Nitric oxide impairs the 17\(\beta\)-estradiol-induced apoptosis in human colon adenocarcinoma cells

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Abstract

Nitric oxide (NO) and 17\(\beta\)-estradiol (E2) are both important in gastrointestinal health and disease. NO contributes to gastrointestinal motility as well as to inflammation and carcinogenic processes. By contrast, E2 reduces the incidence of colon adenoma and carcinoma by about 30%. We report the genomic and non-genomic E2–estrogen receptor (ER) \(\beta\)-induced effects in human colon adenocarcinoma. The effect of NO on ER\(\beta\) activities was also assessed. The E2-ER\(\beta\)-dependent gene transcription was inhibited by exogenous NO, whereas some non-genomic E2-dependent effects (e.g. p38/MAP kinase), important for the activation of the apoptotic cascade, were unaffected by NO. However, NO impaired the E2-induced pro-apoptotic cascade in human colon adenocarcinoma cells by inhibiting caspase-3. The effects of NO may reflect chemical modification(s) of Cys residues present in the DNA recognition domain of ER\(\beta\) as well as in the caspase-3 active site. On the whole, high NO concentrations suppressed the E2 protective effects in the gastrointestinal tract, suggesting that the caspase-dependent apoptotic cascade may become critical under conditions of high redox stress such as occur under specific activation of the immune system by chronic infections or pathogen challenge.

Introduction

Nitric oxide (NO) is a signaling molecule generated by a family of P450-like enzymes, termed NO synthases (Bredt et al. 1991). The lipophilic nature of NO allows it to diffuse quickly, thereby initiating intercellular and intracellular signals. The most characterized downstream NO signaling pathway relates to the soluble guanylate cyclase pathway, with downstream phosphorylation cascades leading to effector functions (Denninger & Marletta 1999). However, in patho-physiologic conditions, NO-mediated signaling may also be independent of guanylate cyclase activation. This regulation appears to occur in part through \(S\)-nitrosylation of Cys residues in target proteins. The reversible regulation of protein function by \(S\)-nitrosylation has led to the proposal that nitrosothiols might function as significant post-translational modifications, analogous to those created by phosphorylation or acetylation (Beckman & Koppenol 1996, Jaffrey et al. 2001, Stanler et al. 2001, Nathan 2004, Ascenzi et al. 2005).

Among proteins regulated by NO, modulation of the estrogen receptor (ER) \(\alpha\) by \(S\)-nitrosylation (Marino et al. 2001, Garban et al. 2005) has important repercussions in cell homeostasis. 17\(\beta\)-Estradiol (E2) binding to the cytosolic ER population (both ER\(\alpha\) and ER\(\beta\)) induces conformational changes that facilitate ER homo/heterodimerization, nuclear translocation, and binding to specific DNA recognition sequences (i.e. estrogen responsive elements; ERE) (Acevedo & Kraus 2004, Marino et al. 2005). In this classical/genomic mode of action, ER\(\alpha\) and ER\(\beta\) promote E2-sensitive gene transcription, ER\(\beta\) being approximately 30% less efficient than ER\(\alpha\) (Nilsson et al. 2001). It is well established that the main role of the plasma concentration of NO is to alter the local redox state. Thus, NO regulates the expression of genes involved in cell proliferation, angiogenesis, and inflammation in the gastrointestinal tract (Marino et al. 2001).
membrane-localized ER population is to generate rapid/non-genomic signal transduction pathways that culminate in the activation of the protein kinase cascade (Levin 2005). The nature of these pathways as well as the role played in cell functions differs between ERα and ERβ. In particular, rapid signals generated from the E2–ERα complex drive cells into the cell cycle and represent the main determinants for the E2 proliferative/survival effects (Marino et al. 2001, 2002, 2003). By contrast, rapid effects generated by the E2–ERβ complex drive cells out of the cell cycle (Acconcia et al. 2005a), representing the key to understanding the E2-induced anti-proliferative effects working both during differentiative processes and in some forms of human cancer (e.g. colon adenocarcinoma) (Weihua et al. 2003, Bardin et al. 2004, Paruthiyil et al. 2004, Strom et al. 2004, Martineti et al. 2005, Koehler et al. 2005). S-nitrosylation of ERα results in the selective inhibition of DNA binding to specific EREs without affecting non-genomic events in transiently transfected HeLa cells (Marino et al. 2001). This may shift the bioactivity of ERα from its major role as a transcription factor toward rapid non-genomic functions, such as the kinase cascade activation, promoting, in turn, cell proliferation. The presence of a similar mechanism able to selectively modulate the activity of ERβ driving cancer cells out of the cell cycle is completely unknown.

