Tumor Necrosis Factor-α Induces Fibronectin Synthesis in Coronary Artery Smooth Muscle Cells by a Nitric Oxide–Dependent Posttranscriptional Mechanism

Catherine A.E. O’Blenes, Caroline Kinnear, Marlene Rabinovitch

Abstract—Postcardiac transplant coronary arteriopathy is associated with tumor necrosis factor-α (TNF-α) induction of fibronectin-dependent smooth muscle cell (SMC) migration into the subendothelium, resulting in occlusive neointimal formation. Because expression of inducible nitric oxide synthase (iNOS) is elevated in neointimal formation after transplantation and upregulated in vascular SMCs by TNF-α, we investigated whether TNF-α induction of fibronectin synthesis in coronary artery (CA) SMCs is mediated by nitric oxide (NO). TNF-α caused a dose-dependent increase in reactive oxygen and nitrogen intermediates in CA SMCs (P<0.05). This correlated with increased NO production (P<0.05) and fibronectin synthesis (P<0.05). TNF-α induction of fibronectin synthesis was abrogated by the NOS inhibitor Nω-monomethyl-l-arginine (L-NMMA) (P<0.05) or the flavonoid-containing enzyme inhibitor diphenyleneiodonium (DPI) (P<0.05) and reproduced with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) (P<0.05). Northern blotting showed no effect of TNF-α on steady-state fibronectin mRNA levels. TNF-α increased expression of light chain 3 (LC-3), a protein shown previously to facilitate fibronectin mRNA translation through its interaction with an adenosine-uracil rich element (ARE) in the 3′-untranslated region of fibronectin mRNA. RNA gel mobility shift and UV cross-linking assays using CA SMC lysates revealed protein binding complexes with radiolabeled oligonucleotide containing the ARE, similar to those generated with recombinant LC-3. One of these complexes increased after TNF-α treatment, an effect inhibited with L-NMMA or DPI. These data demonstrate a novel paradigm whereby cytokines regulate mRNA translation of extracellular matrix proteins through NO-dependent modulation of RNA binding protein interaction with mRNA. (Circ Res. 2001;89:26-32.)

Key Words: nitric oxide ■ fibronectin ■ translation ■ atherosclerosis ■ cardiac transplant

Transplant coronary artery disease, a major complication of cardiac transplantation, is characterized by the development of occlusive neointimal lesions formed by smooth muscle cell (SMC) migration and proliferation and extracellular matrix deposition in the subendothelium. It appears to be initiated by an immune-inflammatory response involving accumulation of activated T cells, expression of the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and upregulation of SMC fibronectin associated with SMC migration into the subendothelium. In cultured porcine coronary artery (CA) SMCs, TNF-α and IL-1β reciprocally coinduce synthesis of soluble fibronectin. TNF-α induction of fibronectin has also been demonstrated in a rabbit heterotopic cardiac transplant model, in which blockade of this effect with soluble TNF-α receptor reduced the number and severity of allograft coronary artery lesions and was associated with a decrease in subendothelial fibronectin expression and in inflammatory cell infiltrate.

Furthermore, in coculture experiments, soluble fibronectin produced by CA SMCs set up a gradient, stimulating transendothelial lymphocyte migration.

The critical importance of fibronectin in SMC migration was demonstrated in the fetal ductus arteriosus (DA), a vessel in which neointimal formation begins as a normal developmental process at 100 days’ gestation in the fetal lamb (term 145 days) to form intimal cushions that facilitate closure of this vessel at birth. In 100-day-gestation DA SMCs, increased synthesis of soluble fibronectin was demonstrated relative to SMCs from the aorta and related to the enhanced migratory phenotype of the DA cells in 3-D collagen gels. Interestingly there was no difference in matrix-associated fibronectin synthesis between the neointima-forming DA SMCs and aortic SMCs. Increased synthesis of soluble fibronectin in 100-day-gestation sheep DA SMCs is regulated at a posttranscriptional level by the binding of light chain 3 (LC-3), a component of
microtubule-associated protein complexes 1A and 1B, to an adenosine-uracil rich element (ARE) in the 3'-untranslated region (3'-UTR) of fibronectin mRNA. LC-3 facilitates fibronectin mRNA recruitment to membrane-bound polyribosomes. By sequestering LC-3 with a decoy RNA containing the fibronectin 3'-UTR ARE, fibronectin synthesis was inhibited and the migratory phenotype was not observed. Subsequent gene transfer experiments in fetal lambs using decoy RNA confirmed that fibronectin production and SMC migration were reduced, and neointimal formation was prevented.

