Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells

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Objective: **The purpose of this study was to simultaneously monitor the transcriptional levels of 12 endothelial growth factor genes in response to alterations in wall shear stress (WSS) under conditions relevant to the development of intimal hyperplasia, a major cause of arterial bypass graft failure.**

Methods: **Human umbilical vein endothelial cells were preconditioned in vitro under steady flow (WSS, 15 dynes/cm^2) for 24 hours before being subjected to WSS at 25 ($\Delta = +10$), 15 ($\Delta = 0$), 5 ($\Delta = -10$), 2.5 ($\Delta = -12.5$), and 0 ($\Delta = -15$) dynes/cm^2 or low magnitude WSS reversal (-2.5 dynes/cm^2) for 6 hours. A focused complementary DNA array was used to simultaneously measure messenger RNA expression levels for END1, endothelial nitric oxide synthase (NOS3), platelet-derived growth factor A, platelet-derived growth factor B (PDGFB), acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor-α, transforming growth factor-β, vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, and angiotensin converting enzyme.**

Results: **Preconditioning significantly ($P < .05$) increased the fold expression of NOS3 (4.1 ± 1.4), basic fibroblast growth factor (3.90 ± 1.16), vascular endothelial growth factor (3.39 ± 1.04), and insulin-like growth factor-1 (2.8 ± 0.7) but decreased END1 (0.47 ± 0.05) and PDGFB (0.70 ± 0.04) messenger RNA expression levels relative to no-flow controls, an effect that was sustained on removal from flow for 6 hours. Notably, the ratio of END1/NOS3 expression was diminished (0.11 ± 0.03) relative to that of cells maintained in static culture. Although few differences in gene expression from baseline (15 dynes/cm^2) were measured in cells exposed to either constant ($\Delta = 0$) or step decreases ($\Delta = -10$, -12.5, or -15 dynes/cm^2) in WSS, marked changes were seen in the group exposed to a step increase in WSS ($\Delta = +10$) or to WSS reversal. Low magnitude retrograde WSS evoked significant ($P < .05$) transcriptional changes in multiple genes, including elevated END1 (4.1 ± 0.5), platelet-derived growth factor A (1.5 ± 0.2), PDGFB (2.3 ± 0.3), and transforming growth factor-β (1.5 ± 0.2) levels, but depressed NOS3 (0.60 ± 0.17) levels, and a marked increase in END1/NOS3 (6.7 ± 1.6) when compared with equal magnitude antegrade WSS (2.5 dynes/cm^2).**

Conclusion: **These results support the implementation of a preconditioning phase for in vitro WSS studies to establish a physiologic baseline. Our findings complement previous macroscale findings and are consistent with a cellular mechanism involving increased END1 and PDGFB levels, but decreased NOS3 levels, leading to intimal hyperplasia at regions of low magnitude reversing WSS. (J Vasc Surg 2003;37:182-90.)**

The response of endothelial cells (ECs) to biomechanical stimuli is of importance in both normal vascular physiology[1-5] and numerous pathologies.[6-8] Growth factors expressed by ECs in response to luminal wall shear stress (WSS)[9-15] may contribute to the pathology of intimal hyperplasia (IH), a major cause of bypass graft failure,[6,16] through their effects on the underlying myoblasts. The extra-anatomic geometry of the end-to-side distal anastomosis[17,18] results in regions of low and reversing flow (WSS) along the vessel floor[19-21] where the ECs are essentially intact. Clinical observations[22] and results from in vitro[20,21] and in vivo[6,18,19,23-25] studies have shown an inverse relationship between WSS and IH and implicated the possible importance of flow reversal.[13,25]

In vitro studies have identified numerous growth factors whose expression in ECs is modulated by WSS,[9-15] both at the transcriptional level and at the protein level (Table I). In general, factors that have opposing effects on vascular smooth muscle cells are oppositely regulated by shear stress, a finding consistent with the development of IH at regions of low WSS. However, this evidence is often contradictory and limited in scope by several experimental factors, including the use of a static culture baseline.[4,23]

In this study, we used a focused complementary DNA (cDNA) array to simultaneously measure the transcriptional levels of multiple endothelial growth factor genes of possible importance in IH over a broad range of clinically relevant WSS. We also described the use of a preconditioning protocol to reset the baseline for EC gene expression from static culture levels.

MATERIALS AND METHODS

Human umbilical vein ECs (HUVECs) were supplied as frozen primaries that were pooled from several donors (lot #P149, Clonetics Corp, San Diego, Calif). They were cultured in endothelial growth medium (Clonetics Corp)

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Table I. Summary of in vitro findings on shear stress regulation of endothelial genes grouped by similar function

Gene	*Biologic activity/function of gene product*	*Shear regulation*
	I. Vasodilatation, inhibition of SMC proliferation	
NOS3	Vasodilatation	Direct[9,13]
	Inhibition of: SMC proliferation, platelet aggregation/adhesion, monocyte adhesion	
TGFB1	Inhibition (stimulation) of SMC proliferation	Direct[11]
	Regulator of differentiation and extracellular matrix production	
	II. Vasoconstriction, stimulation of SMC proliferation/migration	
Endothelin-1	Vasoconstriction	Inverse[10,13] (initial increase)
	SMC proliferation	
	Upregulation of collagen synthesis	
PDGF B	Vasoconstriction	Inverse[12] (conflicting)
	SMC proliferation/migration/chemotaxis	
PDGF A	Vasoconstriction	None[12] (conflicting)
	SMC proliferation/migration/chemotaxis	
FGF2	SMC/EC proliferation	Direct[12] (conflicting)
	Signaling between SMC	
	Angiogenesis	
ACE	Vasoconstriction	Inverse[14] (conflicting)
	SMC proliferation (through angiotensin-I to angiotensin-II)	
	III. Stimulation of EC proliferation/migration	
VEGF	Vasodilatation (via nitric oxide)	Inverse[15]
	EC proliferation	
	Angiogenesis	
	Vascular permeability	

