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Inducible Nitric Oxide Synthase in P11 Cells

EXPRESSION IN THE PRESENCE OF INTERFERON-γ, LIPOPOLYSACCHARIDE, AND MODIFIED SERUM

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ABSTRACT. P11 cells, derived from the transplantable rat pituitary tumor 7315a, have been used previously as a model system to study the regulation of serotonin$_{2A}$ (5-HT$_{2A}$) receptor expression. As our laboratory has been interested in characterizing the interactions between the 5-HT$_{2A}$ receptor and inducible nitric oxide synthase (iNOS), we have analyzed the P11 cell line for iNOS expression. Treatment of P11 cells with interferon-γ and lipopolysaccharide resulted in a 23-fold increase in nitrite production and induced expression of iNOS protein. The increase in nitrite levels was attenuated by the non-selective nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester, but not the neuronal NOS inhibitor 7-nitroindazole. Typically, P11 cells have been grown in either charcoal-stripped or dialyzed serum-containing medium. We have observed that P11 cells grown under these culture conditions express basal iNOS activity, as evidenced by a 5-fold increase in nitrite accumulation over a 48-hr period, compared with that in cells grown in non-modified serum, which was inhibited by the selective iNOS inhibitor L-N^6-(1-iminoethyl)-lysine. Conditioned medium from P11 cells was able to stimulate nitrite accumulation in C6 glioma cells, suggesting that the P11 cells may produce a pro-inflammatory-like factor. As pro-inflammatory cytokines have been shown to modify hormone secretion from the anterior pituitary, the P11 cell line may be a useful *in vitro* model by which to characterize the function of cells from this organ. In addition, our data suggest that alteration of the microenvironment of the anterior pituitary may result in iNOS expression, possibly altering the function of the hypothalamic–pituitary–adrenal axis. BIOCHEM PHARMACOL **59**;5:509–516, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. anterior pituitary; lipopolysaccharide; interferon-γ; dialyzed serum

Administration of pro-inflammatory mediators such as LPS† and the cytokines IL-1β and IFN-γ has been shown to alter the functioning of the hypothalamic–pituitary–adrenal axis [1]. Systemic administration of IL-1β, TNF-α, or IL-6 elevates ACTH levels in rats, an action that is believed to be the result of increased CRF secretion [2–4]. Administration of NOS inhibitors has been shown to modify the action of these pro-inflammatory mediators, suggesting that NO plays a role in the regulation of pituitary functioning [5, 6]. In addition to the *in vivo* models, pro-inflammatory cytokines have been shown to alter the secretory activity of anterior pituitary cells in primary cultures, which is also reversed by NOS inhibitors [7].

NO synthesis is mediated by three different NOSs: nNOS, eNOS, and iNOS [8]. The first two synthases are expressed constitutively and are Ca^{2+}-dependent, whereas iNOS must be induced by pro-inflammatory mediators and is Ca^{2+}-independent. To date, eNOS is believed to mediate the actions of the pro-inflammatory mediators on ACTH secretion *in vivo* at the level of the hypothalamus [9]. iNOS has been shown to modulate the action of the cytokines in primary cultures and to be expressed in non-secreting folliculostellate cells and other non-secreting cells of unknown classification within these cultures [10].

The monoamine neurotransmitters 5-HT, norepinephrine, and dopamine are also known to modulate the functioning of the hypothalamic–pituitary–adrenal axis [11]. 5-HT has been shown to enhance ACTH release via 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{1A}$ receptors [12]. Recently, our laboratory and that of Shimpo *et al.* have shown that 5-HT$_{2A}$ receptor activation reduces cytokine- and LPS-mediated induction of the expression of iNOS in C6 glioma cells and vascular smooth muscle cells, respectively [13–15]. As both the 5-HT$_{2A}$ receptor and iNOS have been shown to regulate hormone expression from the anterior pituitary, we have worked to find an *in vitro* model by which to characterize the interaction between this receptor and iNOS in a pituitary-derived cell line.

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† *Abbreviations*: LPS, lipopolysaccharide; 5-HT, serotonin; CRF, corticotropin-releasing factor; IFN-γ, interferon-γ; L-NAME, N^G-nitro-L-arginine methyl ester; NIL, L-N^6-(1-iminoethyl)-lysine; IL, interleukin; TNF-α, tumor necrosis factor-α; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and PMSF, phenylmethylsulfonyl fluoride.

