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Tumor Necrosis Factor-Alpha-Induced Nitric Oxide Restrains the Apoptotic Response of Anterior Pituitary Cells

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Key Words
Nitric oxide · Nitric oxide synthase · Gonadal steroids · Estrous cycle · Tumor necrosis factor · Apoptosis

Abstract
We previously reported that tumor necrosis factor-α (TNF-α) inhibits cell proliferation whereas stimulates apoptosis of anterior pituitary cells in an estrogen-dependent manner. Also, we showed that nitric oxide (NO) mediates the inhibitory effect of TNF-α on prolactin release. Here, we studied the effect of TNF-α on nitric oxide synthase (NOS) activity and expression in anterior pituitary cells from cycling and ovariectomized (OVX) rats and the role of NO in TNF-α induced apoptosis of anterior pituitary cells. NOS activity was higher in anterior pituitary cells from rats in proestrus than in diestrus and was stimulated by 17β-estradiol (10^{-9} *M*, E2). TNF-α (50 ng/ml) stimulated NOS activity in anterior pituitary cells from rats at both stages of the estrous cycle and in cells from OVX rats cultured either with or without E2. Inducible NOS (iNOS) gene expression was higher in anterior pituitary cells from rats in proestrus than in diestrus and its expression was enhanced by TNF-α. Acute administration of E2 to OVX rats increased endothelial NOS (eNOS) expression in the anterior pituitary gland. Also, E2 increased eNOS mRNA in dispersed anterior pituitary cells from OVX rats, and this effect was blocked by TNF-α. nNOS expression in the anterior pituitary gland was higer at proestrus than at diestrus but eNOS expression was similar at both stages. TNF-α decreased eNOS mRNA in anterior pituitary cells from rats at proestrus or diestrus. In anterior pituitary cells from OVX rats, TNF-α failed to induce apoptosis but was able to induce it when cells were incubated with NAME or NMMA, NOS inhibitors, that did not affect cell viability per se. In the presence of E2, NAME induced apoptosis and enhanced the proapoptotic effect of TNF-α. In conclusion, our study shows that TNF-α upregulates iNOS gene expression whereas it downregulates estrogen-induced eNOS expression in anterior pituitary cells. Endogenous NO may restrain rather than mediate the proapoptotic effect of TNF-α in anterior pituitary cells.

Introduction

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine involved in many biological activities such as cell proliferation, differentiation and death [1]. It is synthesized in many tissues, including the anterior pituitary [2,

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3] where its receptors are also expressed [4–6]. We have previously reported that TNF-α release from anterior pituitary cells is stimulated by 17β-estradiol and is higher in cells from rats killed in proestrus than in diestrus [7, 8]. TNF-α has been shown to affect anterior pituitary hormone secretion [3, 6, 9]. We have previously reported that this cytokine decreases prolactin release [8]. Also, we have shown that TNF-α inhibits anterior pituitary cell proliferation [8] and induces apoptosis of lactotropes and somatotropes [10]. The inhibitory effect of TNF-α on prolactin release and anterior pituitary cell proliferation as well as the proapoptotic effect of this cytokine on lactotropes are estrogen-dependent [8, 10].

Several reports show that nitric oxide (NO) mediates many actions of TNF-α [11–13]. In fact, we have demonstrated that TNF-α inhibits prolactin secretion by a mechanism mediated by NO [14]. NO is a paracrine/autocrine messenger involved in cell proliferation and death in many tissues [15, 16]. NO is synthesized from *L*-arginine by NO synthase (NOS). There are at least three major NOS isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive and Ca^{2+}-calmodulin-dependent enzymes, whereas inducible NOS (iNOS) is Ca^{2+}-independent [17, 18]. These isoforms have been detected in the anterior pituitary gland [19–22]. Gonadotropes and folliculo-stellate cells express nNOS [19–21]. Also, enriched populations of somatotropes have been shown to express iNOS and nNOS, whereas lactotropes express the iNOS, nNOS and eNOS isoforms [22]. iNOS gene expression appears in the anterior pituitary during systemic inflammation [23] and is upregulated by lipopolysaccharide, interleukin-1 and γ-interferon [24, 25]. nNOS gene expression in the anterior pituitary gland has been shown to vary along the estrous cycle reaching a peak in the evening of proestrus [26]. Chronic estrogen treatment decreases the high levels of nNOS mRNA in the anterior pituitary gland of gonadectomized rats [27].

Since it has been demonstrated that NO produces either cytotoxic or cytoprotective effects depending on cell type or experimental conditions [15, 16, 28] and that NO mediates the inhibitory effect of TNF-α on prolactin release [14], we studied whether NO is involved in the apoptosis induced by TNF-α in anterior pituitary cells. Considering that TNF-α induces iNOS gene expression in anterior pituitary cells [14] and that many of the effects of TNF-α on these cells are estrogen-dependent, we also determined the effect of this cytokine on NOS activity and nNOS, iNOS and eNOS expression in anterior pituitary cells from rats killed at selected stages of the estrous cycle or from ovariectomized rats cultured in the presence of 17β-estradiol. Besides, we explored the expression of NOS isoforms in the anterior pituitary gland in rats at proestrus and diestrus and in acutely estrogenized-ovariectomized rats.