Although nitric oxide (NO) exhibits antiatherogenic properties by its inhibitory effects on platelet adhesion and activation as well as vascular SMC proliferation, there is increasing evidence that NO can also play a role in the development of neointimal lesions in inflammatory states in which superoxide levels are elevated. NO readily combines with superoxide (O') to form peroxynitrite anion (ONOO\(^{-}\)), which has been shown to cause LDL and VLDL oxidation leading to lipid accumulation in macrophages of the vessel wall, as well as macrophage apoptosis.

Because inducible NOS (iNOS) is expressed in SMCs during neointimal formation, it has been proposed that NO may regulate acquisition of the migratory SMC phenotype. Expression of NO is elevated in atherosclerotic lesions and as well as in the coronary arteries of human and animal models cardiac allografts. Although iNOS was originally identified in cytokine-activated macrophages, several reports have demonstrated that vascular SMCs in culture also produce iNOS in response to TNF-\(\alpha\) and IL-1\(\beta\). NO-dependent TNF-\(\alpha\) induction of fibronectin expression was demonstrated in a human umbilical vein endothelial cell line. Elevated NO levels in DA SMCs, associated with increased SMC expression of the constitutive nitric oxide synthase (NOS) isoforms endothelial NOS (eNOS) and neuronal NOS (nNOS), both in cultured cells and in sheep, regulate increased fibronectin synthesis through enhanced expression and binding of LC-3 to the 3'-UTR ARE of fibronectin mRNA. Thus, in this developmental paradigm of neointimal formation, NO appears to play a central role in regulating fibronectin-dependent SMC migration into the neointima.

In the present study, we examine whether induction of fibronectin synthesis in primary culture CA SMCs by the cytokine TNF-\(\alpha\) is similarly regulated by NO. We show that TNF-\(\alpha\) induction of fibronectin synthesis requires increased NO production that modulates expression of LC-3 and binding of a protein with properties consistent with LC-3 to the ARE in the 3'-UTR of fibronectin mRNA, a mechanism shown previously to facilitate fibronectin mRNA translation. These are the first experimental data demonstrating that modulation of intracellular NO levels by cytokines can orchestrate specific posttranscriptional mechanisms regulating extracellular matrix expression.

Materials and Methods

Cell Culture

Porcine CA SMCs were isolated for primary cultures by explant technique as previously described and were used at passage 2.

Experimental Conditions

Semiconfluent cultures of CA SMCs were preincubated for 1 hour in serum-free methionine/cysteine-free DMEM containing 1% BSA. To assess the role of reactive oxygen or nitrogen intermediates (ROI/RNI) and specifically NO in TNF-\(\alpha\) induction of fibronectin synthesis, diphenyletheneonidium (DPI, Sigma Chemical Co) (2 \(\mu\)mol/L) or \(N^{6}\)-monomethyl-L-arginine (L-NMMA, Sigma) (250 nmol/L) was added during the 1-hour preincubation. Culture medium was then replaced and recombinant human TNF-\(\alpha\) (R&D Systems) (10, 30, and 100 ng/mL) was added alone or in combination with DPI or L-NMMA for 4 hours. To assess the effect of NO, S-nitroso-N-acetyl-penicillamine (SNAP) (BIOMOL Research Laboratories) was added to the cells for 4 hours.

Fibronectin Synthesis

During the 4-hour incubation described above, 10 \(\mu\)Ci/mL of \([^{35}S]\)-methionine (Amersham) was added. Total protein synthesis was assessed for each culture dish in triplicate by trichloroacetic acid precipitation and liquid scintillation spectrometry. To measure fibronectin synthesis, 1-mL aliquots of culture medium, cell lysates, or extracted extracellular matrix were incubated with 100 \(\mu\)L gelatin 4B-sepharose beads overnight at 4°C. The beads were washed in PBS, and bound fibronectin was eluted into 100-\(\mu\)L SDS-PAGE sample buffer by boiling for 5 minutes. The samples were standardized to total protein synthesis and separated on 6% SDS polyacrylamide gels. Identification of the 220-kDa band on the autoradiograph as fibronectin was previously confirmed by immunoblotting.

