Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 1-8

NITRIC OXIDE SYNTHASE ACTIVITY IN THE PREGNANT UTERUS DECREASES AT PARTURITION

E.S. Natuzzi, P. C. Ursell, M. Harrison,
C. Buscher, and R. K. Riemer*

Departments of Surgery, Pathology, Obstetrics/Gynecology and Reproductive Sciences
University of California at San Francisco
San Francisco, CA 94143

Received May 17, 1993

The mechanisms that mediate changes in uterine activity from a quiescent state during pregnancy to active labor at parturition are unknown. Nitric oxide (NO), a potent mediator of smooth muscle relaxation, and its presence in the uterus is the subject of this report. Nitric oxide synthase (NOS) activity was demonstrated in nerves, blood vessels and decidua of gravid rat uterus by the NADPH-diaphorase staining method. Uterine tissue fixed during labor demonstrated markedly less NOS. Quantitation of NOS activity in subcellular fractions of pregnant and laboring uterus revealed its presence in both the cytosolic and the membranous compartments of uterine homogenates. In both cellular subfractions the enzyme activity decreased significantly from pregnancy to term. We conclude NOS is present in multiple structures within the uterus. Its presence in two cellular compartments suggests more than one form of NOS may be present in the uterus. Reduction in NOS activity at parturition suggests NO may contribute to the maintenance of uterine contractile quiescence during gestation.

The mechanism by which uterine smooth muscle switches from a quiescence state during pregnancy to contraction at labor has been the subject of investigation for years. The role of innervation, neuropeptides, autacoids and hormones in mediating this switch have been extensively explored, yet no clear mechanism for sustained quiescence during pregnancy or the signal mediating the onset of parturition has been identified. Nitric oxide (NO), a potent biologic messenger molecule found in many structures: autonomic nerves in the adrenal gland (1), the corpora cavernosa (2) is best known for its ability to mediate relaxation of vascular smooth muscle(3). This highly reactive and freely permeable molecule is produced from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS, EC1.14.23)(4). In smooth muscle cells, NO has been shown to activate the soluble form of guanylate cyclase, catalyzing intracellular cGMP production (5) leading to decreased myosin-light chain kinase activity (6) and decreased intracellular ionized calcium concentration. These intracellular events culminate in smooth muscle relaxation.

*To whom reprint requests should be addressed at Laboratory of Perinatal Biology, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California at San Francisco, Box 0550, Room HSE-1462, San Francisco, CA 94143-0550.

Abbreviations: L-NMA: N^G-monomethyl-L-arginine; L-NA: N^ω–nitro-L-arginine; SNAP: S-nitro-N-acetylpenicillamine

0006-291X/93 $4.00

Earlier work done in our laboratory has investigated the role of nitric oxide in relaxation of actively laboring gravid uterus. Exogenous NO, given in the form of SNAP arrested laboring uterine contractions in rhesus monkeys (submitted for publication). Furthermore, oxytocin-induced contractions in isolated pregnant rat uterine muscle strips were reversibly blocked by pre-treatment with SNAP (unpublished observations). These studies clearly indicated uterine smooth muscle has the capacity to respond to NO and raised further questions: Does the gravid uterus make its own NO and could this endogenous NO production play a role in mediating uterine smooth muscle quiescence during pregnancy? In this paper, we demonstrate that the uterus has the capacity to produce NO, and that this capacity is regulated in a manner consistent with a role of endogenous NO in contributing to the maintenance of uterine quiescence prior to parturition.

Materials and Methods

Preparation of uterine tissue: Uterine tissue was obtained from time-mated pregnant female Fischer rats. Animals were euthanised by ether gas overdose. The uterus was removed, fetuses, placenta and fetal membranes were separated and discarded. Uterine tissue was rinsed several times in cold isotonic saline, minced into approximately 5 mm cubes, quick frozen in liquid nitrogen and stored at -70°C for later determination of NOS activity. For morphological studies, a 1x1cm sample of full thickness uterus was taken prior to freezing and fixed for two hours in 4% paraformaldehyde then stored in 30% sucrose at 4°C until processed as described below.