Here, the effect of NO on ERβ-mediated rapid and transcriptional activities in human colon adenocarcinoma DLD-1 cells is reported. E2 possesses anti-proliferative effects in DLD-1 cells, stimulating the ERβ-dependent rapid activation of a pro-apoptotic cascade. On exposure to exogenous NO, ERβ undergoes chemical modification, resulting in specific inhibition of the transcriptional activity. Although exogenous NO does not affect some rapid ERβ-induced activity (e.g. p38/MAP kinase (MAPK)), NO inhibits E2-induced caspase-3 activation, thus impairing the protective anti-proliferative effects of E2 in colon adenocarcinoma cells.

Materials and methods

Reagents

4′,6-Diamidino-2-phenylindole (DAPI), E2, L-glutamine, gentamicin, penicillin, RPMI-1640 (without phenol red), charcoal-stripped fetal calf serum, β-estradiol 6-(o-carboxy-methyl)oxime:BSA (E2-BSA), and ±e-4-ethyl-2-[e-hydroxyimino]-5-nitro-3-hexenamide (NOR-3) were purchased from Sigma-Aldrich (St Louis, MO, USA). E2-BSA does not pass through plasma membrane and is much more water soluble than free E2 (Zheng et al. 1996). To ensure the absence of free E2 in E2-BSA preparations, aliquots were preabsorbed with dextran-coated charcoal to remove >99% of free steroid hormone (Marino et al. 2002). NO-deprived NOR-3 (NOR-3*) was prepared as previously reported (Marino et al. 2001). The p38/MAPK inhibitor SB 203,580 was purchased from Calbiochem (San Diego, CA, USA). The pure anti-estrogen inhibitor ICI 182,780 was obtained from Tocris (Ballwin, MO, USA). Lipopectamine reagent was obtained from Gibco-BRL Life Technologies (Gaithersburg, MD, USA). The luciferase kit was purchased from Promega (Madison, WI, USA). GenElute plasmid maxiprep kit was obtained from Sigma-Aldrich. Bradford protein assay was purchased from BIO-RAD Laboratories (Hercules, CA, USA). The polyclonal anti-phospho-p38 and anti-p38 antibodies were obtained from New England Biolabs (Beverly, MA, USA). The polyclonal anti-ERα and anti-ERβ antibodies, the monoclonal anti-caspase-3 antibody, the anti-poly (ADP-ribose)polymerase (PARP) antibody, and the anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The chemiluminescence reagent CDP-Star for western blot was purchased from NEN (Boston, MA, USA). All the other products were from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.

Cell culture and viability

Human colon adenocarcinoma DLD-1 cells were kindly provided by Dr A Cavallini (Biochemistry Laboratory, Scientific Institute for Digestive Diseases IRCCS ‘de Bellis’, Castellana Grotte, Italy). DLD-1 cells contain constitutively only the ERβ-1 isoform and lack any ERα isoform (Fiorelli et al. 1999, Di Leo et al. 2001, Acconcia et al. 2005a). Cells were routinely grown in air containing 5% CO2 in modified, phenol red-free, RPMI-1640 medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2.0 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and the medium was changed every 2 days. They were grown to ~70% confluence in six-well plates, then transfected and, 24 h later, stimulated. Cells were harvested with trypsin and centrifuged at different times after treatment. Cell viability, evaluated by Trypan blue...
exclusion test (Acconcia et al., 2005a), was 90–95% in cells stimulated with 1 and 30 μM NOR-3 and 75–80% with 1000 μM NOR-3. Cells were stained with the Trypan blue solution and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate. The apoptotic response of cells to different concentrations of NOR-3 donor was evaluated by DNA fragmentation. Briefly, 10⁶ cells stimulated with E2, ICI 182,780, SB 203,580, and different NOR-3 concentrations were fixed with 1 ml ice-cold 70% (v/v) ethanol and subsequently stained with 2.0 μg/ml DAPI/PBS solution. DNA fluorescence was measured with a DAKO Galaxy flow cytometer (DAKO Cytomation, Glostrup, Denmark) equipped with a mercury vapor short-arc lamp and the percentage of cells present in sub-G1, G1, S, and G2/M phases as well as the percentage of cellular debris (peak before the sub-G1 phase) was calculated using FloMax software (DAKO).