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P11 cells were derived originally from the transplantable rat pituitary tumor 7315a by the laboratory of Molinoff [16]. The P11 cell line was shown to express the 5-HT_{2A} receptor and has been used extensively to study the regulation of 5-HT_{2A} receptor expression [17–20]. The original 7315a tumor was of a prolactin-secreting lactotroph origin; however, P11 cells have not been reported to release prolactin and thus may have an alternative cellular derivation [16]. To date, it has not been demonstrated whether this cell line expresses any of the NOS enzymes.

To determine if the P11 cell line is a potential model by which to study the regulation of iNOS by 5-HT_{2A} receptors, we have screened this cell line to ascertain whether expression of iNOS occurs following treatment with the pro-inflammatory cytokine IFN-γ and/or LPS. Here, we report that P11 cells express iNOS following LPS and/or IFN-γ exposure. We have also found, however, that the P11 cell line expresses iNOS activity when grown under conditions that maximize 5-HT_{2A} receptor expression. Lastly, we provide evidence that P11 cells may produce a factor that can stimulate iNOS activity in a different cell line.

MATERIALS AND METHODS

Materials

DMEM, FBS, and dialyzed FBS were purchased from HyClone. The iNOS antibody was obtained from Alpha Diagnostics. Penicillin, streptomycin, and L-glutamine solution, oxaloacetate, pyruvate, insulin, nystatin, activated charcoal, NP-40, PMSF, leupeptin, aprotinin, sulfanilamide, *N*-naphthylethylenediamine, sodium nitrite, alkaline phosphatase substrate, the secondary antibody, and LPS (*Salmonella typhosa*) were purchased from the Sigma Chemical Co. IFN-γ was purchased from Genzyme. L-NAME, NIL, and 7-nitroindazole were purchased from Research Biochemical International.

Cell Culture

P11 cells were a gift from the laboratory of Dr. Perry Molinoff (Bristol-Myers Squibb). The cells were grown in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, 150 μg/mL of oxaloacetate, 50 μg/mL of pyruvate, 0.2 U/mL of insulin, 100 U/mL of nystatin, and 10% FBS (see exceptions below) at 37° and 10% CO_2. All experiments were performed when the cells approached confluence. To determine the action of serum modification on iNOS expression in the cells, either 10% charcoal-treated FBS or 10% dialyzed FBS was substituted for unmodified FBS where indicated. The C6 glioma cells were a gift from Dr. Beth J. Hoffman (NIMH) and grown in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Charcoal Treatment of Serum

Five hundred milliliters of serum was incubated with 5 g of activated charcoal for 30 min at room temperature on a stir plate [16]. Then the serum was centrifuged at 16,000 *g* for 30 min at 4°, and decanted into a fresh bottle for storage at −20°. The serum was sterile filtered when the medium was prepared.

Determination of Nitrite Production

The culture supernatants were collected at the indicated times and assayed for the accumulation of NO_2^-, a stable end product of NO formation. A 0.5-mL portion of the sample was added to an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 1% *N*-naphthylethylenediamine), and the absorbance of the resulting azo-dye product was determined with a UV/visible spectrophotometer at 540 nm. Sample concentrations were determined by linear regression analysis using sodium nitrite as the standard. Conversion of nitrate to nitrite using nitrate reductase did not enhance the total level of nitrite in the medium significantly (<10%) so we did not include the reduction process in our subsequent evaluation of nitrite levels for these experiments.

Western Blot Preparation

After various treatments, the cells were washed with ice-cold PBS, and then were solubilized in 0.5 mL of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 1 mM PMSF, 2 mM EDTA, 10 μg/mL of leupeptin, 1 μg/mL of aprotinin, and 1% NP-40. Then the cell lysates were centrifuged for 20 min at 100,000 *g* at 4°. Aliquots of the lysate were taken for the protein concentration determination (see below). Equal amounts of sample lysate (approximately 40 μg) were loaded and separated on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad) using transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3] at 30 V for 8 hr. The membrane was incubated with Tris-buffer saline (TBS) + 0.1% Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk at room temperature for 2 hr to block the nonspecific antibody–membrane interactions.

