Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5212–5216, May 1994
Physiology

Induction of calcium-dependent nitric oxide synthases by sex hormones

(estrogen/testosterone/progesterone/pregnancy/tamoxifen)

CARL P. WEINER*, IGNACIO LIZASOAIN†, SALLY A. BAYLIS, RICHARD G. KNOWLES, IAN G. CHARLES, AND SALVADOR MONCADA

The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom

Communicated by Robert F. Furchgott, January 27, 1994 (received for review August 17, 1993)

ABSTRACT **We have examined the effects of pregnancy and sex hormones on calcium-dependent and calcium-independent nitric oxide synthases (NOSs) in the guinea pig. Pregnancy (near term) caused a >4-fold increase in the activity of calcium-dependent NOS in the uterine artery and at least a doubling in the heart, kidney, skeletal muscle, esophagus, and cerebellum. The increase in NOS activity in the cerebellum during pregnancy was inhibited by the estrogen-receptor antagonist tamoxifen. Treatment with estradiol (but not progesterone) also increased calcium-dependent NOS activity in the tissues examined from both females and males. Testosterone increased calcium-dependent NOS only in the cerebellum. No significant change in calcium-independent NOS activity was observed either during pregnancy or after the administration of any sex hormone. Both pregnancy and estradiol treatment increased the amount of mRNAs for NOS isozymes eNOS and nNOS in skeletal muscle, suggesting that the increases in NOS activity result from enzyme induction. Thus both eNOS and nNOS are subject to regulation by estrogen, an action that could explain some of the changes that occur during pregnancy and some gender differences in physiology and pathophysiology.**

Nitric oxide (NO) synthases (NOSs) constitute a family of isozymes that catalyze the oxidation of L-arginine to NO and citrulline. First identified in the vascular endothelium (1, 2), NO synthesis has subsequently been shown to play important roles in the regulation of vascular and gastrointestinal tone, in cell-mediated cytotoxicity against bacteria and tumors, and in a variety of central and peripheral nervous system activities (for review, see ref. 3). NOSs can be divided into two functional classes based on their sensitivity to calcium (3). The cytokine- or bacterial product-inducible isoenzyme iNOS binds calmodulin tightly at resting intracellular calcium concentrations. The constitutive forms, isozymes eNOS (originally described in endothelial cells) and nNOS (originally described in neuronal tissue), bind calmodulin in a reversible and calcium-dependent fashion. The mechanisms by which their synthesis is controlled are unknown.

The cDNA species encoding the rat, mouse, and human nNOS, the human and bovine eNOS, and iNOS from several species and cell types have been cloned and sequenced (4–17). The three human isozymes characterized to date are distinct, with their deduced protein sequences showing only 50–60% amino acid identity. nNOS, which in rats and humans localizes to neurons in the central and peripheral nervous system (18) and colocalizes with NADPH-diaphorase activity (19), has also been shown to be widely distributed in several nonneuronal tissues including human skeletal muscle (5, 18).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Several observations have suggested that NOS might be regulated by sex hormones. (*i*) Both gastrointestinal tone (for review, see ref. 20) and vascular tone (21) are reduced during pregnancy when the concentrations of estrogen and progesterone are high. (*ii*) The administration of estradiol increases endothelium-dependent relaxations (22, 23). (*iii*) Both the stimulated and basal release of NO from uterine artery of the guinea pig are increased at the end of pregnancy (24, 25), while relaxation of uterine artery by an NO donor (sodium nitroprusside) is reduced (22). (*iv*) Both the urinary excretion and plasma levels of nitrate, the stable metabolite of NO, are elevated in pregnant rats (26). (*v*) The aorta of female rabbits has a higher basal release of NO than does the aorta of male rabbits, and castration eliminates the difference (27). (*vi*) Premenopausal women are less prone than men to coronary artery disease (for review, see ref. 28). This protection is lost with the onset of menopause or after surgical castration. This observation could be linked to reports that NO might slow the development of atherosclerosis by inhibiting the proliferation of smooth muscle cells while stimulating endothelial cell proliferation (29, 30).