FACS Analysis of ROI and RNI

To assess total ROI and RNI levels, dihydrothreodamine 123 (DHR) (2 \(\mu\)mol/L) was added to the cells during the 4-hour incubation period. DHR is converted in the presence of ROI/RNI to the fluorescent dye rhodamine 123 (500- to 540-nm emission spectrum). After DHR loading, all manipulations of the cells were carried out in the dark. CA SMCs were trypsinized (0.5% trypsin, 5 mmol/L EDTA -4Na) (Gibco BRL), washed in PBS, and fixed in 1.5% paraformaldehyde/PBS. Fluorescence intensity was determined for 5000 cells from each sample by fluorescence-activated cell sorting (FACS) analysis using an argon laser with the excitation source emitting at 488 nm as previously described.

Nitrate/Nitrite Analysis

A nitrate/nitrite fluorometric assay kit (Cayman Chemical) was used to measure levels of the stable end products of NO, nitrate and nitrite, in culture medium as described. Cell-free culture medium was harvested from cells treated with the designated conditions, and cell number was counted in triplicate for each condition using a hemocytometer. Culture medium samples were incubated with nitrate reductase and cofactors (Cayman Chemical) for 30 minutes at room temperature to convert nitrate to nitrite. The samples were then incubated with 2,3-diaminonaphthalene (DAN), which reacts with nitrite to form the fluorescent compound (1H)-naphthotriazole. Fluorescence intensity was measured using a fluorometer (excitation and emission wavelengths of 365 and 450 nm).

Northern Blotting

Cells were lysed in Trizol (Life Technologies) (2 mL/100-mm dish extracted with chloroform and total RNA precipitated according to the manufacturer’s directions). RNA samples were separated in 1% agarose gels containing 6% formaldehyde transferred to nitrocellulose membranes and probed with \(^{32}\)P]-dCTP (10\(^{6}\) cpm/mL) (Amer sham) labeled human fibronectin cDNA (1.4 kb) (Gibco BRL) and a control GAPDH cDNA probe (1.2 kb) (ATCC).

Cell Extracts

Semiconfluent cultures of CA SMCs were harvested, and the cells were resuspended in 2 volumes of hypotonic buffer (0.1 mmol/L EDTA, 25 mmol/L Tris-HCl [pH 7.9]) and lysed by 3 cycles of freeze-thaw. Cytosolic proteins were extracted by pelleting the lysed cells for 1 hour at 16 000g. Pellets were either dissolved in Laemmli
sample buffer for SDS-PAGE or subjected to 1 mol/L KCl extraction at 4°C to isolate membrane-associated proteins for RNA gel mobility shift assays. Protein concentrations were determined using a standard Bradford protein assay kit (Bio-Rad) and spectrophotometry at 595 nm.

**Immunoblotting**

Protein extracts (30 μg per lane) from CA SMCs were separated by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 hour in 5% nonfat dry milk/PBS containing 0.5% Tween-20 and then probed with rabbit antiserum to LC-3 (1:3000) (prepared by Dr J. Hammarback, Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Winston-Salem, NC). Blots were washed three times with PBS/0.5% Tween-20 followed by incubation with horse-radish peroxidase–conjugated goat anti-rabbit IgG (1:3000) (Bio-Rad). The blots were washed three times as above and developed using enhanced chemiluminescence (ECL) detection reagents (Amersham).

### RNA Gel Mobility Shift and UV Cross-Linking Assays

Cytosol and KCl extracts from lysed cell pellets were dialyzed against RNA binding buffer (5 mmol/L MgCl₂, 100 mmol/L KCl, 10% glycerol, and 15 mmol/L HEPES [pH 7.9]) in diethyl pyrocarbonate–treated water overnight at 4°C in a Pierce System 100 microdialyzer. For each assay, 15 μg of protein extract from CA SMCs was incubated with 10⁵ cpm of wild-type or mutant fibronectin ARE (underlined) containing RNA oligonucleotide probe (wild type: 5’-ACCUGUUAAUUUMGCAAUU-3’; mutant: 5’-ACCUGGGAGGGACCAAUU-3’; synthesized by Biotechnology Center, University of Calgary, Calgary, Alberta, Canada) in RNA binding buffer containing 2 μg Escherichia coli transfer RNA (Sigma) in a total volume of 20 μL for 30 minutes at 30°C. Samples were separated on 6% native polyacrylamide gels in 0.25×Tris-borate-EDTA (TBE) buffer (90 mmol/L Tris, 90 mmol/L boric acid, and 2 mmol/L EDTA). For competition and specificity analysis of protein-RNA binding excess (500:1), unlabeled RNA probe was incubated with rabbit antiserum to LC-3 (1:3000) (prepared by Dr J. Hammarback, Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Winston-Salem, NC). Blots were washed three times with PBS/0.5% Tween-20 followed by incubation with horse-radish peroxidase–conjugated goat anti-rabbit IgG (1:3000) (Bio-Rad). The blots were washed three times as above and developed using enhanced chemiluminescence (ECL) detection reagents (Amersham).