SMC, Smooth muscle cell.

supplemented with a penicillin (100 U/mL), streptomycin (100 μg/mL), and Fungizone (0.25 μg/mL) mixture (BioWhittaker, Walkersville, Md). Cells (passages 1 through 4) were seeded (5000/cm^2) on 75-mm × 50-mm glass microscope slides (Corning, Inc, Corning, NY), treated with 10 μg/cm^2 human fibronectin (Fisher Scientific, Pittsburgh, Pa), and placed within custom-designed polycarbonate culture chambers (16-cm^2 culture area). At cell confluency, the slides were transferred to parallel-plate flow chambers (gap height, 0.250 mm; width, 32.0 mm; length, 50.0 mm; entrance/exit regions, 3.0 mm each; maximum Reynolds' number, 48; at WSS, 25 dynes/cm^2), for which the fluid mechanics have been well described.[26,27] The chambers were placed within a recirculating flow loop consisting of four independent circuits in parallel (50 mL media/circuit). Flow was driven by a Masterflex L/S peristaltic pumping system (Cole-Parmer Instrument Co, Vernon Hills, Ill).

The shear stress protocol applied to the HUVEC monolayers is shown in Fig 1. Cells first were preconditioned with steady flow at a normalized arterial shear stress[1] of 15 dynes/cm^2 for 24 hours. The flow then was adjusted to apply shear stresses of 25, 15, 5, 2.5, 0, or −2.5 dynes/cm^2 (representing step changes in WSS of Δ = +10, 0, −10, −12.5, −15 dynes/cm^2, or WSS reversal) for 6 hours. Absolute WSS levels were chosen to span the range of values that were shown to be relevant to IH progression in a previous canine study.[25] The "negative" shear level was brought about with flow in the direction opposite that established by the preconditioning protocol. Sixteen slides (representing four replicate runs at each of four passages from the same cell line [HUVEC P149]) were pooled for each shear stress level to yield a single RNA sample per shear level. This pooling was performed to eliminate potential variation from increased passage number while acquiring sufficient RNA for subsequent analysis. Sheared monolayers were rinsed with phosphate-buffered saline solution, the cells were lysed, and messenger RNA (mRNA) was isolated with the Poly(A)Pure mRNA Isolation Kit (Ambion Corp, Austin, Tex). mRNA also was isolated from cells maintained in static culture at passages 1 to 4.

The following sequence-verified human IMAGE Consortium cDNA clones[28] were used in array preparation: β actin, glyceraldehyde-3-phosphate dehydrogenase (GAPD; American Type Culture Collection, Manassas, Va), ribosomal protein S9, END1, endothelial nitric oxide synthase (NOS3), platelet-derived growth factor B chain (PDGFB), platelet-derived growth factor A chain (PDGFA), basic fibroblast growth factor (FGF2), acidic fibroblast growth factor (FGF1), transforming growth factor-α (TGFA), transforming growth factor-β1 (TGFB1), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF1), and epidermal growth factor (EGF; Research Genetics, Carlsbad, Calif). The clone for endothelial angiotensin converting enzyme (ACE) was provided by the Institut National de la Santè et de la Recherche Mèdicale. The phagemid vector pBluescript II KS(+) was purchased from Stratagene (La Jolla, Calif). Target cDNAs (1 μg/spot) and controls were arrayed in triplicate on nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell Protran BA85, Perkin Elmer, Boston, Mass) with the Bio-Dot SF Microfiltration Apparatus (Bio-

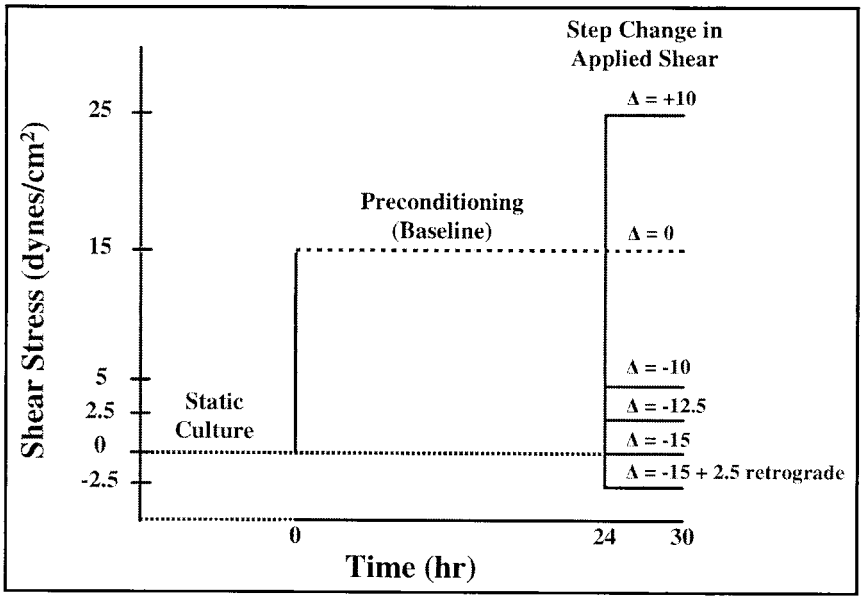

Fig 1. Schematic shows shear stress protocol applied to HUVEC monolayers. On reaching confluency in static culture, cells were preconditioned at normalized arterial WSS of 15 dynes/cm^2 for 24 hours to reset baseline gene expression. They then were exposed to various step changes in WSS for 6 hours.

