Nitric oxide can both stimulate and suppress apoptosis. By reverse transcriptase-polymerase chain reaction and sequencing we show that human breast cancer (MCF-7) cells express endothelial cell nitric-oxide synthase (ecNOS), but not other nitric-oxide synthase isoforms. Inhibition of ecNOS activity in MCF-7 cells increased tumor cell apoptosis, and this effect was also seen following treatment with an NO scavenger. In addition, low concentrations of the NO donor sodium nitroprusside inhibited, whereas high concentrations stimulated MCF-7 cell apoptosis. The ecNOS promoter was found to contain a specific binding site for the apoptosis-regulating protein p53. In co-transfection studies wild-type, but not mutant, p53 down-regulated transcription of an ecNOS promoter-luciferase reporter gene construct. In addition, NO donors up-regulated p53 protein levels in MCF-7 cells. These data point to a previously unrecognized p53-dependent regulation of ecNOS expression that may be important both for regulating apoptosis and for avoiding the generation of genotoxic quantities of NO.

Programmed cell death (apoptosis) is an important mechanism for regulating the growth and shape of the developing organism as well as cell turnover (1). Dysregulation of apoptosis characterizes many human disease states, including cancer (2, 3) and anticancer drugs have been found to induce apoptosis. Interestingly, certain of these drugs have been shown to stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7). Exogenous NO donors or overexpression of NO synthases (NOS)1 in transfected or cytokine-primed cells may stimulate production of the free radical gas transmitter nitric oxide (4–7).

1 The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; ecNOS, endothelial cell NOS; L-NAME, N\(^{\text{\text{-}}}\) nitro-L-arginine methyl ester (L-NAME), N\(^{\text{\text{-}}}\) nitro-o-arginine methyl ester (o-NAME), Tris, nitroblue tetrazolium, and 2-hydroxy-4-chloro-3-indolyl phosphate were from Sigma. Nitrate/nitrite colorometric assay kit, sodium nitroprusside (SNP), 1-hydroxy-2-oxo-3-([N-ethyl-2-aminoethyl]-3-ethyl-1-triazene (NOC-12), and 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (PTIO) were from Alexis (San Diego, CA). TRIZOL reagent was from Life Technologies, Inc., and AmpliTaq polymerase, 10× PCR buffer containing 15 mm MgCl\(\text{2}\), and TagDyeDeoxy Terminator kit were from Perkin Elmer (Foster City, CA). INVaF one cell shot and InVitrogen TA cloning kit were from InVitrogen (NV Leek, Netherlands). T4 polynucleotide kinase, prefection mammalian transfection system-calcium phosphate, pSV-galactosidase control vector, pG1-2-promoter luciferase reporter, luciferase assay system, reporter lysis buffer, β-galactosidase enzyme assay system and poly(dI- dC) were from Promega (Madison, WI). Cell death enzyme-linked immunosorbent assay kit was from Roche Molecular Biochemicals. The pC-p53 SN3 (wild type) and pC-p53 SCX3 (mutant) (36) were kindly donated by Dr. Carol Midgley (CRC Laboratories, University of Dundee, Dundee, UK), and pCMV-Neo-Bam vectors and p53 monoclonal antibody (DO1) were kindly donated by Dr. Jiri Bartek (Danish Cancer Society, Copenhagen, Denmark). γ\(^{\text{32P}}\)ATP was from Amer sham Pharmacia Biotech. Boric acid and EDTA were from Merck, restriction enzymes (XhoI/HindIII) were from Biolabs (Beverly, MA), and SDS, β-mercaptoethanol, SDS-polyacrylamide gels, and nitrocellulose membranes were obtained from Bio-Rad. Skimmed milk powder was from Irma (Copenhagen, Denmark), and alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dako (Glostrup, Denmark).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Eagle’s minimum essential medium and fetal calf serum were from Stataera Serum Institute (Copenhagen, Denmark). Dulbecco’s minimum essential medium, Superscript II kit, and Lipofectin reagent were from Life Technologies, Inc. N\(^{\text{\text{-}}}\) nitro-L-arginine methyl ester (L-NAME), N\(^{\text{\text{-}}}\) nitro-o-arginine methyl ester (o-NAME), Tris, nitroblue tetrazolium, and 2-hydroxy-4-chloro-3-indolyl phosphate were from Sigma. Nitrate/nitrite colorometric assay kit, sodium nitroprusside (SNP), 1-hydroxy-2-oxo-3-[N-ethyl-2-aminoethyl]-3-ethyl-1-triazene (NOC-12), and 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (PTIO) were from Alexis (San Diego, CA). TRIZOL reagent was from Life Technologies, Inc., and AmpliTaq polymerase, 10× PCR buffer containing 15 mm MgCl\(\text{2}\), and TagDyeDeoxy Terminator kit were from Perkin Elmer (Foster City, CA). INVaF one cell shot and InVitrogen TA cloning kit were from InVitrogen (NV Leek, Netherlands). T4 polynucleotide kinase, prefection mammalian transfection system-calcium phosphate, pSV-galactosidase control vector, pG1-2-promoter luciferase reporter, luciferase assay system, reporter lysis buffer, β-galactosidase enzyme assay system and poly(dI- dC) were from Promega (Madison, WI). Cell death enzyme-linked immunosorbent assay kit was from Roche Molecular Biochemicals. The pC-p53 SN3 (wild type) and pC-p53 SCX3 (mutant) (36) were kindly donated by Dr. Carol Midgley (CRC Laboratories, University of Dundee, Dundee, UK), and pCMV-Neo-Bam vectors and p53 monoclonal antibody (DO1) were kindly donated by Dr. Jiri Bartek (Danish Cancer Society, Copenhagen, Denmark). γ\(^{\text{32P}}\)ATP was from American Pharmacia Biotech. Boric acid and EDTA were from Merck, restriction enzymes (XhoI/HindIII) were from Biolabs (Beverly, MA), and SDS, β-mercaptoethanol, SDS-polyacrylamide gels, and nitrocellulose membranes were obtained from Bio-Rad. Skimmed milk powder was from Irma (Copenhagen, Denmark), and alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dako (Glostrup, Denmark).

