Estrogen Induces Nitric Oxide Production via Activation of Constitutive Nitric Oxide Synthases in Human Neuroblastoma Cells

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Although it is becoming increasingly evident that nitric oxide (NO) mediates some of estrogen's actions in the brain, the effects of estrogen on NO production through NO synthases (NOS) in neuronal cells have not yet been identified. Here we assessed changes in NO production induced by 17β-estradiol (E2) in cells of neuronal origin using human SK-N-SH neuroblastoma cells, which we show express all three isoforms of NOS. Involvement of NOS isoforms in E2-induced NO production was examined using isoform-specific NOS inhibitors. E2 (10−8−10−5 M) induced rapid increases in NO release and changes in endothelial NOS (eNOS) expression, which were blocked by ICI 182,780, an antagonist of estrogen receptors, and N5-(1-iminoethyl)-L-ornithine, an neuronal NOS inhibitor, and N5-(1-iminoethyl)-L-ornithine, an eNOS inhibitor, but not by 1400 W, an inducible NOS inhibitor. These results demonstrate that E2-stimulated NO production occurs via estrogen receptor-mediated activation of the constitutive NOSs, neuronal NOS and eNOS. The E2-induced NO increase was abolished when extracellular Ca2+ was removed from the medium or after the addition of nifedipine, an L-type channel blocker, and was partially inhibited using 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester, an intracellular Ca2+ chelator. However, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester itself also caused an increase in NO release that was blocked by 1400 W, suggesting that inducible NOS mediates this response. Together these data reveal that constitutive NOS activities are responsible for E2-induced NO production in neuroblastoma cells and that differential activation of NOS isoforms in these cells occurs in response to different treatments. (Endocrinology 145: 4550–4557, 2004)

NO is catalytically generated by a family of NOSs, including two constitutive Ca2+-calmodulin-dependent isoforms (eNOS), namely nNOS (NOS1) and endothelial NOS (eNOS; NOS3), and one Ca2+-calmodulin-independent isoform, inducible NOS (iNOS; NOS2). The expression of NOS isoforms is related to tissue and cell types. For example, eNOS is widely distributed in the cardiovascular system, including cerebrovascular tissues, where NO production occurs via activation of eNOS in endothelial cells (14, 15). NOS is distributed throughout the central nervous system, where its activity contributes to NO production in neuronal cells (12, 16, 17). iNOS is expressed mainly in activated immune cells, including microglia in the brain (18), and is not usually expressed in neurons. However, increasing evidence has demonstrated that in addition to nNOS, both eNOS and iNOS are present or can be induced in neuronal cells. eNOS has been found in hippocampal neurons (19), motor neurons (20), and neuroblastoma cells (21); the expression of iNOS has also been found in neuroblastoma cells (21) and can be activated in neuronal cells by inflammation, injury, or ischemia (22, 23). Thus, these studies suggest that the presence of eNOS and iNOS in neuronal tissues cannot be ignored, and that the expression and activation of NOS isoforms depend on cell responses to different challenges.

Although it has been shown that estrogen affects NO production through genomic and/or nongenomic actions (12, 17, 24, 25), little information is available about how this steroid hormone influences NO synthesis in neuronal cells, because most studies to date have been carried out using

Abbreviations: BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; cNOS, constitutive nitric oxide synthase; DAN, 2,3-diaminonaphtalene; E2, 17β-estradiol; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; IC50, 50% inhibitory concentration; iNOS, inducible nitric oxide synthase; l-NIO, N2-(1-imino-ethyl)-l-ornithine; NF-κB, nuclear factor-κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; vinyl-l-NIO, N5-(1-imino-3-butenoxy)-l-ornithine.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
endothelial cells or other cells of nonneuronal origin. In the present study we assessed the effects of estrogen on NO production in SK-N-SH human neuroblastoma cells, a model widely used to study the effects of estrogen in neuronal cells (26–28). To characterize the effects of estrogen on NO production, SK-N-SH cells were treated with E2, and the time course of NO release was evaluated using a physiological concentration of E2. Ca\(^2+\)-free medium, nifedipine, an L-type Ca\(^2+\)-channel blocker, or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA/AM), an intracellular Ca\(^2+\) chelator, were used to determine whether E2-induced NO production is Ca\(^2+\)-dependent. Immunoblot analysis was applied to examine whether the different NOS isoforms are expressed in SK-N-SH cells. Finally, we used isoform-specific NOS inhibitors to identify which NOS isoform(s) contributes to E2-induced NO production.