![Image](http://circres.ahajournals.org/)

**Figure 1.** TFN-α induction of ROI+RNI levels and parallel induction of fibronectin synthesis in CA SMCs. A, Mean fluorescence intensity after DHR loading as a measure of ROI and RNI levels after 4-hour treatment with TFN-α (10, 30, and 100 ng/mL). Bars represent mean±SEM of 3 separate experiments in which a total of 5000 cells were examined by FACS analysis. B, Representative autoradiograph (top) and histogram (bottom) illustrating quantitative analysis of affinity-purified metabolically labeled fibronectin (FN) after 4-hour treatment with TFN-α. Bars represent mean±SEM of fibronectin synthesis (cpm) relative to total protein synthesis from 3 separate experiments. *P<0.05 vs control (CON).

### Results

**NO Mediates TFN-α Induction of Fibronectin Synthesis**

We first examined whether TFN-α induction of fibronectin synthesis in CA SMCs is mediated by induction of ROI/RNI or NO specifically. In CA SMCs, treatment with TFN-α caused a dose-dependent increase in intracellular ROI/RNI levels (Figure 1A). Consistent with previous studies in DA SMCs that showed the importance of secreted rather than matrix-associated fibronectin in SMC migration, in that the majority of newly synthesized fibronectin within a 4-hour period was secreted (57%), with 37.5% intracellular, and only 5.5% deposited in the extracellular matrix. TFN-α caused a dose-dependent increase in secreted fibronectin synthesis in CA SMCs (Figure 1B).

Subsequent studies showed that DPI, an inhibitor of ROI and RNI producing flavonoid-containing enzymes such as NADPH oxidase and NO synthase, abrogated the effect of TFN-α on ROI/RNI induction and also caused a decrease in basal levels of ROI/RNI in CA SMCs (Figure 2A). TFN-α induction of fibronectin synthesis was also not observed in the presence of DPI (Figure 2B). However, treatment with DPI alone did not cause a decrease in fibronectin synthesis from basal levels (Figure 2B) suggesting that constitutive expression of fibronectin in cultured CA SMCs is not regulated by ROI/RNI.

Because TFN-α increases iNOS expression in vascular SMCs, we examined whether the TFN-α–induced increase in ROI/RNI levels was due to NO and whether TFN-α induction of fibronectin synthesis was mediated specifically by NO. We first demonstrated that the NO donor SNAP reproduced the effect of TFN-α on CA SMC ROI/RNI levels (Figure 2C) and on fibronectin synthesis (Figure 2D). Next we demonstrated that TFN-α induction of fibronectin synthesis could be abrogated by the specific NOS inhibitor L-NMMA, whereas treatment with L-NMMA alone had no significant effect on basal levels of fibronectin synthesis in CA SMCs (Figure 3). These results indicated that TFN-α induction of fibronectin synthesis is mediated by NOS production of NO.

To confirm that TFN-α was modulating NO in CA SMCs as proposed, NO levels were assessed by measuring the accumulation of its stable end products, nitrite and nitrate, in culture medium (Figure 4). TFN-α caused a 2-fold increase in nitrate/nitrite accumulation, which was abrogated by L-NMMA and partially inhibited by DPI (Figure 4).
NO-Mediated TNF-α Induction of Fibronectin Is Posttranscriptional

Fibronectin mRNA levels did not change appreciably with respect to GAPDH mRNA after treatment with TNF-α, TNF-α and L-NMMA together, or L-NMMA alone (data not shown). These data suggest that NO-mediated TNF-α induction of fibronectin synthesis may be regulated at a posttranscriptional level.