**Cell Culture and Incubations**—MCF-7 cells (passages 18–78) (kindly donated by Dr. N. Brunner, Finsen Laboratory, Copenhagen, Denmark)

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and SAOS-2 cells (passages 30–50) (ATCC, Manassas, VA) were cultured in Eagle’s minimum essential medium and Dulbecco’s minimum essential medium, respectively, containing 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin and were passaged by trypsinization. Cell viability was assessed by trypan blue exclusion. 2 mM NAME, 2 mM AO-NAME, 10–60 μM PTO, 0.125–1 mM SNP, 0.125–1 mM NOC-12, or 3–6 mM sodium butyrate alone or in combinations were added to subconfluent (60%) cultures for 2–24 h. Subconfluent cultures of around 60% confluency were selected because preliminary experiments had established a linear relationship between apoptotic index and cell number up to 80% confluency, beyond which apoptotic index increased dramatically. Subsequently, the cells were analyzed for apoptosis by a cell death detection enzyme-linked immunosorbent assay from Roche Molecular Biochemicals. This assay measures cytosolic histone-associated DNA fragments generated during apoptosis (37–39) and has previously been used for MCF-7 cells (38–40). It was selected because previous studies have demonstrated absence of apoptosis (37–39) and has previously been used for MCF-7 cells (38–40).

Reverse Transcriptase-Polymerase Chain Reaction—MCF-7 cells were harvested using a rubber policeman and total RNA prepared using the TRIZOL reagent. Reverse transcription (RT) employed the SuperScript II kit closely following the supplier’s recommendation for random hexamer RT and PCR except that 2.5 μL of AmpliTaq polymerase was used. Fifteen to forty cycles of PCR (annealing temperature 57 °C) were performed to determine the linearity of the PCR amplification. For specific detection of nNOS, two primers corresponding to positions 2432–2455 and 2645–2666 (GenBank™ accession number U17327) were used. iNOS was detected by primers corresponding to positions 1115–1128 and 1439–1462 (GenBank™ accession number L09210), and eNOS was detected by primers corresponding to positions 2005–2024 and 2330–2350 (GenBank™ accession number M95296). As an internal control primers for amplifying the ubiquitous transcription factor Sp1 (positions 877–899 and 1293–1315, respectively; GenBank™ accession number J03133) were also used. These four primer sets were expected to produce fragments of 236, 351, 346, and 439 bp from the respective cDNA. All primer sets spanned an intron. The PCR products were size fractioned by agarose gel electrophoresis.

TA Cloning and Sequencing—The identity of the PCR products was confirmed by sequencing of cloned PCR fragments. Fragments were cloned using InVitrogen TA Cloning kit and InVitrogen™ One Shot cells and cycle sequenced using either primer M13 forward or M13 reverse and the TaqDyeDeoxy-Terminator kit. Sequence reactions were analyzed on an Applied Biosystems 373A automated DNA sequencer using the GCG Sequence analysis package (Genetics Computer Group, Madison, WI).

