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ORIGINAL ARTICLE

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Dopaminergic modulation of nNOS expression in the pituitary gland of male rat

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Abstract Nitric oxide is an unconventional transmitter since it is not transported and released by exocytosis. In the pituitary gland, nitric oxide is locally synthesised by gonadotroph and folliculo-stellate cells. Dopamine, the principal central inhibitory signal in prolactin release, may exert its inhibitory effects by stimulation of nitric oxide production. However, the effects of dopaminergic modulation on nitric oxide-producing pituitary cells have not been analysed. Therefore, we examined the effects of intraventricular administration of the dopamine antagonist haloperidol (40 μg) on the pituitary expression of neuronal nitric oxide synthase (nNOS) in male adult rats. In untreated and control animals, nNOS-positive cells were very similar. Two types of nNOS-positive cells appeared in the pars distalis: round or polygonal cells and stellate cells. Although some isolated cells were found, the nNOS-positive cells commonly appeared grouped in clusters close to blood vessels. nNOS immunoreactivity appeared as a uniform staining throughout the cytoplasm, including cell prolongations. The number and size of nNOS-expressing cells in the pituitary gland decreased significantly after treatment with haloperidol ($p<0.01$). To evaluate the potential direct effect of dopamine on pituitary cells, pituitary monolayer cultures were treated with dopamine during a time-course of 12 h. Our in vitro studies revealed that dopamine increases the percentage of nNOS-positive cells and augments cellular area ($p<0.05$). These results demonstrate that: (1) treatment of rats in vivo with a dopamine antagonist significantly decreases expression of nNOS in the pituitary and (2) in vitro dopamine exerts a direct effect on pituitary cultures by increasing nNOS-positive cells. Thus, these findings suggest that dopamine may function as a physiological stimulator of nNOS expression in the rat pituitary gland.

Keywords Nitric oxide · Hormonal regulation · Haloperidol · Morphometry

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Introduction

The gas nitric oxide serves as a messenger in the central and peripheral nervous system, where it freely diffuses from its site of production (Bredt et al. 1990; Bult et al. 1990; Hölscher 1997). The expression of the neuronal isoform of nitric oxide synthase (nNOS) in discrete populations of neurones and glial cells is well documented (for review see Alonso et al. 2000). In the pituitary gland, the local production of nitric oxide in two cell types, gonadotroph and folliculo-stellate cells, has been reported (Ceccatelli et al. 1993; Lloyd et al. 1995). Originally, nNOS was thought to be a constitutively expressed enzyme but increasing evidence demonstrates that nNOS expression is regulated by various physiological or pathological conditions (Sasaki et al. 2000). The up-regulation of nNOS may reflect a general response of neuronal cells to stress including changes in temperature, light exposure, and formalin (Foerstermann et al. 1998).

Nitric oxide regulates different pituitary hormones and its inhibitory effects on prolactin release have been demonstrated (Duvilanski et al. 1995, 1996; Vankelecom et al. 1997A; Chiodera et al. 1998). Moreover, nitric

oxide may serve as an auto-paracrine modulator of the prolactin release and thus, may have potential implications for pituitary cell–cell interactions (Okere et al. 1998). The function of nNOS in the human hypophysis remains unclear (Volpi et al. 1998).

Prolactin release is controlled by multiple hypothalamic, peripheral and pituitary signals. Among them, dopamine is the principal central inhibitory signal (Musset et al. 1991). Several observations suggest a role for nitric oxide in mediating the inhibitory effect of GABA and dopamine on prolactin release (Duvilanski et al.,1996; Pinilla et al. 1996). However, the effects of dopaminergic modulation on nNOS-producing pituitary cells have not been analysed yet.

Therefore, the aim of the present study was to determine whether dopamine modifies the immunohistochemical expression of nNOS in the rat pituitary gland via the analysis of nNOS-positive pituitary cells following in vivo treatment with the dopamine antagonist haloperidol. To study the direct effect of dopaminergic regulation on nNOS expression in lactotrophs, pituitary cultures were treated in vitro with dopamine and subsequently examined for nNOS expression by immunohistochemistry.

Material and methods

Animals

Fifteen adult male Wistar rats (175–200 g b.w.) stabled under standard conditions were used (20± 2°C; 55±5% RH; light from 08.00 to 20.00 h; water and equilibrate Panlab maintenance diet ad libitum. Animals were handled according to guidelines of the European Communities Council directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12, 1998).