Materials and Methods

All drugs, media and supplements were obtained from Sigma Chemical Co. (St. Louis, Mo., USA), except fetal bovine serum (GenSa, Buenos Aires, Argentina), rhTNF-α (Promega Co., Madison, Wisc., USA), Dowex AG 50W-X8 resin (Bio-Rad Laboratories, Calif., USA), *L*-[^{14}C]arginine (specific activity: 11.26 GBq/mmol; Amersham Int., Buckinghamshire, UK), all TUNEL reagents (Roche Molecular Biochemicals, Mannheim, Germany) and the materials indicated below.

Animals

Adult female Wistar rats (200–250 g) were kept in controlled conditions of light (12 h light-dark cycles) and temperature (20–25°C). Rats were fed standard lab chow and water ad libitum and kept in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Rats were monitored by daily vaginal smears over 3 consecutive cycles and killed in proestrus or diestrus. Groups of rats were ovariectomized under ether anesthesia 2 weeks before the experiments. Some groups of ovariectomized (OVX) rats were injected subcutaneously with 62.5 μg/rat 17β-estradiol or vehicle (250 μl propylenglycol), 48 and 24 h before sacrifice. Anterior pituitary glands were removed within minutes after decapitation.

Cell Culture

A pool of anterior pituitary cells from 5 to 10 OVX rats or from 3 rats per stage of the estrous cycle was used for each culture. Anterior pituitary glands were washed several times with Dulbecco's modified Eagle's medium (DMEM) and cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in DMEM supplemented with 3 mg/ml bovine serum albumin (BSA), containing 2.5 mg/ml trypsin (type I from bovine pancreas), 1 mg/ml DNase (deoxyribonuclease II, type V from bovine spleen) and 1 mg/ml trypsin inhibitor (type II-S from soybean), and finally dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca^{2+} and Mg^{2+}. Dispersed cells were washed twice and resuspended in DMEM supplemented with 10 μl/ml MEM amino acids, 2 m*M* glutamine, 5.6 μg/ml amphotericin B and 25 μg/ml gentamicin (DMEM-S). Cell viability as assessed by trypan blue exclusion was over 90%. The cells were seeded in 48-well tissue culture plates (25×10^4 cells/0.50 ml/well) for NOS activity determination or 6-well tissue culture plates (3.0×10^6 cells/4 ml/well) for NOS mRNA analysis.

Anterior pituitary cells from cycling rats were cultured for 2 days (37°C, 5% CO_2 in air) in DMEM-S with 10% fetal bovine serum previously treated with 0.025% dextran-0.25% charcoal (FBS-DCC) in order to remove steroids. After this period, cells were washed twice and incubated in DMEM-S without FBS-DCC (0.1% BSA) containing TNF-α (50 ng/ml) for 6 or 24 h.

In the case of OVX rats, cells were cultured in DMEM-S with 10% FBS-DCC for 3 days and then for 1 day in the same fresh medium containing 17β-estradiol (10^{-9} *M*) or vehicle (ethanol, final concentration 1 μl/l). After this period, cells were washed twice and

Table 1. Oligonucleotide sequences of PCR primers

	Oligo	Sequence	Annealing temperature °C	Number of cycles	Product size bp
iNOS	Sense	5′-TAG AAA CAA CAG GAA CCT ACC A-3′	58	33	907
	Antisense	5′-ACA GGG GTG ATG CTC CCG GAC A-3′			
nNOS	Sense	5′-GAA TAC CAG CCT GAT CCA TGG AAC ACC-3′	66	32	599
	Antisense	5′-CTC CAG GAG GGT GTC CAC CGC ATG CC-3′			
eNOS	Sense	5′-CTG TGT CCA ACA TGC TGC TAG AAA TTG-3′	55	59	485
	Antisense	5′-TAA AGG TCT TCT TCC TGG TGA TGC C-3′			
β-Actin	Sense	5′-ACC ACA GCT GAG AGG GAA ATC G-3′	55–66	20–26	281
	Antisense	5′-AGA GGT CTT TAC GGA TGT CAA CG-3′			

the medium was replaced by serum free DMEM-S (0.1% BSA) containing 17β-estradiol or vehicle for 1 day to wash out remaining FBS. Finally, the cells were incubated in the same media with TNF-α (50 ng/ml) for 6 or 24 h.