Plasmids and transfection procedures

2 × 10⁵ DLD-1 cells, ~70% confluence, were transiently transfected with the plasmid containing the promoter of the complement component 3 gene, retaining a natural ERE linked to the gene of luciferase (pC3-luciferase), using lipofectamine reagent according to the manufacturer’s instructions. Plasmid was purified for transfection using the plasmid preparation kit according to the manufacturer’s instructions. A luciferase dose–response curve showed that the maximum effect was observed when 1 μg DNA was transfected in DLD-1 cells together with 1 μg pCR3.1-β-galactosidase (Marino et al., 2003) to normalize transfection efficiency (~55–65%). Six hours after transfection, the medium was changed and 24 h later cells were pretreated with NOR-3 or NOR-3* (final concentration, 1 μM, 30 μM, and 1000 μM). After 30 min, E2 (final concentration, 0.01 μM) or the vehicle (ethanol/PBS 1:10 (v/v)) was added and the reporter plasmid expression was evaluated 6 h thereafter. The cell lysis procedure and the subsequent measurement of the luciferase gene expression were performed using the luciferase kit according to the manufacturer’s instructions, with a Wallac Berthold luminometer apparatus (Perkin-Elmer, Italy).

Electrophoresis and immunoblotting

E2 or E2-BSA (0.01 μM)-stimulated and unstimulated cells were lysed as described (Marino et al., 1998). When indicated, 1 μM ICI 182,780 or 5 μM SB 203,580, the p38/MAPK inhibitor, was added to the medium 15 or 30 min respectively before agonist stimulation. Cells were solubilized in 0.125 M Tris–HCl (pH 6.8) containing 10% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride, and 5.0 μg/ml leupeptin, and boiled for 2 min. Proteins were quantified using the Bradford protein assay. Twenty micrograms of solubilized proteins were resolved using SDS-PAGE at 100 V for 1 h. Proteins were then electrophoretically transferred to nitrocellulose for 45 min at 100 V at 4°C. The nitrocellulose was treated with 3.0% (w/v) BSA in 138 mM NaCl, 26.8 mM KCl, 25.0 mM Tris–HCl (pH 8.0), 0.05% (w/v) Tween-20, and 0.1% (w/v) BSA, and then probed at 4°C overnight with one of anti-ERα, anti-ERβ, anti-phospho-p38, anti-caspase-3, and anti-PARP antibodies. The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-p38 antibody (1 μg/ml). The anti-actin antibody (1 μg/ml) was used to normalize the sample loading. The antibody reaction was visualized with the chemiluminescence reagent for Western blot.

Statistical analysis

A statistical analysis was performed by using Student’s t-test with the INSTAT software system for Windows (GraphPad, CA, USA). Some data were analyzed by one-way ANOVA and post-hoc Bonferroni test (INSTAT software system for Windows). In all cases P values below 0.05 were considered significant.

Results

NO has been shown to selectively inhibit E2-induced gene expression without affecting non-genomic functions in ERα-transfected HeLa cells (Marino et al., 2001). To evaluate the effect of NO on the genomic (transcriptional) response of endogenous ERβ, DLD-1 cells were transiently transfected with a reporter gene system containing an ERE promoter driving the expression of the luciferase gene. DLD-1 cells were incubated with the NO donor NOR-3 (1, 30, and 1000 μM), the NO-deprived NOR-3* (30 μM), and/or E2 (0.01 μM). The transcriptional activation was then determined based on the expression of the luciferase activity by DLD-1 cells after 6 h of treatment. E2 induced the expression of
the transfected reporter gene pC3-luciferase. This E2 effect was totally dependent on the presence of ERβ; in fact, it was completely prevented by the pure anti-estrogen inhibitor ICI 182,780 (Fig. 1A). No effect on reporter gene activity was observed in DLD-1 cells treated with NOR-3 in the absence of E2. By contrast, NOR-3 inhibited the E2-ERβ-induced pC3-luciferase gene expression; this effect was completed at 1 μM NOR-3 (Fig. 1B). These results suggest a significant role of NO in modulating the transcriptional activity of ERβ.