NOS morphology stains: Paraformaldehyde fixed full thickness uterine samples were examined for the presence of NOS using a tetrazolium blue dye method described by Hope and Vincent (7). This method has been shown to be specific for localizing NOS (8, 9). Forty micron thick floating sections of the fixed tissue were incubated for 60 minutes at 37°C in the presence of 0.5mM nitro blue tetrazolium (NBT) dye and 1mM NADPH. The formation of NBT formazan product required the presence of NADPH.

Measurement of NOS enzyme activity: NOS enzyme activity was quantitated using the [^{3}H]-arginine to [^{3}H]-citrulline conversion assay.(10) Previously frozen minced rat uterus was homogenized using a Tissuemizer (Tekmar). Samples were suspended in a volume of 50 mM HEPES, 0.1mM EDTA, 1mM DTT, 1μM leupeptin, 1 μM peptastatin (pH 7.5) that was four times the tissue's wet weight in grams. All homogenization and protein separation steps were performed at 4°C. Crude soluble and membranous subfractions were prepared from homogenates by differential centrifugation: The first centrifugation at 1000g for 20 minutes, followed by centrifugation of the supernatant at 30,000g for 20 minutes. In all preparations, the supernatant (soluble fraction) was decanted from the pellet (membranous fraction). Pellets were then washed to remove residual soluble protein by resuspension in 5 ml of buffer and re-centrifugation. The final pellet was resuspended in 1 ml of buffer. In one experiment purified cytosolic and microsomal subfractions were prepared for the purpose of demonstrating the relative proportion of NOS activity in these two subfractions, and to compare this with the crude separations. Homogenates were centrifuged at 10,000g for 20 minutes and the resulting post-mitochondrial supernatant was subjected to ultracentrifugation at 105,000g for 60 minutes.

Samples of the cellular subfractions (50 to 100 ug protein) were incubated at 37°C for 45 minutes in the presence of 1mM NADPH, 14μM tetrahydrobiopterin, 5μM FAD, 1mM EGTA, 1mM magnesium, 5μM L-arginine and 15nM [^{3}H]-arginine (Specific activity: 77Ci/mmol). Calcium-sensitive NOS activity was determined by the addition of 3 mM CaCl2 (resulting in a total free calcium concentration of 2 mM), and 50 units bovine brain calmodulin (Calbiochem) to the above cofactors. Inhibition of NOS activity was tested by the addition of L-NMA, L-NA and aminoguanidine (0.5mM each) to the incubations. All reactions were stopped by dilution with ice cold stop buffer (50mM HEPES, pH 5.0) and labeled citrulline was separated from labeled arginine by ion exchange chromatography on 1 ml columns of Dowex 50W-X8 (Na form) resin. [^{3}H]-citrulline was quantitated by scintillation counting (Safety Solve, Research Products, Inc.). Total protein concentration was determined using a Coomassiereagent (Bio-Rad). Protein was dissolved in 1.5 N NaOH and bovine serum albumin was the standard. Enzyme activity is reported in pmol [^{3}H]-citrulline/mg protein/minute. Data are reported as means ± SE. One way

ANOVA was used to evaluate differences in enzyme activity at different times in gestation, and the 95% confidence level was used.