Treatment of the animals

The rats were divided into three experimental groups of five animals each: (1) untreated animals; (2) control animals received once, 25 μl of distilled water in the third ventricle using stereotaxis, according to the coordinates of Pellegrino and Cushman (1967), under ketamine anaesthesia (2.5 mg·kg^{-1} b.w.); (3) rats were given a single injection with 40 μg of haloperidol (Syntex Latino, Madrid, Spain) in 25 μl of distilled water, in the third ventricle by stereotaxis, using a protocol similar to control animals. All experimental groups including controls were treated similarly and the stereotaxic injections were performed from 09.00 to 11.00 h.

Sacrifice of animals and sample processing

Thirty minutes after treatment, the animals were killed by decapitation under ketamine anaesthesia. The pituitary glands were carefully dissected and immediately submerged in a mixture containing 0.2% (w/v) picric acid and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 24 h. Tissue was embedded in paraffin to obtain serial frontal sections of 5 µm-thickness.

Pituitary monolayer cultures

Following anaesthesia with isoflurane, male Wistar rats (175–200 g) were killed by decapitation and the anterior pituitary glands were removed and washed in Earle's balanced salt solution. Enzymatic dispersion was carried out by incubation for 15 min at 37°C in Hanks' solution to which 0.15% $MgCl_2$, 0.1% papain, 0.01% DNase and 0.1% neutral protease had been added. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20- to 22-gauge needles. After centrifugation, the supernatant was removed and the cells were re-suspended in an appropriate volume of Dulbecco's modified Eagle medium, supplemented with 10% calf serum, 2.5% foetal calf serum, 2% L-glutamine, 1000 IU/ml of penicillin and 1000 IU/ml of streptomycin. The cells were seeded on chamber slides at a final concentration of 2×10^5 cells/ml and incubated at 37°C in a 5% CO_2/95% air atmosphere for 7 days. On the 4th day of incubation the medium was replaced. On the 7th day of incubation, the medium was replaced by fresh medium in the control dishes and fresh medium plus dopamine (10^{-7}, 10^{-8} M) in the treated dishes. To avoid the oxidation of dopamine, the medium contained 60 μM ascorbic acid. After 1, 3, 6 or 12 h of incubation, the culture medium was removed, the dishes were carefully washed with Dulbecco's sterile PBS, and the cells were fixed in 15% picric acid in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 1 h followed by careful rinsing in PBS.

In order to validate the results, the in vitro studies were carried out in duplicate, with similar values being obtained. Accordingly, the variation coefficients ranged between 0.96 and 1.03 for all the parameters analysed (variation coefficient: culture 1 value/culture 2 value).

Immunohistochemistry

The immunohistochemical study was performed using the avidin–biotin–peroxidase method. Endogenous peroxidase was blocked by incubating the sections in 0.24% (v/v) H_2O_2 in methanol during 20 min. After thorough rinsing, the samples were incubated in a humidified chamber as follows: (a) normal rabbit serum (Dako, diluted 1:30, 80 μg/ml, in TBS:0.8% NaCl in Tris-HCl 0.05 M, pH 7.4) for 30 min at room temperature; (b) sheep polyclonal antiserum against nNOS (K205; Herbison et al. 1996) diluted 1:10,000 in TBS and incubated for 24 h at 4ºC; (c) rabbit biotinylated anti-sheep IgG (H+L) was applied for 40 min at room temperature (1:200 in TBS, 15 μg/ml, Vector, Burlingame, CA, USA); (d) streptavidin–biotin complex (1:200 in TBS, Caltag, San Francisco, CA, USA) was incubated for 40 min at room temperature. Between each step, tissue sections were rinsed in TBS (2×10 min). Tissue-bound peroxidase was visualised by incubating the sections in 0.003% H_2O_2 and 0.025% DAB in 0.05 M Tris-HCl buffer, pH 7.4, the reaction being controlled under the microscope. The samples were counterstained with Mayer's haematoxylin.

As controls for the anti-nNOS reaction, the primary antiserum was substituted by TBS or normal sheep serum (Vector, diluted 1:10,000 in TBS). No residual reaction was detected in any case (Fig. 1a: TBS; Fig. 1b: normal sheep serum). The K205 antibody was generated against purified baculovirus-expressed rat nNOS and its specificity for nNOS has been established by Western blotting and reported previously (Herbison et al. 1996).

