Endocrine, *vol. 11, no. 2, 123–130, October 1999* 0969–711X/99/11:123–130/$12.00

Estrogen Downregulates Neuronal Nitric Oxide Synthase in Rat Anterior Pituitary Cells and GH_3 Tumors

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The anterior pituitary gland produces neuronal nitric oxide synthase (nNOS) and nitric oxide regulates secretion of various anterior pituitary hormones. Estrogen has many functions in anterior pituitary cells including stimulation of prolactin (PRL) cell proliferation and secretion of various anterior pituitary hormones. However, the role of estradiol-17β (E_2) in regulating pituitary nNOS expression has not been previously examined. We studied the regulation of nNOS in normal pituitaries, and neoplastic GH_3 pituitary tumors in order to analyze the effects of E_2 on nNOS in pituitary cells. GH_3 tumors expressed higher levels of nNOS proteins compared to normal pituitaries. Estrogen downregulated nNOS mRNA and protein in both estrogen-treated pituitaries with PRL cell hyperplasia and in GH_3 tumors implanted into the flank of rats treated with E_2 in silastic tubing. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated three alternatively spliced nNOS transcript isoforms — nNOSa, nNOSb, and nNOSc mRNAs — with distinct 5' untranslated first exons that arose from alternative splicing to a common second exon. All three spliced isoforms were found in the normal rat pituitary, whereas nNOSa and nNOSb, but not nNOSc, were expressed in GH_3 tumors implanted into Wistar-Furth rats. E_2 also downregulated the nNOSa alternative mRNA transcript isoform in vivo.

These results indicate that the biological activity of nNOS in the normal rat anterior pituitary and in pituitary tumors is regulated by a complex pattern of alternative splicing and that some of these mRNA isoforms as well as nNOS protein are regulated by estrogen. Our results also indicate that the levels of nNOS and the alternatively spliced nNOS transcript between normal and GH_3 pituitary tumors are different.

Received April 29, 1999; Revised July 2, 1999; Accepted July 7, 1999.

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Key Words: Neuronal nitric oxide synthase; pituitary; mRNA splicing; estrogen.

Introduction

Nitric oxide (NO), an ubiquitous messenger molecule, is synthesized by a family of nitric oxide synthases (NOSs) and participates in diverse cellular processes including neurotransmission, immune regulation, and vascular homeostasis. Three distinct NOS isoforms have been identified: neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS) *(1–3)*. These NOS isoforms have different physiological roles and differ in their tissue distribution. eNOS, which is present in endothelial cells, plays an important role in the control of vascular tone, whereas nNOS, which is present in brain and other tissues, regulates cGMP-mediated neurotransmission. iNOS has been identified in a wide variety of cell types including macrophages, mesangial cells, vascular smooth muscle cells, keratinocytes, chondrocytes, osteoclasts, and hepatocytes *(1–3)*. The role of nNOS in cell regulation has become increasingly important because many diverse biological actions of this molecule have been observed in endocrine cells including the pituitary gland *(4–7)*. NO modulates the secretion of various hormones in the pituitary gland and hypothalamus including corticotropin-releasing hormone *(8,9)*, luteinizing hormone–releasing hormone *(10–11)*, adrenocorticotropin (ACTH) *(7)*, growth hormone (GH) *(12)*, luteinizing hormone *(7,13)*, prolactin (PRL) *(14)*, oxytocin *(13,15)*, and vasopressin *(16–18)*.

Among the three isoforms of NOS, nNOS is responsible for most of NO production *(1–3)*, yet its regulation is not well understood. The genes for the three NOS enzymes have been cloned, and the predominant one expressed in the pituitary is nNOS *(4–7)*. Various studies indicate that NOS may regulate hormone release in specific pituitary cell types *(8–14)*. Estrogen modulates both cell proliferation and synthesis in pituitary lactotrophs *(19)*. The activity of nNOS was found to be downregulated by estrogen in rabbit uterus and vagina *(20)*. Furthermore, transient changes in the levels of expression of nNOS have been observed in the developing rat nervous system and lung

(3,21). However, a possible role of estrogen in regulating pituitary nNOS has not been previously examined.

Recently, different alternatively spliced nNOS transcripts arising from two alternative promoters that differed in their 5'-untranslated regions (5'UTR) have been observed in the human, murine, and rat nNOS genes *(22–25)*. One study demonstrated the existence of at least three transcripts of the rat nNOS gene designated nNOSa, nNOSb, and nNOSc, with distinct 5'-untranslated first exons that arose from alternative splicing to a common second exon *(24,25)*.

A few studies have examined nNOS expression in rat, mouse, and human pituitary tissues *(4,7)*, but little is known about the expression of various nNOS gene transcripts in the rat pituitary. In the present study, we examined the expression of nNOS and the three alternatively spliced nNOS variant mRNAs, as well as specific expression patterns in normal and neoplastic rat anterior pituitary cells, and observed that nNOS expression is modulated by estrogen and specific and complex regulatory mechanisms at the transcriptional level.