LC-3 Expression Is Increased in TNF-α–Treated CA SMCs

We have previously demonstrated NO-dependent posttranscriptional upregulation of fibronectin synthesis by LC-3–mediated recruitment of fibronectin mRNA to the polyribosomes in neointima-forming DA SMCs.13,14 Two forms of LC-3 of distinct molecular weights were identified in DA SMCs by immunoblotting. These proteins could be separated by isolating cytosolic fraction and cellular membrane fractions from freeze-thawed, lysed SMCs. Cytosolic LC-3 has a molecular mass of 16 kDa and codistributes with tubulin.42 However, a lower molecular weight form of LC-3 was identified in the membrane fraction and could be removed from DA SMC membranes by 1 mol/L KCl, suggesting that it is a membrane-associated protein, a characteristic of RNA binding proteins.50 This form exhibited increased binding to the ARE of fibronectin mRNA, suggesting that it is likely the form associated with fibronectin mRNA at the polyribosomes where translation occurs.42 We therefore examined LC-3 expression in cell membrane fractions from CA SMCs treated with TNF-α alone or in combination with L-NMMA or L-NMMA alone (Figure 5). Levels of membrane-associated LC-3 in CA SMCs were increased by TNF-α within 4 hours, and this increase was partially inhibited by L-NMMA (Figure 5).

TNF-α Induces Protein Binding to the ARE of Fibronectin mRNA

We previously demonstrated in DA SMCs that LC-3 binds to an ARE in the 3′-UTR of fibronectin mRNA in RNA gel mobility shift and UV cross-linking assays and that increased binding is associated with increased recruitment of fibronectin mRNA to the polyribosomes.13,14 Therefore, we used RNA gel mobility shift assays to examine protein binding from KCl extracts of CA SMC membranes, to a [32P]-labeled 18-mer oligonucleotide containing the wild-type fibronectin ARE (underlined) (5′-ACCUGUUAAUAUAUCUAUU-3′).
and to a control ARE-mutated 18-mer oligonucleotide (5' ACCUGGGAGGGAGCAAU-3') shown previously to exhibit significantly decreased LC-3 binding activity in DA SMCs.13

From KCl extracts of CA SMC membranes, two prominent, shifted protein-fibronectin [32P]-ARE complexes were identified (Figure 6A). We were unable to demonstrate a supershift of these complexes using an antibody to LC-3, which was not unexpected because this antibody also fails to immunoprecipitate LC-3 from cell extracts. However, two complexes with the same migratory patterns on polyacrylamide gels were detected when recombinant LC-3 was incubated with the fibronectin [32P]-ARE oligonucleotide probe (Figure 6B).

Consistent with fibronectin synthesis data, densitometric analyses revealed that formation of the faster migrating complex was increased in CA SMCs treated with TNF-α, an effect that was inhibited by concomitant treatment with L-NMMA or DPI (Figure 6B). In contrast, binding activity for the larger complex did not appear to be altered appreciably with TNF-α induction of fibronectin synthesis. Furthermore, only the larger complex was detectable after incubation with the ARE-mutated oligonucleotide. This suggests that formation of the smaller complex is more sensitive to the presence of the intact ARE element and that formation of this ARE-specific complex is increased with TNF-α by a mechanism mediated through NO.

In UV cross-linking assays in which the 18-mer RNA oligonucleotide containing the ARE was UV cross-linked to bound proteins from CA SMC extracts, a band at ~22 kDa was resolved on SDS-PAGE (Figure 6D). Detection of the ~22-kDa band is consistent with the proposal that the protein-fibronectin ARE complex contains the LC-3 monomer (~15 kDa) bound to the ~6 to 7 kDa [32P]-labeled fibronectin ARE oligonucleotide. These UV cross-links also confirmed that binding to the fibronectin ARE was enhanced by TNF-α and inhibited by concomitant treatment with L-NMMA (Figure 6D). A protein with the same molecular weight was previously detected in DA SMC extracts and purified as LC-3.13

Discussion

We demonstrated that TNF-α induction of fibronectin synthesis in CA SMCs is mediated by NO and that the mechanism is consistent with binding of the microtubule-associated RNA binding protein LC-3 to an ARE in the 3′-UTR of
fibronectin mRNA. TNF-α induction of NO production and NO-mediated induction of soluble fibronectin synthesis in primary cultured porcine CA SMCs correlates with increased expression of TNF-α, iNOS, and fibronectin in the neointima formed in restenosis, atherosclerosis, and posttransplant coronary arteriopathy.