Quantification and morphometry of immunoreactive cells

From the sections or cultures of each animal, the quantification of nNOS-immunoreactive cells was determined by a double-blind method. 4000 cells (with intact nuclear and cellular profiles) were analysed per animal. For the in vivo studies, the cells were chosen from 20 sections separated from one another by at least 50 µm (200 cells per section) and were selected randomly all parts of the gland. For in vitro studies, the cells were chosen from 10 microscopic fields obtained by zigzag displacement, sampling randomly

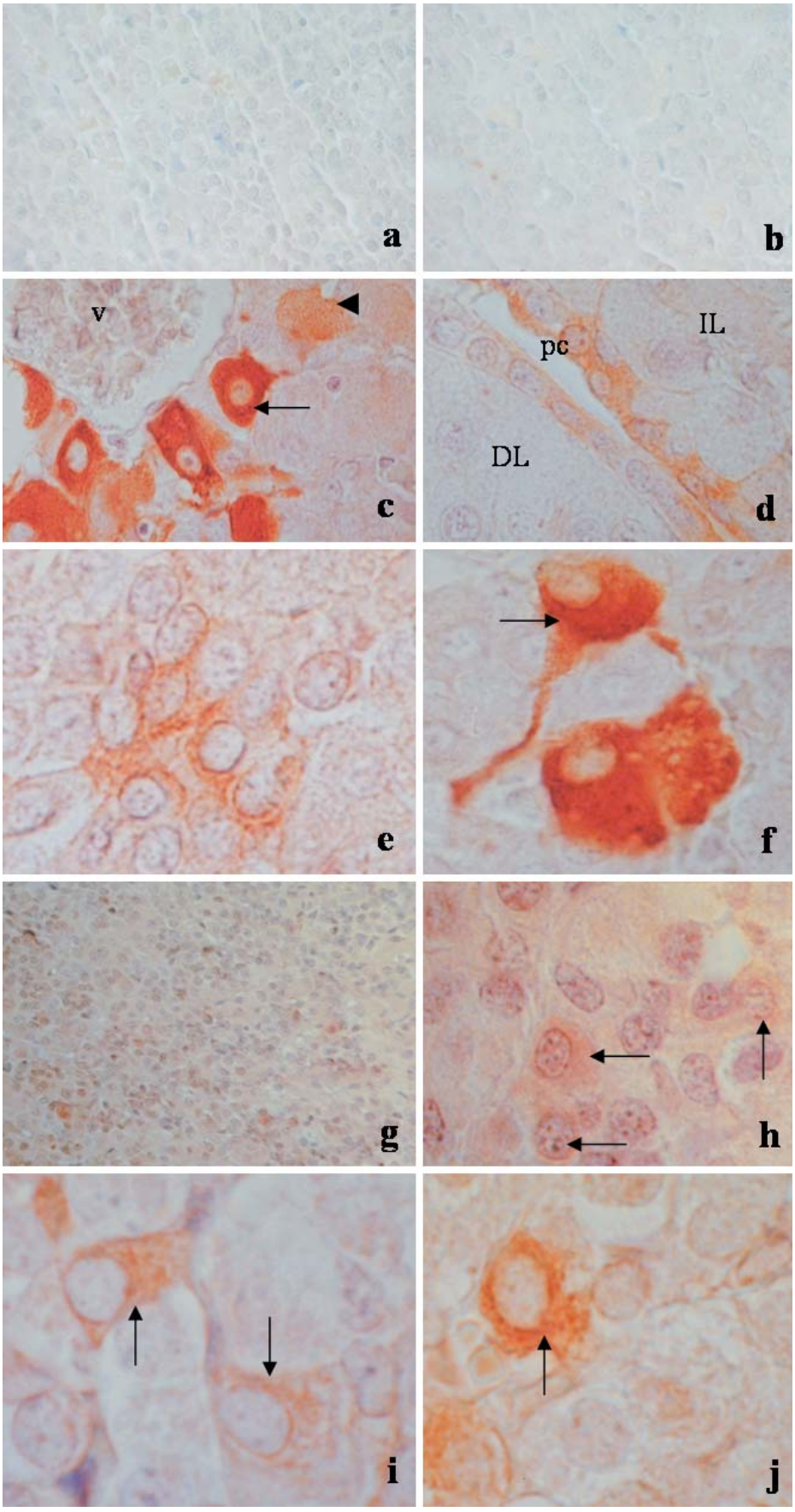

Fig. 1 **a** Micrograph of an untreated control pituitary section in which antiserum was substituted with TBS. Note the absence of immunoreactivity. ×200. **b** Micrograph of an untreated control pituitary gland incubated with normal sheep serum. Note the absence of immunoreactivity. ×200. **c** Typical nNOS immunoreactive cells in the pituitary gland of untreated animals. Note differing intensities of staining: strongly reactive (*arrow*) and weakly reactive (*arrowhead*). (*v* blood vessel). ×400. **d** Representative nNOS-positive cells in the epithelium bordering the pituitary cleft in section of untreated control rat (DL: Distal lobe, IL: Intermediate lobe, pc: Pituitary cleft). ×400. **e** Anti-nNOS immunoreactivity in an islet of glandular cells in an untreated control pituitary. ×400. **f** Micrograph showing a group of nNOS immunoreactive stellate (*arrow*) and glandular cells. ×400. **g** Representative panoramic view of anti-NOS staining of the pituitary gland of a haloperidol-treated rat. Note the weak cytoplasmic staining. ×100. **h** Anti-nNOS immunoreactivity in the pituitary of a haloperidol-treated animal. Note the intensity of cytoplasmic staining (*arrows*) is much weaker than that of the untreated control in *panels* **c** and **f**. ×400. **i,j** Micrographs from the pituitary gland of an animal treated with haloperidol showing the polygonal shape of cytoplasmic nNOS immunoreactive cells (*arrows*) (*v* blood vessel). ×600