Materials and Methods

Human SK-N-SH neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA). All reagents for cell culture were purchased from Invitrogen Life Technologies (Burlington, Canada) unless otherwise stated. E2, nifedipine, BAPTA/AM, and 1400W were purchased from Sigma–Aldrich Corp. (Oakville, Canada). Vinylt-l-NIO was purchased from Alexis (San Diego, CA), ICI 182,780 and t-NIO were obtained from Tocris (Ellissville, MO), and 2,3-diaminonaphthalene (DAN) was purchased from Molecular Probes (Eugene, OR).

Cell culture

SK-N-SH cells were cultured in Eagle’s MEM containing 10% fetal bovine serum, 2 mm l-glutamine, 1 mm MEM nonessential amino acids, 1 mm sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified incubator with 5% CO\(_2\) in air at 37 C. Medium was changed every 2–3 d. Subconfluent cells were harvested and seeded at a density of 1 × 10\(^5\) cells/well in six-well culture plates with phenol red-free MEM containing 5% charcoal/dextran-stripped fetal bovine serum. At 48 h after plating, cells were washed twice with serum-free medium and treated with drugs or vehicle in serum-free medium for different periods according to the experimental design.

Nitrite assay

NO production was determined by measuring nitrite accumulation in culture medium using a modified fluorometric assay (29) with DAN. Briefly, cells cultured in six-well plates were washed twice with phenol red-free and serum-free MEM, 1.2 ml of the same medium containing vehicle or drugs were added to each well, and cells were incubated for the time period designated. In some experiments, Ca\(^2+\)-free Hanks’ medium was used in place of MEM to study the role of extracellular Ca\(^2+\). After incubation, 500 μl medium were removed from each well and mixed with freshly prepared 40 μl DAN (50 μg/ml) in 0.62 M HCl, then incubated for 15 min at room temperature. The reaction was terminated by the addition of 50 μl 2.8 M NaOH. Fluorescence was measured using a spectrofluorometer (SLM-Amino, model 8100, SLM Instruments, Inc., Rochester, NY), with excitation and emission wavelengths set at 365 and 450 nm, respectively. The amount of nitrite in the sample was calculated according to a standard curve prepared with sodium nitrite.

NOS activity

NOS activity in cell lysates was determined by measuring [\(^3\)H]-arginine conversion to [\(^3\)H]-citrulline using an NOS assay kit (Calbiochem, San Diego, CA). Briefly, harvested cells were homogenized in buffer (25 mm Tris–HCl [pH 7.4], 1 mm EDTA, and 1 mm EGTA), and protein concentrations of cell homogenates were determined with the Bradford method using protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Equal quantities of cell proteins (5 μg) were incubated with [\(^3\)H]-arginine (1 μCi/μl, 64 Ci/mmol; Amersham Biosciences, Piscataway, NJ) in reaction buffer containing 1 mm NADPH and 0.6 mm CaCl\(_2\), for 30 min at room temperature. Changes in NOS activities were determined in the presence or absence of selective compounds.

Protein preparation and immunoblot analysis

Cells were harvested and homogenized with lysis buffer (0.1 m PBS (pH 7.4), 0.1% tergitol, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mm sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonylfluoride, and 10 μg/ml aprotinin) and quantified for protein concentrations as described above. Proteins in homogenates were separated by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (20 mm Tris-HCl, 150 mm NaCl, and 0.05% Tween 20) at 4 C, followed by incubation for 1 h with a primary antibody at room temperature. Rabbit polyclonal antihuman eNOS (sc-654, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:1000, and rabbit polyclonal antihuman nNOS (AB5396, Chemicon International, Temecula, CA) was used at a dilution of 1:2000. Mouse monoclonal anti-iNOS (sc2771, Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:500. A horseradish peroxidase-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Mississauga, Canada) or rabbit antimouse IgG (Sigma–Aldrich Corp., St. Louis, MO) was used as a secondary antibody (1:5000). For internal controls, membranes were stripped and probed with monoclonal anti-β-actin (Sigma–Aldrich Corp.) at a dilution of 1:5000. Immunoblots were detected with the enhanced chemiluminescence kit (Amersham Biosciences) and autoradiography film. The levels of proteins were imaged and quantified using the VersaDoc Imaging System (model 3000) with Quantity One software (Bio-Rad Laboratories).

