2-Methoxyestradiol Up-Regulates Death Receptor 5 and Induces Apoptosis through Activation of the Extrinsic Pathway

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ABSTRACT

2-Methoxyestradiol (2ME₂), a natural metabolite of estradiol, is a potent antitumor and antiangiogenic agent. In vitro, 2ME₂ inhibits the proliferation of a wide variety of cell lines and primary cultures, and in numerous models in vivo, it has been shown to be an effective inhibitor of tumor growth and angiogenesis. 2ME₂ is currently in several Phase I and Phase II clinical trials under the name Panzem. Although various molecular targets have been proposed for this compound, the mechanism by which 2ME₂ exerts its effects is still uncertain. This study shows that 2ME₂ uses the extrinsic pathway for induction of apoptosis. 2ME₂ treatment results in up-regulation of death receptor 5 (DR5) protein expression in vitro and in vivo and renders cells more sensitive to the cytotoxic activities of the DR5 ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). 2ME₂-induced apoptosis requires caspase activation and kinetic studies show the sequential activation of caspase-8, caspase-9, and caspase-3. Blockage of death receptor signaling by expression of dominant-negative Fas-associated death domain severely attenuates the ability of 2ME₂ to induce apoptosis. Because 2ME₂ administration has not manifested dose-limiting toxicity in the clinic, DR5 expression may serve as a surrogate marker for biological response.

INTRODUCTION

2ME₂ is a physiological metabolite of estradiol that is formed in vivo by sequential hydroxylation and methylation at the 2-position. Once dismissed as an inert metabolic end product of estradiol, 2ME₂ is now recognized as a potent p.o. active antiangiogenic and antitumor agent, which has virtually no toxicity. Although 2ME₂ has been speculated to be a natural chemopreventative agent (1, 2), the physiological function of this molecule remains undefined. We have recently shown that the actions of 2ME₂ are independent of the ERs. 2ME₂ has low binding affinity for both ERα and ERβ, does not use an ER for its antiproliferative activities, and neither ER agonists nor antagonists influence the activity of 2ME₂ (3). The antiproliferative activities of 2ME₂ have been attributed to several mechanisms, including effects on tubulin polymerization and depolymerization (4–6), up-regulation of p53 (7, 8), and inhibition of superoxide dismutase enzymatic activity (9). Although some of these mechanisms are sufficient to explain the activity of 2ME₂ in certain cases, there is presently no evidence for a common mechanism of action operative in all cells sensitive to 2ME₂.

Apoptosis is an intracellular suicide program that is executed by the activation of caspases, a family of cytoplasmic cysteine proteases. Two major pathways, the intrinsic and the extrinsic apoptotic path-

Received 6/17/02; accepted 11/14/02.

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3 The abbreviations used are: 2ME₂, 2-methoxyestradiol; AFC, 7-amino-4-trifluoromethyl coumarin; DAPI, 4’,6-diamidino-2-phenylindole; DN-FADD, dominant-negative Fas-associated death domain; DR, death receptor; ER, estrogen receptor; HUVEC, human umbilical vein endothelial cells; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor, TRAIL, TNF-related apoptosis-inducing ligand.

MATERIALS AND METHODS

Chemicals. 2ME₂ was purchased from Tetrionics (Madison, WI) and 30 mm stock solutions were made in DMSO and stored in aliquots at −80°C. The compound was diluted in incubation media immediately before each experiment. Thawed stock solutions were used once and discarded. The following reagents were purchased: soluble recombinant human TRAIL protein and anti-His antibody (R&D Systems, Minneapolis, MN or Alexis Biochemicals, San Diego, CA); TNF-α (R&D Systems); anti-Fas clone CH-11 (Upstate Biotechnology, Lake Placid, NY); zVAD-fmk (Alexis Biochemicals); and cycloheximide (Sigma, St. Louis, MO).

Cell Culture. Human breast carcinoma cells MBA-MB-231 and MBA-MB-435, cervical carcinoma cells HeLa, prostate carcinoma cells PC-3, and glioma cells U87-MG were maintained in DMEM:Ham’s F-12 (1:1) medium containing 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1× antibiotic-antimycotic (BioWhittaker, Walkersville, MD). HUVEC were obtained from Clonetics (San Diego, CA). HUVEC cultures were maintained for up to five passages in endothelial growth medium containing bovine brain extract (Clonetics) and 1× antibiotic-antimycotic. Apoptosis Assays. Apoptosis was measured by examination of the presence of cytoplasmic histone-associated DNA fragments. Briefly, cells were seeded in a 24-well plate and after 24 h were exposed to drug for the indicated times. Detection of cytoplasmic histone-associated DNA fragments was performed according to the manufacturer’s instructions (Roche, Indianapolis, IN).

