completing the cell cycle (i.e., no increase in cell number). In this regard, Jahan and associates (37) demonstrated that Ang II did not induce the transition of aortic smooth muscle cells in the G_0 phase to the G_1 phase; rather, it acted as a progression factor stimulating cells remaining in the G_1 phase to synthesize protein and DNA. Additionally, they found that PDGF stimulated the entry of cells in the G_0 phase into the G_1 phase without a further progression into the S and M phases, whereas Ang II incubation stimulated the progression of these PDGF-pretreated G_1 cells to the S and M phases. Perhaps, in our study, DNA repair could also be partly responsible for the increase in [^{3}H]thymidine incorporation into DNA with time in the Ang II-treated cells.

TABLE 1

Effects of Ang II on secretion of biologically active TGF-β_1

Incubation Time with Ang II (*h*)	Active TGF-β_1 (*pg/ml*)
Control	161.6 ± 12.0
8	446.0 ± 3.0
24	393.3 ± 11.9

Biologically active TGF-β_1 was measured in the conditioned medium of human ASM cells treated with Ang II for 8 and 24 h as described in MATERIALS AND METHODS. Values are means ± SEM.

In this study, we chose 60 h of serum-free culture conditions in order to synchronize and growth-arrest human ASM cells. Fluorescent-activated cell sorter analysis verified that more than 85% of cells were in the G_0/G_1 phase of the cell cycle after 60 h of serum deprivation, allowing us to examine the direct effects of Ang II on cell division and growth. Others have reported earlier that 48 h of serum deprivation was successful in growth-arresting ASM cells in culture (10–12, 38). The data presented here demonstrate that Ang II is a weak mitogen and a potent hypertrophic stimulus for human ASM cells *in vitro*. However, the cellular mechanisms involved in Ang II-stimulated mitogenesis in ASM cells are undefined and may be distinct from vascular smooth muscle cells.

Previous studies have established that Ang II can induce a rapid increase of the growth-associated nuclear proto-oncogenes c-myc, c-fos, c-jun, and egr-1 in a variety of cell types (20, 22, 39). Similarly, in human ASM cells, nuclear events associated with Ang II activation appear to be the induction of immediate early genes (c-fos, c-jun, and egr-1). The observed increase in DNA biosynthesis could be attributed to the transient expression of the transcription factors c-fos, c-jun, and egr-1, which have previously been reported to be involved in cell growth and differentiation (18, 20, 21, 39). Additionally, the Fos and Jun proteins form a heterodimeric complex known as the AP-1 transcription factor, which activates gene transcription by binding to an AP-1 site in target genes that may subsequently contribute to cell proliferation and growth (19, 21, 40). Previous studies have demonstrated that Ang II stimulates Ang II type-1 (AT_1) receptor-mediated hypertrophy in cardiac myocytes and hyperplasia in cardiac fibroblasts (18, 41). In vascular smooth muscle cells, proliferative effects of Ang II are attributed to the AT_1 receptor which is coupled to G-proteins and classic intracellular second messenger systems (42). In contrast, the function and the signal transduction pathways for the AT_2 receptor, which exhibits only 32% homology to the AT_1 receptor are not fully understood. However, AT_2 receptor-mediated growth inhibition in endothelial cells treated with Ang II has been reported (43). We might speculate that the Ang II-induced upregulation of immediate early genes in human ASM cells may be mediated via AT_1 receptors.

In the present study we have demonstrated that Ang II induces TGF-β_1 mRNA expression and the secretion of

biologically active TGF-β_1 protein in human ASM cells. Our findings are in agreement with previous reports where Ang II has been shown to stimulate TGF-β_1 both at mRNA and protein levels in vascular smooth muscle cells (17, 26, 34). One explanation for the induction of TGF-β_1 seems to be the positive regulation by the AP-1 transcription factor. The TGF-β_1 promoter region contains an AP-1 site which can modulate TGF-β_1 gene expression, suggesting that the Ang II-induced expression of c-fos and c-jun may participate in the induction of TGF-β_1 gene expression (17, 44). Several groups have shown that the incubation of vascular smooth muscle cells with TGF-β_1 induces cellular hypertrophy and inhibits mitogen-stimulated proliferation (34, 45, 46). Apart from the mechanisms involving induction of growth factors such as TGF-β_1 and PDGF, Ang II-induced hypertrophy in human ASM cells may be attributed partly to changes in the contractile phenotype of these cells in response to Ang II stimulation. Koibuchi and colleagues have shown that vascular smooth muscle cell growth is mediated by the autocrine production of active TGF-β_1 (26). This may also be the case in ASM cells. The autocrine production of biologically active TGF-β_1 is a major determinant of whether vascular smooth muscle cells grow by hypertrophy or hyperplasia (17). It remains to be determined whether the direct incubation of human ASM cells with biologically active TGF-β_1 can stimulate cellular hypertrophy.