Results

nNOS mRNA and Protein Expression in Normal Pituitary and Tumor Cell Lines

Normal rat pituitary and various pituitary tumor cell lines, except for the αT_3-1 cells, all expressed nNOS mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using the primers and internal probes shown in Fig. 1. Various pituitary cell lines were used to determine the distribution of nNOS in different types of hormone-producing pituitary cells. GH_3 and GHRH-CL1 cells expressed higher levels of nNOS mRNA and protein compared to normal pituitary as demonstrated by both RT-PCR and Western blot. Other pituitary tumor cell lines including AtT20 and $L\beta T_2$ had relatively low levels of nNOS mRNA and protein. αT_3-1 did not have detectable mRNA for nNOS. Western blotting showed a 155-kDa band for nNOS in normal pituitary, GH_3, and GHRH-CL1. nNOS protein was not detected by Western blotting in the AtT20, $L\beta T_2$ and αT_3-1 cell lines. When the blot was restained with a monoclonal antibody (MAb) to β-actin to assess equal loading, a 42-kDa band for β-actin was detected (Fig. 2).

Downregulation of nNOS Expression in Estrogen-Treated Rat Pituitary and Implanted GH_3 Tumor

Rats were implanted with E_2 and 0.5×10^6 GH_3 cells at the same time. Tumor growth was monitored, and the rats were then sacrificed after 8 wk of treatment. E_2 treatment increased pituitary weight and decreased implanted GH_3 tumor weight without significant changes in body weights (Table 1). Immunohistochemical analysis (IHC) showed staining of 18 ± 2% cells in the normal control anterior pituitary with monoclonal antineuronal NOS antibody (Fig. 3A), whereas the nNOS-positive cells decreased to 7 ± 1% and showed less intense staining in E_2-induced pituitary hyperplasia (Fig. 3B). Colocalization studies with antibodies to nNOS and pituitary hormones showed nNOS mainly in gonadotroph and folliculo-stellate cells in the normal pituitary (data not shown). A small percentage of GH cells was also positive for nNOS. More than 90% of GH_3 implanted tumor cells were positive for nNOS, and there was a decrease in the intensity of the nNOS immunoreactivity in GH_3 cells after E_2 treatment (Fig. 3C,D). Semiquantitative RT-PCR with Southern hybridization and densitometric analysis showed that there was a significant decrease ($p < 0.01$) of nNOS mRNA in E_2-treated pituitaries compared to normal pituitaries in three separate expriments (Fig. 4). Immunobloting analysis showed a threefold decrease of nNOS protein in E_2-treated pituitaries compared to normal pituitaries (Fig. 4). GH_3 implanted tumors showed a slight decrease in nNOS by immunoblotting after E_2 treatment for 8 wk in vivo (Fig. 4).

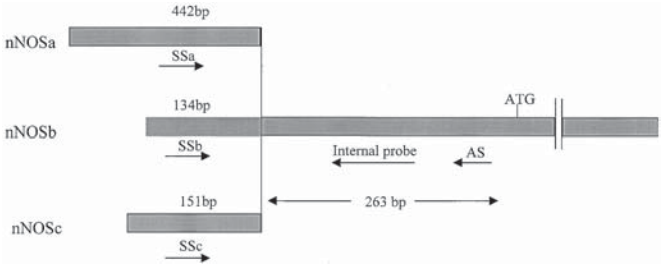


Fig. 1. Schematic presentation of alternative first exons in rat nNOS gene. nNOS cDNA sequence of the rat contained the translation initiation site (ATG). Unique sequences of the alternative 5'UTRs are indicated as nNOSa, nNOSb, and nNOSc. SSa, SSb, and SSc are sense primers for nNOSa, nNOSb, and nNOSc, respectively, and shared common antisense primer used for RT-PCR and internal probe for Southern hybridization with common to all nNOS spliced isoforms (GenBank database accession numbers: AF008911, AF008912, AF008913). Isoform-specific sense oligonucleotides for nNOSa (SSa 5'-AGCGGGATCCACA-GCCCTGGAACT-3'), nNOSb (SSb 5'-GACTGAGGGGCGA-CACTACCATGC-3'), and nNOSc (SSc 5'-CACCACA-GCCTCTGGAATGAAAGA-3'), respectively, were combined with the common antisense primer antisense (5'-GGCGTCATCTGCTCATTCCGATTC-3'). and internal probe (5'-TCAGGGGCAGCAACGGGATGTGTC-3'). The common form nNOS (GenBank accession no. X59949) sense was 5'-CCACCAACAAAGGGAATCTCA-3' (1694–1714); antisense was 5'-GAGTGGTGGTCAACGATGGTC-3' (2286-2306); and internal probe was 5'-CTTGGGGCTTT-CCAGCCCTGCTGTATACAG-3' (1860–1889).