We therefore decided to investigate the hypothesis that sex hormones could regulate constitutive NOSs. We tested this in female and male guinea pigs by determining the effect of pregnancy on NOS activity and by measuring both NOS activity and NOS mRNA before and after sex hormone therapy.

MATERIALS AND METHODS

Hartley guinea pigs of similar chronological age were obtained from a commercial breeder (Charles River Breeding Laboratories): males and nonpregnant females (600–700 g) and pregnant females of >50 days gestation (63 days is full term) were used. Organs and tissues were removed under terminal anesthesia (pentobarbitone, 120 mg/kg i.p.) with the animal breathing spontaneously. These were immediately freeze-clamped and stored at −70°C until studied. Because of their small size, the uterine arteries of five animals were pooled for each assay. Each set of experiments included a separate group of controls randomly selected from the same batch of animals and matched for weight.

Sex Hormone Therapy. The serum concentration of estrogen increases in excess of 20-fold during pregnancy between the nadir at implantation (day 7–8 after copulation) and day 12 (31, 32). To mimic this sustained and rapid increase, gonadal-intact nonpregnant animals were randomly allocated to receive 17β-estradiol acetate (500 μg/kg i.p.) daily for 5

Abbreviations: NO, nitric oxide; NOS, NO synthase.

*Permanent address: Department of Obstetrics and Gynecology, Division of Maternal/Fetal Medicine, Perinatal Research Laboratory, University of Iowa College of Medicine, Iowa City, IA 52242.
†Permanent address: Departamento de Farmacologia, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain.

days (33). The effect of treatment with progesterone (5 mg/kg s.c.) or testosterone (1 mg/kg s.c.) daily for 5 days was also determined. Tissues were removed 12 h after the fifth injection and rapidly frozen. Estradiol was dissolved in absolute alcohol and dispersed in polyethylene glycol (M_r, 400) and double-distilled water (1 ml, 4.5 ml, and 4.5 ml, respectively). Testosterone and progesterone were suspended in peanut oil. Any animal with evidence of intraabdominal infection at the time of organ removal was excluded from study. Gonadal-intact males were also randomly assigned to receive the same dose of estradiol for either 5 or 10 days.

Estrogen Receptor Blockade. To test the role of estradiol on NOS activity during pregnancy, animals at full term received four doses of the estrogen-receptor low-efficacy partial agonist tamoxifen (250 μg i.p. twice a day) and their brains were removed 6 h after the last injection. Animals at the same gestational age were used as controls. Although tamoxifen may have both agonist and antagonist activities in different tissues, in the brain it acts predominantly as an antagonist (34).

NOS Activity. The frozen tissue was homogenized (with an Ystral homogenizer) at 0°C in 3 vol (5 vol for the brain) of buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, phenylmethylsulfonyl fluoride (100 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (100 μg/ml), and aprotinin (2 μg/ml) brought to pH 7.0 at 20°C with HCl. The crude homogenate was then centrifuged at 0°C at 12,000 × *g* for 20 min, the pellet was discarded, and the postmitochondrial supernatant was placed on ice. NOS activity was determined within 1 h of preparation by measuring in duplicate the conversion of L-[U-^{14}C]arginine to [U-^{14}C]-citrulline, as described in detail (35). L-Valine (50 mM) was added to the reaction buffer to minimize any interference from arginase. The total activity was determined as the difference between the [^{14}C]citrulline produced from control samples and samples containing both 1 mM EGTA to bind calcium and 2 mM N^{ω}-monomethyl-L-arginine, an inhibitor of NOS. Calcium-independent activity was determined as the difference between samples containing 1 mM EGTA and samples containing both 1 mM EGTA and 2 mM N^{ω}-monomethyl-L-arginine. Calcium-dependent activity was calculated by subtracting calcium-independent activity from total activity. Intra- and interassay variations were each <8%.

