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PULMONARY VASCULAR MUSCLE PROLIFERATION AS A RESULT OF PROTEIN AND mRNA-eNOS ALTERATIONS IN A RAT MODEL OF CHF

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ABSTRACT

Endothelial Nitric Oxide Synthase (eNOS) produces nitric oxide (NO) from L-arginine and is important for the maintenance of cardiovascular homeostasis. Congestive heart failure (CHF) generally results in increased pulmonary blood flow and if untreated leads to pulmonary hypertension and end stage heart failure. We therefore hypothesized that increased pulmonary flow without changes in pressure would result in hypertrophy of the media (middle layer of vascular wall). NO produced by the lung is regulated by systemic blood flow and in turn adjusts smooth muscle proliferation via altered expression of eNOS. To study this hypothesis, we created an artificial aortocaval shunt in order to increase pulmonary flow for 7 weeks. The shunt resulted in a significant thickening of the media. eNOS Western and Northern blot analysis demonstrated no significant alterations of eNOS protein and mRNA levels in the large-shunted group but in the small shunted one in comparison with sham. We suggested that NO in low concentrations (about > 10μM) caused weak hypertrophy of the media in the small-shunted group and in high concentrations (about > 50μM) caused S-nitrosylation of eNOS protein and deamination of eNOS mRNA and the regulatory genes in the nucleus; thus the media of the vascular wall was significantly thickened in the large-shunted group. In higher concentrations, NO induces apoptosis and decreased cell viability.
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Keywords: Nitric oxide, Shunting, Congestive heart failure (CHF), eNOS.

INTRODUCTION

NO is produced as a result of the enzymatic deamination of L-arginine to L-citrulline by NO synthase (NOS).[1] The reaction occurs mainly in the endothelial cells, brain cells and macrophages.[2] The principal physiologic action of NO is reduction of blood pressure by its vasodilatory effect on blood vessels.[2,3] Much of this effect on vascular smooth muscle cells is mediated by cGMP and its production is increased in response to NO stimulation.[3] NO also seems to be a neurotransmitter and is involved in the host defense mechanism in macrophages.[1,2]

One important effect of NO on vascular smooth muscle is to activate soluble guanylate cyclase.[4] The elevation of intracellular cGMP seems to mediate much of the effects of NO on smooth muscle cell relaxation.

The way in which cGMP blocks cell proliferation is

not well understood. Increased intracellular cGMP inhibits Ca^{2+} influx and activates its efflux. Decreased intracellular [Ca^{2+}] can inactivate protein kinase C (PKC) and thus inhibit PKC dependent cell proliferation.[5] Threshold levels of cGMP may also be important in the activation of cGMP-dependent PKC, particularly when the amounts of these enzymes are decreased. NO does not inhibit DNA synthesis in certain types of mesenchymal cells. For instance, guanylate cyclase in both chinese hamster fibroblast V79 cells[6] and RACS-1 clonal cells[7] becomes insensitive to NO. Increased pulmonary blood flow without changes in pressure results in increased eNOS expression and possibly plays a role in pulmonary vascular remodeling.

A chronic increase in pulmonary flow in the shunted rat model without making any changes in the eNOS protein or mRNA expression results in thickening of the middle layer of the pulmonary arterial vasculature.[8]

The purpose of this study was the stepwise evaluation of the effect of NO on smooth muscle proliferation by an animal model of CHF. Does NOS inhibition lead to a reduction of the smooth muscle proliferation rate? The answer to this question determines the need for more research in this field.

MATERIAL AND METHODS

Aortocaval shunt

The animals were anesthetized using pentobarbital sodium. The abdominal aorta and the inferior vena cava were isolated and the fistula was created by passing a 20 (large shunt)- or 22 (small shunt) gauge sterile needle through the inferior vena cava and into the aorta (number of cases= 10).

The needle was slowly withdrawn, allowing a small hematoma to form at the aortic and venacaval puncture site. Super Glue (S.P. Richards, Atlanta, GA) was applied as a sealant, and the needle was removed. Then the abdominal wall was closed and the animal was allowed to recover. Sham animals were treated similarly without puncturing the aorta or vena cava to assay for changes in vascular smooth muscle proliferation.

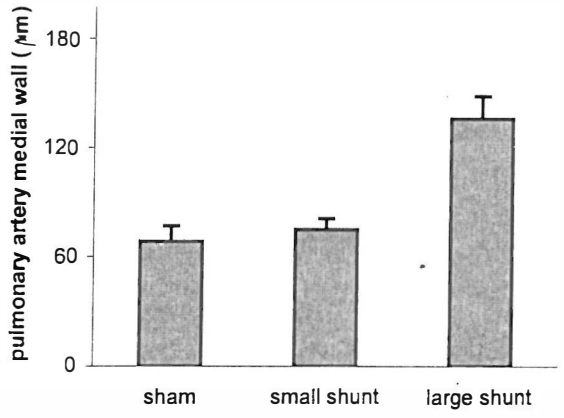

Fig. 1. Measurement of pulmonary arterial medial wall in sham and shunted-lungs ($p<0.05$).