Immunoblot Analysis. Subconfluent monolayers of cells were treated with the indicated concentrations of 2ME₂. Cellular supernatants were removed and collected, cells were scraped in cold PBS, and the total cellular content was collected by centrifugation. Cell pellets were lysed in 50 μl of cold lysis buffer [10 mm Tris (pH 7.5) containing 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mm PMSF, 2 μg/ml aprotonin, 2 μg/ml leupeptin, and 1 mm sodium vanadate], and the lysate was cleared by centrifugation at 4°C. Protein concentration was determined by method of Bradford. The cytosol was mixed with an equal volume of NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) containing 10% 2-mercapto-
ethanol (Sigma) and heated at 95°C for 5 min. Equal protein concentrations of cytosol lysates were subjected to 4–20% Tris-glycine SDS-PAGE (precast NOVEX, Invitrogen). Subsequently, protein was electrophoretically transferred to nitrocellulose membranes in a semidyne blotter and blocked with either a rabbit anti-DR5 polyclonal antibody (Cell Sciences, Inc., Norwood, MA) or a rabbit anti-tau protein monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a rabbit anti-PARP polyclonal antibody (Santa Cruz Biotechnology, Inc.). Proteins were visualized by the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) using a horseradish peroxidase-conjugated antibody against rabbit or mouse IgG (Bio-Rad, Hercules, CA).

**Immunofluorescence.** MDA-MB-231 cells were plated on chamber slides (35 × 10^2 cells/chamber, Falcon Culture Slides) and after 24 h were treated with media alone or containing 1 μM 2ME2 for 24 h. Attached cells were fixed with 4% paraformaldehyde for 25 min at 4°C. Slides were then washed two times in PBS for 5 min. After incubation for 5 min on ice with 0.2% Triton X-100 in PBS, the grid housing was removed and slides were washed twice in PBS. Chamber slides were then stored at −20°C until analysis. Chamber slides were warmed to room temperature and rehydrated in dH2O for 20 min were blocked with 1% fish skin gelatin (Sigma). 0.2% Triton X-100 in PBS for 50 min, rinsed twice in PBS followed by Avidin and Biotin Block (Vector Laboratories, Burlingame, CA). The preparations were then incubated with 1:100 dilution of rabbit antiactive Caspase-3 monoclonal antibody (PharMin-gen, San Diego, CA) at 4°C overnight, rinsed three times sequentially in dH2O, PBS with gelatin/Triton X-100 and PBS, and finally incubated with biotinylated antirabbit secondary antibody for 40 min (1:200 dilution), followed by dark room incubation with Fluorescein Avidin D (1:100 dilution) 40 min (Vector Laboratories). Chamber slides were then incubated with 1% fish gelatin, 0.2% Triton X-100 in PBS for 50 min followed by a rabbit polyclonal anti-DR5 (1:100 dilution; Cell Sciences, Inc.) for 1.5 h in the dark at room temperature followed by direct conjugation with antirabbit Texas Red Alexa 594 for 30 min (Molecular Probes, Inc., Eugene, OR). After the final wash, the slides were mounted and stained with DAPI that produces blue fluorescence. Immunofluorescence microscopy was performed using a BX60 microscope (Olympus).

**Tumor Models and Immunohistochemistry.** Female 8–10-week-old BALB/c nu/nu mice (The Jackson Laboratory, Bar Harbor, ME) received injections in the mammary fat pad with 2 × 10^3 MDA-MB-231 cells. Daily oral administration of 2ME2 was initiated when the tumors became palpable (4–5 mm diameter). A suspension of 2ME2 was prepared by either homogeneous in an aqueous solution of 0.5% methylcellulose (Sigma) using a Kinematica polytron (Brinkmann, Westbury, NY), or 2ME2 was incorporated into liposomes composed of dioleoyl phosphatidylcholine with a molar ratio of phospholipid:2ME2 of 2:1.5. Control animals were given vehicle alone or containing 1 μM 2ME2. Untreated or 2ME2-treated cells by immunoblotting. We found that up-regulation of DR5 protein was a general response of cells exposed to 2ME2, we analyzed whole cell lysates of either untreated or 2ME2-treated cells by immunoblotting. We found that DR5 protein expression is induced in a variety of tumor cell types and in HUVEC primary endothelial cell type (Fig. 1A). The appearance of multiple polypeptides in the DR5 immunoblot has been reported previously (14, 15). This up-regulation is associated with an apoptotic response, as indicated by the appearance in 2ME2-treated cells of the Mr 85,000 PARP cleavage fragment (Fig. 1B). The IC50 values for the antiproliferative activity of 2ME2 are in a narrow range from high nanomolar to low micromolar, and consequently, a 2-μM concentration was selected to evaluate the effects of 2ME2 on the various cell types. However, with HeLa cells, we used a concentration of 1 μM because in these cells the overall cell death is greater, although the IC50 is not particularly different from other cell types. Treatment of HeLa cells with 1 μM 2ME2 for 48 h results in an 88.5% decrease in cell number, whereas treatment of the other cell lines with 1 μM 2ME2 results in only an approximate 65% decrease (Fig. 1C). It is interesting that the appearance of the PARP cleavage fragment varies among cell lines. In MDA-MB-231, MDA-MB-435, and PC3 cells, 2ME2 treatment results in the appearance of the PARP cleavage fragment at 48 h, whereas in HUVEC and U87-MG cells, appearance of detectable level of PARP cleavage fragment occurs at 24 h and does not increase with additional treatment. Consistent with HeLa cells being more responsive to 2ME2, the greatest extent of PARP cleavage in response to treatment occurs with these cells (Fig. 1B).