In order to abrogate fibroblast proliferation, apoptosis was determined in anterior pituitary cells from OVX rats, dispersed as described above and resuspended in minimum essential medium Eagle (MEM-*D*-valine) containing *D*-valine instead of *L*-valine supplemented with 5 μl/ml MEM nonessential aminoacids, 2 m*M* glutamine, 5.6 μg/ml amphotericin B and 25 μg/ml gentamicin (MEM-D-valine-S). Cells were seeded onto coverslides in 24-well tissue culture plates (10×10^4 cells/ 0.5 ml/ well) for the TUNEL method, and cultured in MEM-D-valine-S with 10% FBS-DCC for 3 days. Then, cells were incubated in the same fresh medium containing 17β-estradiol (10^{-9} *M*) or vehicle for 2 days. After this period, cells were washed twice and the medium was replaced by serum free MEM-*D*-valine-S, supplemented with 10 μg/ml insulin, 6.7 ng/ml sodium selenium, 5.5 μg/ml transferrin, 0.02 ng/ml triiodothyronin and 10 μl/ml MEM vitamins (MEM-*D*-valine-SS) containing 17β-estradiol or vehicle and TNF-α (50 ng/ml) and/or NAME (Nω-nitro-*L*-arginine methyl ester hydrochloride, 0.5 m*M*) or NMMA (N(G)-monomethyl-*L*-arginine) for 24 h.

NOS Activity Determination

NOS activity was determined by conversion of *L*-[^{14}C]arginine to *L*-[^{14}C]citrulline, using a modification of the Bredt and Snyder [14] method, as previously described. Briefly, cultured cells were incubated for 24 h in the presence of TNF-α (50 ng/ml). Then, the cells were washed twice with KRB containing 20 m*M* Hepes, pH 7.4, and incubated in the same buffer with 0.1 μCi *L*-[U-^{14}C]arginine. After a 30-min incubation at 37°C, the cells were washed in cold KRB containing 20 m*M* Hepes, 2 m*M* EDTA, 0.2 m*M* EGTA and 2 m*M* NAME and immediately sonicated in the same buffer. Aliquots of sonicate were applied to 1.5-ml columns of Dowex AG 50W-X8 in 20 m*M* Hepes, pH 7.4, loaded with 20 μl of 100 m*M* *L*-citrulline. *L*-[^{14}C]citrulline was eluted with 3 ml of distilled water and the radioactivity quantified by liquid scintillation spectrometry of the flow-through.

NOS mRNA Analysis

NOS mRNA transcripts in whole anterior pituitary glands or cultured anterior pituitary cells from ovariectomized or cycling rats were determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted with TRIZOL (GibcoBRL, Gaithersburg, Md., USA) according to the manufacturer's protocol. 5 μg of total RNA were reverse transcribed with Superscript™ II RNAse H minus Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) in a 20-μl reaction, using 0.2 μg of Oligo $(dT)_{12-18}$ as primer (Invitrogen). Amplification of cDNA was performed with 1–2 μl of cDNA as template in 50 μl PCR reaction containing $MgCl_2$, dNTPs, sense and antisense primers (Transgenomics, Nebraska, USA) and Taq DNA polymerase (Invitrogen) in the buffer provided by the manufacturer. Amplifications were carried out on a thermal cycler (UNO II Biometra, Göttingen, Germany). The number of cycles and the template input for PCR were determined empirically within the linear range of amplification. Synthetic oligonucleotides used for PCR, annealing temperature, number of cycles and product size are listed in table 1. Amplification of iNOS cDNA was performed with 1 μl cDNA, 1.5 m*M* $MgCl_2$, 50 pmoles of each primer and 2.5 U of Taq DNA polymerase. Amplification of nNOS was performed with 1 μl cDNA, 2.5 m*M* $MgCl_2$, 50 pmol of each primer and 2.5 U of Taq DNA polymerase. Amplification of eNOS was performed with 2 μl cDNA, 2 m*M* $MgCl_2$, 20 pmoles of each primer and 1.25 U of Taq DNA polymerase. Amplification of β-actin was performed with 1 μl cDNA, 1.5 m*M* $MgCl_2$, 50 pmoles of each primer and 2.5 U of Taq DNA polymerase. The number of cycles for β-actin amplification was determined for the annealing temperature of each NOS isoform mRNA. PCR products (9.3 μl) were analyzed on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. Experiments always included non-reverse-transcribed RNA samples as negative controls.

Microscopic Determination of DNA Fragmentation by the TUNEL Method

After the culture period, cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized by microwave irradiation [10]. DNA strand breaks were labeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase (0.18 U/μl) according to the manufacturer's protocol. The incorporation of nucleotides into the 3′-OH end of damaged DNA was detected with an anti-digoxigenin-fluorescein antibody. Cells were mounted with an anti-fade mounting medium, 1,4-diazabicyclo[2.2.2]octane (DABCO) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in 90% glycerol wich stains both normal and apoptotic nuclei. Cells were visualized in a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany).

