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# Nitric Oxide Synthase Activity in Genetic Hypertension

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*Summary.* A porphyrinic sensor was used to monitor nitric oxide released from cultured endothelial and vascular smooth muscle cells obtained from genetically hypertensive rats and from a normotensive reference strain of rats. Endothelial cell nitric oxide synthase (the constitutive enzyme) was stimulated with bradykinin, and vascular smooth muscle cell nitric oxide synthase (the inducible enzyme) was induced with interleukin-1ß. Both types of cells from hypertensive rats released less nitric oxide than did cells from normotensive rats. The observed deficient nitric oxide release from endothelial and smooth muscle cells may contribute to the elevated vascular tone and increased cell growth described in hypertension. 

Peripheral vascular resistance normally reflects a balance between factors which cause contraction and those that cause relaxation of vascular smooth muscle (VSM). The elevated arterial pressure of either clinical or experimental hypertension is caused by an increase in total peripheral resistance (TPR). Therefore, the elevated vascular tone in hypertension results from either an increase in the factors that cause contraction or a decrease in those that cause relaxation. This principle is demonstrated by observations that chronic hypertension can be produced experimentally either by infusion of agiotensin II (1), which causes VSM contraction, or by administering nitro-L-arginine (2) which blocks the production of nitric oxide (NO), a normal VSM relaxant.

Recent observations have suggested that the elevated vascular resistance in clinical hypertension may be associated with a deficient production of this dilator, NO. Panza et al. (3) studied the increase in forearm blood flow produced by acetylcholine that increases NO production by endothelial cells. They observed that this increase in flow was less than one-half as great in patients with essential hypertension as compared to age- and sex-matched normotensive control subjects. These investigators postulated that the relaxation response to acetylcholine in the hypertensive subjects reflected a deficient NO production by the vascular endothelial cells. These cells contain a constitutive nitric oxide synthase (cNOS) which is activated by an elevated intracellular calcium concentration in response to acetylcholine (4). This NO diffuses from the endothelium to the medial layer of the vessel wall where it causes VSM relaxation mediated by increased intracellular cGMP (5). The observation by Panza et al. (3) suggests that cNOS is less activated by acetylcholine in patients with essential hypertension than it is in normotensive subjects.

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We recently reached a similar conclusion about NO synthase activity in genetically hypertensive rats (SHRSP; unpublished observation). We treated isolated VSM from SHRSP and from their normotensive reference strain (WKY) with interleukin 1ß (IL-1ß). This cytokine is known to stimulate in VSM the production of "inducible" nitric oxide synthase (iNOS) that is calcium-independent. We studied the effect of IL-1ß-treatment on the contractile response of VSM (endothelial-denuded aortic rings) to phenylephrine. We observed that the depression of contraction resulting from the iNOS production was significantly less in VSM from SHRSP than from WKY. In contrast, sodium nitroprusside, an external source of NO, produced equivalent depressions of contractile responses of VSM from SHRSP and WKY. Therefore, we concluded that iNOS (as well as cNOS) produces less NO in hypertensive than in normal tissue.

One possible interpretation from both the clinical and experimental studies is that a deficit in NO release may contribute to the increase in vascular resistance that causes hypertension. However, the functional evidence for NO release is indirect, permitting alternative interpretations. For instance, the observations made in these studies could have been caused by a diminished sensitivity of the contractile mechanism of SHRSP to IL-1ß-induced changes in alpha adrenergic receptors, receptor/second messenger coupling, or contractile element sensitivity to calcium.

The current study was, therefore, carried out to measure directly NO release from endothelial and VSM cells of normotensive and genetically hypertensive rats. Our hypothesis was that NO production was low in both endothelial and VSM cells from hypertensive rats compared to normotensive rats.