Rad Laboratories, Hercules, Calif), according to the instructions of the manufacturer.

Individual poly(A^+) RNA samples were used as template (1.0 to 2.5 μg) in a reverse transcription reaction containing 0.5 μg/μg RNA oligo$(dT)_{15}$ primer (Promega, Madison, Wis), 1× Moloney murine leukemia virus reaction buffer (Promega), 1 mmol/L dATP/dGTP/dTTP mix (MBI Fermentas), 0.8 U/μL ribonuclease inhibitor (Sigma, St Louis, Mo), 8 U/μL Moloney murine leukemia virus reverse transcriptase (Promega), nuclease-free water (Ambion Corp), and 100 μCi α^{32}P-dCTP (110 TBq/mmol). Sephadex G-50 syringe columns (Sigma) were used to separate unincorporated nucleotides from the labeled cDNA probe. DNA-spotted nitrocellulose membranes were prewet in 6× sodium chloride–sodium phosphate (SSE)–ethylenediamine tetraacetic acid buffer (SSPE), prehybridized at 42° C for 2 hours in 50% formamide, 6× SSPE, 5× Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), and 200 μg/mL denatured herring sperm DNA, and hybridized with the denatured probe at 42° C overnight. Membranes were washed with 5× SSPE/0.1% SDS at room temperature, 1× SSPE/0.5% SDS at 37° C, 0.1× SSPE/1.0% SDS at 65° C, rinsed with 6× SSPE, and imaged on a Betascope 603 Blot Analyzer (Betagen Corp, Waltham, Mass) for 24 hours.

Total raw counts were calculated (three observations per gene, on the basis of a single pooled RNA sample at each shear stress level) and corrected for mean membrane background. Individual gene expression levels were normalized to mean background corrected GAPD levels for a given blot. The END1/NOS3 ratio was calculated by dividing background corrected counts for one gene by the other (three observations per shear level). A single factor analysis of variance was performed for repeated measurements over the six WSS levels for each gene. Differences in expression were assessed with the Student-Newman-Keuls method for a P value of less than .05.

RESULTS

Individual gene expression changes over the six Δ WSS groups are shown in Figs 2 and 3, where statistical differences are indicated relative to baseline gene expression (15 dynes/cm^2) in preconditioned cells only. The mean expression levels ± standard deviation are also presented in tabular form (Table II, online only). Calculated fold changes in gene expression reported in the text are also listed in Table III (online only).

Effect of preconditioning. The effects of preconditioning on baseline gene expression are shown by comparison of mRNA levels for cells maintained at 15 dynes/cm^2 ($\Delta = 0$) with those of cells maintained in static culture (Table III, online only), where differences were seen in six genes. Preconditioning significantly ($P < .05$) increased the fold expression of NOS3 (4.1 ± 1.4), FGF2 (3.90 ± 1.16), VEGF (3.39 ± 1.04), and IGF1 (2.8 ± 0.7) but decreased END1 (0.47 ± 0.05) and PDGFB (0.70 ± 0.04) expression levels when compared with the no-flow controls. The importance of these effects is further shown by comparison of gene expression levels for the static culture (traditional zero) case with the preconditioned 0 dynes/cm^2 ($\Delta = -15$) shear case (Table III, online only). Removal of flow for 6 hours after preconditioning did not