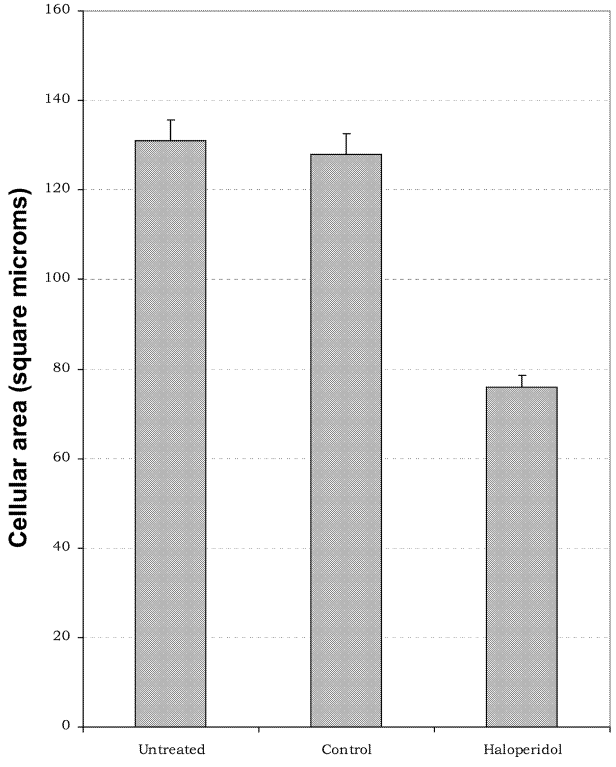

Fig. 2 Graph of the mean pituitary cellular area (μm^2) of the different experimental groups. Note the significant decrease in the area of nNOS-positive cells of rats treated with haloperidol

all parts of the dish. In both the in vivo and in vitro studies, the percentages of reactive cells were calculated. Using a MIP-2 (IMCO-10) image analyser, 100 randomly chosen cells per animal or dish were identified for morphometric study (cells were included only when nuclear and cellular profiles in the plane of section were clearly distinguished). These percentages and morphometric values were analysed statistically and the differences between means obtained were contrasted with double-tail ANOVA, p values less than 0.05 for Scheffé's F-test being considered significant. The results are shown as mean values ± standard error of the mean.

Results

Characterization of the effects of haloperidol administration on the morphology of nNOS-immunoreactive cells in the rat pituitary

To analyse the consequences of haloperidol administration on the nNOS content of pituitary cells, pituitary sections were immunostained with the anti-nNOS antibody K205 (Herbison et al. 1996). No specific staining was observed when the antiserum was substituted with either TBS (Fig. 1a) or normal sheep serum (Fig. 1b). In naïve and control animals, nNOS immunoreactivity was very similar. The nNOS-containing cells appeared in all sections of the pars distalis analysed (Fig. 1c) and in the epithelium bordering the pituitary cleft (Fig. 1d). Two types of cells were distinguished: round or polygonal cells (Fig. 1c) and stellate cells (Fig. 1d). Although some isolated cells were found, the nNOS-immunoreactive cells commonly appeared grouped in clusters or islets close to blood vessels (Fig. 1e,f). nNOS-immunoreactive cells appeared uniformly stained, including cytoplasmic prolongations.