## METHODS

### *Animals*

Stroke-prone spontaneously hypertensive rats (SHRSP) and normotensive Wistar-Kyoto rats (WKY) were obtained from our inbred colonies which have been maintained at the University of Michigan for the past 15 years. Five-week old rats of each strain were decapitated and the mesenteric arteries and aortae were removed under aseptic conditions and placed in ice-cold Hank's balanced salt solution.

### *Isolation and Culture of the VSM Cells*

Cells were harvested following enzymatic dissociation of mesenteric arteries, and cultured by methods we have previously described (6). The cells were verified to be VSMC by electron micrography and by monoclonal antibodies against smooth muscle-specific alpha-actin using an immunofluorescent technique. Cells were studied between their fourth and tenth passages.

### *Culture of Endothelial Cells*

Endothelial cells from the thoracic aorta were isolated and cultured by the method described by McGuire and Orkin (7). Briefly, the vessels were cleaned and cut into rings approximately 2 mm in width. The rings were placed on end on basement membrane components derived from EHS sarcoma (Matrigel). These explants were incubated in complete medium (RPMI 1640) at 37º C in a 95% air/5% $C0_2$ atmosphere. Endothelial cells grew out onto the Matrigel and the aortic explants were removed after four to eight days. The endothelial cells that had grown out onto the Matrigel were passaged with 2% dispase, and used for these studies after two to six passages.

### *The Porphyrinic Electrode*

The NO microsensor was prepared according to the procedure previously described (8). A polymeric film of nickel (II) tetrakis (3-methoxy-4-hydroxy-phenyl) porphyrin was deposited on a

thermally-sharpened carbon fiber electrode (0.7-1.2 μm tip diameter and 2-3 μm tip length) by using a continuous scan cyclic voltammetry. The polymeric porphyrin was sequentially coated (three coats) by dipping the electrode for 5s in 1.25% Nafion and dried for 5 min. A PAR Model 264A voltammetric analyzer and a PAR Model 181 current-sensitive preamplifier were used for the differential pulse voltammetry (DPV) and for amperometry. The DPV was recorded with PAR Model 9002A x-y recorder. The amperometric signal was recorded with a Houston Omniscribe strip chart recorder. A three electrode system consisting of a NO microsensor-working electrode, a platinum wire (0.25 mm diameter) counter electrode and saturated calomel reference electrode was used for the measurement of NO release. NO standard solutions were prepared by saturating a 25 ml degassed-silicon rubber-sealed phosphate buffer with NO gas. Sodium hydroxide (0.1M) was used on-line to trap the other oxides.

*Measurement of NO Release*

Both endothelial and VSM cells were grown on small plastic petri dishes. An electroactive tip of the microsensor was placed on the membrane surface of the endothelium or smooth muscle cell. A Nikon TMS inverted microscope with heated stage (37 ± 0.1°C) and a Stoelting HS6 micromanipulator were used for the microsensor positioning. After preincubation time (10 min) the endothelial cells were stimulated with bradykinin ($5x10^{-7}$M) and the VSM cells were stimulated with IL-1ß (100 U/ml). The release of NO from the endothelial cells was measured by a continuous monitoring of the current for 20 min. NO release from the smooth muscle cell was monitored at intervals for six hours.

## RESULTS

Endothelial cells, between their second and sixth passages were cultured to near confluence (usually three days) in 35 x 10 mm petri dishes. Complete culture media was replaced with Hank's balanced salt solution shortly before NO measurements were made. Release of NO was not detectable from the unstimulated cells. The lower limit of detection of the porphyrinic electrode is 10 nM. Data presented in figure 1 are those obtained 5 min. after treating the cells with bradykinin ($5x10^{-7}$M). These are data from five different preparations of cells from each strain of rats. The mean concentration of NO at the surface of the cells from SHRSP rats was less than one-half of that from WKY rats ($P < 0.05$). When the NOS activity is expressed as the amount of NO released per cell, SHRSP cells released 3.6 ± 0.2 femtomoles whereas WKY cells released 10.4 ± 0.2 femtomoles.