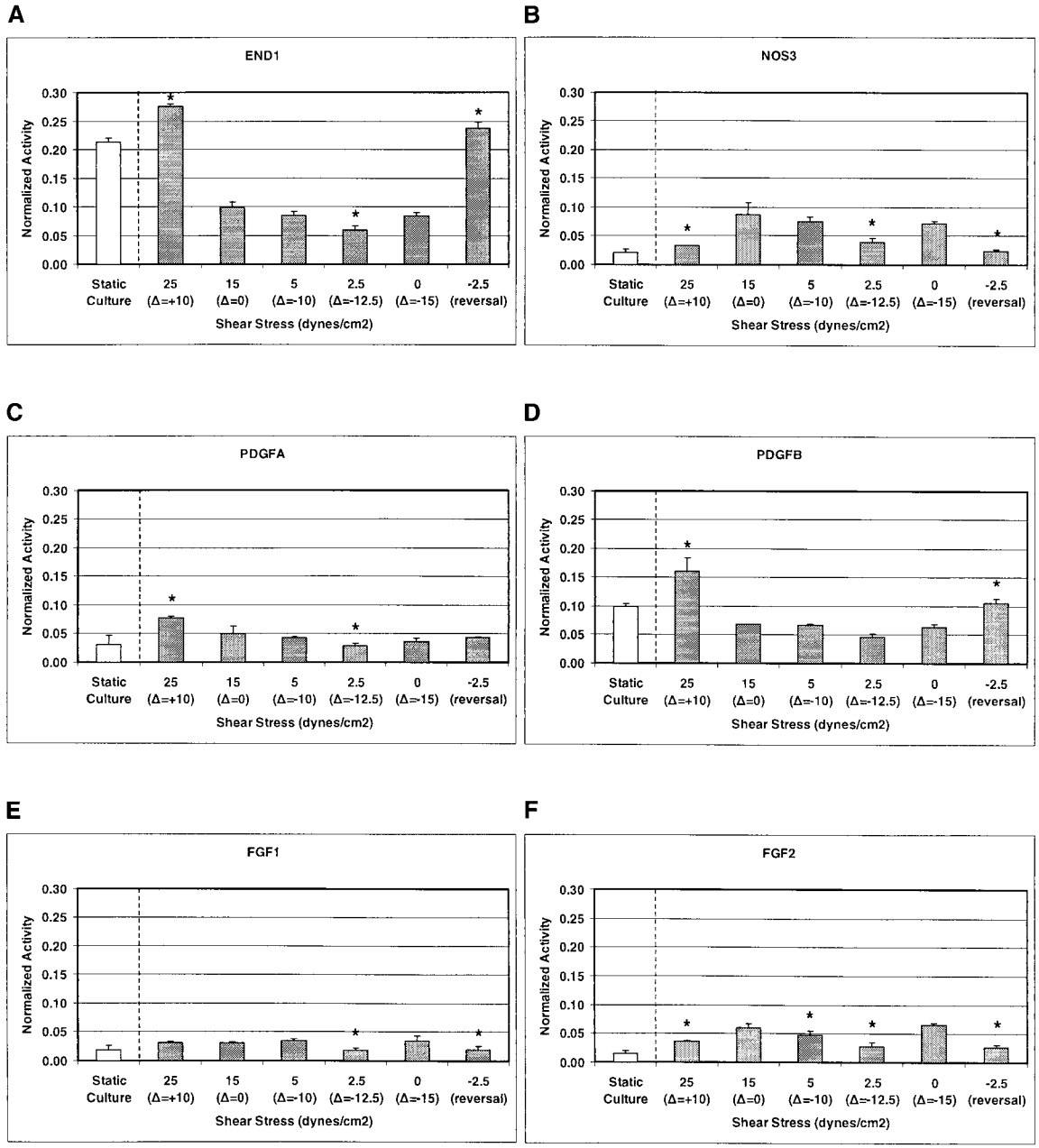

Fig 2. Mean ± standard deviation expression levels for endothelial genes (n = 3 per gene per shear level). **A,** END1; **B,** NOS3; **C,** PDGFA; **D,** PDGFB; **E,** FGF1; and **F,** FGF2. *$P < .05$ from baseline (15 dynes/cm^2) in preconditioned cells. Expression levels in static cultured cells are shown for comparison only.

result in a reversion of gene expression to no-flow levels. Rather, a sustained increase ($P < .05$) was seen in the expression of NOS3 (3.3 ± 0.8), FGF2 (4.26 ± 1.19), VEGF (5.7 ± 1.7), and IGF1 (3.1 ± 0.8) levels and a decrease was seen in END1 (0.40 ± 0.03) and PDGFB (0.65 ± 0.07) levels.

Multigene expression analysis. Most of the genes investigated (END1, NOS3, PDGFA, PDGFB, FGF1, TGFB1, IGF1, and EGF) showed only minor changes in expression from baseline levels in cells exposed to either no change ($\Delta = 0$ dynes/cm^2) in WSS or step decreases ($\Delta = -10$, -12.5, or -15 dynes/cm^2) in WSS (Figs 2 and 3; Table II, online only). PDGFB did not show any differences, and the other genes only differed significantly at the 2.5-dynes/cm^2 ($\Delta = -12.5$) level within this range ($P < .05$). Significant differences in gene expression from base-