Experimental design

To determine the effects of estrogen on NO production, cells were incubated with varying concentrations of E2, ranging from 0.1–1000 nm, and nitrite levels in the medium were examined 30 min after treatment. The time course of changes in nitrite levels was assessed 5, 15, 30, 60, and 120 min after treatment of E2 (10 nm). To examine whether different NOS isoforms are expressed in SK-N-SH cells and to assess acute effects of estrogen on NO expression, immunoblot analyses were performed after cells were treated for 1 h (unless otherwise stated) in the presence or the absence of E2 (0.1–1000 nm).

The following two approaches were used to study the involvement of ERs and NOS in the effects of 10 nm E2 on NO production. 1) Cells were preincubated with medium containing an ER antagonist or NOS inhibitor for 15 min; medium was then replaced with E2 plus the inhibitory compound. 2) Cells were treated for 30 min with E2 in the presence or absence of an ER antagonist or NOS inhibitor, and nitrite levels in the medium and NOS activities in cell extracts were measured thereafter. To evaluate whether the E2-induced NO release is mediated via ERs, we used ICI 182,780, an antagonist of ERs. To identify the NOS isoforms that are responsible for the E2-induced NO production, vinyl-l-NIO, l-NIO, and 1400W were used. The 50% inhibitory concentration (IC\(_{50}\)) values of vinyl-l-NIO for nNOS, eNOS, and iNOS are approximately 0.1, 12, and 60 μmol, making it 120- and 600-fold more selective for nNOS than for eNOS or iNOS, respectively (30). Although highly selective eNOS inhibitors are not currently available, l-NIO has been reported to inhibit eNOS (IC\(_{50}\) = 0.5 μmol) with 8- and 4-fold more selectivity than nNOS and iNOS, respectively (31). 1400W has been shown to be 31-fold more selective for iNOS (IC\(_{50}\) = 0.23 μmol) than nNOS and more than 4000-fold more selective than for eNOS (32).

To evaluate the role of extracellular Ca\(^2+\) in E2-induced NO responses, incubations were carried out in Ca\(^2+\)-free medium or in the presence of nifedipine, an L-type Ca\(^2+\) channel blocker. To determine whether intracellular Ca\(^2+\) plays a role, cells were treated with E2 in the presence or absence of BAPTA/AM, an intracellular Ca\(^2+\) chelator.

Statistical analysis

Data are presented as the mean ± SEM of four to six wells from at least three independent experiments. Depending on the experiment, differ-
ences among groups were determined by one- or two-way ANOVA, followed by the post hoc Student-Newman-Keuls test. Values of $P < 0.05$ were considered statistically significant.

**Results**

**Characteristics of E2-induced NO production**

The dose-response effects of E2 on NO production in SK-N-SH cells are shown in Fig. 1A. After 30 min of treatment with E2 at 0.1–1000 nm, nitrite levels in the medium were increased, with the largest increase occurring with 10 nm E2. We thus used E2 at 10 nm to evaluate the time course of NO release. A significant increase in nitrite levels was observed 5 min after E2 treatment (Fig. 1B). Levels continued to rise until 1 h and were significantly higher than control values.

**Effects of ICI 182,780**

The potential role of ERs in E2-induced NO production was assessed using ICI 182,780 at 1 or 10 μM. These doses of ICI 182,780 have been shown to block E2-induced NO production and NOS activity in endothelial cells (33, 34). ICI 182,780 at 1 μM decreased the E2-induced increases in nitrite levels by 66%, and ICI 182,780 at 10 μM completely inhibited the response (Fig. 2), indicating that stimulation of NO production in SK-N-SH cells is an ER-mediated event.

**Expression of NOS isoforms**

Expression of nNOS, eNOS, and iNOS was detectable in SK-N-SH cells, although the expression level for iNOS was relatively low (Fig. 3). Increases in eNOS protein levels of approximately 30% were found 1 h after treatment with E2 at low concentrations (0.1 and 1 nm), whereas levels of eNOS proteins were decreased by approximately 16% at high concentrations of E2 (1 μM; Fig. 3A). Similar results were obtained when immunoblots were performed 30 min after E2 treatment (data not shown). E2-induced changes in eNOS protein levels were inhibited by 0.1–1000 nm ICI 182,780 (Fig. 3A). Treatment of cells with E2 did not change the expression of nNOS or iNOS (Fig. 3, B and C). These results suggest that E2 induces rapid changes in eNOS protein levels through its receptors.