Results

NOS morphology within the pregnant, laboring and post partum rat uterus: In 16 day gravid rat uterine samples, NOS activity was most prominent in the decidua basalis and the endometrial-decidual interface. NOS was also seen in myometrial varicose nerve bundles, nerve fibers surrounding blood vessels and endothelial cells of arterioles and venules (Figure one). The distribution and density of NOS-containing nerve fibers varied by location throughout the gravid uterus: the greatest number of NOS positive nerve fibers being near the cervix and the least near the ovarian end of the uterine horn. In contrast the distribution of endothelial cell NOS activity appeared uniform throughout the gravid uterus. In uterine samples removed from laboring animals the intensity of NOS staining was greatly decreased in the decidua basalis remnant and myometrial nerve fibers (Figure two B). Blood vessel endothelial cells showed only a mild

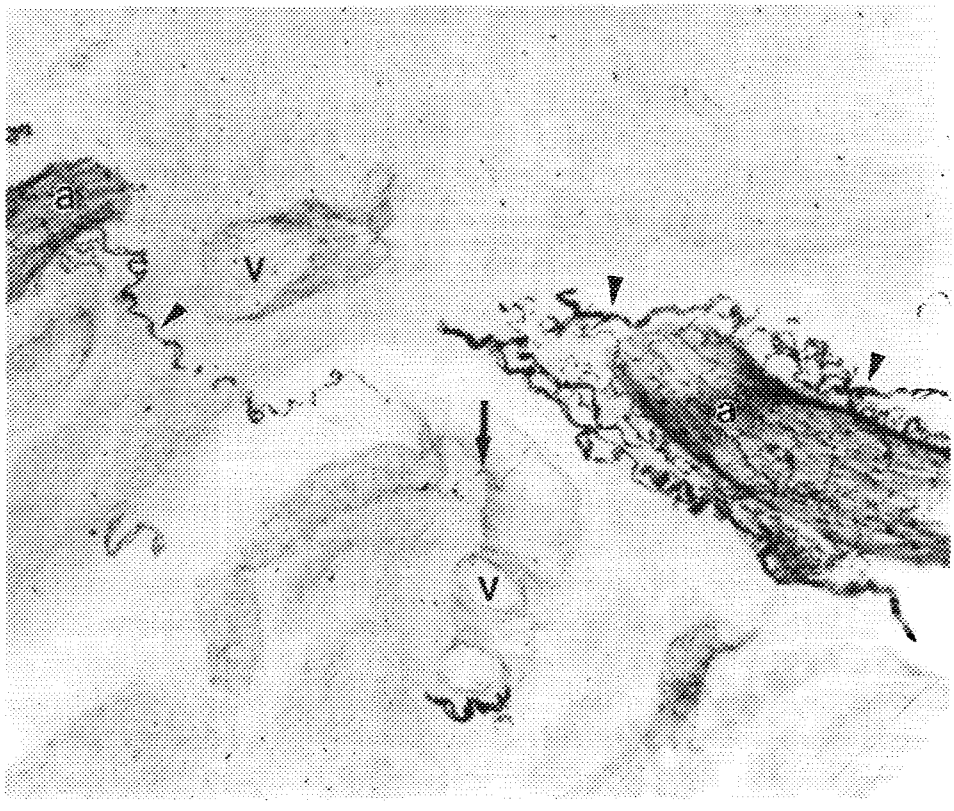

Figure 1. *Nerve fibers containing NOS in the gravid rat myometrium:* High magnification (x 320) of a 16 day gravid rat uterus, mid horn. Using the NADPH-diaphorase method small nerve bundles (arrowheads) and varicose individual nerve processes (arrows) are seen throughout the myometrium. In this section arterioles (a) are seen surrounded by an extensive network of NOS nerve fibers. Veins (v) demonstrate less intense endothelial cell staining and fewer NOS positive nerve processes as compared to arterioles.