Immunoreactivity for nNOS in pituitary glands of haloperidol-treated males decreased considerably (Fig. 1g,h) in comparison with untreated or control animals. The few nNOS-positive cells of haloperidol-treated animals displayed a weak cytoplasmic staining (*arrows* in Fig. 1h,i,j). In contrast to untreated or sham-injected control animals, nNOS-positive stellate cells were not observed in pituitary sections of haloperidol-treated rats.

Morphoplanimetric analysis of cell size

No significant differences were observed in the mean sectioned cellular surface area of nNOS immunoreactive cells between untreated and control animals (131±7.69 vs 128±6.65 μm^2) (Fig. 2). Similarly, the percentages of nNOS immunoreactive cells (1.34±0.24 vs 1.42±0.31%) were comparable between these groups of animals (Fig. 3). However, haloperidol administration decreased significantly ($p<0.01$) cellular area (Fig. 2) compared to naïve and control animals (76±2.34 μm^2 vs 131±7.69 and 128±6.65 μm^2). In addition, the percentage (0.25±0.07%) of nNOS positive cells was significantly lower (Fig. 4) in haloperidol-treated rats ($p<0.01$).

Effects of in vitro dopamine treatment on the morphology of nNOS immunoreactive cultured cells

Our in vivo studies suggest that the dopamine antagonist haloperidol alters cells of the rat pituitary, reducing both cell area and the number of pituitary cells expressing nNOS. However, considering that haloperidol was administered via i.c.v., these observations may reflect the indirect effects on hypothalamic systems such as GnRH. Thus, to more carefully assess the ability of dopamine to directly alter pituitary cells, we prepared primary cultures from rat pituitary. Cultures were then treated with various doses of dopamine for varying times. In untreated control cultures, nNOS-immunoreactive cells were mainly round or polygonal cells with intense cytoplasmic staining (Fig. 4a). Following dopamine treatment, the cells were more rounded in shape and the anti-nNOS immunoreactivity was more intense than in control dishes (Fig. 4b).

Morphoplanimetric findings of in vitro dopamine treatment

Figure 5 summarizes the morphometric data of the in vitro studies. Control dishes displayed a slow and progressive increase in the cellular area from 1 to 12 h of incubation; however, significant differences were not

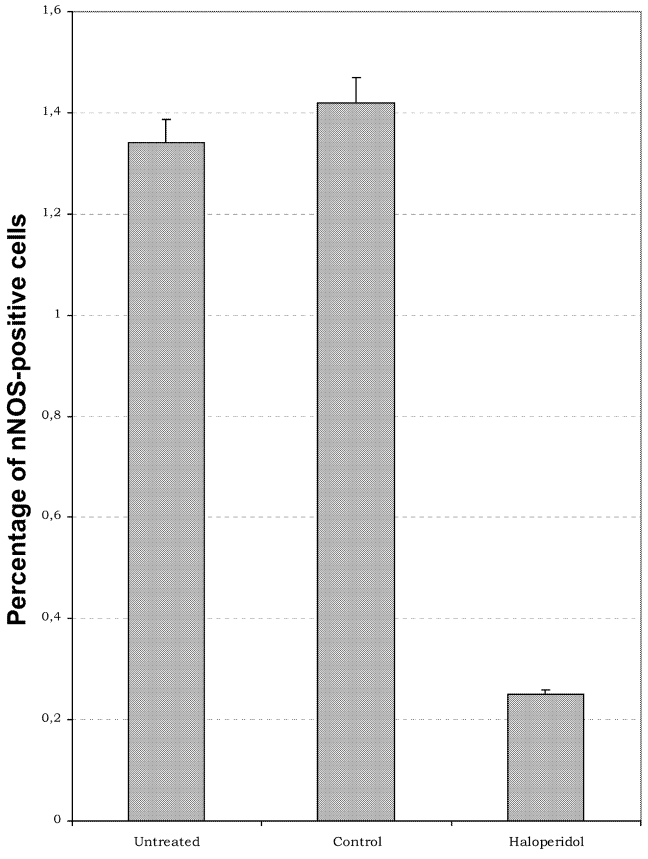

Fig. 3 Plot of the percentage of nNOS-positive pituitary cells calculated from the immunohistochemical analysis of the three experimental groups. Note the significant decrease in the percentage of nNOS-positive cells following treatment with haloperidol