Western blotting

The homogenated lung was separated under denaturing conditions in 7.5% SDS-PAGE gel, followed by blotting of the proteins to nitrocellulose (Bio-Rad Burlingama, CA). Blots were blocked at room temperature for 1h in 50 mM HCl, pH 7.4, 0.15 M NaCl, 2% BSA, and 0.1% Tween 20. Subsequently, blots were incubated with mouse anti-eNOS monoclonal antibody (dilution 1: 500; Transduction Laboratories, Lexington, KY) for 1h at room temperature. Subsequently, membranes were washed at room temperature and incubated with anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) for 1h at room temperature. eNOS immunoreactive protein was detected with enhanced chemiluminescence (ECL; Amersham) and exposure to a film (Hyperfilm-ECL; Amersham) and by using densitometry, signal bands were quantified.

Northern blotting

Total RNA from lungs was extracted after the method of Chomozynski[9] trireagent.[10] Poly(A)+ RNA was isolated from total RNA using Oligo (dT) cellulose (5` prime-3` prime, Boulder, CO). Twenty micrograms of Poly (A)+ RNA per rat was denatured with glyoxal-DMSO, electrophoresed through 0.01M sodium phosphate-buffered agarose gel, and transferred to a nylon membrane (Hybond-Nt; Amersham, Arlington Heights, TL) as previously described.[10] The membranes were hybridized with a 4,091-bp eNOS cDNA (a kind gift of Dr. W.C. Sessa) labeled by random-prime labeling. Hybridization and washing processes were at 65°C.[10] To correct for the amount of RNA loading, RNA transfer efficiency, and gene specific expression, all blots were probed simultaneously with the cDNA for the control gene β-actin. eNOS mRNA signal was detected and quantified using a phosphorimager and Image Quant-Software (Molecular Dynamics, Sunnyvale, CA). Autoradiograms were also obtained by exposure to film (Hyperfilm-MP; Amersham, Arlington Heights, IL).

RESULTS

Creation of an arteriovenous fistula (shunt) between the abdominal aorta superior to the renal arteries and the inferior vena cava described by Garcia and Diebold[11] is a well-tolerated surgical procedure with high animal survival. Importantly, as shown in Fig. 1, there is an increase in muscularity of very small (<50 mM) arterioles of the shunted lungs compared with the sham, whereas

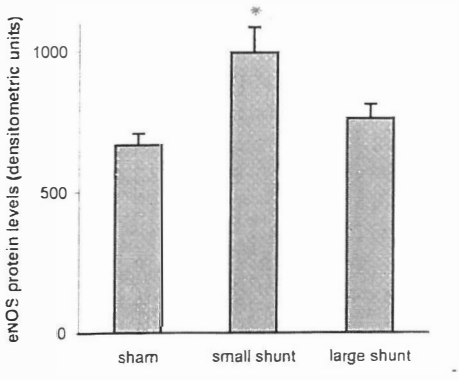

Fig. 2. Western blot analysis of endothelial nitric oxide synthase.

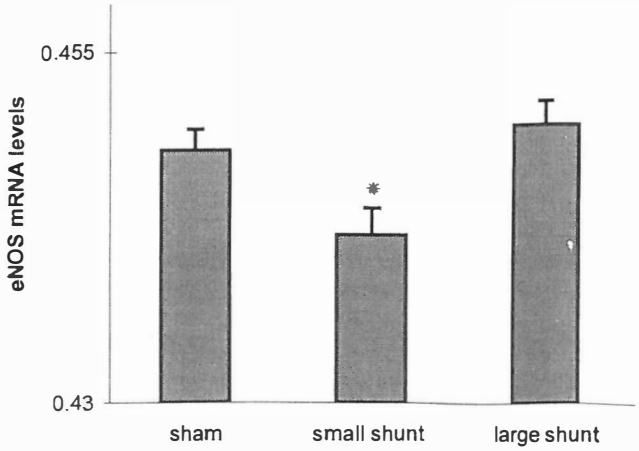

Fig. 3. Northern blot analysis of eNOS mRNA levels in sham and shunted lungs.

the intimal layer remained unchanged, and there was a significant increase in the thickening media from 40 to 100 and 90 to 200 μm in diameter in the small-shunted and large-shunted group respectively, as a conclusion the increase in media thickening was a result of smooth muscle hyperplasia.