iNOS Antibody Labeling

The membrane was probed subsequently for 1 hr at room temperature with polyclonal anti-iNOS antibody (1:1000 in TBST). The antibody used in this study was from a commercial source. The antibody was raised in rabbits against an 18-amino-acid middle section of the human iNOS protein and has been shown to also label the mouse, chicken, and rat isoforms of iNOS. The antibody does not cross-react with either eNOS or nNOS. The western blot positive control was extracted from chickens. The blots

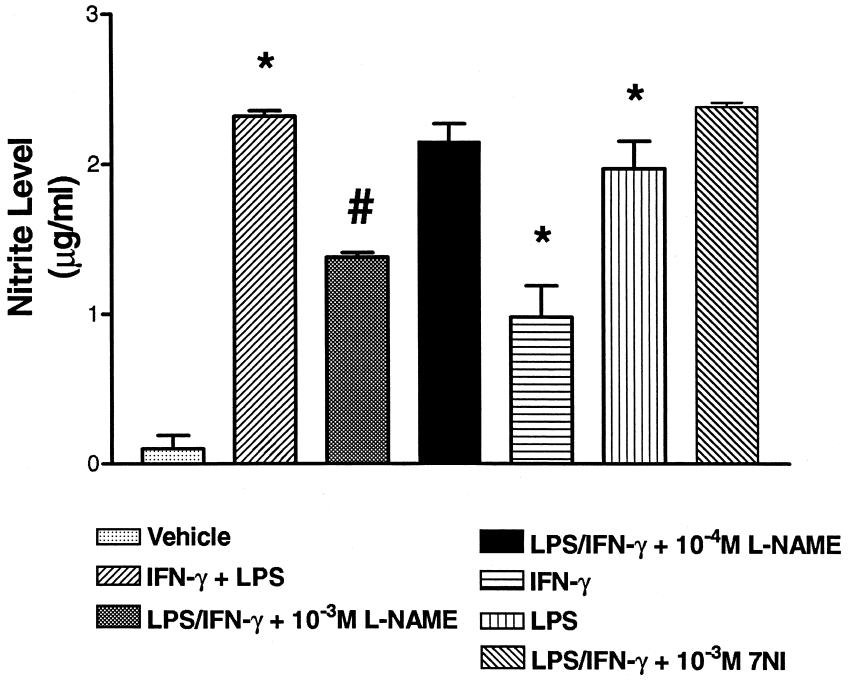

FIG. 1. Activation of iNOS activity by LPS and IFN-γ. Cells were treated with LPS and/or IFN-γ for 24 hr. NOS inhibitors were co-incubated with the inducing agents for the 24-hr time period. Nitrite levels were determined as described in Materials and Methods. Data are expressed as the means ± SEM of three separate experiments. Key: (*) indicates significantly different from vehicle-treated cells as determined by Student's *t*-test ($P < 0.05$); and (#) indicates significantly different from LPS/IFN-γ-treated cells as determined by Student's *t*-test ($P < 0.05$).

were washed three times with TBST and then incubated with the secondary antibody, goat anti-rabbit IgG, conjugated to alkaline phosphatase (1:5000 dilution) for 1 hr. Following washing of the membrane with TBST, a tablet of alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium, BICP/NBT) was dissolved in 10 mL of distilled water, and the blots were incubated with the substrate solution to visualize the bands.

Protein Analysis

Protein levels were determined with bicinchoninic acid protein assay reagent, with bovine serum albumin used as the standard. The absorbance was determined at 560 nm with a UV/VIS spectrophotometer. Then the protein levels of the samples were determined by linear regression analysis.

Statistical Analysis

Results are expressed as means ± SEM. Differences between means were assessed using Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Elevation of Nitrite Levels

To determine if P11 cells could express iNOS activity, we treated the cells with known activators of iNOS. Vehicle-treated cells expressed a low level of basal nitrite production (Fig. 1). Treatment of the cells with a combination of 10 μg/mL of LPS and 50 U/mL of rat IFN-γ produced a 23-fold increase in nitrite production. L-NAME blocked the stimulatory effect of the LPS and rat IFN-γ combination in a concentration-dependent manner. Higher concentrations of L-NAME adversely affected cell viability (not shown). Both 50 U/mL of rat IFN-γ and 10 μg/mL of LPS, when administered individually, increased nitrite production, with LPS being the more effective agent. The nNOS selective inhibitor 7-nitroindazole had no effect on the LPS + rat IFN-γ-mediated increase in nitrite levels.