Differential Expression of Rat nNOS Alternative Spliced Transcripts in Normal, E_2-Treated Pituitaries and GH_3 Tumors

Three different nNOS mRNA spliced isoforms — nNOSa, nNOSb, and nNOSc — were identified in the normal rat pituitary. RT-PCR with isoform-specific sense and shared common antisense primers located at various positions of the cDNA generated three specific nNOS spliced isoform products of 305, 311, and 281 bp for nNOSa,

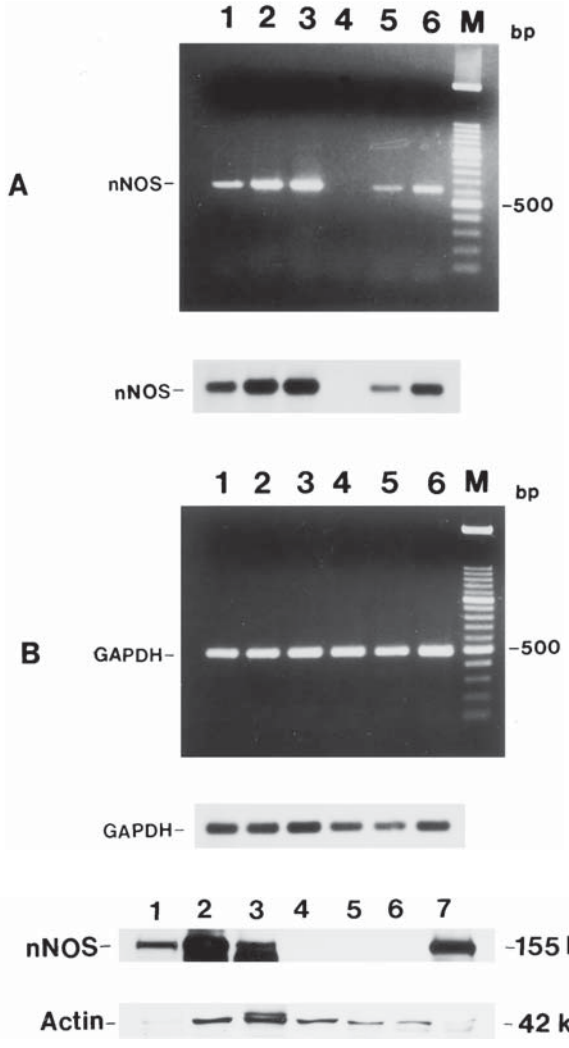


Fig. 2. (A) (Top) nNOS mRNA expression detected by RT-PCR (primers shown in Fig. 1) ethidium bromide–stained gel. Lane 1, normal pituitary; lane 2, GH_3 cells; lane 3, GHRH-CL1 cells; lane 4, αT_3-1 cells; lane 5, AtT20 cells; lane 6, $L\beta T_2$. M, molecular weight markers. nNOS PCR product size: 603 bp. **(Bottom)** Southern hybridization with internal probe. **(B) (Top)** GAPDH external control, 495-bp, ethidium bromide–stained gel; and **(bottom)** Southern hybridization. **(C)** Immunoblot analysis of normal and neoplastic pituitary cells. Proteins were subjected to one-dimensional sodium dodecyl sulfate (SDS)-electrophoresis followed by immunoblotting. Fifty micrograms of protein per lane were used. **(Top)** Immunoblotting with an MAb to nNOS (155 kDa): lane 1, normal pituitary; lane 2, GH_3 cells; lane 3, GHRH-CL1 cells; lane 4, αT_3-1 cells; lane 5, AtT20 cells; lane 6, LβT2; lane 7, human cerebral cortex standard. **(Bottom)** Same blot reimmunoblotted with an MAb to β-actin to assess equal loading of protein in the Western blot (42 kDa).

nNOSb, and nNOSc, respectively. PCR products were visualized by gel electrophoresis followed by Southern blotting with internal probes that hybridized to regions within the amplified sequences (Fig. 5). In addition, specific PCR products were cloned and sequenced to further confirm an nNOS spliced isoform sequence (data not shown). The most abundant spliced isoforms were found in the normal rat

Table 1
Effects of Estrogen Treatment on Pituitary and GH_3 Tumor Weights In Vivo[a]

	Pituitary weight (mg)	Body weight (g)	GH3 tumor weight (g)
Control	13.6 ± 0.29	227 ± 6	7.1 ± 3
Estrogen	46 ± 1.9[b]	240 ± 14	4.1 ± 0.7

[a] $n = 6$ animals per group in two separate experiments.
[b] $p < 0.01$.

pituitary, and abundant nNOSa and low levels of nNOSb, but not nNOSc, were detected in GH_3 implanted tumors. Lower levels of nNOSc mRNA compared to the other isoforms were present in normal rat pituitary (Fig. 5). Analysis by RT-PCR and Southern blotting of RNA from control pituitaries and estrogen-treated pituitaries showed a significant decrease in nNOSa in E_2-treated hyperplastic pituitaries compared to normal pituitaries. However, nNOSb and nNOSc levels were not changed by E_2 treatment (Fig. 5). A decrease of nNOSa, but not nNOSb, mRNA, was observed after E_2 treatment in GH_3 implanted tumors in vivo.

Discussion

These studies demonstrate that E_2 regulates pituitary nNOS protein and mRNA expression. In general, the levels of nNOS were higher in the GH- and PRL-producing pituitary tumor cell lines, GH_3 and GHRH-CL1, compared to normal pituitaries. In the ACTH-producing cell line, AtT20 and the gonadotroph cell line, $L\beta T_2$ and αT_3-1, the levels of nNOS were lower compared to normal pituitaries.