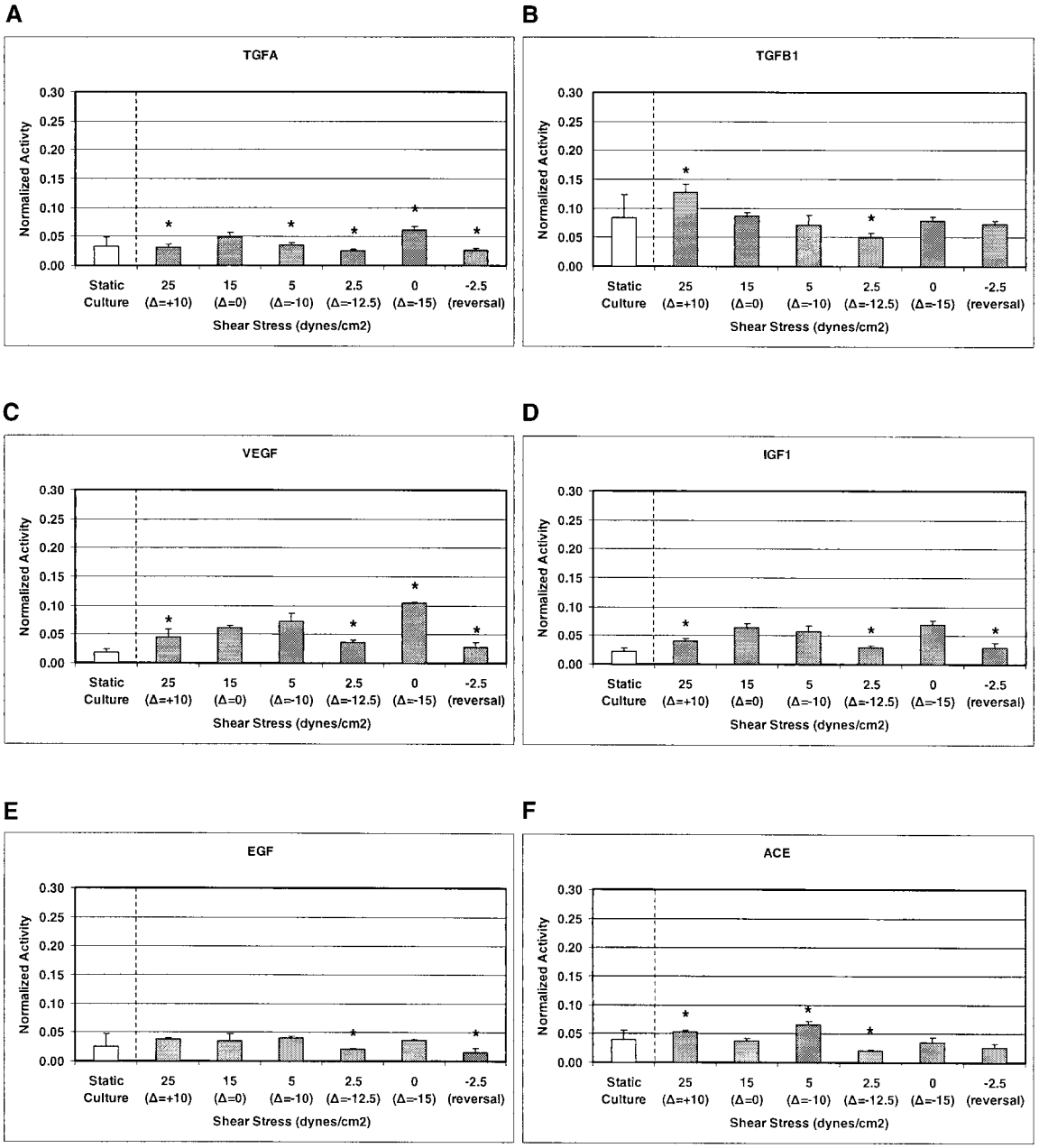

Fig 3. Mean ± standard deviation expression levels for endothelial genes (n = 3 per gene per shear level). **A,** TGFA; **B,** TGFB1; **C,** VEGF; **D,** IGF1; **E,** EGF; and **F,** ACE. *$P < .05$ from baseline (15 dynes/cm^2) in preconditioned cells. Expression levels in static cultured cells are shown for comparison only.

line were more often observed in cells exposed to a step increase in WSS to 25 dynes/cm^2 ($\Delta = +10$) or to reversal of WSS (-2.5 dynes/cm^2) than for other changes in WSS. A Δ of $+10$ resulted in significantly ($P < .05$) elevated END1 (2.8 ± 0.3), PDGFA (1.5 ± 0.4), PDGFB (2.3 ± 0.4), TGFB1 (1.5 ± 0.2), and ACE (1.38 ± 0.18) levels but depleted NOS3 (0.38 ± 0.09), FGF2 (0.60 ± 0.07), TGFA (0.63 ± 0.17), VEGF (0.7 ± 0.2), and IGF1 (0.63 ± 0.09) levels relative to baseline (Table III, online only). WSS reversal (-2.5 dynes/cm^2) resulted in increased ($P < .05$) END1 (2.4 ± 0.2) and PDGFB (1.53 ± 0.10) levels but decreased NOS3 (0.27 ± 0.08), FGF1 (0.64 ± 0.18), FGF2 (0.44 ± 0.08), TGFA (0.56 ± 0.12), VEGF (0.46 ± 0.13), IGF1 (0.47 ± 0.13), and EGF (0.4 ± 0.2) levels relative to baseline (Table III, online only).

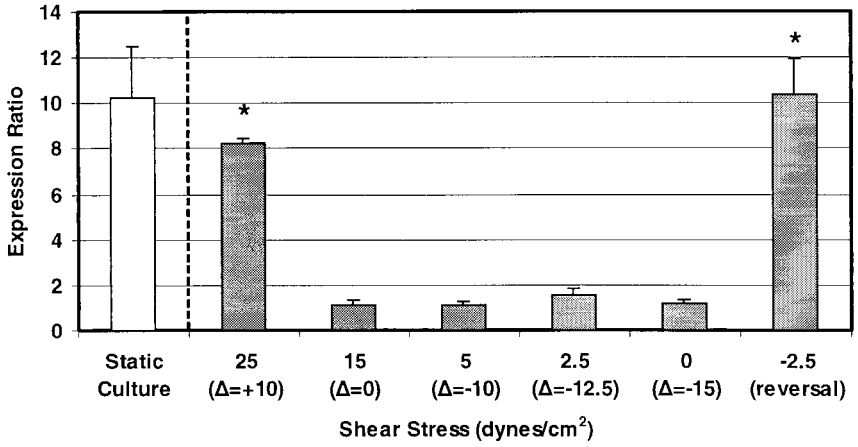

Fig 4. Mean ± standard deviation END1/NOS3 mRNA expression ratio (n = 3 per shear level). *$P < .05$ from baseline (15 dynes/cm^2) in preconditioned cells. END1/NOS3 expression ratio in static cultured cells is shown for comparison only.

END1/NOS3 expression ratio. END1 and nitric oxide have opposing effects on the vessel wall and appear to be oppositely regulated by shear stress[9,10,13] in a manner consistent with finding IH at regions of low WSS (Table I). The balance between these two factors is believed to be important in vascular physiology and pathology.[1] Hence, the expression ratio END1/NOS3, measured within the same biologic sample, may provide valuable insight into interaction between these factors. As shown in Fig 4, preconditioning significantly reduced the END1/NOS3 ratio relative to that of cells in static culture (0.11 ± 0.03). Furthermore, the END1/NOS3 ratio during the 6-hour period after preconditioning was maintained at baseline levels in all groups subjected to either a step decrease or no change of WSS ($\Delta = -15$ to 0 dynes/cm^2), but in cells subjected to a step increase of WSS ($\Delta = +10$) or reversal of WSS (−2.5 dynes/cm^2), END1/NOS3 was restored to near no-flow levels.