Activation of iNOS Protein Expression

We next examined whether treatment with the pro-inflammatory mediators could stimulate iNOS protein expression. Vehicle treatment did not produce any detectable iNOS protein expression (Fig. 2, lane 2). Treatment with the rat IFN-γ and LPS combination produced a robust expression of iNOS protein (lane 3) at the appropriate molecular weight (see positive control in lane 1). The presence of L-NAME, which reduced nitrite levels (see above), did not influence the pro-inflammatory mediator induction of iNOS protein expression (lanes 4 and 5). As demonstrated in the nitrite data above, administration of rat IFN-γ (lane 6) and LPS (lane 7) individually also resulted in the expression of iNOS protein, with LPS producing a greater level of labeling. 7-Nitroindazole did not alter iNOS expression (data not shown).

Time Course of Induction

The combination of LPS and rat IFN-γ induced nitrite formation in a time-dependent manner (Fig. 3). A statistically significant ($P < 0.05$) increase in nitrite production was observed within 6 hr after treatment and began to plateau at 48 hr. The maximal level of nitrite formation was

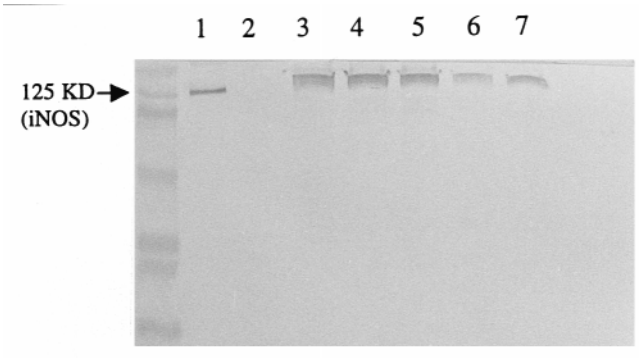

FIG. 2. Western blot analysis of iNOS protein expression. The cells were treated for 24 hr with the following agents: lane 2, vehicle; lane 3, 10 μg/mL of LPS and 50 U/mL of IFN-γ; lane 4, both inducing agents plus 10^{-3} M L-NAME; lane 5, both inducing agents plus 10^{-4} M L-NAME; lane 6, 50 U/mL of IFN-γ; and lane 7, 10 μg/mL of LPS. This figure is representative of three separate experiments. The lane on the far left is a molecular weight standard ladder. Lane 1 is the iNOS protein positive control.

similar to that observed in Fig. 1 following a 24-hr treatment.

Effect of Co-application of LPS and rat IFN-γ

Co-administration of submaximal concentrations of cytokines and LPS has been shown previously to result in synergistic activation of iNOS activity and protein expression in C6 glioma cells. To determine if synergistic activation occurred in P11 cells, we added increasing levels of LPS along with 20 U/mL of rat IFN-γ to the cells for 24 hr (Fig. 4A). We also added increasing amounts of rat IFN-γ along with 5 μg/mL of LPS to the cells (Fig. 4B). In both sets of treatments, an additive and saturable response in nitrite production was obtained, with similar maximal production levels of 2.6 ± 0.3 and 2.7 ± 0.4 μg/mL of nitrite obtained in the respective experiments.

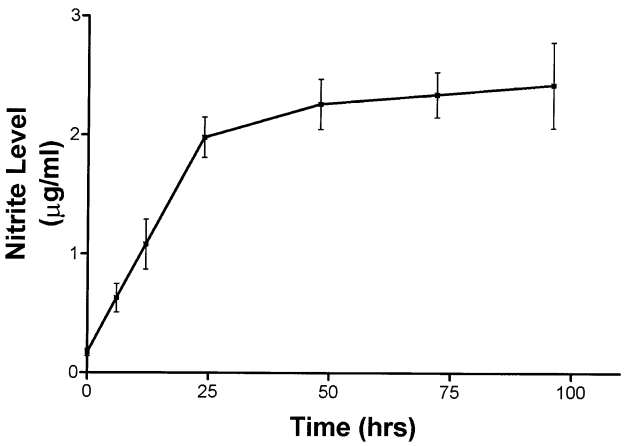

FIG. 3. Time–course analysis of iNOS activity. The P11 cells were treated for the indicated times with 10 μg/mL of LPS and 50 U/mL of IFN-γ. The data presented are means ± SEM of four separate experiments.