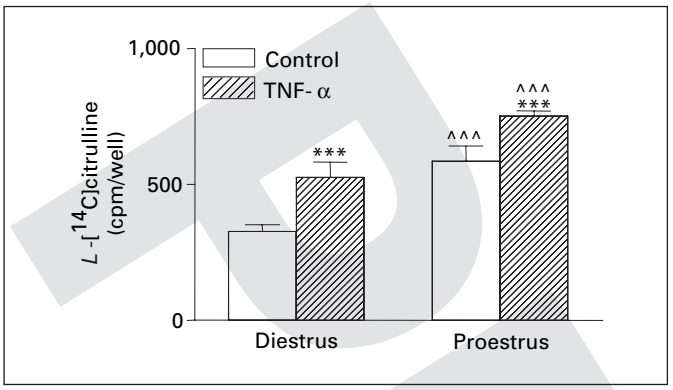

Fig. 1. Effect of TNF-α on NOS activity in anterior pituitary cells from rats killed at selected stages of the estrous cycle. Anterior pituitary cells from rats killed at diestrus or proestrus were incubated with TNF-α (50 ng/ml) for the last 24 h of culture. The columns represent the mean ± SE of 6 wells from 1 of 2 independent experiments. Data were evaluated by two-way ANOVA. *** $p < 0.001$ vs. respective control without TNF-α; ^^^ $p < 0.001$ vs. corresponding control at diestrus.

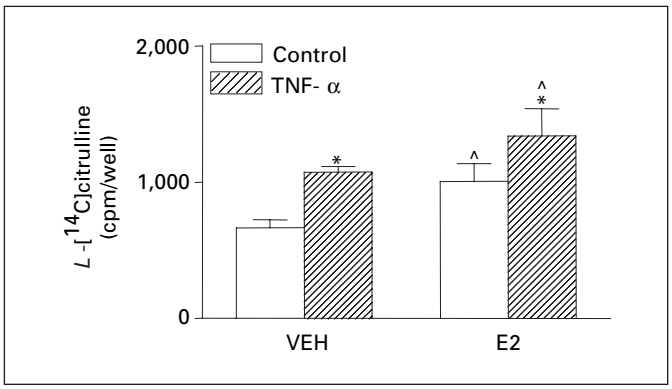

Fig. 2. Effect of TNF-α on NOS activity in anterior pituitary cells from OVX rats cultured with 17β-estradiol. Anterior pituitary cells from OVX rats cultured in the presence of 17β-estradiol (10^{-9} *M*, E2) or vehicle (ethanol 1 μl/l, VEH) were incubated with TNF-α (50 ng/ml) for the last 24 h of culture. The columns represent the mean ± SE of 6 wells from 1 of 5 independent experiments. Data were evaluated by two-way ANOVA. * $p < 0.05$ vs. respective control without TNF-α; ^ $p < 0.05$ vs. respective control without 17β-estradiol.

Statistical Analysis

The densitometric analysis of digitalized gel images were normalized to the internal control and expressed as relative increments of corresponding control group. Data were log transformed when they failed the normality test (Kolmogorov-Smirnov test) or when variances between groups were significantly different (Levene's test for homogeneity of variances). NOS activity and relative NOS mRNA data were expressed as mean ± SE and evaluated by paired t test or two-way analysis of variance (ANOVA). Post hoc analyses were performed using Sheffe's F test. The number of apoptotic cells (as identified by the TUNEL method) was analyzed in duplicate slides for each cell preparation, and each group included at least three independent experiments. Results were expressed as the percentage of apoptotic cells of the total number of cells counted of each specific population or condition. Differences between proportions were analyzed by the chi-square test. $p < 0.05$ was used as the cut-off point for significance. All experiments were performed at least twice.

Results

Effect of TNF-α on NOS Activity in Cultured Anterior Pituitary Cells

We have reported that TNF-α stimulates NO synthesis in anterior pituitary cells from intact female rats by inducing iNOS gene expression [14]. In order to determine whether the stimulatory effect of TNF-α on NOS activity is variable throughout the estrous cycle, we studied the effect of TNF-α on NOS activity in cultures of anterior pituitary cells from rats sacrificed in the morning of proestrus or diestrus. Basal NOS activity was higher in cells from rats sacrificed in proestrus than in diestrus. TNF-α (50 ng/ml) increased NOS activity in anterior pituitary cells from rats at both stages of the estrous cycle. However, TNF-α-induced NOS activity was significantly higher in anterior pituitary cells from rats sacrificed in proestrus than in diestrus (fig. 1). To investigate whether estrogens modulate NO synthesis induced by TNF-α, NOS activity was assayed in anterior pituitary cells from OVX rats cultured in the presence of 17β-estradiol (10^{-9} *M*). 17 β-estradiol significantly increased NOS activity. TNF-α stimulated NOS activity in anterior pituitary cells cultured with or without 17β-estradiol. Nevertheless, TNF-α-induced NO synthesis was higher in the presence of 17β-estradiol (fig. 2).