Effect of shear stress directionality. Comparison of normalized gene expression levels for the 2.5 dynes/cm^2 (antegrade) with the −2.5 dynes/cm^2 (retrograde) shear cases (equal in magnitude but opposite in direction to established flow) revealed differences in five of 12 genes (Table III, online only). Retrograde shear resulted in significantly ($P < .05$) elevated END1 (4.1 ± 0.5), PDGFA (1.5 ± 0.2), PDGFB (2.3 ± 0.3), and TGFB1 (1.5 ± 0.2) levels but depressed NOS3 (0.60 ± 0.17) levels when compared with the antegrade case. Furthermore, the END1/NOS3 ratio (Fig 4) was 6.7 ± 1.6 fold higher in the retrograde case.

Comparison of normalized gene expression levels at 15 dynes/cm^2 (baseline) with the −2.5 dynes/cm^2 (retrograde) shear case revealed differences in 10 of 12 genes (Table III, online only). Retrograde shear resulted in significantly ($P < .05$) increased END1 (2.4 ± 0.2) and PDGFB (1.53 ± 0.10) levels and decreasing NOS3 (0.27 ± 0.08), FGF1 (0.64 ± 0.18), FGF2 (0.44 ± 0.08), TGFA (0.56 ± 0.12), TGFB1 (0.85 ± 0.09), VEGF (0.46 ± 0.13), IGF1 (0.47 ± 0.13), and ACE (0.71 ± 0.15) levels relative to baseline. The END1/NOS3 ratio was 9.0 ± 1.9 fold higher for the retrograde shear case (Fig 4).

DISCUSSION

Effect of preconditioning. Exposure of ECs to arterial levels of flow (shear stress) results in changes in morphology[1,29] and cellular architecture,[30,31] which are consistent with observations in native arteries and appear to be crucial for adhesion under flow. Ott and Ballermann[32] showed that EC adhesion and retention in seeded vascular grafts were markedly enhanced with preconditioning of the cells to shear stress. Although several papers have pointed out limitations in the use of a static culture control,[4,23] few in vitro studies on gene expression in response to shear stress have adopted a flow preconditioning step.[33]

Our results support the implementation of a preconditioning phase for WSS studies in vitro. Preconditioning HUVEC monolayers at arterial levels of shear stress for 24 hours resulted in changes in cell shape and alignment consistent with previous observations. Comparison of gene expression levels between a physiologic baseline (preconditioned cells) and the traditional static culture baseline revealed significant differences in six of the 12 genes studied (END1, NOS3, PDGFB, FGF2, VEGF, and IGF1) and in the END1/NOS3 ratio (Table III, online only; Fig 4). Furthermore, these differences were maintained for 6 hours on removal of flow from preconditioned cells (Table III, online only), showing a fundamental difference between our zero-shear level and the traditional static culture zero. Our results indicate that studies with static culture as a baseline may tend to overestimate or underestimate the effects of shear stress on certain genes. For example, in preconditioned cells, we found END1 mRNA expression to be less (0.47 ± 0.05) and NOS3 mRNA expression to be

greater (4.1 ± 1.4) than that in static culture (Table III, online only), resulting in an END1/NOS3 ratio that was dramatically reduced (0.11 ± 0.03) in preconditioned cells. This observation is consistent with the inhibition of EC growth by laminar shear stress previously reported.[3] Although previous studies have clearly established a dynamic response of ECs to mechanical forces (ie, direct or inverse relationship), the magnitude of these responses (relative to a static baseline) may in fact be more reflective of adaptive changes as the cells reacclimate to more physiologic WSS levels. Similarly, apparent transients in mRNA expression[2,4] may simply be reflective of this adaptation. These effects may mask subtle long-term changes that may be of clinical significance in a chronic process such as IH.

Multigene expression analysis. The six WSS groups studied (25, 15, 5, 2.5, 0, and −2.5 dynes/cm^2) are representative of those observed in normal vascular physiology (including under arterial, venous, and low flow conditions) and in the pathology of IH (at near zero, zero, and low magnitude retrograde flow).[25] Our results indicate that the direction of an applied change in WSS from normal physiologic levels may be of greater interest than absolute WSS levels. Analysis of gene expression over six WSS levels revealed few differences from baseline in cases where cells were exposed to either no change in WSS ($\Delta = 0$ dynes/cm^2) or to step decreases in WSS ($\Delta = -10$, -12.5, or -15 dynes/cm^2) for 6 hours (Figs 2 & 3; Table II, online only). Most of the genes studied either showed no differences or differed from baseline only at the 2.5 dynes/cm^2 shear level ($\Delta = -12.5$). Analysis at this shear level was complicated by the choice of GAPD as a control, which led to expression levels reading consistently low (data not shown). There is no justification to believe that this level should differ significantly from both the neighboring 5 ($\Delta = -10$) and 0 ($\Delta = -15$) dynes/cm^2 levels (which did not differ from each other). Furthermore, no differences were seen in the END1/NOS3 expression ratio over the shear range of $\Delta = 0$ to $\Delta = -15$ dynes/cm^2 (Fig 4), which, by virtue of its independent means of normalization, lends support to the validity of these observations for the individual genes.

The range in WSS considered in our study (0 to 15 dynes/cm^2) closely corresponds to that considered by most previous in vitro studies (with the exception of preconditioning applied), which showed dramatic effects of shear stress on gene expression.[9-15] Thus, a relative lack of differences over this shear range further underscores the importance of the preconditioning step in this study and shows the importance of the proper choice of baseline and zero-shear levels in the experimental design. However, a comparison made in our study that more closely matches those made in previous studies is that for cells maintained in static culture versus those exposed to preconditioning (15 dynes/cm^2; Table III, online only), which revealed a decrease in END1 and PDGFB and an increase in NOS3 and FGF2, results that are consistent with previous findings (Table I).