Cell Electrophoretic Mobility Shift Assay—Oligonucleotides (5’ to 3’, with a BamHI-BglII (GATC) overhang) corresponding to base pairs –112 to –147 of the upstream region of the human ecNOS gene (GenBank™ accession number D26607; GATC 5’–147/CTTCAGAGCTTCAGCAGGGCTTGTCCCTGTCCCA-3’ (–112)), and GATC 5’–(–112)GGGACAGAACAAGCCGGCTTGAGGAAGCTTCAAAGG– 3’ (–147) were labeled with [γ-32P]ATP using T4 polynucleotide kinase and annealed. 5-μg aliquots of nuclear extracts (44) from SAOS-2 cells (p53 negative) or MCF-7 cells (p53 positive) were incubated for 20 min at 25 °C with 10 fmol [32P]labeled double-stranded oligonucleotide and/or monoclonal p53 antibodies in a final volume of 20 μl of DNA binding buffer (44), containing 200 ng of poly(dI-dC). The gel was electrophoresed for 90 min at 200 V with 1 TBE as running buffer, stained with 0.125–1 mM NOC-12, or 3–6 mM sodium butyrate alone or in combination with a NO scavenger (PTIO; 30 μM; 18 h) on the apoptotic index of MCF-7 cells. Note that both the scavenger and the NO synthase inhibitor significantly increase apoptosis in the MCF-7 cells, whereas low concentrations of the NO donor significantly decrease apoptosis in the MCF-7 cells (p < 0.001). In contrast, nNOS is without effect.

Lipofectin or the calcium phosphate co-precipitation method. The cells were transfected with 3 μg of either the 791-bp or the 1197-bp ecNOS luciferase reporter plasmid construct, together with 3 μg of pSβ-galactosidase control vector and 3 μg of pC-p53 SN3 (wild-type), 3 μg of pC-p53 SCX3 (mutant), or 3 μg of pCMV-Neo-Bam plasmid DNA for 16 h. After 48 h the transfected cells were resuspended in 700 μl of reporter lysis buffer and centrifuged at 20,000 × g for 2 min. Supernatants were used for measurement of luciferase and β-galactosidase activity with the respective kits. Luciferase activity was normalized relative to β-galactosidase activity. Expression of p53 in the transfected cells was verified using Western blotting.

Western Blotting—Cells were scraped off with a rubber policeman in 75 μl sample buffer (62.5 μM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.5% bromphenol blue), boiled for 3 min, and sonicated for 1 min, and 25-μg protein aliquots were applied to 10% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto nitrocellulose membranes in 4.1 mM Tris containing 96 mM glycine and 10% methanol. Membranes were dried, preblocked with 3% skimmed milk powder in phosphate-buffered saline and exposed to a mouse monoclonal p53 (DO1) antibody diluted 1:1000, and the site of

Fig. 1. Sequence of the PCR product obtained from MCF-7 cell RNA (top row) as compared with the sequence of human ecNOS (bottom row; GenBank™ accession number M95296). Sequence uncertainties are indicated by N.
antigen-antibody reaction was detected with an alkaline phosphatase rabbit anti-mouse Ig antiserum. Following development in nitroblue tetrazolium-bromochloroindolyl phosphate medium, the blots were densitometrically analyzed.

RESULTS

Our initial experiments examined whether cultured MCF-7 cells express one or more NOS isoforms. RT-PCR of MCF-7 cell RNA yielded a band of the expected size (346 bp) when the ecNOS primer set was used, whereas no band could be detected when the nNOS or iNOS primer sets were used. 35 cycles were needed to obtain a visible amplicon with the ecNOS primer set. After 40 cycles a correspondingly stronger ecNOS amplicon was obtained, whereas the nNOS and iNOS primer sets failed to produce an amplicon even after 40 cycles. The quality of the latter primers was documented by amplification of genomic DNA. The absence of genomic DNA contamination in the RNA prepared from MCF-7 cells was attested both by the fact that a band of the size expected from DNA was obtained neither with the ecNOS nor with the Sp1 primer sets, which both spanned an intron. The identity of the ecNOS RT-PCR product from MCF-7 RNA was further verified by sequencing (Fig. 1).