The low levels of nNOS in $L\beta T_2$ and αT_3-1 cells, unlike the gonadotroph cells in the intact pituitary, which have high levels of nNOS, may be related to the uniqueness of the simion virus-40 T-antigen targeted oncogenesis that was used to create these cell lines *(26)*. Although nNOS was localized mainly in rat gonadotroph and folliculo-stellate cells in this study and in a previous study *(7)*, other reports have shown an important role of NO in regulating hormone secretion from many types of anterior pituitary cells including GH *(12)* and PRL cells *(14)*. However, the effects of NO on GH and PRL secretion may be mediated by paracrine effects from folliculo-stellate and gonadotroph cells. Increased levels of expression of nNOS in tumors compared to normal cells have been reported by various investigators *(27,28)*. Although the significance of these finding is uncertain, they suggest an increase in metabolic activity along with the increase in nNOS levels in neoplasms compared to their normal tissues. The physiological significance of downregulation of nNOS in the pituitary after estrogen treatment is uncertain, but may be related to a decrease in PRL release in the presence of PRL cell hyperplasia resulting from estrogen treatment *(29)*.

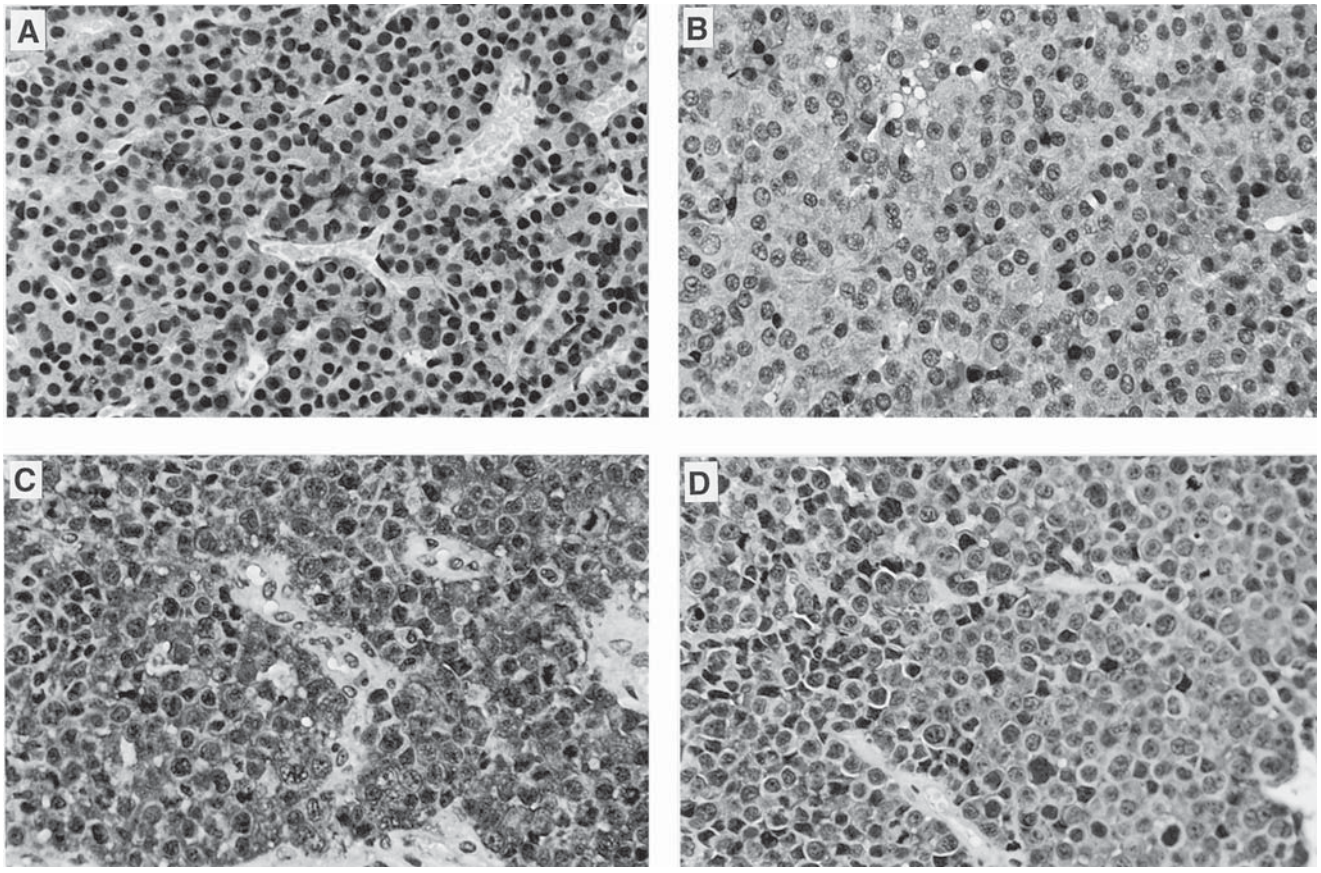


Fig. 3. Localization of neuronal NOS protein by immunohistochemistry in normal pituitaries (normal pituitary control group), E_2-treated pituitary, and GH_3 implanted tumors in Wistar-Furth rats. **(A)** Using a peroxidase-diaminobenzidine chromogen (brown cytoplasmic staining with blue nuclear staining with the hematoxylin counterstain), 18 ± 2% of anterior pituitary cells stained positively for nNOS. **(B)** nNOS-positive cells decreased to 7 ± 1% in E_2-treated pituitary secondary to PRL cell hyperplasia. Positive cells showed less intense staining. **(C)** Most cells in the GH_3 implanted tumors show strong positive immunoreactivity for nNOS (90%). **(D)** After E_2 treatment, there was less intense staining in individual cells. Similar results were obtained with four samples of pituitary and GH_3 cells.

