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Nitric oxide decreases surfactant protein gene expression in primary cultures of type II pneumocytes

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Submitted 7 June 2004; accepted in final form 30 December 2004

INHALED NITRIC OXIDE (NO) is a selective pulmonary vasodilator effective in treating elevated pulmonary vascular resistance in newborns with persistent pulmonary hypertension (10, 50) and in children with pulmonary hypertension following congenital heart disease (CHD) surgery (8, 43). However, multiple in vivo and in vitro studies have shown a negative effect of NO on surfactant activity as well as surfactant protein gene expression. Although the relationship between NO and surfactant has been studied previously, the data has been hard to interpret due to the model systems used. The objective of the current study was to characterize previous experiments exploring the role of NO on surfactant gene expression difficult. In whole lung or mixed cell cultures, surfactant gene expression has been very difficult to interpret due to the model systems used. In vivo and in vitro studies have shown a negative effect of NO on surfactant activity as well as surfactant protein gene expression. Although the relationship between NO and surfactant has been studied previously, the data has been hard to interpret due to the model systems used.

Previous experiments exploring the role of NO on surfactant synthesis, secretion, and/or metabolism have focused on the role of toxic intermediates of NO such as peroxynitrite (25–26, 28, 42, 61), methemoglobin (29–30, 32), and nitrogen dioxide (NO2) (45). In vitro, peroxynitrite has been shown to decrease surfactant surface activity through lipid peroxidation and nitration of tyrosine residues on surfactant protein (SP)-A and SP-B (25–26, 28, 42, 61). Haddad et al. (27) also have shown that, in freshly isolated adult rat type II cells, several NO donors decreased the rate of surfactant synthesis as measured by the incorporation of methyl-3H into phosphatidylcholine and reduction in cellular ATP levels. In this same study, however, ventilating the rats with exogenous inhaled NO at 80 ppm did not affect surfactant synthesis or ATP levels (27). The conflicting results between in vivo and in vitro studies may be explained by the presence of various antioxidants within epithelial lining fluid in vivo not present in vitro, various antioxidants that inactivate the toxic intermediates of NO, and toxic intermediates that affect surfactant function.

Recently, several studies have looked at the role of NO on surfactant protein gene expression. Stuart et al. (58) found that ventilating 4-wk-old lambs with inhaled NO at 40 ppm increased SP-A and SP-B mRNA levels at both 12 and 24 h with a corresponding increase in SP-A and SP-B protein contents at 12 h. However, by 24 h, there was a decrease in SP-A and SP-B protein contents of 70% and 65%, respectively (58). Bhandari et al. (4) found, in freshly isolated fetal type II cells, that exposure to S-nitroso-N-acetylpenicillamine (SNAP) and hyperoxia decreased surfactant protein mRNA levels. Ayad and Wong (2) showed that in vitro exposure to SNAP decreased SP-A mRNA and protein content in a human lung tumor cell line representative of distal respiratory epithelium. Salinas et al. (52) also demonstrated that SNAP decreased SP-B gene expression in H441 cells, which are characteristic of bronchiolar (Clara) cells, and MLE-12 cells, an SV40 transformed epithelial cell line.

In addition, in a lamb model of CHD with increased pulmonary blood flow and pressure, the interaction between NO and endothelin (ET)-1 signaling was found to mediate the dynamic changes in vascular tone following acute changes in fetal pulmonary blood flow (5–6, 47) and was also associated with a gradual decrease in surfactant protein gene expression (23, 37).

Although the effect of NO on surfactant has been studied previously, the model systems used whether in vivo or in vitro have made the interpretation of the effect of NO on surfactant protein gene expression difficult. In whole lung or mixed cell...
culture studies, it has been difficult to attribute specific direct effects of NO on type II cells because type II cells comprise only 15% of all lung cells. In primary cultures, adult rat type II pneumocytes cultured in a substrate that promoted attachment and spreading resulted in the rapid loss of differentiated phenotype, specifically surfactant protein gene expression. Lastly, most cell lines did not express the full spectrum of type II cell differentiated characteristics. A recent modification of the type II cell culture model system by Dobbs et al. and other investigators (3, 14–17, 41, 54–55, 60) has been developed that maintains differentiated characteristics such as cuboidal in shape, presence of lamellar bodies, and, most importantly, surfactant protein gene expression in culture.

