TNF-α is a mitogen in skeletal muscle

Yi-Ping Li

Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

Submitted 30 September 2002; accepted in final form 16 April 2003

Li, Yi-Ping. TNF-α is a mitogen in skeletal muscle. Am J Physiol Cell Physiol 285: C370–C376, 2003. First published April 23, 2003; 10.1152/ajpcell.00453.2002.—Emerging evidence suggests that tumor necrosis factor (TNF)-α plays a role in muscle repair. To determine whether TNF-α modulates satellite cell proliferation, the current study evaluated TNF-α effects on DNA synthesis in primary myoblasts and on satellite cell activation in adult mouse muscle. Exposure to recombinant TNF-α increased total DNA content in rat primary myoblasts dose-dependently over a 24-h period and increased the number of primary myoblasts incorporating 5-bromo-2′-deoxyuridine (BrdU) during a 30-min pulse labeling. Systemic injection of TNF-α stimulated BrdU incorporation by satellite cells in muscles of adult mice, whereas no BrdU was incorporated by satellite cells in control mice. TNF-α stimulated serum response factor (SRF) binding to the serum response element (SRE) present in the c-fos gene promoter and stimulated reporter gene expression controlled by the same element. Our data suggest that TNF-α activates satellite cells to enter the cell cycle and accelerates G1-to-S phase transition, and these actions may involve activation of early response genes via SRF.

Cytokine; cell cycle; satellite cells; serum response factor; c-fos

SKELETAL MUSCLE REGENERATION is essential for muscle adaptation. Despite considerable efforts to understand the complex mechanism that controls muscle regeneration, the complete profile of intrinsic and extrinsic cues that regulate muscle regeneration remains unclear (18, 20). Inflammation is a key response to muscle injury and is essential for muscle regeneration (49). Soon after injury, inflammatory cells infiltrate muscle and release inflammatory mediators. The mediators released by infiltrating macrophages are capable of stimulating myoblast proliferation and differentiation (4, 5). Macrophage-released cytokines including leukemia inhibitory factor, IL-6, and IL-15 have emerged as part of the muscle repair mechanism (20, 53). In addition, it is increasingly clear that tumor necrosis factor (TNF)-α, a major proinflammatory cytokine produced by activated macrophages, has an important role in muscle repair (28, 54).

In addition to macrophages, myocytes are also a source of TNF-α. Myocytes constitutively synthesize TNF-α (38). During muscle injury, TNF-α is not only released in large quantity by infiltrating macrophages but also synthesized at increased levels by injured muscle fibers. Strong expression of TNF-α by injured muscle fibers is detected in human muscles biopsied from patients of inflammatory myopathies or Duchenne muscular dystrophy (10, 48) and in the injured muscles of experimental animals during the course of regeneration (8, 54, 56). The level of TNF-α expression in injured muscle fibers is not correlated to the grade of inflammation (48), suggesting that upregulation of TNF-α expression in injured muscle fibers is not a simple response to inflammation. Similar responses may be evoked in the absence of overt pathology. In healthy humans, acute increases in circulating TNF-α levels occur after strenuous exercise (35) and possibly originate from skeletal muscle (45). Clearly, muscle regeneration proceeds in a high-TNF-α environment, which makes TNF-α a relevant factor in muscle regeneration.

TNF-α has pleiotropic functions (50). In addition to mediating inflammatory, cytotoxic/apoptotic, and muscle protein catabolic responses, TNF-α modulates growth and differentiation in various cell types. TNF-α and its receptors are detected in embryos and neonatal animals (16, 24, 34, 55). Repeated injections of neutralizing antibodies to TNF-α into pregnant mice resulted in growth retardation of the fetus (11). The seemingly normal development of TNF-α or TNF-α receptor knockout mice does not refute this observation, because the compensatory increase of other cytokines such as IL-1, IL-12 and IFN-γ can largely replace the role of TNF-α (12, 21, 46). Nevertheless, closer examinations of TNF-α signaling-impaired mice revealed that TNF-α is important for muscle development and regeneration. Mice deficient in TNF-α receptor-associated factor 2, an important signaling molecule for TNF-α receptor activation (22, 43), are born with a systematically smaller muscle mass (30). TNF-α gene knockout in dystrophin-deficient mice (TNF−/−mdx) resulted in a significantly lower muscle mass than TNF−+/−mdx mice (44) after a period of active muscle regeneration (26, 31). TNF-α receptor double knockout impairs muscle strength recovery after freeze-induced injury in adult mice (54). These observations suggest a physiological role for TNF-α in muscle development and regeneration.