Our studies show, for the first time, that nNOS in the rat anterior pituitary occurs in three isoforms characterized by differences in exon one *(24,25)*. The three isoforms of nNOS showed differential expression patterns in normal rat pituitary and GH_3 tumor cells. Our findings demonstrate that nNOS mRNA levels in normal and neoplastic rat pituitary are composed of different amounts of nNOS mRNA variants. The relative abundance of nNOS mRNA spliced isoforms in the normal rat pituitary was higher compared to GH_3 tumor cells, and the expression levels of nNOSc were much lower compared to the other two isoforms in normal rat pituitary.

The potential significance of the differential expression of the three nNOS isoforms may be related to various modes of regulatory control of pituitary cell function. Support for this possibility comes from our observation that normal pituitary cells had more abundant nNOS mRNA spliced isoforms compared to the neoplastic GH_3 cells, and tumors usually have decreased regulatory control compared to normal cells.

The newly identified first exons 1a, 1b, and 1c of the rat nNOS gene are located upstream of the coding sequence and, therefore, do not result in a modification of the encoded protein. Recently, two nNOS mRNAs with alternative 5'UTRs were identified in the mouse as well as humans *(22–25)*. Rat nNOSa shows a close homology to one of the mouse mRNA isoforms (nNOS); however, no similarity was detectable between nNOSb and nNOSc and the second 5'UTR (nNOS) in the murine nNOS gene *(24)*. Likewise, no homology was found among any of the three first exons in the rat and the two reported alternative exons in humans. This may simply reflect species-specific differences, or point to the presence of further yet to be identified alternative 5' ends in the rat that are homologous with the alternative first exon in mouse and the two human isoforms.

The present study showed that estrogen downregulates the alternative transcript isoforms and nNOS protein expression in E_2-treated pituitaries and E_2-treated implanted GH_3 tumor. E_2 treatment stimulates PRL cell hyperplasia *(29)*. Because PRL cells expressed very little nNOS in our study, the decreased levels of nNOS in hyperplastic pituitaries is most likely related to an absolute decrease in nNOS levels.