Cloning of Guinea Pig cDNAs Encoding Partial nNOS and eNOS Sequences. The cDNA sequences for either guinea pig nNOS or eNOS, to our knowledge, have not been reported. We amplified fragments of NOS cDNA from reverse-transcribed guinea pig skeletal muscle poly(A)$^+$ mRNA by using the PCR and primers based on the rat brain (5′-TGTGATGAATTCTGCGTGGGGGATGACGTCAA-CATC-3′ and 5′-GACGGGAAGCTTTCCAGCACCTC-CACCATTGTGGGGTTCT-3′) and the human endothelial (5′-CAAGTTCCCTCGTGTGAAGAACTG-3′ and 5′-GGAGCTGTAGTACTGGTTGATGAAGTC-3′) NOS sequences. PCR was carried out using a GeneAmp reverse transcriptase PCR kit (Perkin–Elmer/Cetus) under the following conditions: 95°C, 35 sec; 56°C, 2 min; 72°C, 2 min for 35 cycles.

The PCR product of the eNOS primers was ≈212 bp and of the nNOS primers was ≈630 bp. Both fragments were purified by agarose gel electrophoresis and cloned into pT7 Blue (Novagen) by using standard procedures (36). The identities of the cloned PCR products were confirmed as encoding eNOS and nNOS, respectively, by DNA sequencing (37) using T7 DNA polymerase (38), which revealed >90% identity with the published sequences from rat and human cDNAs (4–9).

Northern Blot Analysis. In preliminary experiments we observed that, of the tissues studied, the guinea pig skeletal muscle showed relatively high expression of both nNOS and eNOS mRNA. Others have also observed high expression of nNOS mRNA in human skeletal muscle (5). Because of this, all the following experiments were carried out using mRNA extracted from this tissue. Poly(A)$^+$ mRNA was isolated by oligo[d(T)] chromatography (Invitrogen), fractionated on formaldehyde/agarose gels, and transferred to nylon membranes (36). Blots were hybridized with either the guinea pig-specific eNOS or nNOS probes or a human β-actin cDNA probe. The blots were washed under stringent conditions with 0.5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.2)/0.1% SDS at 65°C and autoradiographed at −70°C.

Statistical Analyses. The results are presented as the mean ± SEM expressed as percent of the control or nonpregnant values. For example, the mean pregnant value was calculated as percent of the mean nonpregnant value, [mean (pregnant value)/mean (nonpregnant value)] × 100, and the SEM was similarly calculated as [SEM (pregnant value)/mean (nonpregnant value)] × 100.

The day of copulation was considered to be the first day of pregnancy. Student's *t* test and analysis of variance (ANOVA) were employed to assess statistical significances. If a significant *F* value resulted from an ANOVA, Scheffe's test for multiple comparisons was used to identify differences among groups. $P \leq 0.05$ was considered to indicate either a significant correlation or difference among means.

RESULTS

NOS Activity. *Pregnancy.* Pregnancy caused a >4-fold increase in the activity of calcium-dependent NOS activity in the uterine artery (Fig. 1). Significant increases in calcium-dependent NOS activity were also found in the heart, kidney, skeletal muscle (the vastus muscle group), esophagus, and cerebellum. Calcium-independent NOS activity was observed in several tissues, but on average it was <10% of the total activity and was not altered by pregnancy [e.g., kidney, pregnant 5 ± 3 (n = 21) vs. nonpregnant 4 ± 3 (n = 14) pmol of citrulline per min per g; skeletal muscle, pregnant, 10 ± 6 (n = 21) vs. nonpregnant 13 ± 4 (n = 14) pmol of citrulline per min per g].

Estrogen receptor antagonism during pregnancy. Tamoxifen reduced cerebellar calcium-dependent NOS activity in the pregnant animal to the activity level of the nonpregnant animal (Fig. 1).