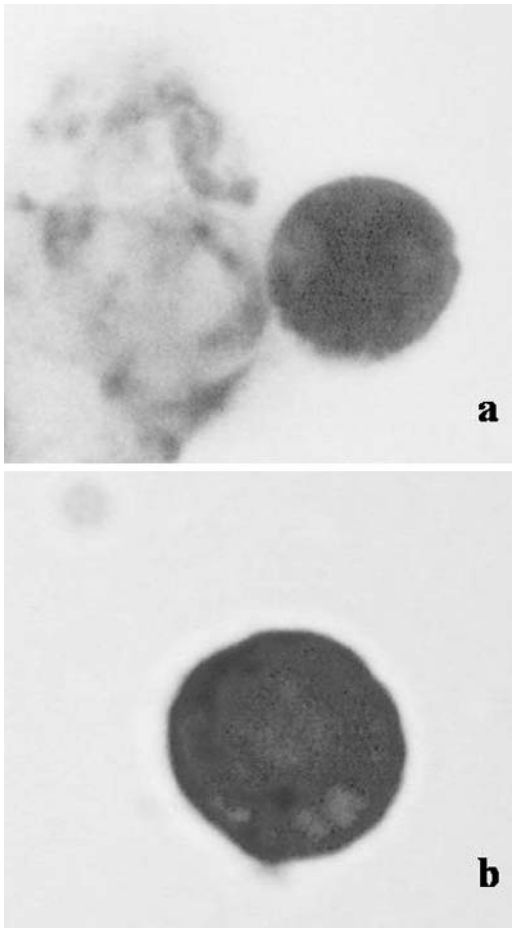

Fig. 4 **a** Micrograph of an anti-nNOS immunoreactive pituitary cell obtained from untreated control cultures (1 h). ×1000. **b** Typical anti-nNOS immunoreactivity in pituitary cultures treated with dopamine. (10^{-7} M dopamine, 1 h). ×1000

noted between the various points of the time-course (1 h:111.5±0.46; 3 h:112.28±4.24; 6 h:115.57±4.04; 12 h: 117.66±4.82 μm^2).

The area of nNOS-positive cells was increased in the cultures treated with either 10^{-7} M or 10^{-8} M dopamine in comparison to control dishes (Fig. 5). After 1 h of treatment, a significant increase of the cellular area was observed for both doses (DA 10^{-7} M:132.89±5.18 μm^2, $p<0.01$; DA 10^{-8} M:126.27±4.42 μm^2, $p<0.05$). Following 3 h of treatment, the effects of the low dose of dopamine were not significant, but the higher dose maintained an enhancement of ($p<0.05$) cellular area (DA 10^{-7} M:121.36±4.85 μm^2; DA 10^{-8} M:111.63±3.79 μm^2). After 6 h of treatment, the positive effect of dopamine on the cellular area of nNOS-positive cells was no longer observed.

The percentage of nNOS-positive cells in untreated control cultures changed very little during the 12 h time-course (Fig. 6). Dopamine augmented the number of nNOS-expressing cells in a dose-dependent manner. At 10^{-8} M, dopamine increased significantly these percentages ($p<0.05$) but only at 1 (8.13±0.28) and 3 h (7.24±0.25) of treatment (Fig. 6). However, at a concentration of 10^{-7} M, dopamine increased significantly ($p<0.01$) the percentages of positive cells at all point of the 12 h time-course. (1 h:10.22±0.15; 3 h:9.97±0.19; 6 h:8.54±0.17; 12 h:8.14±0.19). These in vitro results suggest that dopamine is capable of directly up-regulating the expression of nNOS in pituitary cultures.

Discussion

In the present work, we have demonstrated that the intraventricular injection of the dopamine inhibitor haloperidol affects the expression of nNOS in the hypophysis of the rat. After treatment with the drug, the expression of the enzyme is significantly decreased as compared with control animals, based on the percentage of anti-nNOS immunoreactive cells in the pituitary gland. Moreover, the cell size of nNOS-positive cells decreased with haloperidol treatment.