Effect of TNF-α on NOS mRNA Expression in Cultured Anterior Pituitary Cells

In view of the variations observed in NOS activity in anterior pituitary cells throughout the estrous cycle, we studied the effect of TNF-α on NOS expression in anterior pituitary cells from rats killed at proestrus or diestrus.

iNOS gene expression was significantly higher in anterior pituitary cells from rats killed in proestrus than in diestrus. TNF-α significantly increased iNOS mRNA lev-

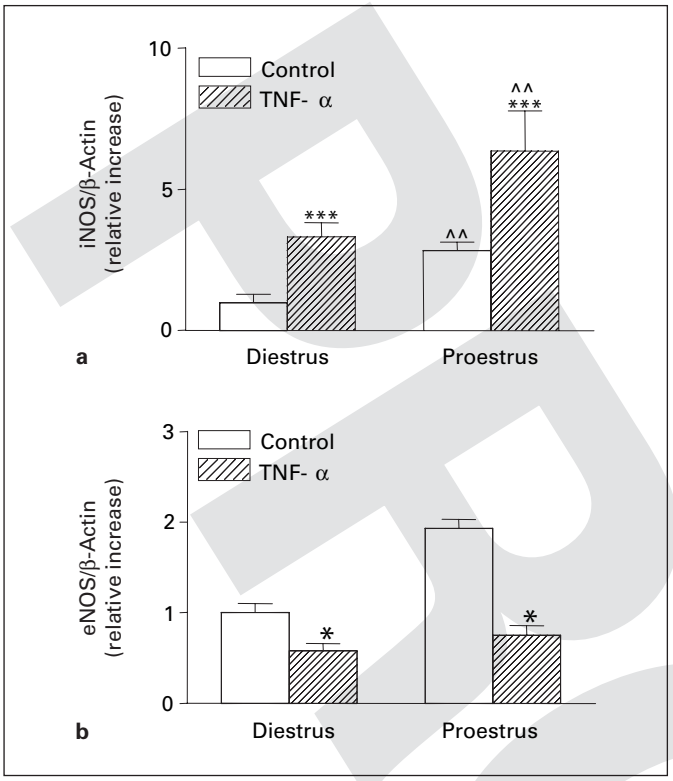

Fig. 3. Effect of TNF-α on gene expression of iNOS (**a**) and eNOS (**b**) in anterior pituitary cells from rats killed at selected stages of the estrous cycle. Anterior pituitary cells from rats killed at diestrus or proestrus were incubated with TNF-α (50 ng/ml) for the last 6 h of culture. RNA was extracted and analyzed by RT-PCR using the primer pairs specific for iNOS (**a**), eNOS (**b**) and β-actin. Columns represent the mean ± SE of relative increase in NOS mRNAs (normalized to β-actin) with respect to control at diestrus of 2 separate experiments performed in duplicate. Data were evaluated by two-way ANOVA. * $p < 0.05$; *** $p < 0.001$ vs. respective control without TNF-α; ^^ $p < 0.01$ vs. diestrus.

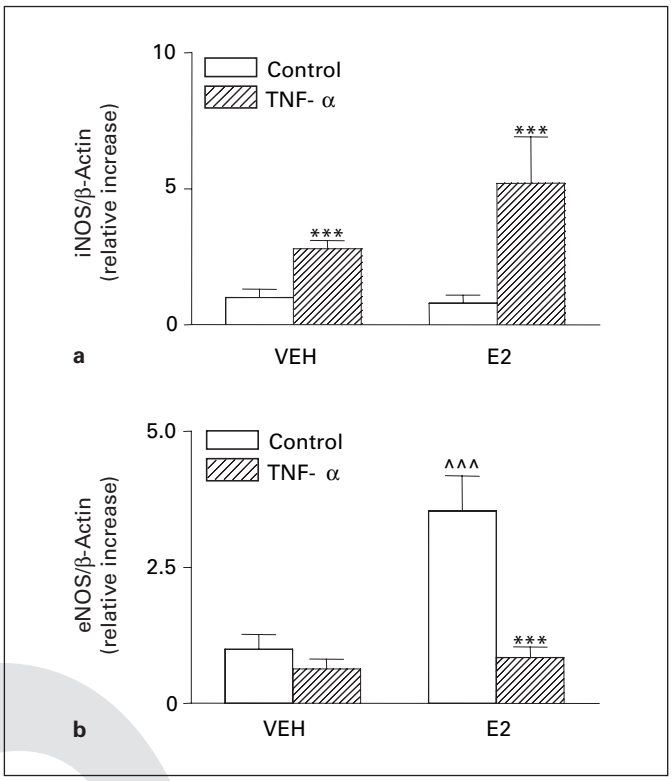

Fig. 4. Effect of TNF-α on gene expression of iNOS (**a**) and eNOS (**b**) in anterior pituitary cells from OVX rats cultured with 17β-estradiol. Anterior pituitary cells from OVX rats cultured in the presence of 17β-estradiol (10^{-9} *M*, E2) or vehicle (ethanol 1 μl/l, VEH) were incubated with TNF-α (50 ng/ml) for the last 6 h of culture. RNA was extracted and analyzed by RT-PCR using the primer pairs specific for iNOS (**a**), eNOS (**b**) and β-actin. Columns represent the mean ± SE of relative increase in NOS mRNAs (normalized to β-actin) with respect to corresponding control without 17β-estradiol of 2–5 separate experiments performed in duplicate. Data were evaluated by two-way ANOVA followed by Sheffe's F test. *** $p < 0.001$ vs. respective control without TNF-α; ^^^ $p < 0.001$ vs. respective control without 17β-estradiol.