In this study, we examined the effect of a NO donor on surfactant protein gene expression on primary cultures of type II cells cultured on a substrate that promoted maintenance of type II cell phenotype. This model of differentiated type II cells in steady state has allowed us to investigate the signal transduction pathways involved in the regulation of surfactant apoprotein expression.

MATERIALS AND METHODS

Isolation of rat type II pneumocytes. Alveolar type II cells were isolated from pathogen-free adult male rats by digestion with elastase and purified by differential adhesion on rat IgG-coated plates as previously described (3, 14–17, 41, 54–55, 60). Type II cells were removed from this resultant population by negative selection using monoclonal antibodies and magnetic beads (MiniMACS Separation Columns, Miltenyi) specific to type II cells (22). The number of type II cells was quantitated in each preparation by immunohistochemistry using an antibody for RTI40 for type I cells and an antibody for RTII70 for type II cells (18, 20, 24). Both antibodies were a kind gift from Dr. Leland Dobbs. The resultant population contained roughly 20 × 106 cells/rat of which 85–90% were type II cells. The contaminating cells included macrophages, fibroblasts, and lymphocytes. To maintain type II differentiated characteristics, the cells were incubated at 37°C, 10% CO2 in DMEM (Specialty Media, Phillipsburg, NJ) with 1% rat serum, 100 U/ml insulin (Glyco-SNAP-1 (SNAP) (Calbiochem, San Diego, CA) at concentrations of 0.0, 0.1, 1.0, 10.0, and 100.0 nM for 24 h. The supernatants were removed, and the cells were harvested for RNA isolation or protein extraction.

ET-1. After 7 days in culture, type II cells were exposed to ET-1 (Peptide Institute, Osaka, Japan) at concentrations of 0.0, 0.1, 1.0, 10.0, and 100.0 nM for 24 h. The supernatants were removed, and the cells were harvested for RNA isolation or protein extraction.

UO-126. After 7 days in culture, type II cells were exposed to 0.0 or 100 μM UO-126 (Cell Signaling, Beverly, MA) (1, 59) at 0, 4, 12, and 24 h. UO-126 is a specific MEK1/2 inhibitor that does not affect other protein kinases such as c-Abl, Raf, MEKK, extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), MKK3, MKK4/stress-activated kinase-1, MKK6, c-Jun-dependent kinase (ckd) 2, or cdk4. After the exposure, the supernatants were removed, and the cells were harvested for RNA or protein.

RNA isolation and ribonuclease protection assay. To isolate RNA, 0.75 ml of RNA-STAT (Tel-Test, Friendswood, TX) was applied directly onto the cells within the matrix and mixed into a liquid consistency. Total cellular RNA was extracted with phenol-chloroform, precipitated with isopropanol, and quantitated spectrophotometrically. Total RNA, 0.5 μg per sample, was hybridized to an in vitro transcription maxiscript mixture (BD Biosciences), which contained antisense RNA probes for SP-A, SP-B, SP-C, RTI40, GAPDH, cavelin, L-32 (RiboQuant Custom Rat Template; Pharmingen, BD Biosciences), and 18S rRNA. RNA from day 0 and day 7 controls and treated cells were hybridized to the RNA probes, prelabeled with 32P-UTP, before RNase digestion of unprotected single strands. The resultant double-stranded products were separated on denaturing polyacrylamide gels. The gels were then dried before undergoing autoradiography. Phosphorimaging was used to quantify the level of surfactant protein gene expression, normalized to 18S rRNA to account for loading discrepancies.