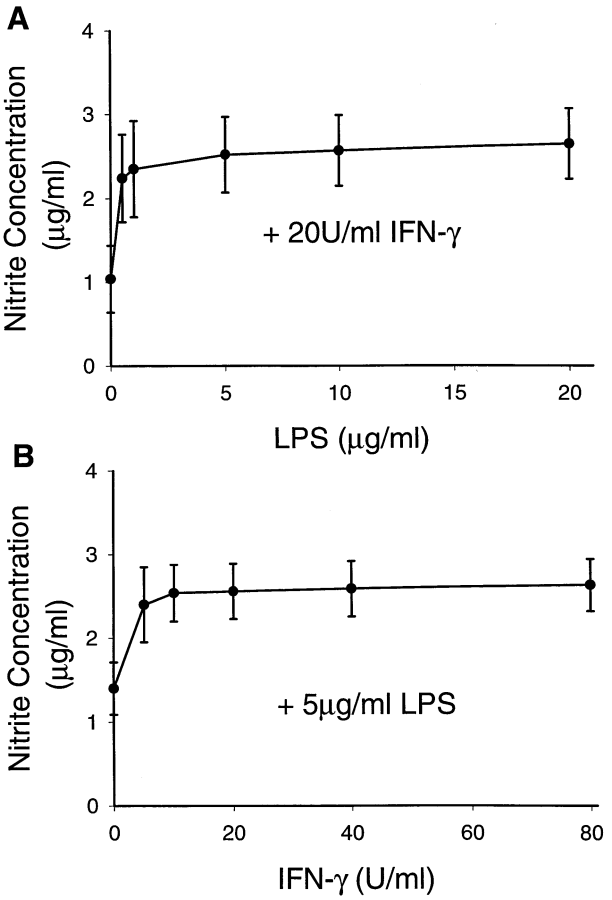

FIG. 4. (A) Influence of IFN-γ on LPS-mediated increases in iNOS activity. (B) Influence of LPS on IFN-γ-mediated increases in iNOS activity. The P11 cells were treated with the indicated concentrations of inducing agents for a period of 24 hr. Data are expressed as means ± SEM of three separate experiments.

Effect of Serum Modification

P11 cells are usually grown in the presence of modified serum, with charcoal treatment or dialysis performed to remove endogenous 5-HT, in order to allow for maximum expression of 5-HT receptors. We examined whether the use of modified serum could alter the expression and activity of iNOS in the P11 cell line. P11 cells (75% confluent) were grown for 48 hr with medium containing either 10% non-modified, 10% dialyzed, or 10% charcoal-treated FBS. Cells grown in the modified sera exhibited a significant increase in nitrite formation (Fig. 5) and protein expression in comparison with those cells grown in non-modified serum, with the cells in dialyzed serum exhibiting the greatest increase in protein expression (Fig. 6, left and right, respectively). Those cells grown in charcoal-stripped medium produced a barely detectable increase in iNOS protein expression (Fig. 6, center). L-NAME and the selective iNOS inhibitor NIL at a concentration of 1 mM and 100 μM, respectively (Fig. 7), blocked the increase in nitrite formation in the cells grown in dialyzed medium. 7-Nitroindazole at 1 mM did not alter the nitrite production in the cells grown in the presence of dialyzed serum.