els in cultured cells of both groups. However, TNF-α induced iNOS mRNA levels were significantly higher in anterior pituitary cells from rats killed in proestrus than in diestrus (fig. 3a). Basal nNOS expression in cells from rats in proestrus was similar to that of cells from rats in diestrus. TNF-α failed to modify nNOS expression in anterior pituitary cells from rats killed either in proestrus or diestrus (data not shown). Basal eNOS expression was almost significantly higher ($p = 0.07$) in anterior pituitary cells from rats in proestrus than in diestrus. TNF-α significantly decreased eNOS mRNA levels in cells from both groups (fig. 3b).

To clarify whether differences in the expression of NOS isoforms in cultured cells were estrogen-dependent we explored the effect of TNF-α on NOS gene expression in anterior pituitary cells from OVX rats incubated with 17β-estradiol. The presence of 17β-estradiol did not significantly modify iNOS expression. TNF-α significantly increased iNOS mRNA levels in cells cultured either in the presence or absence of 17β-estradiol (fig. 4a). Neither 17β-estradiol nor TNF-α had any effect on nNOS expression in anterior pituitary cells from OVX rats (data not shown). Nevertheless, 17β-estradiol significantly increased eNOS mRNA levels whereas TNF-α significantly

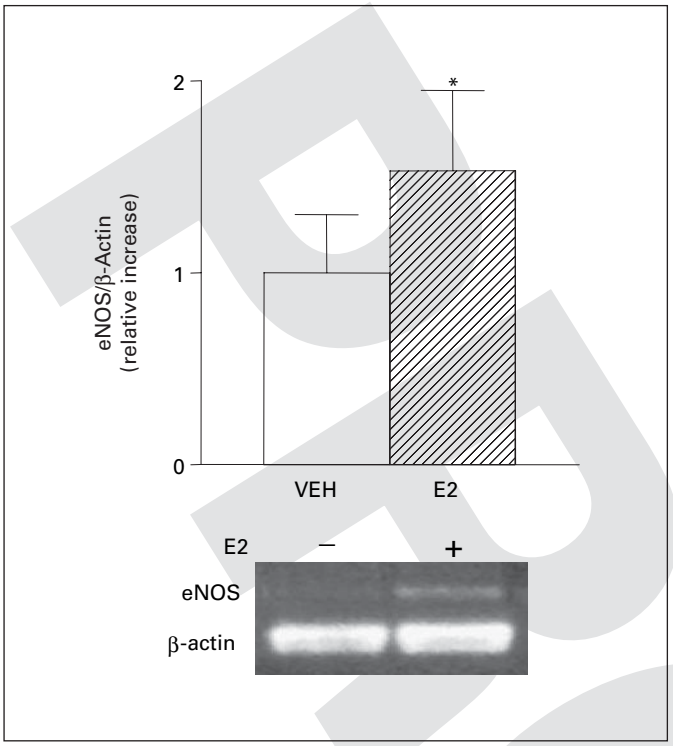

Fig. 5. Gene expression of eNOS in the anterior pituitary gland of acutely estrogen-treated OVX rats. eNOS expression was determined in anterior pituitary glands from OVX rats injected with 17β-estradiol (E2) or vehicle (OVX) 48 and 24 h before death. RNA was extracted and used for RT-PCR with primer pairs specific for eNOS and β-actin. Columns represent the mean ± SE of relative increase in eNOS mRNA (normalized to β-actin) with respect to OVX of 3 separate experiments performed in duplicate. Data were evaluated by paired t test. * $p < 0.05$. Gel shows a representative experiment of RT-PCR for eNOS and β-actin mRNA.

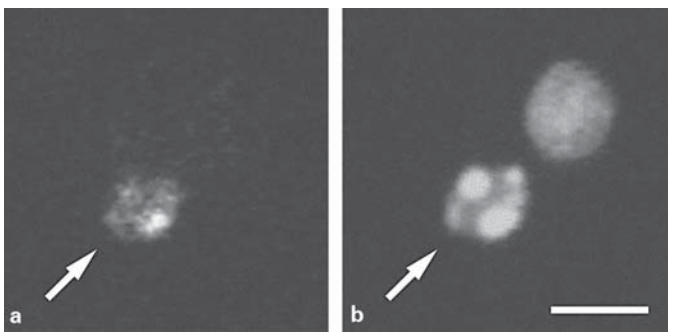

Fig. 6. Apoptotic anterior pituitary cells. **a** Representative anterior pituitary cell showing TUNEL staining. **b** Two anterior pituitary cells stained with DAPI. Arrows show a TUNEL-positive cell exhibiting nuclear chromatin fragmentation. Scale bar = 10 μm.

blocked the stimulation of eNOS expression induced by 17β-estradiol (fig. 4b).