**Identification of NOS isoforms by NOS inhibitors**

Vinyl-l-NIO (a highly selective nNOS inhibitor) and l-NIO (an eNOS inhibitor) were used to determine whether constitutive NOS's are responsible for E2-induced NO production. E2-induced increases in nitrite levels were inhibited by 92% with 1 μM vinyl-l-NIO and were completely inhibited with 100 μM vinyl-l-NIO (Fig. 4A). Vinyl-l-NIO also inhibited E2-induced increases in NOS activity (Fig. 5).

![Fig. 1. Effects of E2 on NO production. A, Dose response of E2-induced nitrite production in SK-N-SH cells. Cells were treated with E2 (0.1–1000 nM) or vehicle. The nitrite levels in the culture medium were measured 30 min after treatment. B, Time course of E2-induced nitrite production. The nitrite levels in the culture medium were measured at 5, 15, 30, 60, and 120 min after treatment of cells with E2 (10 nM) or vehicle. * $P < 0.05$; ** $P < 0.01$ (vs. vehicle control).](https://academic.oup.com/endo/article-abstract/145/10/4550/2499775)

![Fig. 2. Effects of ICI 182,780 on E2-induced NO production. Nitrite levels in the culture medium were measured 30 min after treatment of cells with E2 (10 nM) in the presence or absence of ICI 182,780 (1 or 10 μM) or vehicle. ## $P < 0.01$ (E2 vs. vehicle control); ** $P < 0.01$ (E2 vs. E2 plus ICI 182,780).](https://academic.oup.com/endo/article-abstract/145/10/4550/2499775)
Similarly, the addition of L-NIO (1 or 5 μM) prevented E2-induced increases in nitrite levels (Fig. 4B) and NOS activities (Fig. 5). These results suggest that nNOS and eNOS are both involved in E2-induced NO production in SK-N-SH cells.

To determine whether iNOS activity is involved in E2-induced NO production, 1400W, a highly selective iNOS inhibitor, was used at 5 μM, a dose previously shown to completely inhibit cytokine-induced NO production in cultured cells (35). In contrast to vinyl-L-NIO and L-NIO, 1400W had no significant effects on E2-induced increases in nitrite levels (Fig. 4B) or NOS activity (Fig. 5), suggesting that iNOS activity does not contribute to E2-induced NO production.

Role of Ca²⁺

To verify that E2-induced NO production is mediated by constitutive NOSs, we determined the role of Ca²⁺ in the NO production.
response. When Ca\textsuperscript{2+}-free medium was used, basal NO levels were inhibited by 25\%, and the addition of E2 (10 nM) did not induce increases in nitrite levels (Fig. 6A). Similarly, basal NO levels were inhibited by 8\% with nifedipine (1 \mu M) in Ca\textsuperscript{2+}-containing medium, and E2 did not increase nitrite levels in the presence of nifedipine (Fig. 6B). Finally, the addition of BAPTA/AM (50 \mu M) inhibited E2-induced nitrite production by approximately 56\% (Fig. 7A). These results suggest that both intra- and extracellular Ca\textsuperscript{2+} influences E2-induced NO production.

**Discussion**

The data presented in this study demonstrate that estrogen at concentrations in the physiological range stimulates NO release in human neuroblastoma cells. This response occurs rapidly and can be blocked with ICI 182,780. The response is also inhibited with BAPTA/AM alone, showing an increase in nitrite production compared with vehicle-treated controls (Fig. 7B). To determine whether BAPTA/AM-induced nitrite production involves activation of iNOS, 1400W (5 \mu M) was applied. Compared with BAPTA/AM alone, a significant drop in nitrite levels was observed after treatment with BAPTA/AM plus 1400W (Fig. 7C), indicating that iNOS activity is involved in BAPTA/AM-induced nitrite production.

**BAPTA/AM-induced NO release**

Cells treated with BAPTA/AM alone showed an increase in nitrite production compared with vehicle-treated controls (Fig. 7B). To determine whether BAPTA/AM-induced nitrite production involves activation of iNOS, 1400W (5 \mu M) was applied. Compared with BAPTA/AM alone, a significant drop in nitrite levels was observed after treatment with BAPTA/AM plus 1400W (Fig. 7C), indicating that iNOS activity is involved in BAPTA/AM-induced nitrite production.