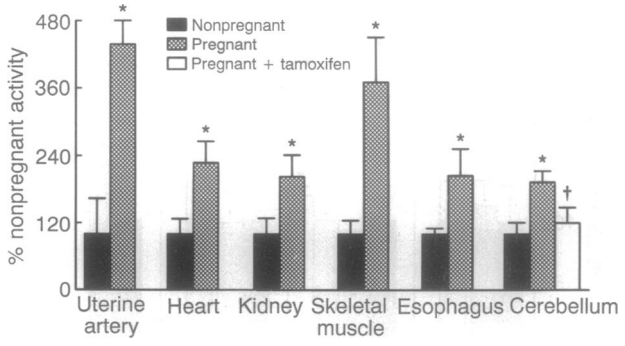

FIG. 1. Effect of pregnancy on calcium-dependent NOS activity as a percent of nonpregnant values (mean ± SEM) in various tissues obtained from near-term pregnant guinea pigs (n = 21). The nonpregnant activities were as follows (units = pmol of citrulline per min per g; n = 14): uterine artery, 31 ± 20; heart, 18 ± 5; kidney, 360 ± 61; skeletal muscle, 60 ± 16; esophagus, 306 ± 36; cerebellum, 8730 ± 1170. Pregnancy increased NOS in all tissues studied. Tamoxifen (250 μg/kg i.p. twice a day for a total of four doses) reduced cerebellar NOS to nonpregnant levels (n = 6). *, $P \leq 0.05$ from nonpregnant activity; †, $P \leq 0.05$ from pregnant activity.

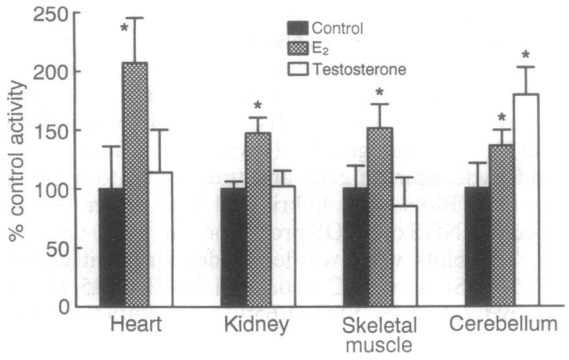

FIG. 2. Effect of treatment of nonpregnant female guinea pigs with 17β-estradiol (E_2; 500 μg/kg i.p. for 5 days) or testosterone (1 mg/kg s.c. for 5 days) on calcium-dependent NOS activity in tissues, illustrated as the percent of untreated values (mean ± SEM, four to six animals). The control activities were as follows (units = pmol of citrulline per min per g): heart, 10 ± 3; kidney, 963 ± 64; skeletal muscle, 89 ± 16; cerebellum, 8730 ± 1170. Testosterone increased NOS activity only in the cerebellum. *, $P \leq 0.05$ from control.

Sex hormone therapy. Estradiol ($n = 6$) significantly increased calcium-dependent NOS activity in the heart, kidney, skeletal muscle, and cerebellum, which were the four tissues selected for further study from the female guinea pig (Fig. 2). Progesterone caused no significant increases in NOS ($n = 5$, data not shown), whereas testosterone ($n = 4$) increased calcium-dependent NOS only in the cerebellum. In the same tissue extracts calcium-independent NOS activity in the female was unaltered by any sex hormone (data not shown).

NOS activity in the tissues obtained from male guinea pigs was not affected by a 5-day course of estradiol in any tissue studied except the cerebellum (Fig. 3). However, when the period of treatment with estradiol was extended to 10 days, calcium-dependent activity was also significantly increased in the heart, kidney, and skeletal muscle. Calcium-independent activity in the male was not altered by any sex hormone (data not shown).

NOS mRNA. Bands of 4.2-kb (eNOS) or 10-kb (nNOS) were identified using the guinea pig eNOS- and nNOS-specific probes on Northern blots (Fig. 4 *A* and *B*, each representative of two experiments involving a total of three to eight animals per group). Whereas in controls the 4.2-kb eNOS mRNA was present at the limit of detection and the 10-kb nNOS mRNA was present as a faint band, pregnancy increased both eNOS and nNOS mRNA in skeletal muscle. The 5-day course of estradiol also increased eNOS and nNOS mRNAs. A second band was sometimes seen in the nNOS Northern blots at ≈6 kb; this may be an alternatively spliced form of the nNOS mRNA, like that reported for murine nNOS (15), which does not code for an active NOS enzyme (39).