Protein isolation and Western blotting. Total cellular protein was extracted from the cells within the matrix using 0.25 ml of lysis buffer per well containing 4% Triton X-100, 80 mM Tris base (pH 8.0), 548 mM NaCl, 40% glycerol, 2 mM vanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM Pefabloc and mixed into a liquid consistency. Each sample was centrifuged for 10 min twice at 14,000 rpm at 4°C, and the resultant supernatant was removed. Protein content was measured by the bicinchoninic acid method (Pierce, Rockford, IL) (57). Each sample was reduced and denatured with a sample buffer and run on a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA), 20 μg of protein per lane, using a MOPS SDS buffer (Invitrogen, Carlsbad, CA) at 100 V for ~1.5 h. The proteins were then transferred to a nitrocellulose membrane and blocked with 3% milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h. The nitrocellulose membrane was than exposed to the primary antibody (either phosphorylated ERK1/2 or total ERK1/2, Cell Signaling, Beverly, MA) at 1:1,000 dilution for 2 h. The nitrocellulose blot was washed repeatedly with TBS-T and then exposed to the secondary antibody, an horseradish peroxidase-labeled antibody at 1:2,000 dilution for 30 min. Antibodies to phosphorylated or total ERK1/2 recognized two bands on the Western, one at 44 kDa for ERK1 MAPK and the other at 42 kDa for ERK2 MAPK. After repeated washing with TBS-T, the protein bands were then visualized with a chemiluminescence reagent, ECL+ (Amersham, Piscataway, NJ), and quantitated with phosphorimaging.

RT-PCR and ET mRNA Level. Total RNA was isolated as previously described. Multiplex RT-PCR was used for analysis of ET-1 mRNA in combination with 18S rRNA levels in type II cells treated with 0.0, 0.1, 1.0, and 3.0 mM SNAP for 24 h utilizing CompetiGene technology (Ambion, Austin, TX). Total RNA was reverse transcribed using random primers. cDNA was amplified with specific oligonucleotide primers for ET-1 as previously described (56) and 18S rRNA.
Dot blot analysis for SP-A and SP-B protein levels. For type II cells treated with SNAP or UO-126, dot blot analyses for SP-A and SP-B protein levels were performed as previously described (37). In short, 0.5 μg/well of protein for each condition was run in triplicate on nitrocellulose (Bio-Dot Slot Format; Bio-Rad Laboratories, Richmond, CA). A gentle vacuum was applied to the dot blot apparatus until all the proteins were pulled through. The nitrocellulose was incubated in 15% H2O2 for 15 min to quench any endogenous peroxidase activity, and nonspecific binding was blocked by incubation with 3% milk in TBS-T at room temperature for 1 h. The blots were then washed with TBS-T for 5 min at room temperature. The blots were incubated in primary rabbit antibody (1:2,000 dilution) against SP-A or SP-B for 30 min. We used rabbit polyclonal antibodies against sheep SP-A and SP-B that were also immunoreactive to rat SP-A and SP-B, respectively, by immunohistochemistry and Western blot analysis (24, 35). Both antibodies were a kind gift from Dr. Samuel Hawgood. The blots were repeatedly washed in TBS-T for 1 h and then incubated in peroxidase-labeled donkey anti-rabbit secondary antibody (1:2,000 dilution, Amersham) for 20 min. Unbound secondary antibody was removed with TBS-T over 1 h. Bound secondary antibody was detected by exposure to a luminol (ECL Western Blotting Detection System, Amersham) for 5 min and autoradiography. Relative light units were measured in a plate luminometer (Packard Instrument, Downers Grove, IL).

Statistics. All comparisons among treated cells were made against day 7 controls treated with 0.0 mM SNAP, 0.0 mM ET-1, or 0.0 μM UO-126, not day 0 controls, due to the significant initial drop in surfactant protein gene expression among type II cells plated. Comparisons between treated and control cells were made by repeated measures of analysis of variance (ANOVA) using the Bonferroni correction for multiple-comparison testing. Statview (SAS Institute) was the software used to perform the test.

RESULTS

Effect of concentration of SNAP on type II pneumocyte viability. There was a significant increase in cell death at 3.0 mM SNAP (6.95 ± 2.26% vs. 1.81 ± 0.73% for day 7 controls treated with 0.0 mM SNAP, P < 0.05, n = 3). However, overall cell viability remained >90% for all concentrations of SNAP at 24 h (Fig. 1).

Effect of increasing dosage of SNAP on surfactant protein gene expression. SNAP decreased SP-A, SP-B, and SP-C mRNA contents as normalized to 18S rRNA in a dose-dependent manner (Fig. 2). The decrease in mRNA became statistically significant at 0.1 mM SNAP for SP-A and at 1.0 mM SNAP for SP-B and SP-C compared with day 7 controls treated with 0.0 mM SNAP at 24 h.