**Discussion**

The data presented in this study demonstrate that estrogen at concentrations in the physiological range stimulates NO release in human neuroblastoma cells. This response occurs rapidly and can be blocked with ICI 182,780. The response is also inhibited with BAPTA/AM, upon removal of Ca\textsuperscript{2+} from the medium, and after the addition of nifedipine. These results suggest that E2-induced NO production in neuroblastoma cells involves ER receptor-mediated and Ca\textsuperscript{2+}-sensitive events. Furthermore, we show that nNOS, eNOS, and iNOS are expressed in SK-N-SH cells, in agreement with a previous study that showed that SK-N-MC neuroblastoma cells express these NOS isoforms (21). Expression of the different NOS isoforms in these neuroblastoma cells allowed us to determine whether E2-induced NO production involves selective activation of an NOS isoform(s). Using specific NOS inhibitors, we show that E2 stimulates the production of NO in SK-N-SH cells through activation of the cNOS isoforms, eNOS and nNOS.

One of the challenges in demonstrating the involvement of individual NOS isoforms in E2-induced NO production is that no highly selective eNOS inhibitors are currently available. Although the finding that l-NIO inhibited E2-induced NO production supports the involvement of eNOS, we can...
not exclude the possibility that nNOS activity may have been slightly affected by l-NIO, because the degree of selectivity of l-NIO for eNOS over nNOS is not large. In contrast, vinyl-l-NIO at 1 μM effectively inhibits nNOS (36), but does not appreciably affect eNOS activity (30). Thus, our finding that 1 μM vinyl-l-NIO did not completely inhibit estrogen-induced NO release supports our contention that in addition to nNOS, eNOS is involved in the effects of E2. In contrast to l-NIO and vinyl-l-NIO, 1400W had no effect on the E2-induced NO release. These data, taken together, suggest that the induction of NO production by estrogen occurs via activation of the two cNOSs, nNOS and eNOS, but not via iNOS. These findings are consistent with our recent in vivo study that showed that estrogen replacement in ovariectomized female rats modulates cardiovascular responses to psychological stress through NO release in the brain via activation of cNOS (4). Thus, by showing that estrogen stimulates NO release through cNOS activities, our data contribute to the fundamental understanding of estrogen’s actions on NO production in neuronal cells.

The finding that E2-induced NO production occurs through activation of cNOS is also supported by our data obtained using Ca^2+ blockers; removal of Ca^2+ from the medium or addition of nifedipine decreased the basal level of NO release and prevented an increase in NO production induced by E2. These results suggest that extracellular Ca^2+ is critical to the maintenance of basal levels of NO production in neuroblastoma cells. We also demonstrate that BAPTA/AM inhibits E2-induced NO production, supporting the idea that intracellular Ca^2+ is required in E2-induced activation of cNOS. Consistent with our results, previous studies have shown that an E2-induced transient rise in the intracellular Ca^2+ concentration is followed by NO release in monocytes (37); an increase in eNOS phosphorylation was more pronounced in the presence of ionomycin, a Ca^2+-mobilizing agent, in uterine artery endothelial cells (38). The mobilization of Ca^2+ by estrogen is mediated through membrane ERs (37, 38), but the source of the Ca^2+ may differ among cell types, because increases in intracellular Ca^2+ occurred through activation of L-type, voltage-gated Ca^2+ channels in female colonic epithelium (39), whereas Ca^2+ was released from intracellular Ca^2+ stores in astrocytes (40).

The mechanism by which estrogen causes activation of cNOS in neuronal tissues is unclear. Previous studies have indicated that E2 may act through several different pathways, including ER-mediated genomic and nongenomic pathways and non-ER-mediated, nongenomic pathways (41–43). In endothelial cells, a rapid increase in E2-induced NO production occurred through activation of eNOS, which required the ERα ligand-binding domain, but did not require the hormone’s nuclear effects (44), and could be blocked by ICI 182,780 (44, 45). In mollusk pedal ganglia, acute NO release induced by E2 has been reported to act through an ERβ expressed on the cell surface (46). These data suggest that the effects of estrogen on NO production involve an ER-mediated, nongenomic pathway. Similarly, we show that