Many lines of evidence implicate the involvement of nitric oxide in regulation of pituitary function, including the modulation of secretion of adrenocorticotropin hormone (Rivier 1995; Turnbull and Rivier 1996), luteiniz-

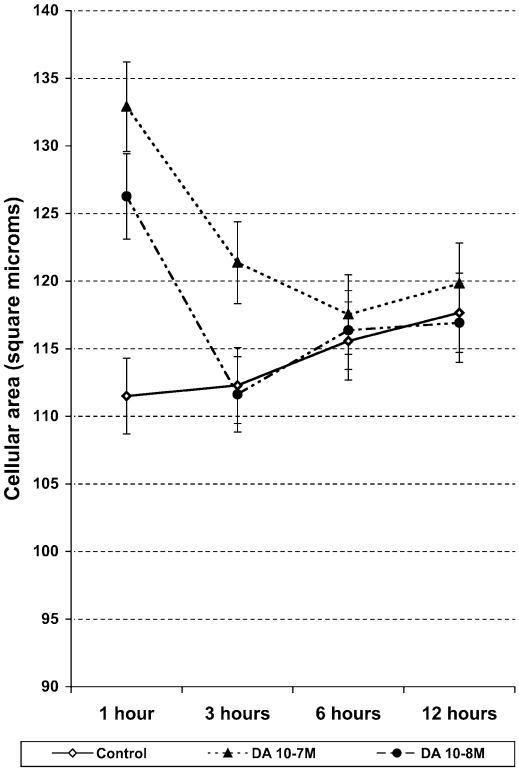

Fig. 5 Graph of the mean cellular area (μm^2) of control or dopamine-treated pituitary cells in vitro. Note the increase in the area of nNOS-positive cells following treatment with dopamine

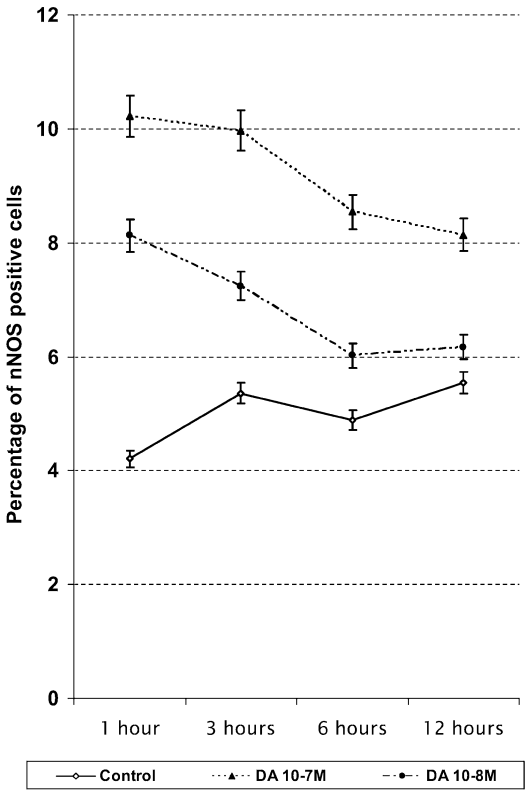

Fig. 6 Plot of the percentage of nNOS-positive cells detected in control and dopamine-treated cultures of rat pituitary gland. Note the significant increases in the percentage of nNOS-positive cells following treatment with dopamine

ing hormone or follicle-stimulating hormone (Ceccatelli et al. 1993; Kaiser et al. 1996; Yamada et al. 1997; Yu et al. 1997; Gobbetti and Zerani 1998), growth hormone (Tena-Sempere et al. 1996; Cuttica et al. 1997), prolactin (Duvilanski et al. 1995, 1996; Pinilla et al. 1996; Vankelecom et al. 1997A; Chiodera et al. 1998) and thyrotropin hormone (Haluzik et al. 1998). Nitric oxide may serve as an auto-paracrine modulator because the presence of nNOS in the gonadotroph and folliculo-stellate pituitary cells has been reported (Ceccatelli et al. 1993; Lloyd et al. 1995) and clusters of nNOS-positive cells located near pituitary blood vessels have been found (Okere et al. 1998). This is consistent with our current study where nNOS immunoreactivity was detected in both glandular and folliculo-stellate cell types. The differing intensities of anti-NOS reactivity observed in pituitary cells of the present study most likely reflect different functional moments in the nNOS-expressing pituitary cells, although we can exclude that this may be due to the orientation of the cells within the pituitary section. Thus, the variations in levels of nNOS expression may be related to cell age, stage of cell cycle, or expression of certain types or numbers of receptors.