To examine the effect of the endogeneous NO production on apoptosis, MCF-7 cells were incubated in the presence of the NOS inhibitor l-NAME or its inactive stereoisomer d-NAME for 2 h. l-NAME significantly increased apoptosis in the cells, whereas d-NAME was without effect (Fig. 2). To further prove the involvement of endogenous NO in MCF-7 cell apoptosis we added an NO scavenger and an NO donor to the cells. At all doses tested, the scavenger PTIO significantly increased apoptosis in the MCF-7 cells, whereas low concentrations of the NO donor SNP significantly reduced apoptosis (Fig. 2). At higher concentrations (0.25–1 mM) SNP stimulated apoptosis. Another NO donor SNP significantly reduced apoptosis (Fig. 3). The increased apoptosis obtained with NOC-12 was reduced by simultaneous incubation with the scavenger PTIO (Fig. 4). To study the effects of NO on apoptosis induced by other agents, we exposed MCF-7 cells to sodium butyrate, an agent previously demonstrated to induce apoptosis in MCF-7 cells and other cell types (40). Addition of sodium butyrate to the cells produced a significant increase in the apoptotic index that was of much greater magnitude than that seen after modulation of the NO levels (Fig. 5). NOC-12 dose-dependently decreased the sodium butyrate-induced apoptosis of MCF-7 cells (Fig. 5). Interestingly, this NO-mediated decrease in apoptosis was obtained also with concentrations of NO donors that by themselves stimulated apoptosis in MCF-7 cells. In fact, the antipapoptotic effect was most marked at such high (0.2–1 mM) concentrations. Addition of the NO scavenger PTIO increased the rate of apoptosis induced by sodium butyrate, suggesting that also endogenously produced NO protected against sodium butyrate-induced apoptosis (Fig. 6).

Because NO-induced apoptosis may involve p53, we next examined possible interactions between the NO and p53 pathways. Inspection of DNA sequences upstream of the transcription initiation site identified a putative p53 binding site (Fig. 7). A double-stranded probe corresponding to this site (Fig. 7) was synthesized, 32P-labeled, and used for electrophoretic mo-
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bility shift assay. Nuclear extracts from MCF-7 cells that produce wild type p53 but not from SAOS-2 cells, which do not produce p53, yielded a band that could be competed out by excess unlabeled probe and that was eliminated by addition of a monoclonal p53 antibody (Fig. 8). We next cloned sequences upstream of the ecNOS gene that incorporated the p53 binding site (Fig. 7). Subcloning these into a luciferase reporter plasmid and transfecting the constructs to SAOS-2 cells revealed low basal activity with a short (791 bp) and higher basal activity with a longer (1197 bp) sequence. Co-transfections with a wild-type or mutant p53 expression vector under the control of the CMV promoter or with a control vector revealed that wild-type but not mutant p53 significantly reduced the luciferase activity of both the 791-bp (data not shown) and 1197-bp (Fig. 9) constructs. This effect could be seen with cells transfected by either the calcium phosphate or Lipofectin methods and was seen in both SAOS-2 and MCF-7 cells. That p53 was indeed expressed by the transfected cells was verified by Western blotting (data not shown).

To study the influence of NO on p53 expression in MCF-7 cells we exposed cells to graded concentrations of the NO donor NOC-12 and then examined p53 expression by densitometric analysis of Western blots. The analysis showed that at all concentrations tested (0.125–1 mM; 4 h) NOC-12 increased the levels of p53 protein (Fig. 10).

DISCUSSION

Our RT-PCR and sequencing results definitely document the presence of ecNOS mRNA in MCF-7 cells. This agrees with previous data on cultured breast cancer cells and mammary carcinomas (34, 35). In addition, our data show that mRNAs encoding the other two NOS isoforms (iNOS and nNOS) were not detectable in MCF-7 cells. Accordingly, results obtained by the specific NOS-inhibitor L-NAME reflect inhibition of MCF-7 cell ecNOS rather than inhibition of iNOS or nNOS. In these cells L-NAME treatment stimulated apoptosis, whereas the inactive stereoisomer D-NAME was without effect. In addition, the NO scavenger PTIO stimulated apoptosis, whereas low concentrations of the NO donor SNP inhibited apoptosis. Together, these results show that endogenous ecNOS-mediated production of NO reduces basal apoptosis in breast cancer cells. We next examined the effect of different concentrations of NO donors on MCF-7 cell apoptosis. Although low doses of the least effective donor (SNP) inhibited apoptosis, higher doses of SNP and all doses of the more effective donor NOC-12 stimulated apoptosis. Thus, low levels of NO protects MCF-7 cells against apoptosis, whereas high levels of (exogenous) NO promotes apoptosis. We were interested in determining whether apoptosis induced by sodium butyrate could be modulated by NO. In agreement with previous studies (40), this reagent induced a marked increase in the apoptotic index of MCF-7 cells. Exposure to the NO donor NOC-12 dose-dependently reduced sodium butyrate-stimulated apoptosis. Interestingly, concentrations of NOC-12 that by themselves stimulated apoptosis afforded the most effective protection against sodium butyrate-induced apoptosis. Thus, this result documented that the effects of NOC-12 and sodium butyrate were not additive. This was further verified by the use of the scavenger PT10, which potentiated the effect of sodium butyrate on apoptosis, suggesting that also endogenous NO reduced the high level of apoptosis induced by sodium butyrate.