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**FIG. 7.** Effects of BAPTA/AM on NO production. A, Effects of BAPTA/AM on E2-induced nitrite production. Cells were treated with E2 (10 nM) in the presence or absence of BAPTA/AM (50 μM) or vehicle. B, Increases in nitrite production stimulated by BAPTA/AM. Cells were treated with BAPTA/AM (0.5, 5, or 50 μM) or vehicle. C, Effects of 1400W on BAPTA/AM-induced nitrite production. Cells were treated with BAPTA/AM (5 μM) in the presence or absence of 1400W (5 μM) or vehicle. The nitrite levels in the culture medium were measured 30 min after treatment. A: ##, P < 0.01 (E2 vs. vehicle control); *, P < 0.05 (E2 vs. E2 plus BAPTA/AM). B and C: #, P < 0.05; ##, P < 0.01 (BAPTA/AM vs. vehicle controls); *, P < 0.05 (BAPTA/AM plus 1400W).
stimulation of NO release in neuroblastoma cells by E2 occurs as early as 5 min after exposure to E2, that this response is Ca\(^{2+}\) dependent, and that it could be blocked by ICI 182,780. These results suggest that the acute effects of estrogen on NO release in neuroblastoma cells probably involve an ER-mediated and Ca\(^{2+}\)-dependent nongenomic pathway. Finally, we also demonstrate that changes in levels of eNOS protein occur as early as 30 min after the addition of E2, consistent with the observation that E2 stimulates eNOS internalization, which occurs immediately after enzyme activation and is blocked by ICI 182,780 in endothelial cells (47). Moreover, translocation of eNOS from the lipid-anchored membrane to the cytoplasm increases enzyme phosphorylation and solubilization (48, 49). Thus, it is likely that acute changes in eNOS proteins induced by E2 involve posttranslational modifications, including protein-protein interactions and subsequent induction of eNOS subcellular trafficking, recycling, and degradation (49–51).

It is worth noting that BAPTA/AM, an intracellular Ca\(^{2+}\) chelator, did not completely inhibit E2-induced NO production even at a relatively high concentration (50 μM). The reason for this response is probably that BAPTA/AM itself stimulated an increase in NO release through activation of iNOS, because the response could be blocked by 1400W. This result is consistent with the idea that iNOS activity is Ca\(^{2+}\) independent. Because activation of iNOS in neuronal tissues is correlated with incidence of tissue injury and damage, our finding that NO levels are augmented by BAPTA/AM via activation of iNOS may explain the toxic effects of BAPTA/AM described by other investigators (52, 53).

Unlike a large increase in NO release induced by tissue injury and inflammation, we show that E2-induced enhancement in NO levels is relatively small, supporting the idea that changes in NO production are stimulus dependent. Furthermore, increasing concentrations of E2 do not stimulate greater release of NO. Although the reason for this phenomenon is not known at this time, it is possible that the ERs required for NO production from eNOS are fully occupied at the lower concentrations of E2.

Estrogen has been shown to exert an important neuroprotective effect (43, 54), which has been suggested to be mediated by cNOS-derived NO release (55) and its related cGMP-dependent protein kinase pathway (56). This hypothesis is based on the observation that basal levels of NO produced by cNOS provide a protective effect in neural tissues through suppression of nuclear factor-κB (NF-κB) and subsequent inhibition of relevant immune responses. In contrast, β-amyloid, a major constituent of senile plaques in Alzheimer’s disease, has been shown to decrease basal NO levels by inhibiting cNOS activities (57), but to increase iNOS activity through activation of TNFα and NF-κB signaling pathways (58). We now show that E2 induces NO production through activation of cNOS, rather than iNOS, in neurons-like cells, supporting the idea that activation of cNOS may contribute to estrogen’s neuroprotective effects.

In summary, we have shown for the first time that concentrations of E2 in the physiological range cause rapid increases in NO production in neuroblastoma cells through activation of cNOS, but not iNOS. By demonstrating that nNOS and eNOS both contribute to E2-induced NO synthesis in cells of neuronal origin, our results bring into question the common assumption that estrogen affects NO release in neuronal cells only through nNOS activation. We also demonstrate that the rapid effects of estrogen on NO and eNOS proteins occur through membrane-mediated nongenomic events, rather than classic genomic pathways. In addition, we have shown that BAPTA/AM stimulates NO release in neuroblastoma cells through activation of iNOS, suggesting that differential activation of NO isoforms can occur in response to different treatments in the same cells. This finding is important, because activation of different NO isoforms leads to production of different levels of NO, which, in turn, can exert either beneficial or pathological effects in cells and tissues. Finally, we have already shown in vivo that estrogen’s actions on blood pressure responses to psychological stress are mediated by NO in the brain (4). Although it is important to emphasize that our current results in neuroblastoma cells do not necessarily translate directly to responses in vivo, the present study provides new insight into the possible mechanisms for interactions between estrogen and NO in neuronal cells.

Acknowledgments

Received March 15, 2004. Accepted June 28, 2004.

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This work was supported by the Canadian Institutes of Health Research (MT-14462).

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