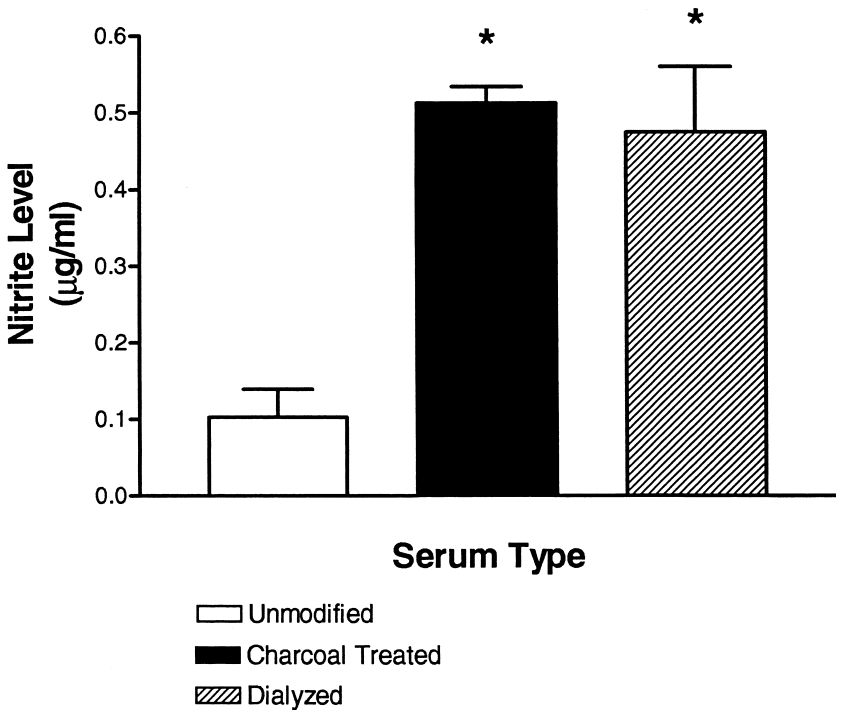

FIG. 5. Influence of serum modification on nitrite accumulation. Confluent P11 cells were cultured in DMEM containing the indicated sera at a concentration of 10% for a period of 48 hr. The medium was removed and analyzed for nitrite. The data are expressed as means ± SEM of three separate experiments. Key: (*) indicates significantly different from unmodified serum-containing medium ($P < 0.05$).

Stimulation of iNOS in C6 Glioma Cells

Alterations in culture conditions or microenvironments such as pH can activate the synthesis and release of iNOS-activating agents in some cell lines. To determine if such a mediator was produced, P11 cells were cultured in dialyzed serum-containing medium for 4 days. The conditioned medium was subsequently collected and a portion analyzed for nitrite levels. The remaining conditioned medium, or control medium, was transferred to confluent cultures of C6 glioma cells. The original C6 medium was removed, with the nitrite levels assayed to make sure that iNOS was not active, and the cells were washed with PBS. After a 24-hr incubation with the C6 cells the medium was collected again and assayed for nitrite. Application of control medium produced a 2.4-fold increase in nitrite levels in the C6 glioma cells, indicating that the mechanical perturbation of the cells alone could activate nitrite formation (Fig. 8). Application of the conditioned P11 medium produced a 2.1-fold increase in C6 glioma cell nitrite levels over the control medium. Co-incubation with the iNOS selective inhibitor NIL and the conditioned medium prevented the increase in nitrite levels over the

FIG. 6. Effect of serum modification on iNOS protein expression. Confluent cultures were treated as described in the legend of Fig. 5. The cells were processed for western blot analysis as described in Materials and Methods. Left panel: Expression of iNOS in P11 cells grown in unmodified serum (lane 2). Center panel: P11 cells grown in charcoal-treated medium (lane 2). Right panel: P11 cells grown in the presence of dialyzed serum-containing medium (lane 2). Lane 1 in each picture is the positive control protein.