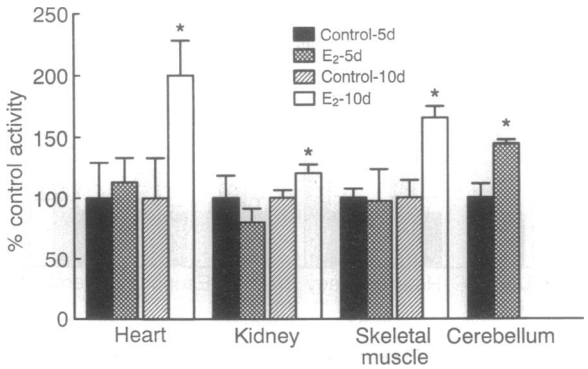

FIG. 3. Effect of treatment of male guinea pigs with 17β-estradiol [E_2; 500 μg/kg i.p. for either 5 or 10 days (d)] on calcium-dependent NOS activity in tissues, illustrated as the percent of untreated values (mean ± SEM, four to six animals). The 5-day control activities were as follows (units = pmol of citrulline per min per g): heart, 23 ± 7; kidney, 644 ± 124; skeletal muscle, 66 ± 5; cerebellum, 6210 ± 720. The 10-day controls were as follows: heart, 12 ± 4; kidney, 737 ± 64; skeletal muscle, 58 ± 8. *, $P \leq 0.05$ from control.

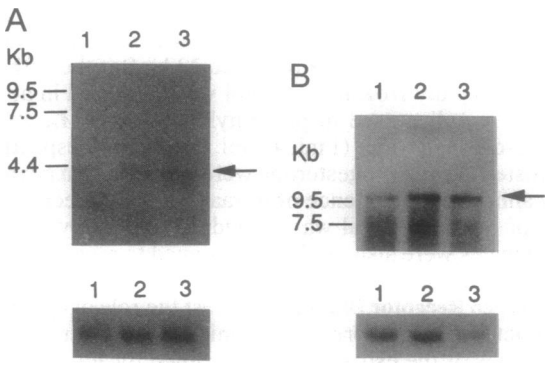

FIG. 4. (*A*) Northern blot containing equal loadings (1.4 μg) of poly(A)$^+$ mRNA extracted from skeletal muscle obtained from a nonpregnant female (lane 1), an estradiol-treated female (5 days of treatment, lane 2), and a pregnant female (lane 3) guinea pig performed using the cloned eNOS cDNA fragment as a probe. The eNOS probe was labeled with [α-^{32}P]dATP by random priming and hybridized overnight at 65°C. The blot was washed under high-stringency conditions (65°C, with a 0.5% SSC final wash) and autoradiographed at −70°C for 4 days. The arrow indicates the position of the eNOS band. Hybridization with the eNOS probe shows increased eNOS mRNA after estradiol treatment and during pregnancy compared to the nonpregnant animal. The same filter (stripped of the eNOS signal) probed with a β-actin fragment as an internal control to demonstrate equal RNA loading and transfer to the membrane in each track and is shown below. A repeat experiment with mRNA pooled from two or three animals from each treatment group gave a similar result. (*B*) Northern blot containing similar loadings (3.6 μg) of poly(A)$^+$ mRNA extracted from pooled skeletal muscle obtained from nonpregnant female (lane 1, $n = 7$), estradiol-treated female (5 days of treatment; lane 2, $n = 5$), and pregnant (lane 3, $n = 5$) guinea pigs performed using the cloned nNOS cDNA fragment as a probe. The nNOS probe was labeled with [α-^{32}P]dATP by random priming and hybridized with the membrane as described for *A*. The membrane was autoradiographed at −70°C for 12 days. An arrow indicates the position of the nNOS band. Control hybridizations of the same membrane with the β-actin probe (below) show that less mRNA had been loaded and transferred in the case of the mRNA from pregnant animals (lane 3). Despite this, there is clearly more nNOS mRNA in lanes 2 and 3 (from estradiol-treated and pregnant animals, respectively). A repeat experiment with mRNA from single animals from each treatment group gave a similar result.