The time-course of E2-stimulated DLD-1 cell growth is shown in Fig. 2A. E2 stimulation induced a decrease in cell growth with respect to unstimulated ones. This hormone effect needs the presence of ERβ, being completely prevented by ICI 182,780 (Fig. 2A). Furthermore, we analyzed, by flow cytometry, the DLD-1 cell cycle distribution at 30 h after E2 treatment. The typical plot of DLD-1 cell population is illustrated in Fig. 2B (CONTROL). The first peak indicates the cell number in the G1 phase of the cell cycle (45.4 ± 5.0%), followed by the S phase (10.3 ± 3.2%), and by the peak of the G2/M phase (44.3 ± 2.8%). Thirty hours after E2 stimulation, a peak in the sub-G1 region appeared (7.5 ± 1.0%) (Fig. 2B), indicating the presence of DNA fragmentation. Cell pretreatment with ICI 182,780 prevented the E2-induced sub-G1 peak appearance (Fig. 2B).
Figure 2 Dose-dependent effect of NOR-3 (i.e. NO) on DLD-1 cell growth. (A) DLD-1 cells were grown in the presence of E2 (0.01 μM) and/or ICI 182,780 (ICI, 1 μM) and counted at the indicated times. The data are means ± S.D., n = 5 of duplicate analyses. *P < 0.001, calculated with Student’s t-test, compared with respective unstimulated values (control; PBS) (*). (B) Flow cytometric analysis of DLD-1 cells after 30 h of E2 treatment in the presence or the absence of ICI 182,780 (ICI, 1 μM) compared with unstimulated cells (CONTROL). The plots indicate cell cycle distribution present in sub-G1, G1, S, and G2/M phases respectively. In (C) the dose-dependent effect of 30 h of stimulation of E2 in the presence or the absence of NOR-3 on DLD-1 cell growth is shown. The data are the means ± S.D., n = 4 of duplicate analyses. *P < 0.001 was calculated with Bonferroni’s test: a, significantly different from control value (open bar); b, significantly different from NOR-3*; c, significantly different from 1 μM NOR-3; and d, significantly different from 0.01 μM E2. (D) Flow cytometric analysis of DLD-1 cells after 30 h of E2 treatment in the presence or the absence of different concentrations of NOR-3 compared with unstimulated cells (CONTROL). The plots indicate cell cycle distribution present in sub-G1, G1, S, and G2/M phases respectively. For details see the text.
Stimulation of DLD-1 cells with the E2 cell membrane-impermeable E2-BSA, a well-known agent able to discriminate between non-genomic versus genomic effects of ER(s) (Marino et al. 2003), affected DLD-1 cell growth like E2. This indicated the pivotal role of plasma membrane starting signals in E2-induced anti-proliferative effects. As expected, ICI 182,780 reversed the E2-BSA effect. Cell pretreatment with 30 and 1000 μM NOR-3 blocked the E2-induced effects, whereas the effects of E2 were not impaired by 1 μM NOR-3 concentration (Fig. 2C and D). NOR-3 (1 and 30 μM) did not affect cell growth in the absence of E2 (Fig. 2C and D). However, cell growth was affected by 1000 μM NOR-3, a small increase in cellular debris (~10-20%, see above) and no cells in the sub-G1 phase were observed (Fig. 2D).

We previously showed in transiently transfected HeLa cells that E2-ERβ-induced decrease in cell growth was dependent on the rapid non-genomic activation of p38/MAPK (Acconcia et al. 2005a). To verify that the NOR-3 effect on DLD-1 cell growth was associated with the block of this non-genomic ERβ activity, the effect of NOR-3 on E2-induced p38/MAPK activation was investigated. E2 stimulation induced a rapid (15 min) and persistent (24 h) increase of p38/MAPK phosphorylation in DLD-1 cells (Fig. 3A and B). This effect was mimicked by the E2 membrane-impermeable E2-BSA and prevented by pretreatment with ICI 182,780 or with the p38/MAPK inhibitor SB 203,580 (Fig. 3C and D). DLD-1 cell pretreatment with the p38/MAPK inhibitor SB 203,580 completely prevented the E2-induced sub-G1 peak appearance (Fig. 3E). Therefore, both ERβ-transfected HeLa cells (Acconcia et al. 2005a) and endogenous ERβ-containing DLD-1 cells (present study) require the activation of rapid signal (i.e. p38/MAPK) for E2-induced anti-proliferative effects. ERβ-NOR-3 did not impair kinase activation either 15 min (Fig. 3F and G) or 24 h (data not shown) after E2 stimulation. This strongly suggests that high NO concentrations impair E2 anti-proliferative effects by inhibiting p38/MAPK downstream pathways without affecting ERβ-dependent rapid activity.