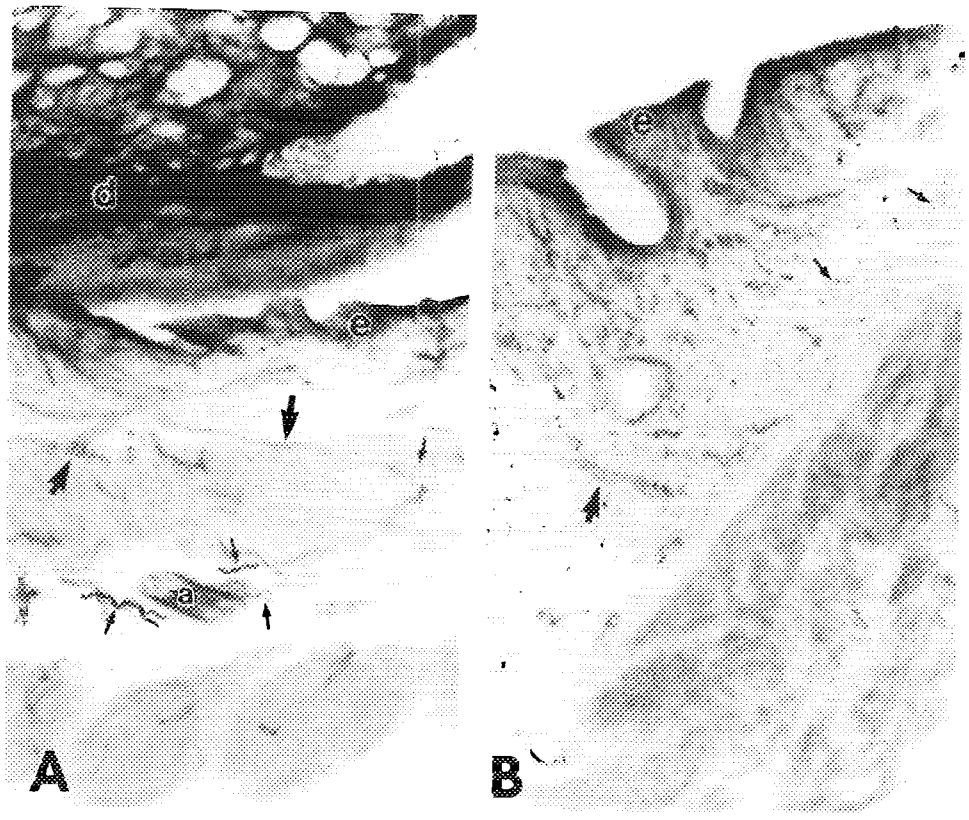

Figure 2A. *Gravid rat uterus:* Intense staining of the decidua (d) and the endometrial-decidual interface (e) as well as staining of endothelial cells of small arterioles (a) and other vessels (large arrows) scattered throughout the endometrium and myometrium. Intense staining is demonstrated in small nerve processes (small arrows) seen individually throughout the myometrium and surrounding blood vessels. Magnification x 160.
Figure 2B. *Laboring rat uterus:* Decreased NOS activity throughout the myometrium and endometrium. Endometrial lining (e) and nerve fibers (small arrows) stain weakly for NOS. Blood vessels (large arrows) demonstrate only a mild decrease in NOS staining. Magnification x 160.

decrease in staining when compared to pregnant tissue. Post partum uterus, removed 12 hours after delivery, had a staining pattern similar to that seen in the laboring uterus.

NOS enzyme activity during pregnancy, labor and post partum: NOS enzyme activity was present in crude soluble and membranous subfractions of uterine homogenates. The production of [^{3}H]-citrulline was linear with time for up to 60 minutes. Enzyme activity in the presence of L-NMA was 20% of the total uninhibited activity, L-NA was 26% of total and aminoguanidine was 34% of total (Inset figure three). Ultracentrifugation of the post-mitochondrial supernatant fraction to resolve cytoplasmic and microsomal uterine subfractions verified that NOS activities measured in the more crude subfractions consisted of both cytoplasmic as well as microsomal membranous isoforms of NOS.