During muscle regeneration, normally quiescent satellite cells are activated to enter the cell cycle and proliferate. A subset of the daughter satellite cells then...
differentiates and becomes part of the repaired or enhanced muscle fibers. Certain growth factors or cytokines can modulate satellite cell proliferation by activating satellite cells to enter the cell cycle (competence factor) or enhancing satellite cell proliferation once it has been initiated (progression factor) (7, 47). Previous studies evaluated TNF-α effects on myocyte proliferation in vitro using myogenic cell lines. However, the results were inconsistent with both a stimulatory effect reported in the C2C12 myoblast cell line (25) and an inhibitory effect reported in the L8 myoblast cell line (23). More importantly, there are no data on whether TNF-α affects satellite cell activation. The present study was designed to determine whether TNF-α affects DNA synthesis and cell cycle progression in primary myoblasts and whether TNF-α stimulates satellite cell activation in adult mice. Here we show evidence that TNF-α is a mitogen in skeletal muscle and that TNF-α stimulates expression of the early response gene c-fos via activating serum response factor (SRF).

METHODS

Myogenic cell culture. Limb skeletal muscles were excised from neonatal rats (2–4 days old), minced with razor blades in a minimal volume of PBS, and enzymatically dissociated in dissociation buffer (0.1% trypsin, 0.1% collagenase type 2, and 0.025% DNase in PBS) at 37°C for 5 min. The slurry was centrifuged in 20 ml of DF20 (20% fetal bovine serum in DMEM) at 150 g for 1 min at 4°C, and the supernatant was discarded. The dissociation process was repeated three times. Each time, the pelleted cells were incubated in dissociation buffer for 15 min and the supernatant was collected by centrifuging at 300 g for 7 min at 4°C. The dissociated cells were pelleted and resuspended in 1.082 g/ml Percoll (Pharmacia, Piscataway, NJ) for purification through a density gradient (1.050, 1.060, and 1.082 g/ml) by centrifugation at 2,000 g for 25 min at room temperature. The Percoll gradient was made in a buffer containing 6.8 g/l NaCl, 0.4 g/l KCl, 0.1 g/l MgSO4, 1.5 g/l NaH2PO4, 1.0 g/l dextrose, and 4.76 g/l HEPES (pH 7.3). The band containing myocytes at the interface between 1.060 and 1.082 g/ml Percoll layers was collected and washed twice in the gradient buffer plus 0.02 g/l phenol red. Cells were resuspended in F-10/DMEM supplemented with 20% fetal bovine serum and preplated in a noncoated dish for 1 h to remove contaminating fibroblasts. The unattached cells were resuspended in Ham’s F-10 nutrient mixture (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum and then plated in Primaria culture dishes and grown at 37°C in the presence of 5% CO2. The purity of the myoblast culture was determined to be 90.3 ± 2.8% by immunoperoxidase labeling with the D3 desmin antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). C2C12 myoblasts were cultured in DMEM supplemented with 20% newborn bovine serum at 37°C in the presence of 5% CO2.

DNA determination. Total DNA was isolated from primary myoblasts by using the DNAzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. DNA concentration was determined by measuring the fluorescence emission of DNA in a Hoechst dye solution containing 0.1 μg/ml Hoechst 33258, 0.2 M NaCl, 10 mM Tris-Cl, and 1 mM EDTA (pH 7.4) using the DyNA Quant 200 fluorometer (Pharmacia).

Flow cytometry analysis of cell cycle in myoblasts. Murine recombinant TNF-α (Roche, Indianapolis, IN) or vehicle was incubated with proliferating rat primary myoblasts (~50% confluent) for 16 h, followed by a 30-min pulse labeling with 10 μM BrdU. Myoblasts were harvested by brief trypsinization and fixed in cold 70% ethanol overnight. Cells were pelleted, resuspended, and incubated in 0.1 M HCl containing 0.5% Triton X-100 on ice for 10 min. Cells were pelleted, resuspended in distilled water, and boiled for 5 min and quickly chilled on ice. After being washed in PBS containing 0.5% Triton X-100, cells were incubated with 5 μg/ml anti-BrdU-FITC (Roche) in PBS containing 0.1% BSA in the dark at room temperature for 30 min. Cells were then stained with 5 μg/ml propidium iodide containing 200 μg/ml RNase and analyzed with an EPICS XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL).