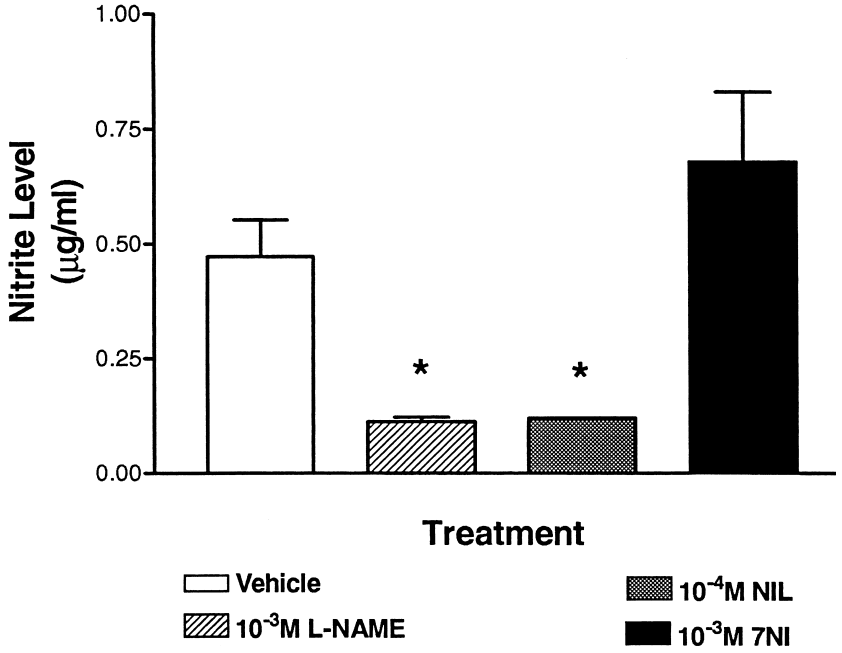

FIG. 7. Effect of NOS inhibitors on nitrite accumulation. The P11 cells were grown in the presence of dialyzed serum-containing medium, with or without the indicated levels of NOS inhibitors, for 48 hr. The data are means ± SEM of three separate experiments. Key: (*) indicates significantly different from dialyzed serum-containing medium (vehicle; $P < 0.05$).

control medium, indicating that the elevation was due to iNOS activity. An increase in iNOS protein expression following treatment with the conditioned medium, however, was not detectable by western blot analysis. Interestingly, the concentration of nitrite in the conditioned medium was less than that observed at 48 hr of culture (see Fig. 5), suggesting a desensitization response to the dialyzed serum.

DISCUSSION

In the present study, we have found that P11 cells express iNOS following activation by the pro-inflammatory mediators LPS and rat IFN-γ and by growth in modified-serum-containing medium. The expression of iNOS activity was time dependent, and NOS inhibitors, including an agent selective for iNOS, blocked the observed increases in nitrite production. We also found that conditioned medium from the P11 cells was capable of stimulating iNOS activity in C6 glioma cells.

iNOS expression has been observed previously in primary anterior pituitary cell cultures from rats treated with IFN-γ and in the mouse anterior pituitary tumor cell line AtT20/D16 following stimulation with IL-1β [10, 21]. In the primary culture model, the majority of iNOS-expressing cells were classified as non-secretory folliculostellate cells, while the remaining iNOS-expressing cells could only be described as non-secretory cells [10]. The P11 cell line was derived originally from a prolactin-secreting tumor but has

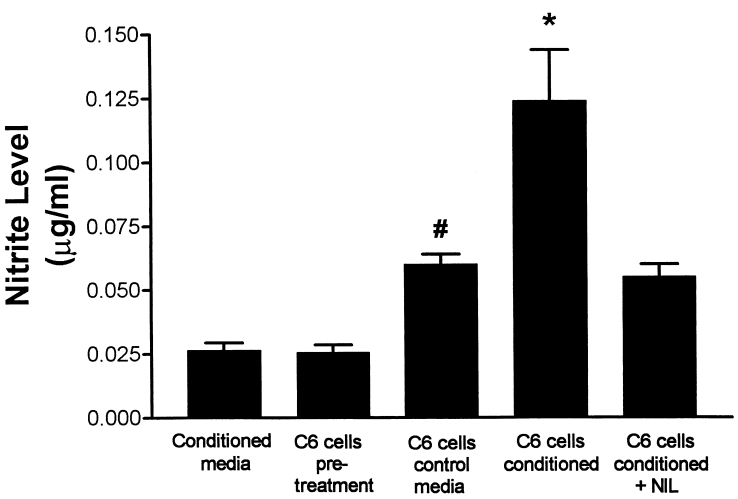

FIG. 8. Induction of nitrite accumulation in C6 glioma cells treated with P11 conditioned medium. P11 cells were cultured in dialyzed serum-containing medium for 96 hr. The medium was removed from the P11 cells, with a portion being assayed for nitrite (conditioned medium), and transferred to confluent cultures of C6 glioma cells. The original C6 glioma cell culture medium had been removed and tested for nitrite (C6 cells pretreatment), and the cells were washed twice with PBS. The C6 glioma cells were cultured in either control medium (C6 cells control medium), conditioned medium (C6 cells conditioned) or conditioned medium plus 10^{-5} M NIL (C6 cells conditioned + NIL) for 24 hr after which the medium was removed and analyzed for nitrite accumulation. The data are means ± SEM of three separate experiments done in duplicate. Key: (#) indicates significantly different from the untreated C6 glioma cells ($P < 0.05$); and (*) indicates significantly different from C6 glioma cells treated with control medium ($P < 0.05$).

not been shown to secrete this hormone [16]. Thus, it is possible that the P11 cell line may have a folliculostellate origin, a conclusion that must be tested further by immunocytochemical methods.