Expression of NOS Isoforms in the Anterior Pituitary Gland

In order to evaluate whether estrogen circulating levels affect the expression of NOS and considering the estrogenic effect on the activity and expression of NOS in anterior pituitary cells, we determined transcript levels of NOS isoforms in the anterior pituitary gland from rats at proestrus or diestrus and from acutely estrogenized-OVX rats. eNOS expression in the anterior pituitary gland of rats proestrus was not significantly different to that at diestrus (diestrus 1.00 ± 0.27 arbitrary units; proestrus: 0.73 ± 0.34; 3 independent experiments performed in duplicate). Tallying with data reported by others [26], nNOS expression was significantly higher in the anterior pituitary gland at proestrus than at diestrus (diestrus: 1.00 ± 0.43; proestrus: 2.84 ± 0.66, $p < 0.05$). iNOS mRNA was not detected in the anterior pituitary gland of cycling rats (data not shown).

The subcutaneous administration of 17β-estradiol (62.5 μg/rat) to OVX rats, 48 and 24 h before sacrifice significantly enhanced eNOS mRNA levels (fig. 5), but failed to significantly modify nNOS expression in the anterior pituitary gland (control: 1.00 ± 0.26; E2: 1.26 ± 0.36). iNOS mRNA was detected neither in control nor in estrogen-treated OVX rats (data not shown).

The Blockade of NO Synthesis Increases TNF-α-Induced Apoptosis

To evaluate whether NO is involved in TNF-α-induced apoptosis of anterior pituitary cells, we studied the apoptotic effect of this cytokine in the presence of NAME, a NOS inhibitor. Staining with TUNEL permitted the observation of nuclear morphological features of apoptosis in anterior pituitary cells (fig. 6). Anterior pituitary cells from OVX rats cultured in the presence of 17β-estradiol or vehicle were incubated with TNF-α and NAME (0.5 m*M*) for the last 24 h. In the absence of 17β-estradiol, TNF-α failed to induce apoptosis of anterior pituitary cells. The blockade of NOS activity with NAME did not modify the percentage of apoptotic anterior pituitary cells but triggered the apoptotic effect of TNF-α in these cells (fig. 7a). As we previously reported [10], TNF-α induced apoptosis of anterior pituitary cells incubated in the presence of 17β-estradiol (fig. 7b). In the presence of 17β-estradiol, NAME significantly increased the percentage of anterior pituitary apoptotic cells and enhanced the proapoptotic action of TNF-α (fig. 7b). Similar results

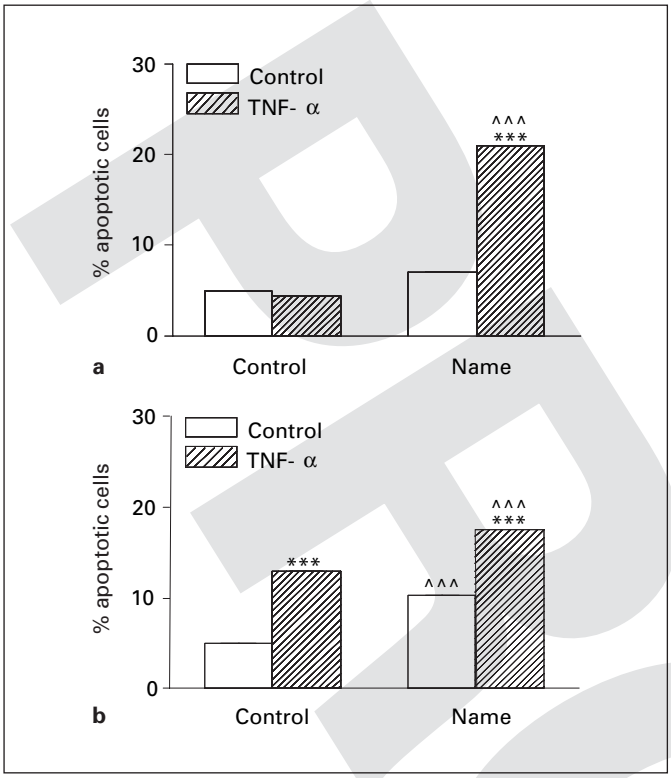

Fig. 7. Effect of TNF-α and NAME on the percentage of apoptotic anterior pituitary cells from OVX rats cultured in the absence (**a**) or presence (**b**) of 17β-estradiol. Cells from OVX rats cultured with vehicle (ethanol 1 μl/l, **a**) or 17β-estradiol (10^{-9} *M* , **b**) were incubated with TNF-α (50 ng/ml) in the presence or absence of NAME (0.5 m*M*) for the last 24 h of culture. Each column represents the percentage of apoptotic cells (TUNEL), n > 2,000 cells from at least three independent experiments. Data were evaluated by the χ^2 test. *** $p < 0.001$ vs. respective control without TNF-α; ^^^ $p < 0.001$ vs. respective control without NAME.

were obtained when anterior pituitary cells were incubated in the presence of another NOS inhibitor, NMMA (data not shown).