In conclusion, we have established that Ang II induces DNA biosynthesis and protein biosynthesis, with the net effect being the accumulation of protein over DNA indicating hypertrophy in human ASM cells. Caution must be exercised in extrapolating from *in vitro* studies to *in vivo* observations, but our data suggest a potentially important role for Ang II in ASM growth in airway remodeling in asthma. This possibility is supported by the findings that plasma levels of Ang II are elevated in patients with acute severe asthma (13, 14). Our results also demonstrate that the ASM cells are activated by Ang II and that egr-1, c-fos, and c-jun appear to be the primary response genes. The transient expression of these transcription factors is evidence in favor of induction of the nuclear transcriptional machinery that can subsequently be involved in cellular hypertrophy, since egr-1, c-fos, and c-jun upregulation generally precede the induction of growth-associated genes in a wide variety of cells. Furthermore, the late and sustained induction of TGF-β_1 in response to Ang II could contribute, in an autocrine manner, to the process of hypertrophy in human ASM cells. Hence, Ang II activates a proliferative pathway (increase in DNA synthesis), as well as a possible antiproliferative and hypertrophic pathway (TGF-β_1 upregulation), such that the net effect is hypertrophy in human ASM cells.

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References

1. Lambert, R. K., B. R. Wiggs, K. Kuwano, J. C. Hogg, and P. D. Pare. 1993. Functional significance of increased airway smooth muscle in asthma and COPD. *J. Appl. Physiol.* 74:2771–2781.
2. Dunnill, M. S., G. R. Massarella, and J. A. Anderson. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24:176–179.
3. Jeffrey, P. K. 1992. Pathology of asthma. *Br. Med. Bull.* 48:23–39.
4. Stewart, A. G., P. R. Tomlinson, and J. Wilson. 1993. Airway wall remodelling in asthma: a novel target for the development of anti-asthma drugs. *Trends Pharmacol. Sci.* 14:275–279.
5. Okazawa, M., P. D. Pare, J. C. Hogg, and R. K. Lambert. 1994. Airway and Vascular Remodelling in Asthma and Cardiovascular Diseases. Academic Press, London. 91–101.
6. Ebina, M., T. Takahashi, T. Chiba, and M. Motomiya. 1993. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma: a 3-D morphometric study. *Am. Rev. Respir. Dis.* 148:720–726.
7. Ebina, M., H. Yaegashi, R. Chiba, T. Takahashi, M. Motomiya, and M. Tanemura. 1990. Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles: a morphometric study. *Am. Rev. Respir. Dis.* 141:1327–1332.
8. Glassberg, M. K., A. Ergul, A. Wanner, and D. Puett. 1994. Endothelin-1 promotes mitogenesis in airway smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 10:316–321.
9. Hirst, S. J., P. J. Barnes, and C. H. C. Twort. 1996. PDGF isoform-induced proliferation and receptor expression in human cultured airway smooth muscle cells. *Am. J. Physiol.* 270:L415–L428.
10. Hirst, S. J., P. J. Barnes, and C. H. C. Twort. 1992. Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am. J. Respir. Cell Mol. Biol.* 7:574–581.
11. Stewart, A. G., P. R. Tomlinson, D. J. Fernandes, J. W. Wilson, and T. Harris. 1995. Tumor necrosis factor-α modulates mitogenic responses of human cultured airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* 12:110–119.
12. Panettieri, R. A., P. A. Yadvish, A. M. Kelly, N. A. Rubinstein, and M. I. Kotlikoff. 1990. Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am. J. Physiol.* 259:L365–L371.
13. Millar, E. A., J. E. Nally, and N. C. Thomson. 1995. Angiotensin II potentiates methacholine-induced bronchoconstriction in human airway both in vitro and in vivo. *Eur. Respir. J.* 8:1838–1841.
14. Millar, E. A., R. M. Angus, G. Hulks, J. J. Morton, J. M. C. Connell, and N. C. Thomson. 1994. Activity of the renin-angiotensin system in acute severe asthma and the effect of angiotensin II on lung function. *Thorax* 49: 492–495.
15. Itoh, H., M. Mukoyama, R. E. Pratt, G. H. Gibbons, and V. J. Dzau. 1993. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *J. Clin. Invest.* 91:2268–2274.
16. Sharma, H. S., H. A. A. van Heugten, M. A. Goedbloed, P. D. Verdouw, and J. M. J. Lamers. 1994. Angiotensin II induced expression of transcription factors precedes increase in transforming growth factor-1 mRNA in neonatal cardiac fibroblasts. *Biochem. Biophys. Res. Commun.* 205:105–112.
17. Gibbons, G. H., R. E. Pratt, and V. J. Dzau. 1992. Vascular smooth muscle cell hypertrophy vs. hyperplasia: autocrine transforming growth factor-β_1 expression determines growth response to angiotensin II. *J. Clin. Invest.* 90:456–461.
18. Sadoshima, J. I., and S. Izumo. 1993. Molecular characterization of angiotensin II induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT-1 receptor subtype. *Circ. Res.* 73: 413–423.
19. Halazonetis, T. D., G. Georgopoulos, M. E. Greenberg, and P. Leder. 1988. C-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 55:917–924.
20. Kim, S., M. Kawamura, H. Wanibuchi, K. Ohta, A. Hamaguchi, T. Omura, T. Yukimura, K. Miura, and H. Iwao. 1995. Angiotensin II type-1 receptor blockade inhibits the expression of immediate-early genes and fibronectin in rat injured artery. *Circulation* 92:88–95.
21. Herschman, H. R. 1991. Primary response genes induced by growth factors and tumour promoters. *Annu. Rev. Biochem.* 60:281–319.
22. Naftilan, A. J., G. K. Gilliland, C. S. Eldridge, and A. S. Kraft. 1990. Induction of the proto-oncogene c-jun by angiotensin II. *Mol. Cell Biol.* 10:5536–5540.
23. Lyons, R. M., L. E. Gentry, A. F. Purchino, and H. L. Moses. 1990. Mechanism of activation of latent recombinant transforming growth factor β1 by plasmin. *J. Cell Biol.* 110:1361–1367.
24. Sporn, M. B., and A. B. Roberts. 1992. Transforming growth factor-β: recent progress and new challenges. *J. Cell Biol.* 119:1017–1021.
25. Villarreal, F. J., and W. H. Dillmann. 1992. Cardiac hypertrophy-induced changes in mRNA levels for TGF-β_1, fibronectin, and collagen. *Am. J. Physiol.* 262(6, Pt. 2):H1861–H1866.
26. Koibuchi, Y., W. S. Lee, G. H. Gibbons, and R. E. Pratt. 1993. Role of transforming growth factor-β1 in the cellular growth response to angiotensin II. *Hypertension* 21:1046–1050.
27. Twort, C., and C. van Breeman. 1988. Human airway smooth muscle in culture. *Tissue Cell* 20:339–344.
28. Chamley-Campbell, J., G. R. Campbell, and R. Ross. 1979. The smooth