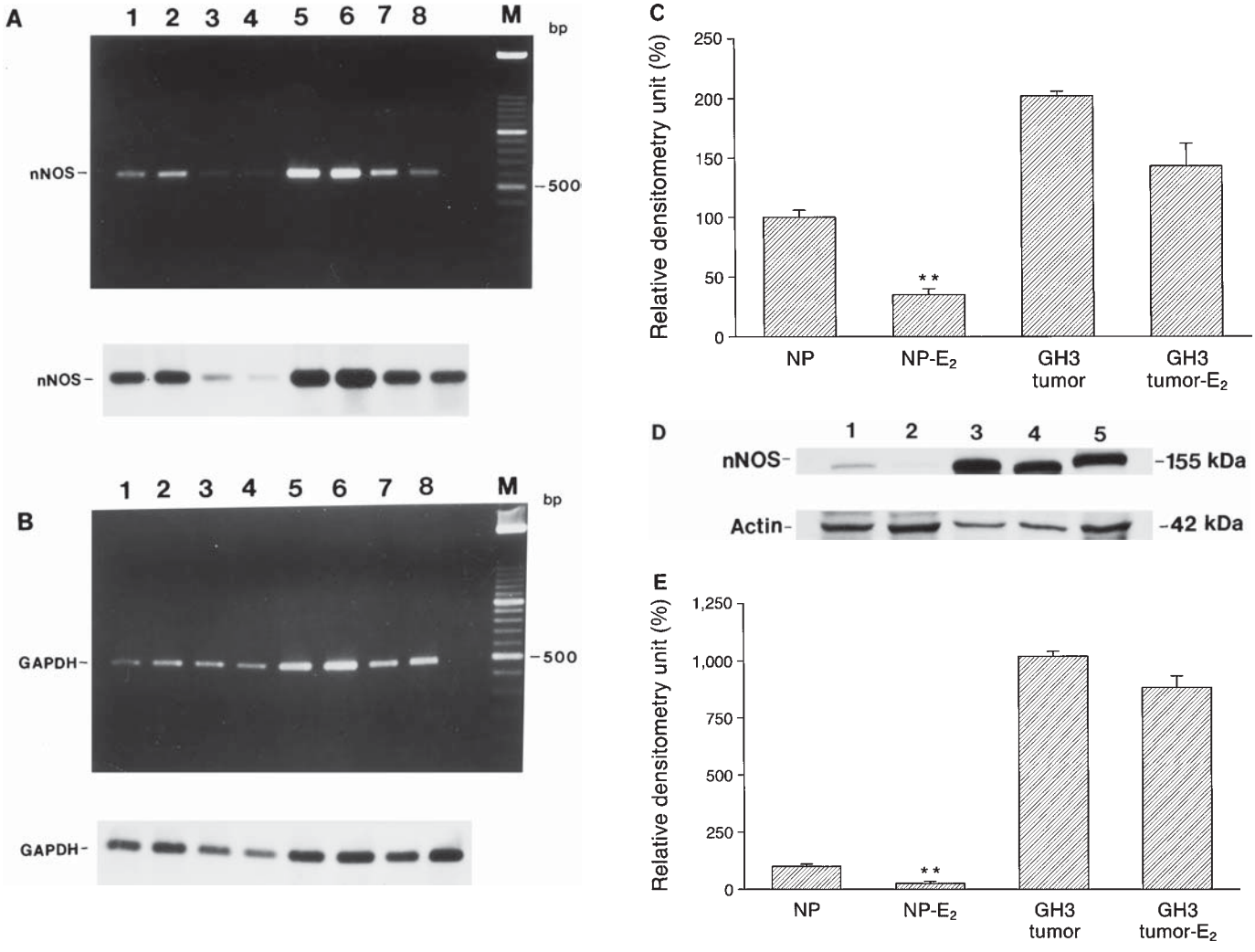
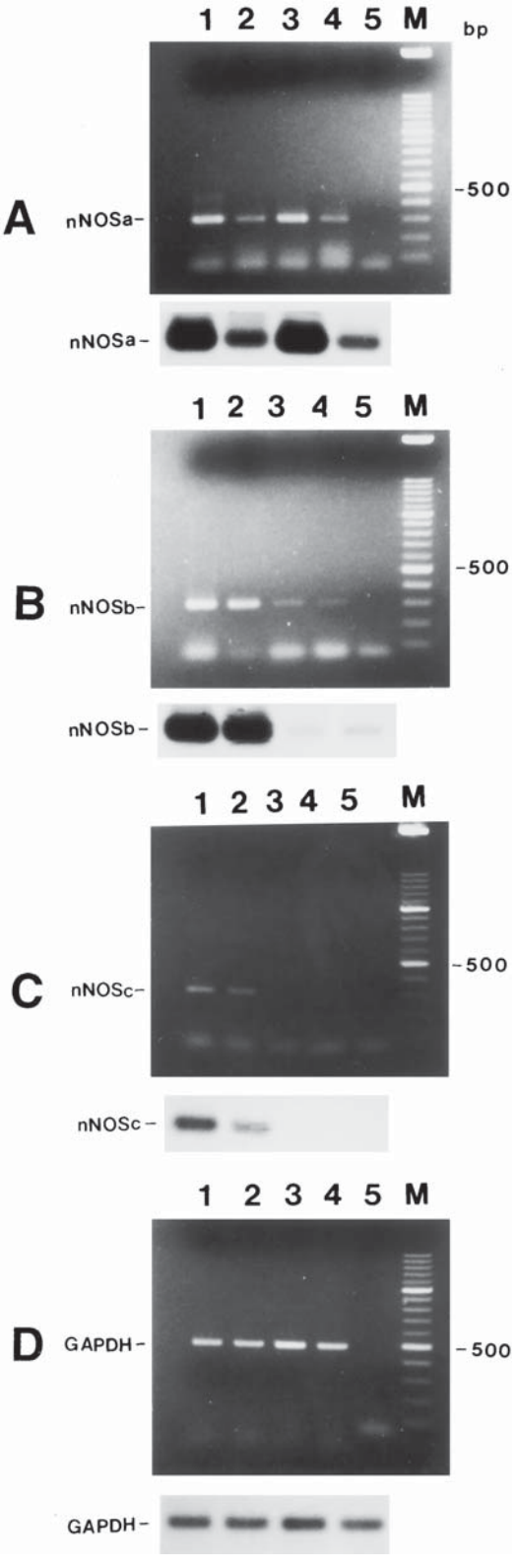


Fig. 4. The effect of estrogen on nNOS in pituitary and implanted GH_3 tumors analyzed by RT-PCR and Western blotting. **(A)** Ethidium bromide stained gel from RT-PCRand Southern hybridization. **(Top)** nNOS: lanes 1 and 2, control pituitary; lanes 3 and 4, estrogen-treated pituitary hyperplasia; lanes 5 and 6, control implanted GH_3 tumors; lanes 7 and 8, E_2-treated implanted GH_3 tumors; M, molecular weight markers. **(Bottom)** Southern hybridization with nNOS internal probe. **(B)** GAPDH ethidium bromide–stained gel **(top)** and Southern hybridization with GAPDH internal probe **(bottom)**. **(C)** Densitometric analysis of nNOS mRNA in pituitary and implanted GH_3 tumors (n = three separate experiments, $^{**}p < 0.01$). **(D)** Proteins were subjected to one-dimensional SDS-electrophoresis followed by immunoblotting. Fifty micrograms of protein per lane for pituitary and 10 μg of protein for GH_3 tumor were used. **(Top)** Immunoblotting with an MAb to brain NOS: lane 1, control pituitary; lane 2, estrogen treated hyperplasia pituitary; lane 3, control implanted GH_3 tumors; lane 4, E_2-treated implanted GH_3 tumors; lane 5, human cerebral cortex standard **(bottom)**. Same blot reimmunoblotted with a monoclonal antibody to β-actin to assess equivalent loading of protein in the Western blot. **(E)** Densitometric analysis of nNOS protein in pituitary and GH_3 tumors (n = three separate experiments $^{**}p < 0.01$).

The presence of much higher levels of nNOS in neoplastic GH_3 cells suggests an upregulation of nNOS during transformation from hyperplasia to neoplasia and is consistent with the reported increased levels of nNOS expression in tumors compared to normal cells *(26,27)*.