Differences in gene expression from baseline were more often observed in cells exposed to a step increase in WSS ($\Delta = +10$) or low magnitude reversal of WSS (−2.5 dynes/cm^2). In fact, results for these two WSS levels were similar for a particular gene or ratio (Figs 2 and 3). One might expect gene expression at the 25 dynes/cm^2 shear level ($\Delta = +10$; on the high end of normal but well below levels that would be damaging to the cells) to deviate from baseline (arterial) levels less than that for a low magnitude reversing WSS. The 25 dynes/cm^2 case was unusual in that this was the only case where the cells were exposed to a step increase in WSS relative to baseline (by a factor of 2/3; Fig 1). This essentially represents a step increase from baseline conditions whose magnitude is similar to the step increases from static levels described in previous studies. Similarly, the −2.5 dynes/cm^2 shear case was the only case where cells were exposed to flow (WSS) in the direction opposite to that established by the preconditioning phase. The similarity in outcomes may reflect common adaptive responses taking place under these two conditions. It is also noteworthy that the expression of β actin (included as one of our internal controls) was elevated in these two cases relative to baseline (2.13 ± 0.16 at 25 dynes/cm^2 and 1.49 ± 0.10 at −2.5 dynes/cm^2, respectively), which may be reflective of associated adaptive changes taking place in cytoskeletal architecture.

Effects of directionality-retrograde shear. The importance of the flow directionality effect is supported by comparison of the low magnitude retrograde shear case (−2.5 dynes/cm^2) with the low magnitude antegrade shear case (2.5 dynes/cm^2), where significant differences were seen in the expression of five of the 12 genes (including an increase in END1 and PDGFB mRNA expression and a decrease in NOS3 mRNA expression; Table III, online only) and in the END1/NOS3 ratio (Fig 4). Furthermore, comparison of gene expression for retrograde shear with baseline levels (15 dynes/cm^2) revealed dramatic differences, which were observed in 10 of the 12 genes (including increased END1 and PDGFB mRNA levels and decreased NOS3 and TGFB1 mRNA levels; Table III, online only) and in the END1/NOS3 ratio (Fig 4). Additional differences that were observed in comparison of the baseline with the retrograde shear case may represent additional effects of shear magnitude in addition to directionality alone. These observations are in agreement with the functional dependence of gene expression on WSS previously reported (Table I)[9-15] and are consistent with a cellular mechanism involving IH progression at regions of low magnitude reversing shear stress as seen in vivo[25] on the basis of the effects of these factors on vascular smooth muscle cells.

The arterial bypass graft anastomosis creates a dynamic environment, with complex oscillatory flow patterns and a shifting stagnation point. Although there is an important distinction between oscillatory flow and the steady retrograde flow applied in our model, it should be noted that time-averaged flow is reversed in the mean at several anastomotic sites (ie, heel, toe, and artery floor). Our results indicate that flow (WSS) directionality may play an important role in the pathology of IH, leading to graft failure at a

cellular level. This point is supported by in vitro studies that have used an oscillatory (ie, flow reversing) shear stress, such as that of Ziegler et al,[13] which showed dramatic differences in END1 and NOS3 expression when comparing a nonreversing pulsatile shear stress with a purely oscillatory one. Furthermore, this was suggested by the in vivo results of Keynton et al[25] in which IH correlated best with an oscillatory shear index. Thus, retrograde shear stress may present a particularly challenging environment to ECs, requiring a more dramatic adaptive response than other low but antegrade stresses. This is consistent with the observation that a spectrum of morphologic and cytoskeletal changes seem to nonuniformly adapt the EC to flow in a given direction.[1,29-31] Although we would expect that ECs should readapt to a new flow direction over time and that this should be reflected in gene expression, complete adaptation may not be possible within the dynamic environment of the anastomosis.

Several comments are appropriate regarding the conduct of this study. First, we chose to primarily focus on measuring the transcriptional levels of numerous genes over a range of relevant shear stresses (on the basis of results of a detailed in vivo study in our laboratory[25]) rather than obtaining a wealth of time-dependent data. Because IH is a chronic process leading to graft failure (beyond 30 days), it is likely that gene expression over an extended period of time contributes to this process. Being practically limited as to the duration of our experiments by the in vitro model, we chose to measure gene expression at 6 hours after shear stress changes from an established arterial baseline. This choice was based on indications that gene expression appears to reach steady state values within this time frame.[2,4] Differences observed in the $\Delta = +10$ and the retrograde shear cases at 6 hours may serve as motivation to track changes further out in time in future experiments.

Second, our focus has been on the expression of multiple growth factors at the level of transcription. Although transcriptional changes do not necessarily reflect changes at the level of protein, they often do. Several of the genes evaluated in our study (including END1, NOS3, and TGFB1) have been shown to be consistently regulated at the protein level for similar shear stress levels (Table I). With results from this study, at this time, we are not able to determine the relative importance of transcriptional versus post-transcriptional regulatory processes for the genes involved. Future studies will evaluate the contributions of mRNA and expressed protein levels that contribute to IH.

Third, we have chosen to investigate the role of gene expression in the mechanically responsive endothelium, given the complex hemodynamic environment to which the ECs are exposed in the arterial bypass graft anastomosis. However, IH is a complex process in which other cell types participate as well. For example, oscillatory flow patterns at the distal end-to-side anastomosis have the tendency to elevate the EC overlapping processes and allow platelets, leukocytes, and blood-borne elements to enter the subintima, also without endothelial injury or disruption. Ultimately, the products of altered gene expression in ECs and other sources bring about the process of IH, through the effects on the underlying myoblasts, including cell proliferation, migration, differentiation, and matrix production. These complex interactions are best sorted out in an in vivo model and are the subject of future work.