The mechanism by which NO modulates apoptosis is unknown. However, high levels of NO may cause nitrosylation and inactivation of caspases (20). Moreover, NO has been found to stimulate accumulation of p53 (10, 16, 17, 28). We therefore investigated whether p53 had effects on ecNOS transcription. Inspection of sequences upstream of the human ecNOS transcription initiation site identified a putative p53 binding site. Gel band shift analysis revealed that this site represented a specific p53 binding site. Upstream sequences containing this site were cloned and ligated to a luciferase reporter gene. Upon co-transfection with a vector expressing wild-type p53, the luciferase activity was reduced 3-fold, whereas co-transfection with a vector expressing mutant p53 did not decrease the luciferase activity. Co-transfections of MCF-7 cells produced similar results. Transcriptional down-regulation by p53 characterizes many genes involved in cell cycle regulation and apoptosis (46–53). Different mechanisms have been considered to cause such down-regulation including interactions with the basal transcriptional machinery in both TATA-containing and TATA-less promoters as well as interactions with Sp1-stimulated transcription (46–53). Because the TATA-less ecNOS promoter contains several Sp1 binding sites (54) and is regulated
and lanes 3

luciferase reporter construct plus a control plasmid (p

ecNOS promoter activity by 3-fold (p

with vectors expressing either wild-type (SN3)

lanes 2

tion with unlabeled oligonucleotide (50-fold,

band (arrow) from p53-positive MCF-7 cells (lanes

lanes 1–4

nuclear extracts from p53-negative SAOS cells (lanes

extracts (lane 5) with the oligonucleotide that can be displaced by compe-

lanes 3 and 7) or shifted by addition of p53 monoclonal antibody (lanes

4 and 5).

FIG. 9. SAOS cells co-transfected with the ecNOS promoter-luciferase reporter construct plus a control plasmid (pCMV) or with vectors expressing either wild-type (SN3) or mutant (SCX3) p53. Note that the vector expressing wild-type p53 decreases ecNOS promoter activity by 3-fold (p < 0.001), whereas there is no difference between the control plasmid and the vector expressing mutan p53. Closely similar results were obtained both by the calcium phosphate and Lipofectin methods and by transfection of MCF-7 cells (data not shown).

by Sp1 (55), either or both of these mechanisms may account for the observed down-regulation. A more remote possibility is that the p53 binding site detected by us in the ecNOS promoter is responsible for the down-regulation. However, this is unlikely because similar such sites detected in other promoters have been associated with transcriptional activation rather than inactivation (56, 57). Future studies will be needed to delineate the effects of this site on the transcription of the ecNOS gene. However, the overall effect of p53 on the promoter is that of down-regulation.

Together, our results clearly demonstrate that low endoge-

nous ecNOS-driven NO production protects against apoptosis in cultured breast cancer cells, whereas high exogenous levels promote apoptosis of the same cells. The overall effect of low endogenous NO levels is that of protection against basal and induced apoptosis. The p53-mediated down-regulation of ec-

NOS may therefore serve to enhance apoptosis. This mecha-

nism could work in concert with the previously observed effects of p53 on the apoptosis-regulating genes bcl-2 (46) and bax (57). In agreement with previous studies (10, 16, 17, 28) we also found that NO donors caused an up-regulation of p53. Thus, in addition to the mechanism discussed above, the p53 pathway may also serve to protect the NO-producing cells against the effects of excessive ecNOS activity. Interestingly, iNOS transcrip-

tion has also been described to be down-regulated by p53 (29) and may similarly safeguard iNOS expressing cells against the effects of excessive NO production. High exogenous produc-

tion of NO (delivered by chemical NO donors or activated cells) may induce apoptosis in appropriate target cells. Although p53 will also serve to down-regulate endogenous ecNOS (and iNOS) transcription in such target cells, this would not affect NO production by the exogenous source of NO. Thus, endogenous NO production may protect the producing cell against apoptosis, whereas release of NO onto invading or parasitic cells may induce apoptosis of the latter (58, 59).

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FIG. 10. Western blot showing the effect of increasing concen-

trations of NOC-12 on MCF-7 cell levels of p53 protein. Lanes 1

and 5, 0 mm; lanes 4 and 8, 0.0625 mm; lanes 3 and 7, 0.25 mm; lanes

2 and 6, 1 mm; 4 h. M denotes prestained molecular mass markers (scale to the left).
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