Tamoxifen has been reported to inhibit nNOS *(30)*, and estrogen was noted to increase the expression of nNOS mRNA through the rostrocaudal extent of the ventromedial nucleus of the hypothalamus *(31)*. By contrast, E_2 inhibited the induction of nNOS by a receptor-mediated system *(32,33)*. Although downregulation of cytosolic nNOS by estrogen was noted in the uterus and vagina *(20)*, a biphasic effect of estrogen on nNOS in rabbit cerebellum with a low concentration enhancing and a high concentration attenuating nNOS activity has been reported *(34)*. These studies highlight the variable regulatory effects of estrogens on nNOS.

In summary, in rat pituitary cells estrogen downregulates nNOS expression and various transcripts of nNOS show differential expression patterns in normal and neoplastic anterior pituitary tissues. These alternatively spliced nNOS variants may represent another mechanism for the regulatory modulation of nNOS expression.



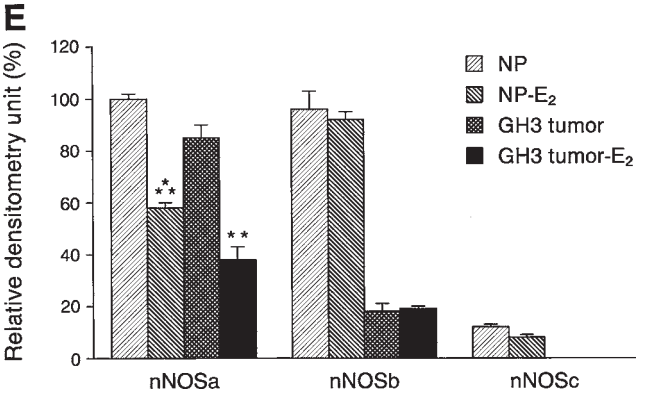


Fig. 5. Analysis of nNOS spliced isoforms by RT-PCR. Expression patterns of nNOSa, nNOSb, and nNOSc were examined using 2 μL of cDNA (equal to 200 ng of total RNA) from pituitary and GH_3 implanted tumors. Sense primers SSa, SSb, and SSc (specific for nNOSa, nNOSb, and nNOSc, respectively) were combined with common antisense (*see* Fig. 1). The sizes of the PCR fragments for nNOSa, nNOSb, and nNOSc were 305, 311, and 281 bp, respectively. A 495-bp fragment corresponds to GAPDH. Regulation of nNOS spliced isoforms in rat pituitary and GH_3 tumor cells by estrogen. Lane 1, control pituitary; lane 2, E_2 treated pituitary; lane 3, GH_3 tumor; lane 4, E_2-treated GH_3 tumor; lane 5, Negative control with omission of RTase. **(Top)** Ethidium bromide gel and **(bottom)** Southern hybridization for nNOSa **(A)**. nNOSb **(B)**, nNOSc **(C)**, and GAPDH **(D)**, respectively. **(E)** Densitometric analysis shows that estrogen significantly downregulates nNOSa, but not nNOSb and nNOSc in pituitary and GH_3 implanted tumor. $^{**}p < 0.01$, $^{***}p < 0.001$); n = three separate experiments. , normal pituitary; , normal pituitary = E_2; , GH_3 tumor; ■, GH_3 tumor = E_2.

Materials and Methods

Animals and Implanted GH_3 Tumors

Normal pituitaries were obtained from 60-d-old female Wistar-Furth rats (Harlan, Indianapolis, IN). In vivo studies of estrogen regulation were done by inserting a Silastic capsule with 10 mg of E_2 subcutaneously as previously reported *(29)*. Empty Silastic capsules were inserted into control animals. Some experimental groups of rats were injected with 0.5×10^6 GH_3 cells in the right flank. Rats were maintained in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were sacrificed 8 wk after tumor implantation *(34)*. Pituitaries and GH_3 tumors were fixed in 10% neutral phosphate-buffered saline (PBS) for 2 h and then embedded in paraffin. Aliquots of pituitary and tumor tissues were used for RNA and protein extraction *(35,36)*.

Immunohistochemistry

Paraffin sections were immunostained as previously reported, using the avidin-biotin-peroxidase (Vector kit, Burligame, CA) method *(4)*. MAbs to neuronal NOS (Transduction, Lexington, KY) were used at a 1:1000 dilution. Before incubation with the primary antibody, the paraffin sections were microwaved for 10 min in 10 m*M* citric acid, pH 6.0. Positive controls consisted of staining rat hypothalamus with the antibody. Negative controls consisted of sections in which PBS was substituted for the primary antibodies. The specificity of the antibody was also checked by immunoblot analysis. Positively stained cells were enumerated by counting a minimum of 1000 cells/slide, and the

results were expressed as the percentage of positive cells determined by immunohistochemistry.

Colocalization studies for nNOS and pituitary hormones were done as previously reported *(4,36)* using pituitary antibodies from the National Hormone and Pituitary Program.

Cell Culture

The rat GH_3 cell line, which secretes PRL and GH, and the mouse AtT20 pituitary cell line, which secretes ACTH, were obtained from the American Type Culture Collection (Rockville, MD). The mouse GHRH-CL1 cell line, which produces PRL and GH, was developed from a GHRH transgenic mouse pituitary tumor *(35)*. The αT_3-1 and $L\beta T_2$ gonadotropic cell lines were obtained from Dr. P. Mellon (University of California, San Diego, CA). These cell lines were studied to examine the relationship between specific hormone secretion and nNOS expression.