Total NOS enzyme activity (Figure three) was highest in the preterm (16 day) pregnant uterus (2.7 pmols/mg/min ± 0.68) and declined significantly in term laboring (1.18

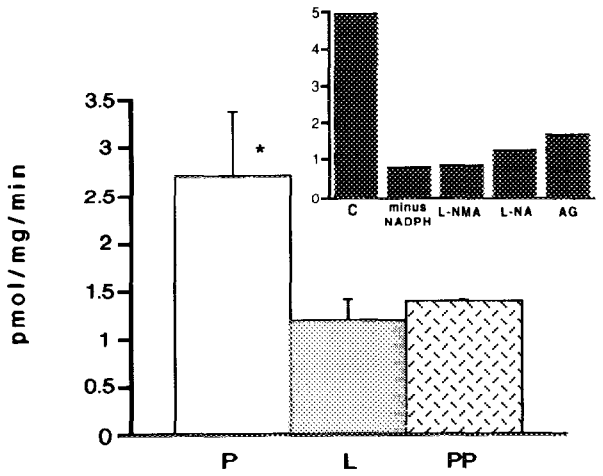

Figure 3. *Total NOS activity in pregnant, laboring and post partum rat uterus:* Enzyme activity was measured using the [^{3}H]-arginine to [^{3}H]-citrulline conversion assay described in the methods section. The decrease in NOS activity measured in pregnant tissue (N=5) was significant (*p<0.05) when compared to that measured in laboring and post partum uterine tissue. *Inset:* inhibition of NOS activity by L-arginine analogs. Addition of 0.5mM L-NMA, L-NA or aminoguanidine to enzyme preparations decreased NOS activity to 20, 26, 34%, respectively, when compared to that of the control activity.

pmols/mg/min ± 0.22) or post partum tissue (1.4 pmols/mg/min ± 0.13). In both subfractions, NOS activity could be increased by the addition of calcium and calmodulin. Figure four shows enzyme activity in both the cytosolic sub fraction (A) and the membrane bound subfraction (B) at each time point. The greatest portion of the total enzyme activity was measured in the

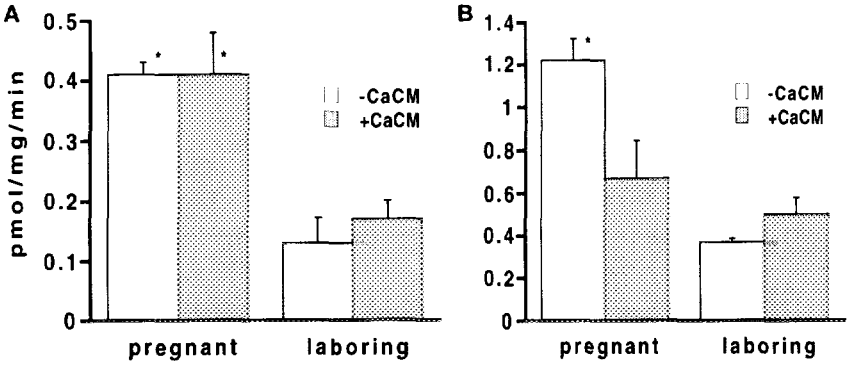

Figure 4A. *NOS activity found in the cytosol:* NOS activity (N=5) in the cytosolic subfraction was measured independently of calcium and calmodulin (-CaCM in white). Activity decreased significantly from pregnancy to labor (*). NOS activity measured in the presence of 3mM calcium and 50U calmodulin (+CaCM in gray) represents additional activity that is dependent on the presence of calcium. The decrease from pregnancy to labor in this group was also significant (*). *p<0.05.

Figure 4B. *NOS activity found to be membrane bound:* NOS activity (N=5) in the particulate subfraction was measured in the absence (-CaCM in white) and presence of (+CaCM in gray) 3mM calcium and 50U calmodulin. The decrease in NOS activity in the -CaCM group was significant (*), while NOS activity that was dependent upon the presence of calcium and calmodulin did not change significantly from pregnant to laboring tissue. *p<0.05.