Steady-state levels of fibronectin mRNA in CA SMCs cultured in serum-free medium were not affected appreciably by a 4-hour exposure to TNF-α. Previous SMC culture studies have demonstrated that there is reciprocal coinduction of fibronectin by TNF-α and IL-1β. Because IL-1β induction of fibronectin synthesis is associated with increased steady-state mRNA levels, it appears that TNF-α and IL-1β may regulate fibronectin synthesis at transcriptional and posttranscriptional levels.

Increased elastase activity has also been linked to posttranscriptional regulation of increased fibronectin synthesis in postcardiac transplant arteriopathy. Our recent data suggest that elastin peptides mediate an increase in fibronectin synthesis by a posttranscriptional mechanism, because the selective elastase inhibitor elafin reduces elastin-peptide–mediated induction of fibronectin synthesis without affecting steady-state mRNA levels (authors’ unpublished data, 1998). Because elafin also abrogates the development of postcardiac transplant coronary arteriopathy in experimental animals, posttranscriptional induction of fibronectin gene regulation may be critical in this model of occlusive neointimal formation.

We have demonstrated that a membrane-associated form of LC-3, which facilitates fibronectin mRNA translation in 100-day-gestation DA SMCs, a vessel in which neointimal formation occurs, also appears to be present in porcine CA SMCs and increased by TNF-α. In this report, we demonstrate by gel shift and UV cross-linking assays that TNF-α increases the formation of a complex between the [32P]-labeled fibronectin ARE and a membrane-associated protein from CA SMCs. Evidence suggesting that the protein component is LC-3 was provided by the following additional data: The complex could be reproduced by incubating recombinant LC-3 with the fibronectin ARE. In addition, UV cross-linking assays demonstrated that the molecular weight of the protein binding to the ARE is the same as LC-3. Our results with L-NMMA and DPI suggest that NO may mediate TNF-α induction of fibronectin synthesis by increasing binding of this protein consistent with LC-3 to the fibronectin ARE.

Interestingly, in DA SMCs, although several complexes were detectable in the cytosolic fraction of the cells, only the smaller complex was detectable in the membrane fraction of the cells, which is the site where LC-3 appears to act to facilitate mRNA translation at the polyribosomes. The membrane-associated smaller complex has been postulated to represent a phosphorylated form of LC-3. It is feasible that phosphorylation of LC-3 could result in its monomerization, hence a smaller complex size, with increased binding efficiency to the fibronectin 3′-UTR ARE, which in turn facilitates mRNA translation. From the present studies in CA SMCs, we have found that binding of this postulated phosphorylated/monomeric form of LC-3 appears to be ARE-sensitive, which would explain our previous gene transfer studies demonstrating the importance of the ARE in fibronectin upregulation and SMC migration in neointimal formation.

Neointimal formation in the fetal ductus arteriosus can be abrogated by inhibiting SMC fibronectin synthesis by gene transfer of plasmid-encoding decoy RNA containing the 3′-UTR of fibronectin mRNA. Our demonstration that cytokine induction of fibronectin synthesis is likely mediated by a similar posttranscriptional mechanism suggests that the same gene transfer strategy might be useful in inhibiting occlusive neointimal formation of vascular disease.

Acknowledgments

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR) PG-13920. M.R. is a Distinguished Scientist of the CIHR and C.O. was supported by a CIHR MD PhD studentship. M.R. was also supported by the Heart and Stroke Foundation of Ontario Research Chair. We are grateful to Joan Jowlabar and Judy Edwards for their assistance in preparing this manuscript and to Dr James Hammarback for providing the antisera to LC-3.

References

32 Circulation Research July 6, 2001


47. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnock JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982;126:131–138.


Tumor Necrosis Factor-α Induces Fibronectin Synthesis in Coronary Artery Smooth Muscle Cells by a Nitric Oxide–Dependent Posttranscriptional Mechanism
Catherine A. E. O'Blenes, Caroline Kinnear and Marlene Rabinovitch

*Circ Res.* 2001;89:26-32; originally published online June 21, 2001; doi: 10.1161/hh1301.093631
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/1/26

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/