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Table II, online only. Mean expression levels ± standard deviation for EC genes over varying WSS

		Preconditioned Cells					
Gene	*Static culture*	*25 dynes/cm² (Δ = +10)*	*15 dynes/cm² (Δ = 0)*	*5 dynes/cm² (Δ = −10)*	*2.5 dynes/cm² (Δ = −12.5)*	*0 dynes/cm² (Δ = −15)*	*–2.5 dynes/cm² (reversal)*
Endothelin-1	0.212 ± 0.007	0.276 ± 0.004*	0.099 ± 0.009	0.084 ± 0.008	0.059 ± 0.007*	0.085 ± 0.005	0.238 ± 0.010*
NOS3	0.022 ± 0.005	0.033 ± 0.001*	0.09 ± 0.02	0.074 ± 0.009	0.039 ± 0.008*	0.071 ± 0.004	0.023 ± 0.004*
PDGFA	0.031 ± 0.017	0.078 ± 0.002*	0.050 ± 0.013	0.044 ± 0.001	0.029 ± 0.005*	0.037 ± 0.005	0.043 ± 0.001
PDGFB	0.098 ± 0.006	0.16 ± 0.03*	0.069 ± 0.001	0.067 ± 0.002	0.046 ± 0.006	0.064 ± 0.005	0.105 ± 0.007*
FGF1	0.018 ± 0.009	0.031 ± 0.003	0.030 ± 0.002	0.035 ± 0.003	0.019 ± 0.004*	0.035 ± 0.008	0.019 ± 0.005*
FGF2	0.015 ± 0.004	0.036 ± 0.002*	0.060 ± 0.007	0.048 ± 0.007*	0.028 ± 0.008*	0.066 ± 0.003	0.026 ± 0.004*
TGFA	0.032 ± 0.018	0.031 ± 0.006*	0.049 ± 0.009	0.035 ± 0.004*	0.025 ± 0.003*	0.061 ± 0.006*	0.027 ± 0.003*
TGFB1	0.08 ± 0.04	0.127 ± 0.014*	0.086 ± 0.007	0.072 ± 0.016	0.050 ± 0.007*	0.079 ± 0.007	0.073 ± 0.005
VEGF	0.018 ± 0.006	0.046 ± 0.014*	0.062 ± 0.003	0.073 ± 0.013	0.037 ± 0.004*	0.106 ± 0.001*	0.029 ± 0.008*
IGF1	0.023 ± 0.005	0.040 ± 0.004*	0.064 ± 0.007	0.058 ± 0.009	0.030 ± 0.004*	0.071 ± 0.006	0.030 ± 0.007*
EGF	0.03 ± 0.02	0.038 ± 0.003	0.036 ± 0.011	0.041 ± 0.003	0.021 ± 0.001*	0.036 ± 0.002	0.016 ± 0.007*
ACE	0.039 ± 0.017	0.053 ± 0.004*	0.038 ± 0.004	0.066 ± 0.005*	0.021 ± 0.001*	0.035 ± 0.009	0.027 ± 0.005

*Indicates $P < .05$ from baseline (15 dynes/cm²) in preconditioned cells (n = 3). Static culture values are given for comparison only.

Table III, online only. Fold changes in gene expression ± standard deviation

Gene	*15 dynes/cm² (Δ = 0) versus static culture*	*0 dynes/cm² (Δ = −15) versus static culture*	*25 dynes/cm² (Δ = +10) versus 15 dynes/cm² (baseline)*	*–2.5 dynes/cm² (retrograde) versus 2.5 dynes/cm² (antegrade)*	*–2.5 dynes/cm² (retrograde) versus 15 dynes/cm² (baseline)*
Endothelin-1	0.47 ± 0.05*	0.40 ± 0.03*	2.8 ± 0.3*	4.1 ± 0.5*	2.4 ± 0.2*
NOS3	4.1 ± 1.4*	3.3 ± 0.8*	0.38 ± 0.09*	0.60 ± 0.17*	0.27 ± 0.08*
PDGFA	1.7 ± 1.0	1.2 ± 0.7	1.5 ± 0.4*	1.5 ± 0.2*	0.9 ± 0.2
PDGFB	0.70 ± 0.04*	0.65 ± 0.07*	2.3 ± 0.4*	2.3 ± 0.3*	1.53 ± 0.10*
FGF1	1.7 ± 0.9	1.99 ± 1.15	1.01 ± 0.11	1.0 ± 0.4	0.64 ± 0.18*
FGF2	3.90 ± 1.16*	4.26 ± 1.19*	0.60 ± 0.07*	0.9 ± 0.3	0.44 ± 0.08*
TGFA	1.5 ± 0.9	1.94 ± 1.10	0.63 ± 0.17*	1.08 ± 0.17	0.56 ± 0.12*
TGFB1	1.0 ± 0.5	0.9 ± 0.4	1.5 ± 0.2*	1.5 ± 0.2*	0.85 ± 0.09*
VEGF	3.39 ± 1.04*	5.7 ± 1.7*	0.7 ± 0.2*	0.8 ± 0.2	0.46 ± 0.13*
IGF1	2.8 ± 0.7*	3.1 ± 0.8*	0.63 ± 0.09*	1.0 ± 0.3	0.47 ± 0.13*
EGF	1.4 ± 1.2	1.39 ± 1.14	1.1 ± 0.4	0.7 ± 0.3	0.4 ± 0.2
ACE	1.0 ± 0.4	0.9 ± 0.4	1.38 ± 0.18*	1.3 ± 0.2	0.71 ± 0.15*

*Indicates $P < .05$ difference in mean expression levels (n = 3).