The cleavage of the caspase-3 proform (32 kDa band), which results in the production of the active subunit of the protease (17 kDa band), was examined. Twenty-four hours of E2 stimulation induced the production of the caspase-3 active subunit in DLD-1 cells even in the presence of 1 and 30 μM NOR-3 (Fig. 4A), whereas pretreatment of the cells with 1000 μM NOR-3 did not allow the E2-induced caspase-3 activation (Fig. 4A). To confirm that the appearance of the 17 kDa band was associated with the increase of the caspase-3 activity, we monitored the cleavage of PARP, a well-known caspase-3 substrate (Wesierska-Gadek et al. 2004). The 116 kDa DNA repair enzyme PARP was cleaved by caspase-3, producing the inactive 85 kDa fragment (Fig. 4B) when DLD-1 cells were stimulated with E2 in both the presence or the absence of 1 μM NOR-3. Although 30 μM NO donor allowed the E2-induced 17 kDa caspase-3 formation, the E2-induced PARP cleavage was completely prevented, suggesting an impairment of caspase activity. On the contrary, 1000 μM NO donor concentration did not allow the E2-induced cleavage of both caspase-3 and PARP. The E2 effects were dependent on the presence of ERβ; in fact, E2-induced caspase-3 activation and PARP cleavage were completely prevented by ICI 182,780 (Fig. 4). These results are consistent with the idea that NO impairs the E2-induced anti-proliferative effects without affecting ERβ non-genomic activities.

Under all the experimental conditions, NO-deprived NOR-3 (NOR-3*; 1, 30 and 1000 μM) did not affect E2-induced effects (Figs 1–4).

**Discussion**

Epidemiologic, clinical, and experimental evidence shows that estrogen hormones confer protection against colon cell proliferation and malignant transformation, reducing the incidence of colon adenoma and carcinoma by about 30% (Foley et al. 2000, al-Azzawi & Wahab 2002, Burkman 2002, Qiu et al. 2002, 2004, Konstantinopoulos et al. 2003, Bardin et al. 2004). The molecular mechanisms underlying these effects are starting to be clarified. Among others (e.g. epigenetic mechanisms) (Qiu et al. 2004), the specific tissue distribution and the intracellular-generated signals of ERα and ERβ have opened new avenues for understanding these protective effects of E2 (Weihua et al. 2003, Acconcia et al. 2005b).

The present data indicated that E2-induced rapid signal transduction pathways in DLD-1 cells appear to play a major role in mediating the protective properties of this steroid hormone against colon cancer. The action of E2 in these cells results from binding to ERβ which, in turn, acutely promotes the rapid and persistent phosphorylation of p38/MAPK, triggering downstream the activation of a
Figure 3 Dose-dependent effect of NOR-3 (i.e. NO) on p38/MAPK activation in DLD-1 cells. Analysis of p38/MAPK phosphorylation was performed on (A and B) untreated (0; control) and E2-treated (0.01 μM) DLD-1 cells at the indicated times or (C and D) cells treated for 15 min with E2 (0.01 μM) or E2-BSA (0.01 μM) or ICI 182,780 (ICI, 1 μM) or 30 min p38/MAPK inhibitor, SB203,580 (SB, 5 μM) or (F and G) in the presence of different concentrations of NOR-3 before E2 stimulation. The protein levels were normalized by comparison with actin expression. (A, C, and F) Typical blots and (B, D, and G) the densitometric analysis. The data are the means ± S.D., n = 3 of duplicate analyses. *P < 0.001, calculated with Student’s t-test, compared with respective unstimulated values (0, CONTROL) (*) or with E2-stimulated values (8). (E) Flow cytometric analysis of DLD-1 cells after 30 h of E2 treatment in the presence or the absence of the p38/MAPK inhibitor SB203,580 (SB, 5 μM) compared with unstimulated cells (CONTROL). The plots indicate cell cycle distribution present in sub-G1, G1, S, and G2/M phases respectively. For details see the text.
pro-apoptotic cascade (Acconcia et al. 2005a, present data).

We have here demonstrated that the NO donor NOR-3 selectively inhibits the gene transcriptional activity of ERβ without affecting some rapid non-genomic effects (e.g. p38/MAPK). This result is reminiscent of that reported for ERα when over-expressed in ER-devoid HeLa cells (Marino et al. 2001). More recently, it has been confirmed that the NO-dependent S-nitrosylation of Cys residues, predominantly in the ERα DNA-binding domain, does occur (Garban et al. 2005).