membrane bound sub fraction; pregnant 70%, laboring 75% and post partum 74%, with significant decreases in NOS activity from pregnant to laboring samples (54%). The portion of total enzyme activity found in the cytosol was less than that measured in the membrane bound subfraction; 30%, 25% and 26% respectively. However, as was seen in the membrane bound activity the total cytosolic activity exhibited a significant decrease in NOS activity from pregnant to laboring samples (63%). Table one summarizes the uterine NOS activities in pregnant and laboring uterine tissue based upon location in the cell and dependence on calcium. The reduction in NOS activity from pregnancy to labor was significant ($p<0.05$) in the calcium-independent form of the enzyme present in both cytosolic and membranous subfractions, and in the calcium-stimulated activity present in the cytosol subfraction. Calcium-stimulated activity present in the membranous fraction decreased but was not statistically significant.

Discussion

This study demonstrates by histochemical and biochemical assays the presence of NOS activity in the pregnant rat uterus and a significant decline in this enzyme activity at term. The total NOS activity is reduced by nearly 70% in laboring rat uterus, and is consistent with the decreased NOS staining of laboring uterus nerves and decidua basalis. The reduced capacity for endogenous NO production in laboring tissue, therefore, occurs in a manner consistent with a causal role in the increased uterine contractions seen at parturtition, although no relation can yet be shown.

Histochemical studies of NOS activity in the pregnant uterus demonstrate the presence of high enzyme concentrations in uterine vasculature, nerves and decidua, while its presence in the myometrium is inconclusive. The presence of NOS in multiple uterine structures is consistent with previous reports of NOS in other tissues: vascular endothelial cells (11), and the myenteric plexus of the gut wall (12, 13) The presence of NOS in these different structures suggests the probable presence of multiple molecular forms of NOS in the uterus. NOS isoforms are defined

Table 1

Decrease in NOS Activity from Pregnancy to Labor

Location	Calcium Dependence	% decrease in activity
cytosol	-	68% *
cytosol	+	59% *
membrane	-	69% *
membrane	+	25%

* $p<0.05$ by one way ANOVA.

by their molecular weight, location within the cytosolic or membrane bound compartments of the cell, sensitivity to stimulation by calcium and calmodulin; and constitutive verses inducible regulation of enzyme activity.(14, 15) The enzyme characterization data presented in this paper suggests there may be as many as four isoforms present in the uterus and that changes in the activity in certain NOS isoforms from pregnancy to parturition could facilitate labor. Inhibition of NOS activity by the arginine analogs; L-NMA and LNA, and aminoguanidine confirm the activity measured is NOS.

The NOS activities that underwent the greatest decline between the quiescent and laboring state of pregnancy were the calcium-independent activities present in the cytosol and membrane particulate subfractions. These activities correspond to isoforms that have been found in macrophages. Calcium-independent membrane-bound NOS activity has been demonstrated in macrophages (14) but a cDNA has not as yet been isolated. The isozyme whose cDNA sequence has been isolated and expressed from macrophages is located in the cytosol (16). The activity of these two "macrophage-like" isoforms measured in pregnant and laboring uterine samples decreased by 69% in the soluble subfraction and 68% in membrane bound subfraction, suggesting that macrophages could play a role in NO mediation of uterine quiescence during pregnancy and uterine contraction at parturition. It is known that the decidua basalis is rich in macrophages. De and Wood have demonstrated an increase in the number of macrophages in the mouse decidua basalis during pregnancy and a subsequent regression in the number of these cells at term (17).

The calcium-sensitive NOS activities found in the uterine cytosolic and membrane bound subfractions could correspond to isoforms whose cDNAs were previously isolated and verified from neurons found in the rat CNS (18) and bovine aortic endothelial cells, respectively (19). The calcium-sensitive cytosolic enzyme activity has been found in neural tissue by Bredt and Snyder (20, 21). This NOS activity, significantly decreased when the uterus was actively contracting, may correspond to the NOS demonstrated in nerve fibers and varicosities. These nerve fibers stain avidly for NOS during pregnancy but lack NOS staining at term and may reflect down regulation of NOS production in the nerve or degeneration of the nerve itself.