Discussion

The present study indicates that estradiol exerts a direct stimulatory effect on NOS activity in anterior pituitary cells. Previous studies have shown that chronic estrogenization reduces anterior pituitary nNOS expression by inhibiting hypotalamic GnRH release, which up-regulates the expression of nNOS in gonadotropes [19, 26, 27]. As others have reported, we observed that nNOS expression in the anterior pituitary gland is higher at proestrus than at diestrus [26]. Therefore, gonadal steroids may have a dual effect on NOS activity in the anterior pituitary. Estrogens indirectly reduce NO synthesis by inhibiting GnRH release and directly stimulate NO production in the anterior pituitary. The acute administration of 17β-estradiol failed to modify nNOS expression in the anterior pituitary gland but enhanced the expression of eNOS suggesting that estradiol could directly stimulate NOS activity in the pituitary by inducing eNOS expression. However, no differences were detected in pituitary eNOS expression at different stages of the estrous cycle suggesting that other mechanisms occurring during the estrous cycle could mask or restrain the upregulation of eNOS induced by estrogens. In fact, the presence of 17β-estradiol in the culture medium did increase eNOS mRNA levels in anterior pituitary cells. Direct upregulation of pituitary eNOS mRNA and NOS activity by estrogens could play a role in the modulation of pituitary function. The stimulation of eNOS-derived NO by estrogens could be important in physiological and pathological conditions considering that eNOS is expressed in lactotropes [22]. In accordance with previous studies, we failed to detect iNOS gene expression in the anterior pituitary gland but observed iNOS mRNA in cultured anterior pituitary cells [22, 27].

Considering that 17β-estradiol directly stimulates NO synthesis in anterior pituitary cells, it can be suggested that the variation in NOS activity in cells from rats at different stages of the estrous cycle may depend on estrogens. Although 17β-estradiol failed to modify iNOS mRNA levels in cells from OVX rats, iNOS expression was higher in anterior pituitary cells from rats in proestrus than in diestrus. Therefore, the higher NOS activity observed in anterior pituitary cells from rats in proestrus may result from an enhancement of NO synthesis by estrogen-dependent eNOS and by iNOS, which may not depend on estrogens.

Infectious and inflammatory stimuli have been shown to decrease eNOS expression and activity, especially through TNF-α [29]. TNF-α negatively regulates eNOS mRNA and protein levels and NOS activity in endothelial cells [30–32]. This is the first report to show that TNF-α downregulates eNOS mRNA in anterior pituitary cells. It was recently shown that TNF-α exerts transcriptional as well as post-transcriptional effects on eNOS expression [33]. Since TNF-α enhances iNOS mRNA levels in anterior pituitary cells, our results suggest that the stimulatory effect of TNF-α on NOS activity in these cells may result from a balance between two opposite effects of this cytokine, which downregulates eNOS but upregulates

iNOS expression. Nevertheless, the overall effect of TNF-α is an enhancement of NO synthesis in anterior pituitary cells, which may be responsible for some of the effects of TNF-α on pituitary function. Thus, we observed that NO is involved in the inhibitory effect of TNF-α on prolactin release [14].

Cell death by apoptosis plays a key role in the maintenance of normal tissue homeostasis. The rate of both cell proliferation and death controls cell number in tissues [34]. It is well established that NO affects cellular decisions of life and death either by turning on or shutting off apoptotic pathways, depending on cell type and condition [15]. Long-term treatment with NO was recently reported to induce apoptosis of anterior pituitary cells, mainly lactotropes [35]. However, in our experimental conditions, the inhibition of NO synthesis with NAME or NMMA increased the number of apoptotic anterior pituitary cells when they were cultured in the presence of 17β-estradiol, suggesting that endogenous NO may exert an antiapoptotic action on anterior pituitary cells. Since estrogens stimulated NOS activity and eNOS gene expression it can be suggested that eNOS-derived NO could contribute to the regulation of homeostasis in the anterior pituitary. It has been suggested that eNOS-derived NO is anti-inflammatory, playing a role in key features of inflammation such as angiogenesis and apoptosis [36, 37]. We have previously reported that TNF-α induces apoptosis of anterior pituitary cells in an estrogen-dependent manner [10]. Since NAME or NMMA enhanced the apoptotic effect of TNF-α on anterior pituitary cells, it can be speculated that NO synthesis induced by TNF-α may restrain rather than mediate the apoptotic events triggered by this cytokine. In fact, it has been shown that NO can protect some cells from apoptosis induced by many different stimuli such as TNF-α [38], oxidative stress and serum deprivation [39].

In conclusion, our study indicates that 17β-estradiol stimulates eNOS gene expression in the anterior pituitary gland. It also shows that TNF-α upregulates iNOS gene expression, while it downregulates estrogen-induced eNOS expression in anterior pituitary cells. Endogenous NO produced by anterior pituitary cells may protect them from apoptosis.

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