DISCUSSION

Until now, it had been thought that both nNOS and eNOS were purely constitutive enzymes, although recent studies suggest eNOS may be induced by shear stress (40). Our studies demonstrate that these NOSs can be induced in several tissues during pregnancy and in nonpregnant female and male animals by estradiol and that in skeletal muscle it is accompanied by an increase in NOS-specific mRNA.

Evidence emerging from various laboratories shows that there is an increase in the release of NO from the vasculature during pregnancy (21, 24, 25). Our results are consistent with this and provide an explanation for these observations, for they clearly show that there was an increase in the calcium-dependent NOS in all tissues studied. Furthermore, treatment of pregnant animals at the end of gestation with tamoxifen reduced NOS activity in the cerebellum, an organ where tamoxifen acts as a pure estrogen-receptor antagonist (34). Thus, our results suggest that the increase in calcium-

dependent NOS activity during pregnancy is mediated by estrogen.

This conclusion is supported by the fact that treatment of nonpregnant females and male animals with estradiol also increased calcium-dependent NOS activity in all tissues studied. Interestingly, testosterone treatment also increased cerebellar NOS activity without affecting other tissues. The reason(s) for this remain to be investigated in detail; however, testosterone may increase brain NOS by directly binding estrogen receptors as has been reported (41). Furthermore, the cerebellum was the only tissue in the male to respond to a 5-day course of estradiol, suggesting that it may have a larger number and/or a greater availability of estrogen receptors than other tissues. In addition, the brain is rich in aromatase, which converts testosterone into estradiol (42). This, together with the observation that progesterone does not induce NOS, indicates that the induction of both nNOS and eNOS is specific for estrogen and not a characteristic of all sex steroids. These experiments do not exclude the possibility that the addition of progesterone might modify the estradiol effect.

Our results suggest that the increases in NOS activity are the result of augmented enzyme synthesis (enzyme induction) since they are accompanied by increases in the specific mRNAs for both eNOS and nNOS. It is not, however, possible to tell at this stage whether the increases in mRNA are caused by an upregulation of mRNA synthesis (transcriptional induction) or decreased mRNA breakdown.

Although calcium-dependent NOS activity was increased by estradiol in tissues obtained from both female and male guinea pigs, a longer duration of treatment was necessary in the male. The most likely explanation for this observation is that the number or availability of estrogen receptors is initially too low in most tissues of the male and requires a period of estrogen priming (43). Although other factors may play a role, the duration of exposure may well explain the observation that the effect of pregnancy on NOS-specific mRNA is greater than estradiol alone.

The observation that estradiol induces calcium-dependent NOSs has several important implications. An increase in release of NO from the endothelium would decrease vascular tone and contractility, events that are characteristic in pregnancy. Heterogeneity among tissue endothelium regarding the effects of estrogen on basal NO release could explain the selective redistribution of maternal cardiac output to organs important for a successful pregnancy. Consistent with this possibility is the observation that the effect of pregnancy on endothelium-derived NO is greatest in the uterine artery, followed by the mesenteric artery and then renal arteries (24, 25).

An alternative hypothesis to explain the adaptation of smooth muscle to pregnancy is that it is caused by prostacyclin (44). Prostacyclin is increased during pregnancy and contributes to the observed reduced contractility of the ovine uterine artery to angiotensin II (45). However, estradiol does not increase the synthesis of prostacyclin by the endothelium (46, 47), nor does inhibition of prostacyclin synthesis prevent the effects of pregnancy on smooth muscle (48, 49).