The band labeled by the iNOS antibody was observed to run higher than the control protein in our western blots. This may be due to the fact that the positive control protein is from chickens (125 kDa), an isoform that is known to be 11 amino acids shorter than the rat isoform (see the detailed description in Materials and Methods). The rat variant is known to run between 135 and 150 kDa. We are confident that we are not also labeling the other forms of NOS, as our pharmacological data indicate that neither eNOS nor nNOS is contributing to the production of nitrite. The inability of 7-nitroindazole to alter nitrite levels in either the pro-inflammatory mediator-stimulated cells or the cells grown in modified serum indicates that nNOS is not responsible for our observations. In addition, the iNOS selective inhibitor NIL produced an equal level of inhibition of nitrite accumulation as did the non-selective NOS inhibitor L-NAME. If eNOS were contributing to the increase in nitrite, L-NAME would have produced a greater level of inhibition than NIL.

Both LPS and IFN-γ were effective in activating iNOS protein expression and activity in the P11 cell line, with LPS being more effective than rat IFN-γ at the levels tested. The two agents together produced a greater level of iNOS induction as evidenced by both nitrite levels and protein expression. Co-stimulation with various combinations of pro-inflammatory mediators has been shown to result in synergistic activation of iNOS in some cell lines such as C6 glioma cells [22]. We found in the P11 cells, however, that co-application of low concentrations of LPS and IFN-γ only resulted in an additive effect on nitrite formation and quickly reached a ceiling equaling the level of nitrite produced by a high concentration of LPS alone. What may be the possible mechanisms that contribute to this observation? Induction of iNOS can occur via the activation of numerous transcription factors including nuclear factor-κB, C/EBPβ, and AP-1, all of which have transcription binding sites on the iNOS promoter [23]. The presence of multiple transcription sites on the iNOS promoter allows for varying levels of activation. The observation that LPS and IFN-γ stimulation produces a plateau of nitrite formation that cannot be surpassed by higher concentrations of either compound or by increasing the time of stimulation may indicate that the two agents may be working through the same transcription factor, reaching a maximal level of activation. Indeed, analysis of the promoter region of rat iNOS has demonstrated that each transcription factor-binding region contributes a submaximal level of activity for the initiation of transcription [23]. Alternatively, it may be possible that an increase in substrate availability (L-arginine) may contribute to the increase in nitrite formation. Subsequently, the plateau in nitrite formation could be the result of a plateau in substrate availability. Another factor that could potentially contribute to the increase in nitrite formation and iNOS expression is the possibility that the turnover of the enzyme is decreased. Detailed analysis of transcription, L-arginine transport, and enzyme turnover will be necessary to determine which factor plays the greatest role in the elevation of iNOS protein expression and nitrite formation.

The observation that the P11 cells can express iNOS activity following culture in modified serum could be the result of the loss of small molecules and/or proteins, resulting in stress to the P11 cells. The stress event may then result in the P11 cells producing and releasing agents such as cytokines. Our evidence suggests that the release of a small molecule from the P11 cells may be occurring, as we were able to transfer an iNOS-stimulating "factor" to C6 glioma cells. Although the application of medium itself to the C6 glioma cells produced an increase in nitrite, the application of the conditioned medium produced a further enhancement of nitrite accumulation that was blocked by the iNOS-selective inhibitor. Identifying the factor will require further research. Macrophages have been demonstrated to respond in a similar manner to low pH levels [24]. The low pH level results in the stimulation of TNF-α production and secretion followed by an increase in iNOS activation.

In summary, the present data demonstrated that P11 cells express iNOS in response to LPS and IFN-γ, that they can be activated to express iNOS activity following exposure to modified serum, and that this activation results in the formation of an iNOS-inducing factor that can be transferred to another cell line. Thus, the P11 cell line may provide a novel *in vitro* system by which to examine the regulation of iNOS activity in the anterior pituitary as well as providing another system by which to examine the regulation of iNOS induction by serotonin 5-HT_{2A} receptors. The data also suggest that stressful changes in the microenvironment of the anterior pituitary may result in the expression of iNOS, thereby altering the secretion of hormones from this organ.

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