Animal use. Experimental protocols were approved in advance by the Animal Protocol Review Committee of the Baylor College of Medicine. Six- to eight-week-old adult male mice (ICR) were injected intraperitoneally with 50 μg/kg murine recombinant TNF-α (BD Biosciences) and 16 h later Bio-Rad mg-kg BrdU kit. Mice were deeply anesthetized by intraperitoneal injection of 85 mg/kg pentobarbital sodium. Soleus and diaphragm muscles were excised for immunofluorescence staining, and the anesthetized animals were killed by rapid exsanguinations.

Immunofluorescence staining. Frozen muscle sections (5 μm) were prepared and submerged in 3.7% formaldehyde for 15 min. After three 10-min washes in PBS, an incubation in 2 M HCl was carried out for 20 min at 37°C and again washed three times. Muscle sections were blocked in a blocking buffer (5% chicken serum in PBS) for 1 h at room temperature. Incubation with primary antibodies, anti-BrdU-FITC (Roche) and anti-laminin (Sigma, St. Louis, MO), was done for 1 h at 37°C with 1:20 and 1:25 dilution, respectively. After three washes, incubation with a rhodamine-conjugated anti-rabbit second antibody was carried out for 1 h at 37°C. The sections were washed again three times and incubated with 0.1% 4,6-diamidino-2-phenylindole (DAPI) at room temperature for 1–3 min. After a final three washes, the sections were mounted. A Zeiss Axiosphot fluorescence microscope coupled to a digital camera utilizing Adobe Photoshop software was used to acquire the images.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was carried out in a binding assay buffer containing 5 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.3 mM dithiothreitol, 5 mM MgCl2, 10% glycerol, 2 μg/ml of BSA, 0.2% NP-40, and 1 μg of poly(dG-dC)-poly(dG-dC). A DNA probe replicating the serum response element (SRE) present in the c-fos gene and the flanking sequence (5′-ACAGG-ATGTCCTATTAGGACATCTGCCG-3′) was labeled with [α-32P]dATP (3,000 Ci/mmole; Amersham Pharmacia, Arlington Heights, IL) using the Klenow fragment. After 20-min preincubation of 5 μg of nuclear extract prepared according to Andrews and Faller (1) in the assay buffer, 1 ng (10,000–15,000 cpm) of labeled probe was added and incubation was continued for 30 min on ice. For supershift assay, a specific SRF (Santa Cruz Biotechnology) or a control antibody was added for an additional 20-min incubation. The reaction mixtures were resolved on 4.5% polyacrylamide gels. Protein concentration of the nuclear extracts was determined by the Bradford protein assay.

Reporter gene assay. C2C12 myoblasts were transfected with either a plasmid construct containing the minimal promoter of the c-fos gene without the SRE (c-fosΔ56) or a construct containing the c-fos promoter including the SRE (c-fos-SRE) by using the Lipofectamine reagent (Invitrogen).
These constructs were gifts from Dr. Robert J. Schwartz (Baylor College of Medicine). At 20 h posttransfection, myoblasts were incubated with TNF-α and then lysed at 3, 6, and 9 h for luciferase assay. Light intensity was measured for 10 s in a Turner Designs TD-20/20 Luminometer and expressed in arbitrary relative light units (RLU). Experiments were carried out in duplicate. RLU were normalized to total protein content determined by the Bio-Rad protein assay kit.