Effect of duration of exposure of SNAP on surfactant protein gene expression. The effect of 1.0 mM SNAP on type II pneumocyte expression of surfactant protein mRNAs was apparent by 6 h for SP-A and 4 h for SP-B compared with day 7 controls treated with 0.0 mM SNAP. Although the SP-C mRNA level dropped by 56% by 6 h on exposure to 1.0 mM SNAP compared with day 7 controls, the decrease was not statistically significant by ANOVA. One millimolar SNAP significantly decreased SP-A mRNA content by 60% at 6 h compared with day 7 controls. One millimolar SNAP significantly decreased SP-B mRNA content by 38% at 4 h compared with day 7 controls. Further increased exposure to 1.0 mM SNAP decreased all surfactant protein mRNA levels (Fig. 3).

Effect of ET-1 on surfactant protein gene expression. ET-1 decreased SP-A, SP-B, and SP-C mRNA contents as normalized to 18S rRNA in a dose-dependent manner (Fig. 4). The decrease in mRNA became statistically significant at 0.1 mM

Fig. 1. Effect of increasing concentration of S-nitroso-N-acetylpenicillamine (SNAP) on cell viability. A: microscopy of type II pneumocytes at 7 days in culture. B: fluorescent (red) highlights dead cells at 3.0 mM SNAP at 24 h. C: % of type II pneumocytes alive following treatment with increasing concentration of SNAP for 24 h. *P < 0.05 vs. day 7 controls treated with 0.0 mM SNAP.
ET-1 for all surfactant protein mRNA levels compared with day 7 controls treated with 0.0 nM ET-1 at 24 h.

Influence of ET-1 antagonist pretreatment on the effect of SNAP on surfactant protein gene expression. Pretreating type II pneumocytes with PD-156707, an ETA antagonist, or bosentan, a mixed ETA and ETB antagonists, partially prevented the decrease in surfactant protein gene expression by SNAP at 24 h (Fig. 5).

Effect of SNAP on ET-1 secretion and mRNA level. Exposure to 1.0 mM SNAP led to a significant increase in ET-1 secretion compared with the corresponding untreated cells at 12 and 24 h. There was a constitutive release of ET-1 secretion in untreated cells that remained within 5% of untreated cells at 4 h at all time periods examined. There was a significant increase in ET-1 secretion from 4 to 12 h and from 12 to 24 h in cells treated with 1.0 mM SNAP (Fig. 6).

Using multiplex RT-PCR, we found no difference in ET-1 mRNA content between type II cells treated 24 h with 0.1 mM SNAP (1.21 ± 0.09 vs. 1.09 ± 0.08, P = 0.22, n = 3), 1.0 mM SNAP (1.13 ± 0.05 vs. 1.09 ± 0.08, P = 0.63, n = 3), and 3.0

Fig. 2. Effect of increasing concentrations of SNAP on rat type II pneumocyte expression of surfactant protein (SP)-A, SP-B, and SP-C mRNA contents at 24 h. Ribonuclease protection assays (RPAs) were performed on total RNA, and autoradiograms were obtained before radioactivity was quantified by phosphorimager analysis. RPA values were area under the curve (AUC) standardized per 18S rRNA. Values are % of day 7 controls treated with 0.0 mM SNAP ± SD for each condition. *P < 0.05 vs. day 7 controls treated with 0.0 mM SNAP.

![Graph showing effect of SNAP on surfactant protein expression](image)

Fig. 3. Effect of increasing duration of exposure to 1.0 mM SNAP on rat type II pneumocyte expression of SP-A, SP-B, and SP-C mRNA contents. RPAs were performed on total RNA, and autoradiograms were obtained before radioactivity was quantified by phosphorimager analysis. A: autoradiogram of RPA. B: values are % of day 7 controls treated with 0.0 mM SNAP (AUC/18S rRNA) ± SD for each condition. *P value is significant by according to ANOVA, the Bonferroni correction.
Effect of duration of exposure of SNAP on phosphorylated ERK1/2 MAPK. One millimolar SNAP decreased the expression of phosphorylated ERK1/2 MAPKs as early as 15 min of exposure. Increasing the duration of exposure of type II pneumocytes to 1.0 mM SNAP further decreased both phosphorylated ERK1/2 MAPKs (Fig. 7). The decrease in the activated form of the MAPK was not due to a decrease in total MAPKs, phosphorylated and unphosphorylated ERK1/2 MAPKs.