In the shunted lung, increased pulmonary flow results in changes in eNOS expression, lung eNOS mRNA and protein levels compared with sham. As shown in Fig. 2 Western blot analysis demonstrated significant differences in eNOS protein levels in the lungs of animals with both small and large shunts compared with the sham. As shown in Fig. 3, eNOS protein levels in the small-shunted group had a significant difference compared with the sham but not in the large-shunted group. NO causes deamination of mRNA and conversion of cytosine to uracil that in turn leads to miscoding of mRNA and inhibition of protein synthesis. On the other hand an increased amount is likely to cause S-nitrosylation of cysteine in eNOS and this in turn causes inactivation of this protein.

DISCUSSION

As presented in the study, eNOS protein, mRNA and immuno-histochemical localization were not changed in the lungs of the shunted animals. eNOS could play two potential roles in modulating the pulmonary vascular response to increase pulmonary blood flow. First, by increasing gene expression in the small arteries, it facilitates vascular recruitment and maintains flow tolerance and second, it adjusts smooth muscle cell growth.

Therefore increased blood flow alone is not a significant regulator of pulmonary eNOS, and probably blood flow regulation of eNOS is a result of the difference between the PaO_2 of the aorta and the pulmonary arteries.[7] Similarly, Nadaud et al.[8] demonstrated that aortic eNOS mRNA and protein were increased in the rats after creation of an A-V shunt. it is unclear how reduction of pulmonary arterial oxygen content resulting from shunt construction regulates the amount of eNOS protein. Chronic hypoxemia can increase the eNOS protein and gene expression in the rat lung.[10]

NO stimulates soluble guanylate cyclase in the smooth muscle and plays diverse roles. It decreases Ca^{2+} concentrations and vasodilatory effects. It is well known that high concentrations of NO are proapoptotic and cytotoxic for different cells. In contrast, low concentrations of NO have been shown to be protective against apoptosis.[12,13] In addition to the known cGMP dependent effects, NO modifies proteins with cysteine residue via S-nitrosylation. S-nitrosylation of reactive cysteine groups by NO is a well-known post-translative modification of proteins. The cysteine protease caspase-3 which has been shown to be essential for TNF-α-mediated apoptosis can be modified by NO. NO may directly induce DNA damage by deamination of cytosine.

NO in concentrations of 10 and 100 μM did not affect cell viability and apoptosis in the absence of TNF-α. However, higher concentrations (about 500 μM) induced apoptosis and decreased cell viability.[17] NO is released as NO or an even co-product of NO and O_2, and this co-product may lead to the generation of ONOO *in vivo* and inhibition of apoptosis induction. The eNOS gene does appear to be regulated by increasing blood flow in the aorta as suggested by Sessa et al.[7] They demonstrated that exercise for 10 days resulted in increased aortic endothelial eNOS mRNA and increased NOS activity in epicardial coronary arteries of dogs. Similarly, Nadaud et al.[8] showed that aortic eNOS mRNA and protein were increased in rats after creation of an A-V shunt.

In summary, this study suggests that the difference between the PaO_2 of the aorta and pulmonary arteries increases blood flow. NO is produced by the eNOS protein in

low concentrations (about >10 μ M) and causes weak hypertrophy of the media in the small-shunted group. In high concentrations (about >50 -100 μM) NO caused S-nitrosylation of eNOS protein and deamination of eNOS mRNA and the regulatory genes of proliferation in the nucleus. Proto-oncogen or tumor-suppressor genes are two types of genes that changed via deamination. So, decreased translation of eNOS-mRNA to eNOS protein is caused by deamination of eNOS-mRNA. As well as the media, the turnover of the endothelial cells was significantly increased in the large-shunted group.

The creation of a large systemic arteriovenous shunt not only caused increased proliferation of smooth muscle cells, but also cardiac heart mass, heart-to-body mass and lung-to-body weight ratios. All last figures were significantly increased (>2-fold; $p<0.05$) in the shunted animals compared with the sham (Table I). However, higher concentrations (500 μM) of NO may directly induce DNA damage by deamination of cytosine, and thus may trigger the apoptotic cell death pathway.

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Table I. Changes in tissue weight with the shunt.

Tissue	Sham	Case (shunted animals)	
		Small	Large
Body weight(g)	380±9	368±9	377±4
Lung's wet weight(g)	1.14±0.15	1.38±0.15	1.8±0.2
Heart's wet weight(g)	1.09±0.04	1.5±0.14	2.3±0.14
Lung/body weight	$(3\pm0.3)\times10^{-3}$	$(3.7\pm0.4)\times10^{-3}$	$(4.7\pm0.6)\times10^{-3}$
Heart/body weight	$(2.86\pm0.15)\times10^{-3}$	$(4.1\pm0.5)\times10^{-3}$	$(6.1\pm0.5)\times10^{-3}$

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