The great propensity for nitrosothiol and mixed disulfide bridge formation represents a modulation mechanism of (macro)molecules containing NO-reactive Cys residues at their active center(s), recognition region(s), and/or allosteric site(s) (Beckman & Koppenol 1996, Jaffrey et al. 2001, Stamler et al. 2001, Nathan 2004, Ascenzi et al. 2005). As reported for ERα (Marino et al. 2001), the prevalence and high reactivity of thiols over other nucleophiles suggest that Cys residue(s) may also represent NO targets in ERβ. The present findings support the notion that Cys residues co-ordinating zinc atoms in the DNA-binding domain of ERβ, important in modulating the ER transcriptional function (Pace et al. 1997, Nilsson et al. 2001), are susceptible to NO-mediated chemical modifications. This adds to emerging data indicating that Cys residues in ER may undergo several chemical modifications, such as S-nitrosylation and S-palmitoylation, to regulate the biologic activity of ER in vivo (Marino et al. 2001, Accconcia et al. 2005b).

S-nitrosylation seems to selectively modulate the bioactivity of ER, shifting the receptor from its role as a transcription factor toward rapid functions. These E2–ERβ-induced rapid signals, such as the p38/MAPK-dependent activation of apoptotic cascade, might enhance the pro-apoptotic effects of E2. Thus, as reported for the cardioprotective role of E2 (Kim & Bender 2005), the E2-mediated biogeneration of NO, reported also in colon (Shah et al. 2001), could be one of the key features underlying the protective effects of E2 against colon cancer. In the occurrence of NO concentrations in the nanomolar to micromolar range, achievable in the gastrointestinal system in vivo by different chemical messengers (Felley-Bosco et al. 2002, Shah et al. 2004), E2 still induces the ERβ-dependent caspase-3 activation and the cleavage of its downstream substrate, PARP, preserving its protective/anti-proliferative role.

When over produced (30–1000 μM) NO worsens its effects. Although the ERβ-dependent phosphorylation of p38/MAPK is still present, 30 μM NOR-3 inhibits the caspase-3 catalytic activity (i.e. the cleavage of its substrate PARP; Fig. 4). Furthermore, 1000 μM NOR-3 impairs pro-caspase-3 to caspase-3 activation and blocks the enzyme action. This suggest that NO does not affect caspase-3 activation at <30 μM NOR-3, inhibiting the enzyme activity at >30 μM NOR-3. High levels of NO, superoxide, and peroxynitrite are produced in vivo during infectious diseases by the activated phagocytes expressing inducible-NO synthase to inhibit the growth of pathogenic viruses, bacteria, fungi, and parasites. However, nitrogen and oxygen reactive species may contribute to pathogenic processes during infection (Maeda & Akaike 1998, Colasanti et al. 1999, Akaike & Maeda 2000, Ascenzi et al. 2003, Fang 2004, Zaki et al. 2005).

The cytotoxic effect of NO is realized through chemical modifications of biomolecules, such as proteins, nucleic acids, and membrane lipids (Jaffrey et al. 2001, Stamler et al. 2001, Ascenzi et al. 2005). Besides these molecules, the present
data indicate that high NO levels impair caspase-3 activity in DLD-1 cells.

In vitro caspase-3 inhibition by NO is a generally accepted phenomenon. Caspases play a crucial role in the execution of apoptosis (Wang et al. 2005). The processing of pro-caspase-3 to its active form is considered to be a point of no return in the death signaling cascade (Green 2005). In fact, caspase-3 represents the execution enzyme of the caspase cascade that cleaves the inhibitor of caspase-3. This processing of pro-caspase-3 to its active form, the execution enzyme of the death signaling cascade (Green 2005), is considered to be a point of no return in the cell physiology of a whole variety of tissues.

In conclusion, the regulatory effects of NO reported here could represent a general mechanism by which cell physiology of a whole variety of tissues is fine tuned by NO via the control of the status of hormone receptors as well as some execution steps of the caspase cascade. In such a model, the susceptibility of the cell to death signals transmitted by E2 via ERβ-dependent caspase-3 activation or to cell proliferation signals transmitted by E2 via ERα-dependent MAPK activation is prone to an additional regulatory influence dependent on the cellular and exogenous NO levels. In view of the ambivalent capabilities of NO to act either in a pro-apoptotic or in an anti-apoptotic fashion depending on its level, a complex spectrum of NO-mediated control of E2-induced effects is conceivable.

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