RESULTS

In the present study, we chose to use rat primary myoblasts to evaluate TNF-α effect on DNA synthesis and cell cycle progression. Primary myoblasts were prepared from neonatal rats by using a long-established protocol in our laboratory (29). To examine TNF-α effect on DNA synthesis, proliferating rat primary myoblasts were incubated with murine recombinant TNF-α for 24 h, and total DNA content in lysed myoblasts was determined. TNF-α exhibited a dose-dependent stimulatory effect on total DNA content (Fig. 1). From 2 to 6 ng/ml, TNF-α progressively increased DNA content. Further increase of TNF-α to 20 ng/ml reversed the stimulatory effect, which is likely due to cell death caused by the cytotoxicity of TNF-α at this very high dose (39). These results suggest that TNF-α at a concentration range from 2 to 6 ng/ml has a stimulatory effect on DNA synthesis.

To verify this finding, we incubated proliferating primary myoblasts with 6 ng/ml TNF-α for 16 h and subjected them to a 30-min pulse labeling with 10 μM BrdU. Myoblasts were then stained with anti-BrdU-FITC and propidium iodide. The stained myoblasts were analyzed by flow cytometry. TNF-α increased the number of myoblasts incorporating BrdU, which is represented by cells in quadrant 2 of Fig. 2A, from ~21% of total cells in control to 35% (n = 3, Fig. 2B). These results suggest that TNF-α stimulates DNA synthesis by accelerating cell cycle progression from the G1 phase to S phase. This increase is comparable to the increase of total DNA content illustrated in Fig. 1.

To determine whether TNF-α is capable of activating quiescent satellite cells to enter the cell cycle, we evaluated DNA synthesis in satellite cells by monitoring BrdU incorporation in vivo. Murine recombinant TNF-α (50 μg/kg) was administered via intraperitoneal injection to 6-wk-old adult mice and the mice were pulse labeled 16 h later with intraperitoneally injected 50 mg/kg BrdU for 2 h. By modifying the methods published by Mozdziak et al. (32) and Cornelison and Wold (9), immunofluorescence staining with a specific anti-BrdU antibody was performed to detect proliferating satellite cells in frozen muscle sections. To determine whether an anti-BrdU-stained cell is satellite cell, we took advantage of the characteristic localization of satellite cells, which is underneath or embedded in the basal lamina of myofibers (3), by costaining

![Fig. 1. TNF-α increases total DNA content in rat primary myoblasts. Recombinant mouse TNF-α or vehicle was incubated with proliferating myoblasts (~50% confluent) for 24 h. DNA was extracted using DNAzol reagent (Invitrogen). DNA concentration was determined using a DyNA Quant 200 fluorometer (Pharmacia). Experiments were carried out in duplicate or triplicate. Values are means ± SE. Data analysis with ANOVA indicates a difference with increasing TNF-α (P < 0.001).](http://ajpcell.physiology.org/10.1152/ajpcell.00151.2003/)

![Fig. 2. TNF-α stimulates cell cycle progression from the G1 to S phase. TNF-α (6 ng/ml) or vehicle was incubated with proliferating rat primary myoblasts (~50% confluent). After 16 h of incubation, 10 μM BrdU was added for a 30-min pulse labeling. Harvested cells were stained with anti-BrdU-FITC and propidium iodide and then analyzed using an EPICS XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL). A: histograms generated by flow cytometer that identify cells in the S phase of the cell cycle (quadrant 2) on the basis of anti-BrdU staining. B: summary of TNF-α effect on BrdU incorporation by primary myoblasts. Values are means ± SE from 3 separate experiments. Data were analyzed using paired t-test (*P < 0.05).](http://ajpcell.physiology.org/10.1152/ajpcell.00151.2003/)
muscle sections with anti-laminin to outline the basal lamina. In addition, costaining with DAPI verifies that the anti-BrdU staining belongs to nuclei of single cells, which rules out the possibility that these cells are vascular endothelial cells. Figure 3 shows an example of a BrdU-incorporated satellite cell. We observed no satellite cell that incorporated BrdU in nine soleus muscle sections from three control mice, consistent with the notion that adult satellite cells are quiescent. However, an average of 6 ± 1.3 BrdU-stained satellite cells per section were observed in nine soleus sections from three TNF-α-injected mice. Similar results were observed in diaphragm sections (data not shown). The morphological integrity was monitored by hematoxylin and eosin staining of the muscle sections, and no abnormality suggesting muscle injury was seen.