Membrane bound calcium-dependent NOS activity was not significantly decreased from pregnancy to labor and probably corresponds to the consistent staining of endothelial NOS activity in pregnant and laboring tissue. It is not possible to draw absolute conclusions regarding the relation between enzyme activities and the structures staining positively for NOS until the enzyme activity present in each tissue structure can be directly evaluated and compared via molecular weight and cDNA sequencing.

In summary, we find that uterine NOS activity is markedly higher in pre-term pregnant uterus than at or after parturition. Taken together with our previous observation that exogenous NO relaxes uterine smooth muscle and abates preterm labor in the rhesus monkey, these studies suggest that NO could play a key role in the maintenance of the uterine quiescence during pregnancy and that reduced capacity for its production could promote the initiation of labor at term. Further studies are needed to determine whether NO serves a critical role in any of these processes.

Acknowledgments

The authors greatly appreciated the technical assistance provided by Margaret Mayes and the advice provided by Dr. Michael Heyman.

This work was supported by National Institute of Health Grants: HD26152 and 5T32 DKO7573, and a grant from the UCSF Academic Senate Committee on Research.

References

1. M. Palacios, R. Knowles, R. Palmer and S. Moncada. (1989) Biochemical Biophysical Research Communications. 165, 802-809.
2. A. Burnett, C. Lowenstein, D. Bredt, T. Chang and S. Snyder. (1992) Science. 257, 401-403.
3. L. Ignarro. (1991) The Western Journal of Medicine. 154, 51-62.
4. R. M. J. Palmer, D. S. Ashton and S. Moncada. (1988) Nature. 333, 664-666.
5. L. Ignarro. (1990) Annual Review of Pharmacology and Toxicology. 30, 535-560.
6. R. A. Word, M. L. Casey, K. E. Kamm and J. T. Stull. (1991) American Journal of Physiology. 260, C861-7.
7. B. T. Hope and S. T. Vincent. (1989) Journal of Histochemistry and Cytochemistry. 37, 653-661.
8. B. Hope, G. Michael, K. Knigge and S. Vincent. (1991) Proceedings of the National Academy of Sciences. 88, 7797-7801.
9. T. M. Dawson, D. S. Bredt, P. M. Fotuhi, P. M. Hwang and S. H. Snyder. (1991) Proceedings of the National Academy of Sciences. 88, 7797-7801.
10. P. Bush, N. Gonzales and L. Ignarro. (1992) Biochemical and Biophysical Research Communications. 186, 308-314.
11. J. Edwards, L. Ignarro, A. Hyman and P. Kadowitz. (1984) Journal of Pharmacology and Experimental Therapeutics. 228, 33-42.
12. D. Bredt, S. Hwang and S. Snyder. (1990) Nature. 347, 768-770.
13. Z. Grozdanovic, H. Baumgarten and G. Bruning. (1992) Neuroscience. 48, 225-235.
14. U. Forstermann and H. Schmidt. (1991) Biochemical Pharmacology. 42, 1849-1857.
15. D. Stuehr and O. Griffith. (1992) Advances in Enzymology and Related Areas of Molecular Biology. 65, 286-346.
16. C. Lyons, G. Orloff and J. Cunningham. (1992) Journal of Biological Chemistry. 267, 6370-6374.
17. M. De and G. Wood. (1991) Journal of Leukocyte Biology. 50, 381-392.
18. D. S. Bredt and S. H. Snyder. (1990) Proceedings of the National Academy of Sciences. 87, 682-685.
19. U. Forstermann, J. S. Pollock, H. W. Schmidt, M. Heller and F. Murad. (1991) Proceedings of the National Academy of Sciences. 88, 1788-1792.
20. S. H. Snyder and D. S. Bredt. (1992) Scientific American. 266, 68-77.
21. S. Vincent and H. Kimura. (1992) Neuroscience. 46, 755-784.