muscle cell in culture. *Physiol. Rev.* 59:1–61.
29. Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* 201:134–139.
30. Kapuscinski, J., and B. Skoczylas. 1977. Simple and rapid fluorimetric method for DNA microassay. *Anal. Biochem.* 83:252–257.
31. Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–253.
32. Denziot, F., and R. Lang. 1986. Rapid colorimetric assay for growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89:271–277.
33. Winick, M., and A. Noble. 1965. Quantitative changes in DNA, RNA, and protein during prenatal and postnatal growth in the rat. *Dev. Biol.* 12:451–466.
34. Stouffer, G. A., and G. K. Owens. 1992. Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-1. *Circ. Res.* 70:820–828.
35. Geisterfer, A. A. T., M. J. Peach, and G. K. Owens. 1988. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* 62:749–756.
36. Naftilan, A. J., R. E. Pratt, and V. J. Dzau. 1989. Induction of platelet-derived growth factor A-chain and c-myc gene expression by angiotensin II in cultured rat vascular smooth muscle cells. *J. Clin. Invest.* 83:1419–1424.
37. Jahan, H., S. Kobayashi, J. Nishimura, and H. Kanaide. 1996. Endothelin-1 and angiotensin II act as progression but not competence growth factors in vascular smooth muscle cells. *Eur. J. Pharmacol.* 295:261–269.
38. Hirst, S. J., and C. H. C. Twort. 1992. The proliferative response of airway smooth muscle. *Clin. Exp. Allergy* 22:907–915.
39. Sachinidis, A., P. Weisser, Y. Ko, K. Schulte, K. Meyer zu Brickwedde, L. Neyses, and H. Vetter. 1992. Angiotensin II induces formation of the early growth response gene-1 protein in rat vascular smooth muscle cells. *FEBS Lett.* 313:109–112.
40. Adcock, I. M., C. R. Brown, H. Shirasaki, and P. J. Barnes. 1994. Effects of dexamethasone on cytokine and phorbol ester stimulated c-Fos and c-Jun DNA binding and gene expression in human lung. *Eur. Respir. J.* 7:2117–2123.
41. Sadoshima, J. I., Z. Qiu, J. P. Morgan, and S. Izumo. 1995. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes: the critical role of calcium-dependent signalling. *Circ. Res.* 76:1–15.
42. Unger, T., O. Chung, T. Csikos, J. Culman, S. Gallinat, P. Gohlke, S. Hohle, S. Meffert, M. Stoll, U. Stroth, and Y. Z. Zhu. 1996. Angiotensin receptors. *J. Hypertens. Suppl.* 14:S95–S103.
43. Stoll, M., U. M. Steckelings, M. Paul, S. P. Bottari, R. Metzger, and T. Unger. 1995. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.* 95:651–657.
44. Kim, S. J., F. Denhez, K. Y. Kim, J. T. Holt, M. B. Sporn, and A. B. Roberts. 1989. Activation of the second promoter of the transforming growth factor beta-1 gene by transforming growth factor beta-1 and phorbol ester occurs through the same target sequences. *J. Biol. Chem.* 264:19373–19378.
45. Assoian, R. K., and M. B. Sporn. 1986. Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* 102:1217–1223.
46. Bjorkerud, S. 1991. Effects of transforming growth factor-beta 1 on human arterial smooth muscle cells in vitro. *Arterioscl. Thromb.* 11:892–902.