SRF is known to stimulate c-fos gene expression by binding to the SRE motif present in the c-fos gene promoter, and c-fos is an important early response gene involved in cell growth regulation (36). We therefore evaluated TNF-α effect on the binding activity of SRF to the SRE in the c-fos gene promoter by using EMSA. We took advantage of C2C12 myoblasts that respond to TNF-α with increased proliferation (25) and tolerate standard transfection procedures better than primary myoblasts. TNF-α rapidly stimulated binding activity in nuclear extracts of C2C12 myoblasts. SRF binding to a DNA fragment that replicates the c-fos SRE was increased within 15 min of TNF-α exposure and returned to control level at 60 min (Fig. 4). To determine whether enhanced SRF binding results in a stimulation of c-fos gene expression, TNF-α effect on c-fos SRE-controlled luciferase reporter gene expression in C2C12 myoblasts was studied by transient transfection. We observed a stimulation of c-fos SRE-controlled luciferase activity by TNF-α, most notably at 6 h of TNF-α exposure (Fig. 5), whereas the minimal c-fos promoter without SRE did not respond to TNF-α. These results suggest that TNF-α stimulation of satellite cell proliferation may involve activation of c-fos gene expression.

DISCUSSION

Previous studies using myoblast cell lines resulted in contradictory results on TNF-α effect on myoblast proliferation (23, 25). The present study used primary myoblasts that replicate the properties of satellite cells more closely than do myoblast cell lines (2). The data demonstrate that TNF-α stimulates DNA synthesis and that this effect is achieved via accelerating G1-to-S phase transition. These results clearly support a stimulatory effect of TNF-α on proliferation as observed in C2C12 myoblasts by Langen et al. (25). More importantly, our animal studies demonstrate for the first time that TNF-α is capable of activating satellite cells to enter the cell cycle from the normally quiescent state. Thus TNF-α appears to be both a progression factor (enhancing satellite cell proliferation once it has been initiated) and a competence factor (activating satellite cells to enter the cell cycle). These results suggest that TNF-α generated from infiltrating macrophages and injured muscle fibers may exert a mitogenic effect on satellite cells and contribute to muscle regeneration.

Fig. 3. TNF-α activates adult mouse muscle satellite cells in vivo. Male adult mice (~25 g) were injected with TNF-α (50 μg/kg ip) or vehicle as a control. Sixteen hours later, both groups were injected with BrdU (50 mg/kg ip) for a 2-h pulse labeling, and then muscle samples were surgically removed under anesthesia. Frozen cross sections (5 μm) were prepared and double-stained with monoclonal anti-BrdU-FITC and polyclonal anti-laminin. Anti-BrdU-FITC was visualized by its green color under a fluorescence microscope, and anti-laminin was visualized through the attachment of rhodamine-conjugated second antibody (red). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). A Zeiss Axioshot fluorescence microscope coupled to a digital camera utilizing Adobe Photoshop version 5.0 software was used to acquire the images. The merged image shows a typical anti-BrdU-stained satellite cell (arrow) present in soleus muscle sections prepared from mice injected with TNF-α. The BrdU-incorporated satellite cell is embedded in the basal lamina of myofibers.
cell activation is a response to muscle injury. Although TNF-α level in injured muscle is unknown, the acute surge of TNF-α expression after injury could create a local TNF-α concentration high enough to activate satellite cells. After acute muscle injury, TNF-α expression increases within 5 h and reaches the peak level around 24 h before returning to the basal level gradually (54); the onset of peak TNF-α level parallels the time when satellite cells are activated (15), supporting a causal relationship between the two events.

We observed 6 ± 1.3 activated satellite cells in soleus sections from TNF-α injected mice. To put this number in perspective, we estimated the number of total satellite cells possibly present in a section. The adult soleus contains about 5,000 satellite cells/mm³ (40). The cross-sectional area of our soleus samples averaged 0.65 mm². Each 5-μm transverse section gives an average volume of 0.0033 mm³. Thus each section averaged 16.5 satellite cells (5,000 × 0.0033), suggesting that a substantial number of satellite cells were activated by TNF-α.