Effect of MEK1/2 inhibitor UO-126 on surfactant protein gene expression. After 7 days in culture, the addition of 100 μM of UO-126, an MEK1/2 inhibitor, for 24 h significantly decreased SP-A mRNA level by 75%, SP-B mRNA level by 66%, and SP-C mRNA level by 53% compared with day 7 controls treated with 0.0 μM UO-126. The effect of UO-126 was apparent as early as 12 h for all surfactant protein mRNA levels (Fig. 8).

Dot blot analysis for SP-A and SP-B protein levels. For type II cells exposed to 1.0 mM SNAP, both SP-A and SP-B protein levels progressively decreased from 0 to 24 h and correlated closely with mRNA levels but were not statistically significant. SP-A protein level progressively decreased by 24% at 24 h, and SP-B protein level by 33% at 24 h compared with day 7 controls treated with 0.0 mM SNAP. For type II cells exposed to 100 μM UO-126, there was no change in SP-A or SP-B protein levels by 24 h compared with day 7 controls (data not shown).

DISCUSSION

In models of CHD, increased pulmonary blood flow and pressure have been shown to affect both endothelial cell function, such as the secretion of NO and ET-1, and epithelial cell function, such as the expression of surfactant proteins. Although the relationship between NO and surfactant protein gene expression has been studied previously, the data has been hard to interpret due to the model systems used. Type II cells quickly lose their differentiated function when cultured on a substratum that promotes attachment and spreading. Specifically, type II cells cultured on fibronectin tissue culture plastic expressed 5% of surfactant protein mRNAs compared with freshly isolated cells after 48 h (24). We used type II cells, cultured on EHS matrix for 1 wk, which maintained type II cells differentiated characteristics. Type II cells were cuboidal in shape and formed three-dimensional aggregates on the substratum with their apical surfaces directed inward. Characteristic lamellar bodies could be identified. More significantly, by 1 wk in culture, type II pneumocytes expressed SP-A, SP-B, and SP-C mRNAs at 40–60% of preculture levels (21).

Using this model, we found that an NO donor, SNAP, decreased the expression of surfactant protein in a time- and dose-dependent manner. Increasing the duration of exposure to...
1.0 mM SNAP or increasing the concentration of SNAP from 0.1 mM to 3.0 mM progressively decreased all surfactant protein mRNA levels. The significant decrease in surfactant protein gene expression was not caused by a decrease in cell viability and was apparent in primary cultures of rat type II pneumocytes exposed to 1.0 mM SNAP by 6 h for SP-A and 4 h for SP-B. Although SP-C mRNA dropped by 56% by 6 h on exposure to 1.0 mM SNAP, the decrease was not statistically significant by ANOVA. In addition, by dot blot analysis, SP-A protein level progressively decreased by 24% and SP-B protein level by 33% at 24 h of exposure to 1.0 mM SNAP. Although not statistically significant, the decrease in surfactant apoprotein levels was in the same direction as and correlated closely with mRNA levels at all time points examined. The results seemed to substantiate the negative effect of NO on surfactant protein gene expression seen in previous in vivo and in vitro experiments. In the lamb model of CHD following an in utero placement of aortopulmonary graft, the increased pulmonary blood flow and pressure may have increased NO synthesis, which led to the decrease in surfactant protein gene expression (5–6, 23, 37, 47).

NO was also found to increase ET-1 secretion but not ET-1 mRNA content. Type II pneumocytes exposed to 1.0 mM SNAP had an increase secretion of ET-1 over time but not an increase in mRNA level of the vasoactive peptide by RT-PCR. ET-1 exposure alone decreased surfactant protein mRNA expression in a dose-dependent manner similar to NO.