In addition, both the incidence of esophageal reflux and the gastrointestinal transit time are increased during pregnancy (21). Although this phenomenon has previously been attributed to a direct effect of progesterone (50, 51), NO is a powerful dilator of the gastrointestinal smooth muscle (52, 53). If the increase in NOS activity observed in the esophagus applies to the bowel, enhanced NO might be the mechanism underlying both increased esophageal reflux and transit time. The biological significance of an estradiol-dependent increase in the NOS in the central nervous system is of great interest and deserves further investigation. Furthermore, an estradiol-mediated increase in NOS in the vasculature could be the mechanism whereby premenopausal women are protected from coronary artery disease (28) since increased NOS may slow the development of atherosclerosis (54) and reduce the contractile response to acute thrombosis (55).

Finally, the induction of calcium-dependent NOS enzymes by estradiol suggests that the present classification of this family of enzymes into constitutive and inducible types needs to be revised (56), since eNOS and nNOS enzymes at least are both constitutive and inducible.

This work was sponsored in part by grants from the U.S. Public Health Service—HD22294 (C.P.W.) and HL49041 (C.P.W.)—and the Commission of the European Community (IS/ERB 4001GT921179).

1. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327**, 524–526.
2. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature (London)* **333**, 664–666.
3. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43**, 109–142.
4. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351**, 714–718.
5. Nakane, M., Schmidt, H. H. H. W., Pollock, J. S., Forstermann, U. & Murad, F. (1993) *FEBS Lett.* **316**, 175–180.
6. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. & Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6348–6352.
7. Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B. & Bloch, K. D. (1992) *J. Biol. Chem.* **267**, 14519–14522.
8. Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R. & Peach, M. J. (1992) *J. Biol. Chem.* **267**, 15274–15276.
9. Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S. & Michel, T. (1992) *FEBS Lett.* **307**, 287–293.
10. Lyons, C. R., Orloff, G. J. & Cunningham, J. M. (1992) *J. Biol. Chem.* **267**, 6370–6374.
11. Lowenstein, C. J., Glatt, C. S., Bredt, D. S. & Snyder, S. H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6711–6715.
12. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. (1992) *Science* **256**, 225–228.
13. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H. & Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3491–3495.
14. Charles, I. G., Palmer, R. M. J., Hickery, M. S., Bayliss, M. T., Chubb, A. P., Hall, V. S., Moss, D. W. & Moncada, S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11419–11423.
15. Ogura, T., Yokoyama, T., Fujisawa, H., Kurashima, Y. & Esumi, H. (1993) *Biochem. Biophys. Res. Commun.* **193**, 1014–1022.
16. Wood, E. R., Berger, H., Jr., Sherman, P. A. & Lapetina, E. G. (1993) *Biochem. Biophys. Res. Commun.* **191**, 767–774.
17. Nunokawa, Y., Ishida, N. & Tanaka, S. (1993) *Biochem. Biophys. Res. Commun.* **191**, 89–94.
18. Springall, D. R., Riveros-Moreno, V., Buttery, L., Suburo, A., Bishop, A. E., Merrett, M., Moncada, S. & Polak, J. M. (1992) *Histochemistry* **98**, 259–266.
19. Vincent, S. R. & Kimura, H. (1992) *Neuroscience* **46**, 755–784.
20. Everson, G. T. (1992) *Gastroenterol. Clin. North Am. USA* **21**, 751–776.
21. Naden, R. P. & Rosenfeld, C. R. (1985) *Am. J. Obstet. Gynecol.* **153**, 417–425.
22. Gisclard, V., Miller, V. M. & Vanhoutte, P. M. (1988) *J. Pharmacol. Exp. Ther.* **244**, 19–22.
23. Miller, V. M. & Vanhoutte, P. M. (1991) *Am. J. Physiol.* **261**, R1022–R1027.
24. Weiner, C. P., Martinez, E., Chestnut, D. H. & Ghodsi, A. (1989) *Am. J. Obstet. Gynecol.* **161**, 1605–1610.
25. Weiner, C. P., Zhu, L. K., Thompson, L., Herrig, J., Chestnut, D. H. & Ghodsi, A. (1991) *Am. J. Physiol.* **261**, H1275–H1283.
26. Conrad, K. P., Joffe, G. M., Kruszyna, H., Kruszyna, R., Rochelle, L. G., Smith, R. P., Chavez, J. E. & Mosher, M. D. (1993) *FASEB J.* **7**, 566–571.