TNF-α stimulation of myoblast proliferation appears to involve multiple signaling pathways. Guttridge et al. (19) demonstrated previously that NF-κB stimulates cell cycle progression from the G1 to S phase by regulating the expression of the cyclin D1 gene. TNF-α activates NF-κB in skeletal muscle myoblasts (28, 41), and TNF-α stimulation of cell cycle progression is likely to involve mediation by NF-κB. We have further demonstrated that the mitogenic effect of TNF-α may also involve signaling through SRF. SRF, a transcription factor, activates gene transcription by binding as a homodimer to a DNA motif known as the SRE (or

![Image](http://ajpcell.physiology.org/) Downloaded from

Fig. 4. TNF-α stimulates serum response factor (SRF) binding to the c-fos serum response element (SRE). C2C12 myoblasts were incubated with TNF-α and harvested at indicated times (A). Nuclear extracts (NE) were prepared, and nuclear protein binding to a 32P-labeled DNA probe replicating the SRE present in the c-fos gene and the flanking sequence (5′-ACAGGATGTCATATTAGGACATCTGCG-3′) was analyzed by EMSA. The specificity of the protein-DNA interaction was verified by successful competition with unlabeled probe in 100-fold excess (data not shown) and by supershift assay using anti-SRF (B). C: optical density of the DNA-protein complex on autoradiograph was quantified using densitometry software (Image-Quant 5.2, Molecular Dynamics).

We have demonstrated that TNF-α stimulates DNA synthesis at a concentration range from 2 to 6 ng/ml, which was shown previously to evoke signaling events mediated by TNF-α receptor activation in cultured myocytes without inducing necrotic or apoptotic death (27, 29). The fall of the stimulatory effect of 20 ng/ml TNF-α on DNA content is consistent with the reported cytotoxicity of TNF-α on myocytes at high concentrations (39). These observations reiterate the concentration dependence of TNF-α actions.

The TNF-α dose (50 μg/kg ip) we used to evoke satellite cell activation is lower than those used to induce muscle protein loss or apoptosis, which require repeated doses of 100–500 μg/kg via the same route (6, 13, 14). Histological study did not see signs of muscle damage, so we can rule out the possibility that satellite

Fig. 5. TNF-α stimulates luciferase reporter gene activity directed by the c-fos SRE. C2C12 myoblasts were transiently transfected with either a plasmid construct containing the minimal promoter of the c-fos gene without the SRE (c-fos56) or a construct containing the c-fos promoter including the SRE (c-fos-SRE). At 24 h posttransfection, myoblasts were treated with 6 ng/ml TNF-α. At indicated times, myoblasts were lysed and assayed for luciferase activity. Data are expressed as arbitrary relative light units (RLU) normalized to protein. Values are means ± SE. Data were analyzed using paired t-test (*P < 0.05) from the sample within the time group that was transfected with the same plasmid without exposure to TNF-α.
CARG box) (33). SRE mediates increased expression of immediate-early genes, including c-fos, in cells treated with growth factors or cytokines (51). Our observations that TNF-α stimulates SRF binding to the SRE of the c-fos gene promoter and c-fos SRE-directed luciferase reporter gene expression suggest that the mitogenic effect of TNF-α is likely to involve activation of specific immediate-early genes. Our experiments were carried out in a growth medium supplied with 20% newborn bovine serum, SRF responds to serum growth factors (51). The basal activity of SRF seen in our transfection experiments may reflect the presence of serum growth factors. Growth factors regulate SRF activity via cofactors that interact with SRF (52) without changing its DNA-binding activity (17, 42, 51). The ability of TNF-α to enhance SRF binding to SRE is unique compared with growth factors.

The mitogenic effect of TNF-α described in the present study may be an integrated part of a comprehensive tissue remodeling effect of TNF-α in skeletal muscle, in addition to the previously reported TNF-α stimulation of muscle catabolism (37) and differentiation (28). The catabolic effect is likely to promote the degradation of necrotic tissue, whereas the proliferative and differentiation-stimulatory effects promote regeneration. Such a scenario suggests that TNF-α has a physiological role in the maintenance of skeletal muscle and that caution needs to be exercised in using anti-TNF-α strategies to control inflammatory and other conditions.

I thank James Agan, Fei Han, and Eric Gerken for technical support and Michael B. Reid for helpful discussion.

DISCLOSURES

This work was supported by grants from the National Heart, Lung, and Blood Institute (HL-59878), American Heart Association-Texas, and American Lung Association-Texas.

REFERENCES