Tumor cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% horse serum, 2.5% fetal calf serum (FCS), 1 μg/mL of insulin, and 1% antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of fungizone). At the start of each experiment, 2×10^6 GH_3 cells were plated in 75-mm dishes and grown in DMEM for 5 d at 37°C in an atmosphere of 5% CO_2-95% air. An aliquot of cells was used to make cytospins (1×10^5 cells/slide), and the remainder (10×10^6 cells/group) was used for RNA and protein extraction.

Immunoblot Analysis

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 17% gel using the discontinuous buffer system of Laemmli, as previously reported *(4)*. The electrophoresed proteins were transferred to a polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA) and subjected to immunoblot analysis with nNOS MAb at 1:2000 dilution. The reaction was detected by an enhanced chemiluminescence system, and semiquantitative estimates by densitometry were made using X-ray film. A linear response was obtained using different amounts of protein on the gel.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

nNOS mRNA expression and alternatively spliced isoforms were examined by RT-PCR *(35,36)*. First-strand cDNA was prepared from total RNA by using a first-strand synthesis kit (Stratagene, La Jolla, CA). The RT reaction was performed in a final volume of 50 μL with 5 μg of total RNA, 300 ng of oligo (deoxythymidine) primer, 1X RT buffer, 1.0 m*M* each deoxyribonucleotide (dATP, dCTP, dTTP, and dGTP), 40 U of RNase inhibitor, and 50 U of Moloney murine leukemia virus RTase at 37°C for 60 min, then heated at 95°C for 5 min, and immediately placed on ice.

PCR was performed in 50-μL final reaction volumes containing 2 μL of RT reaction product as template DNA corresponding to cDNA synthesized from 200 ng of total RNA, 1X PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleotide, 100 ng of each sense and antisense primer *(37)*, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI). Primers and probes for PCR and Southern blot were synthesized from the published sequence of neuronal NOS using a Model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) in the biochemistry department at the Mayo Foundation. The specificity of the probes and primers was confirmed by checking GenBank (Madison, WI). Programmable temperature cycling (Cetus 9600, Perkin-Elmer, Norwalk, CT) was performed with the following cycle profile: 95°C for 5 min, 60°C for 2 min, followed by 30 cycles for nNOS, 35 cycles for the nNOS isoforms for nNOS; and 26 cycles for GAPDH of 72°C for 2 min, 94°C for 1 min, and 60°C for 1 min. After the last cycle, the elongation step was extended by 10 min *(36)*. To ensure specific amplification, omission of the RT enzyme was employed as negative control. A 20-μL aliquot of PCR product was analyzed by gel electrophoresis using a 2% agarose gel and stained with ethidium bromide. A 100-bp ladder (Boehringer Mannheim, Indianapolis, IN) was used as the mol wt standard. The separated PCR amplification products were transferred to nylon membrane filters, and Southern hybridization with internal probes that hybridized to regions within the amplified sequences was performed. Hybridization was performed with 1×10^6 cpm/mL of ^{33}P dATP-labeled probe at 42°C for 18 h. After washing with 6X sodium chloride/sodium citrate 0.1% SDS at 23°C for 20 min and 42°C for 20 min, autoradiography was performed at –70°C with Kodak Omat-AR film (Eastman Kodak, Rochester, NY) with intensifying screens. The film was determined by scanning densitometry of the autoradiography with a CS9000U densitometer (Shimadzu, Tokyo, Japan). The results were expressed as a ratio of the nNOS spliced isoforms relative to external GAPDH standard. Analysis of various concentrations of nNOS and GAPDH cDNA was performed to ensure amplification in the linear portion of the curve. The linearity of the densitometric analysis of the Southern hybridization products was determined using varying concentrations of PCR products and different exposure periods.

TA Cloning and Automated Sequencing

The PCR products of nNOS spliced isoforms were comfirmed by automated sequencing. PCR-generated DNA fragments were ligated into the Topo TA cloning vector using standard protocols (Invitrogen, Carlsbad, CA). Cloning of PCR-amplified products was performed with at least four plasmid clones that were then sequenced using forward and reverse M13 primers that yielded sequences from both strands. Sequence data analyses were performed using the GCG program (University of Wisconsin, Madison). The sequence of the nNOS spliced fragments from normal pituitaries and tumor cell lines was compared to the original sequence in the GenBank database (accession no.

AF008911, AF008912, AF008913 for rat nNOSa, nNOSb, and nNOSc, respectively). Automated sequencing was done in the Molecular Biology Core at the Mayo Clinic using an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer).

Statistical Analysis

A minimum of three independent experiments using three or more replicates per treatment group were concluded. Results were expressed as the mean ± SE. Statistical analysis was done with the Student's *t*-test.

Acknowledgement

The authors thank Shuya Zhang for technical assistance; the National Hormone of Pituitary Program for the antibodies to pituitary hormones; and Dr. P. Mellon, University of California, San Diego, for the αT_3-1 and $L\beta T_2$ cell lines. This work was supported by National Institutes of Health Grant CA 37231.

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