27. Hyashi, T., Fukuto, J. M., Ignarro, L. J. & Chaudhuri, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11259–11263.
28. Stampfer, M. J. & Colditz, G. A. (1991) *Prev. Med.* **20**, 47–63.
29. Dubey, R. K. & Overbeck, H. W. (1993) *FASEB J.* **7**, 340 (abstr.).
30. Ziche, M., Morbidelli, L., Heppette, P., Amerini, S., Ledda, F. & Granger, H. J. (1993) *FASEB J.* **7**, 126 (abstr.).
31. Challis, J. R. G., Heap, R. B. & Illingworth, D. V. (1971) *J. Endocrinol.* **51**, 333–345.
32. Thapar, M., Kumari, G. L., Shrivastav, T. G. & Pandey, P. K. (1988) *Steroids* **52**, 85–108.
33. Bell, C. (1973) *Br. J. Pharmacol.* **49**, 595–601.
34. McKenna, S. E., Simon, N. G. & Cologer-Clifford, A. (1992) *Horm. Behav.* **26**, 536–544.
35. Salter, M., Knowles, R. G. & Moncada, S. (1991) *FEBS Lett.* **291**, 145–149.
36. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
37. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
38. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4767–4771.
39. Ogura, T., Yokoyama, T., Fujisawa, H., Kurashima, Y. & Esumi, H. (1994) *Proceedings of Third International Conference on the Biology of Nitric Oxide* eds. Moncada, S., Feelisch, M., Busse, R. & Higgs, A. (Portland, London), in press.
40. Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W. & Murphy, T. J. (1992) *J. Clin. Invest.* **90**, 2092–2096.
41. Westley, B. R. & Salaman, D. F. (1977) *Brain Res.* **119**, 375–388.
42. Mooradian, A. D., Morley, J. E. & Korenman, S. G. (1987) *Endocr. Rev.* **8**, 1–28.
43. Rosser, M., Chorich, L., Howard, E., Zamorano, P. & Mahesh, V. B. (1993) *Biol. Reprod.* **48**, 89–98.
44. Gerber, J. G., Payne, N. A., Murphy, R. C. & Nies, A. S. (1981) *J. Clin. Invest.* **67**, 632–636.
45. Magness, R. R., Rosenfeld, C. R., Faucher, D. J. & Mitchell, M. D. (1992) *Am. J. Physiol.* **263**, H188–H197.
46. David, M., Griesmacher, A. & Muller, M. M. (1989) *Prostaglandins* **38**, 431–438.
47. Steinleitner, A., Stanczytk, F. Z., Levin, J. H., d'Ablaing, G., III, Vijod, M. A., Shahbazian, V. L. & Lobo, R. A. (1989) *Am. J. Obstet. Gynecol.* **161**, 1677–1681.
48. Baylis, C. (1987) *Am. J. Physiol.* **253**, F158–F163.
49. Conrad, K. P. & Colpoys, M. C. (1986) *J. Clin. Invest.* **77**, 236–245.
50. Ryan, J. P. & Bhojwani, A. (1986) *Am. J. Physiol.* **251**, G46–G50.
51. Ryan, J. P. & Pellecchia, D. (1982) *Gastroenterology* **83**, 81–83.
52. Sanders, K. M. & Ward, S. M. (1992) *Am. J. Physiol.* **262**, G379–G392.
53. Calignano, A., Whittle, B. J. R., Di Rosa, M. & Moncada, S. (1992) *Eur. J. Pharmacol.* **229**, 273–276.
54. Cooke, J. P. & Tsao, P. (1992) *Curr. Opinion Cardiol.* **7**, 799–804.
55. Badimon, L., Badimon, J. J., Penny, W., Webster, M. W., Chesebro, J. H. & Fuster, V. (1992) *J. Hypertens. Suppl.* **10**, S43–S50.
56. Knowles, R. G. & Moncada, S. (1994) *Biochem. J.* **298**, 249–258.