Vascular smooth muscle cells were studied between their fourth and tenth passages and were also grown to near confluence (seven days) in 35 x 10 mm petri dishes. Prior to NO

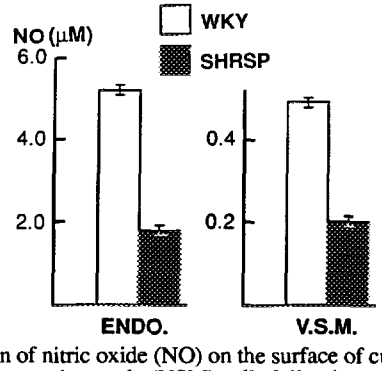


Figure 1. Concentration of nitric oxide (NO) on the surface of cultured endothelial (ENDO) and vascular smooth muscle (VSM) cells following stimulation, respectively, with bradykinin ($5x10^{-7}$M) and interleukin-1ß (100 U/ml). Both types of cells from the hypertensive rats (SHRSP) release significantly less ($P< 0.05$) NO than do those from the normotensive reference strain (WKY) of rats.

measurements the culture media was replaced with Hank's balanced salt solution. Again, NO concentration at the surface of the cultured cells was not measurable prior to stimulation. Figure 1 presents the mean concentrations of NO at the surface of eight different cultures of vascular smooth muscle of SHRSP and eight of WKY, 60 min. after treatment with interleukin-1ß (100 U/ml). Similar to the endothelial cells, the concentration of NO at the surface of SHRSP cells was significantly less than that at the surface of the WKY cells. The amount of NO released by cell was $4.0 \pm 0.2$ femtomoles for the SHRSP cells and $9.9 \pm 0.7$ for the WKY cells.

## DISCUSSION

The current observations establish the validity of implications made from indirect observations that cNOS and iNOS are less active in releasing NO in hypertensive than in normal blood vessels. Cultured endothelial cells from SHRSP, when stimulated with bradykinin, release only one-third as much NO as do those from WKY. This direct measure of cNOS activity indicates that the enzyme is depressed in this tissue from the genetically hypertensive rat compared to that from a normotensive control rat.

Similarly, when iNOS is induced with IL-1ß in cultural VSM cells, the cells from SHRSP release less than one-quarter as much NO as do these cells from WKY.

These two inbred strains clearly differ genetically in the physiological trait of blood pressure. Based on the current direct evidence, the two strains also differ in the biochemical trait of NOS activity. This is a difference that has persisted through numerous passages of the cultured cells. Therefore, it appears to represent an inbred genetic trait that is not secondary to the blood pressure difference between the two strains.

In the genetic model of hypertension that we have studied there is a similar depression of the activities of the two types of the enzyme studied. Both cNOS and iNOS have been cloned (9) and a 50% homology has been found between the two, suggesting that there could be a genetic basis for the similar depressions.

Several results of the deficient NOS activity could contribute to the increase in TPR in hypertension. Most obvious is the reduction in the normal vasodilator action of NO. The potency of this action is demonstrated by the observation that the severe hypotension of endotoxic shock results from the induction of excess NO (10). This hypotension can be reversed by nitro arginine that block NOS activity.

The increase in TPR in hypertension is the result of two related vascular changes. In addition to the increase in vascular smooth muscle contraction, an augmented proliferation of this muscle causes thickening of the vessel wall so that it encroaches on the lumen (11). The presence of NO normally places constraints on vascular smooth muscle growth (12), so that in hypertension when NO is reduced, increased vessel thickness and, hence, increased TPR is expected.

Evidence of the role played by NO in the normal regulation of blood pressure is seen when NO production is reduced in the normal animal by the administration of nitro arginine which blocks the production of NO by NOS. This procedure causes an elevation in arterial pressure (13), demonstrating that NO is part of the overall homeostatic system responsible for normal blood pressure.

In the current study we observed that the production of NO is deficient in two tissues from genetically hypertensive rats.

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