Previous studies demonstrated a relationship between ET-1 and surfactant secretion as well as NO and ET-1 expression. Sen et al. (53) demonstrated that, in a primary culture of adult rat type II cells, ET-1 activated protein kinase C, which increased Ca^{2+} influx through L-type calcium channels, which resulted in the secretion of phosphatidylcholine. However, surfactant protein mRNAs were not measured. In other experiments using primary cultures of vascular endothelial cells isolated from adult animals, stimulation of endogenous NO led to a decrease in ET-1 secretion (7). Recently, Kelly et al. (34) found that in primary cultures of pulmonary arterial endothelial cells from juvenile lambs, exposure to exogenous NO led to a decrease in ET-1 gene expression through activation of soluble guanylate cyclase and cGMP.
Pretreating type II pneumocytes with either the ETA antagonist PD-156707 or the ETA and -B antagonist bosentan before exposure to SNAP partially prevented the decrease in surfactant protein gene expression. The partial ablation of the NO-mediated decrease in surfactant protein gene expression by ET-1 receptor antagonists suggested a possible novel mechanism through which NO suppressed the expression of surfactant proteins via ET-1 secretion.

The next objective was to begin to characterize the underlying mechanism. Previous studies exploring the relationship between NO and surfactant protein emphasized the role of toxic intermediates of NO such as peroxynitrite, methemoglobin, and NO2 on the nitration of crucial tyrosine amino acids. However, in endothelial cell cultures, NO was shown to activate the Ras-ERK1/2 MAPK intracellular pathway, which stimulated protein tyrosine phosphorylation and led to the activation of transcription factors Myc and Elk1 (44, 46).

The MAPKs are a group of serine-threonine kinases that integrate and process various extracellular signals through phosphorylation events. Four distinct MAPK pathways have been described: ERKs, JNK, p38 MAPK pathways, and big mitogen-activated protein kinase-1/ERK5. In general, the ERKs are activated by mitogenic and proliferative stimuli, and JNKs and p38 MAPKs respond to environmental stress (9, 36).

We found that primary cultures of type II pneumocytes exposed to 1.0 mM SNAP had a time-dependent decrease in phosphorylated ERK1/2 MAPKs. The significant drop in phosphorylated MAPKs was apparent as early as 15 min of exposure. Ultimately, phosphorylated ERK1 dropped by 69% and phosphorylated ERK2 by 66% by 4 h of exposure to SNAP. Exposure to NO decreased the activated form of the intracellular messenger, phosphorylated ERK1/2, which preceded the decrease in surfactant protein gene expression. The effect was not caused by a decrease in the total ERK1/2 but by a decrease in the activated form of the enzymes. The results were substantiated by the use of MEK1/2 inhibitor, UO-126, which prevented the phosphorylation or activation of ERK1/2 MAPK. Exposure to 100 µM UO-126 alone significantly decreased the expression of SP-A mRNA by 75%, SP-B mRNA by 66%, and SP-C mRNA by 53% at 24 h vs. day 7 controls. However, by dot blot analysis, SP-A and SP-B protein levels remained unchanged at 24 h of exposure to UO-126. The underlying mechanism through which a NO donor decreases phosphorylated ERK1/2 MAPKs will need to be further elucidated. However, in several other model systems, the interaction between NO and MAPK inactivation was associated with an increase in MAPK phosphatase-1, a dual specificity phosphatase that dephosphorylates ERK1/2 (9, 33, 49).

The major limitation to the current study is the lack of investigation into the role of reactive oxygen species (ROS) in the downregulation of surfactant protein gene expression through increase in ET-1 secretion and decrease in ERK1/2 MAPKs. NO can increase ROS production, which can inhibit endothelin-converting enzyme-1, decreasing ET-1 secretion (38). In addition, ROS can lead to peroxynitrite production, which can nitrate protein-bound tyrosines, such as on surfactant proteins (25–62, 28, 42, 61). The relationship between peroxynitrite and other ROS and MAPK needs to be further elucidated.

In conclusion, our study showed that an NO donor, SNAP, significantly decreased the expression of SP-A, SP-B, and SP-C mRNAs in a time- and dose-dependent manner in type II pneumocytes whose phenotype was maintained in culture. The effect was mediated at least in part by an increase in ET-1 secretion and the decrease in the intracellular MAPK, phosphorylated ERK1/2. The results seemed to substantiate the previous in vivo and in vitro work concerning NO and surfactant and alluded to a novel mechanism by which NO suppressed surfactant protein gene expression, at least in part by the secretion of ET-1.

**REFERENCES**