Although nNOS has historically been considered a constitutively expressed enzyme, accumulating evidence indicates that nNOS expression is, in fact, highly-susceptible to regulation by various physiological or pathological conditions (Sasaki et al. 2000). Modifications in the pituitary expression of immunological nNOS can be induced by ageing or endocrine manipulations (Vankelecom et al. 1997B; McCann, 1997). For example, castration or treatment with GnRH (Shi et al. 1998; Garrel et al. 1998) alters nNOS expression in the pituitary and these modifications are mainly detected in gonadotroph cells. It is known that nitric oxide inhibits the release of prolactin (Duvilanski et al. 1996) and inhibits VIP-stimulated secretion of the hormone (Chiodera et al. 1998). However, studies analysing changes in nNOS expression following experimental manipulation of mechanisms involved in the regulation of the secretion of prolactin are not available.

Dopamine induces the in vitro activity of nNOS and could inhibit secretion of prolactin by increasing nitric oxide and cGMP (Duvilanski et al. 1996). Moreover, it has been demonstrated that kainate injection inhibits prolactin secretion, which is dependent on both endogenous nitric oxide and dopamine release (Pinilla et al. 1996). However, it is not known whether nNOS expression in pituitary cells is regulated by dopamine. The present study demonstrates that dopamine antagonist haloperidol down-regulates nNOS expression in the rat pituitary, implicating a role for dopaminergic system in regulating the expression of this enzyme. Similar mechanisms of regulation by dopamine have been observed in

other systems. For example, in the corpus striatum, the histochemical staining of the NOS-related NADPH-diaphorase is decreased by an antagonist of the dopamine D1-like receptor and increased when an antagonist of the dopamine D2-like receptor is administered (Morris et al. 1997). Furthermore, Melis et al. (1997) have demonstrated that nitric oxide production within the hypothalamic paraventricular nucleus is increased in projecting oxytocinergic neurons when D2 receptors are stimulated.

Whether D1 or D2 receptors are involved in the dopaminergic regulation of nNOS expression in pituitary cells remains to be elucidated. Although haloperidol binds to all types of dopamine receptors (D1 to D5), it is classically considered an antagonist for D2 receptors. Thus, the inhibition of nNOS expression in haloperidol-treated rats suggests that dopamine most likely signals via the D2 receptor to enhance production of nitric oxide in the pituitary. Moreover, D1 receptors appear not to be involved in the basal regulation of prolactin and other pituitary hormones (Grodum et al. 1998).

In our studies, haloperidol was administered to rats by intraventricular (3rd ventricle) injections. Based on the route of administration, this antagonist could alter different hypothalamic systems given that dopamine is involved in the regulation of various endocrine loops including the GnRH and somatostatin producing neurons of the hypothalamus (McMahon et al. 1998; West et al. 1997; Hileman and Jackson 1999; Tortonese 1999). Thus, considering that GnRH or GHRH which is influenced by somatostatin activates nNOS in pituitary cells (Chen et al. 1999; Ferrini et al. 2001; Tsumori et al. 2002), we cannot totally exclude the possibility that the haloperidol effects which we have observed on pituitary nNOS-positive cells reflect the indirect effects of hypothalamic systems.

However, our in vitro results demonstrate that dopamine is able to directly up-regulate nNOS in primary pituitary cultures. Transcripts of the two D2 s and D2L variants of the D2 receptor are highly expressed in melanotroph cells of the pituitary intermediate lobe and in the anterior lobe of the gland, localized mostly within lactotrophs and to a less extent somatotrophs (Bunzow et al. 1988; Dal Toso et al. 1989; Meador-Woodruff et al. 1989; Giros et al. 1989; Monsma et al. 1989; Kukstas et al. 1991; Soyoola et al. 1994). Therefore, the results of the in vitro stimulation with dopamine suggest that the in vivo intraventricular treatment with haloperidol may act directly on D2 receptor-containing cells in the anterior lobe of the pituitary gland.

In summary, the decrease in the percentage of cells expressing nNOS and the accompanying reduction in cell size noted after treatment with haloperidol suggest that dopamine regulates nNOS levels in populations of pituitary cells. These effects are supported by our in vitro study demonstrating that dopamine induces increases in the number and size of nNOS-positive pituitary cells in culture. Thus, our observations are consistent with a role for nitric oxide in the dopaminergic regulation of the rat pituitary gland. Moreover, the current study indicates that nitric oxide may function as an auto-paracrine modulator of the inhibitory effects of dopamine on hormonal secretion in the pituitary.

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