We also assessed other DRs. Although 2ME2 treatment did result in up-regulation of DR4 protein in PC3 cells, this up-regulation was not a general response to 2ME2 in all of the tumor cell lines that we assessed. Consistent with previous reports, in endothelial cells we found up-regulation of Fas in response to 2ME2 treatment in HUVEC.
However, 2ME₂ had no effect on Fas in tumor cell lines. Similarly, up-regulation of TRAIL was observed in 2ME₂ treated HUVEC but not in tumor cell lines (data not shown).

**2ME₂ Treatment Sensitizes MDA-MB-231 Cells and HUVEC to TRAIL-induced Apoptosis.** TRAIL, the ligand for DR5, has cytotoxic activity on a wide variety of transformed cell lines in vitro and is a potent antitumor agent in vivo (17, 18). However, up-regulation of DR5 does not always predict susceptibility to TRAIL-induced apoptosis (19–21). Because 2ME₂ up-regulates DR5, we investigated whether 2ME₂ treatment sensitizes tumor and endothelial cells to TRAIL-induced apoptosis. Treatment of either MDA-MB-231 cells or HUVEC with 50 ng/ml TRAIL alone or 2ME₂ alone induces apoptosis. Exposure of both cell types to TRAIL in combination with 2ME₂ results in a marked increase in apoptosis as compared with either treatment alone (Fig. 2, A and B). The strong induction of apoptosis by the combination treatment makes it appear that 2ME₂ alone does not significantly induce apoptosis. However, with more prolonged incubation during the color development of the ELISA, a dose-dependent increase in apoptosis in response to 2ME₂ is observed (Fig. 3). These data are consistent with the DR5 expressed in these cells being able to induce apoptosis and indicate that 2ME₂ treatment further sensitizes cells to TRAIL-induced cell death.

**2ME₂-induced Caspase Activation and Kinetics Are Consistent with DR-mediated Apoptosis.** DR5 activated apoptosis has been shown to be mediated via a caspase signaling cascade in which caspase-8, an initiator caspase, becomes activated and, in turn, activates other initiator caspases such as caspase-9 followed by activation of executioner caspases such as caspase-3 (11). To determine whether caspase activation is required for 2ME₂-induced cell death, we assessed the ability of 2ME₂ to cause apoptosis in the presence of zVAD-fmk, a broad specificity caspase inhibitor. MDA-MB-231 cells or HUVEC pretreated with 50 μM zVAD-fmk were incubated for 48 h with increasing concentrations of 2ME₂ or without the inhibitor, and the levels of apoptosis were assessed. As shown in Fig. 3, 2ME₂-induced apoptosis in MDA-MB-231 cells and HUVEC was greatly attenuated in the presence of zVAD-fmk. These data suggest that both in tumor and endothelial cells 2ME₂ requires the activation of caspases to induce apoptosis.

To further characterize the apoptotic pathway activated by 2ME₂, we determined the kinetics of caspase activation. The activation of
caspases in 2ME₂-treated cells was assessed using the fluorogenic tetrapeptide substrates, IETD-AFC, LEHD-AFC, and DEVD-AFC, which have been shown to be selective for caspase-8-, caspase-9-, and caspase-3-like enzymatic activities, respectively. Treatment of MDA-MB-231 cells with 2 µM 2ME₂ results in the detection of caspase-8 activation as early as 2 h followed by caspase-9 activation at 4 h and caspase-3 activation at 8–14 h (Fig. 4). The level of caspase-8 and caspase-9 activation increased up to 16 h of treatment. Caspase-3 activation continued to intensify over time and resulted in a much greater fluorescence signal than either caspase-8 or caspase-9 (Fig. 4). Our observation that caspase-8 is the first caspase activated by 2ME₂ is consistent with an apoptotic pathway initiated by DR activation.

2ME₂ Activates Caspase-3 in Cells that Up-Regulate DR5. To identify whether there is a relationship between DR and caspase activation, we assessed the up-regulation of DR5 and the activation of caspase-3 by immunofluorescence in cultured cells. MDA-MB-231 cells were either left untreated or treated with 1 µM 2ME₂ for 24 h, the adherent cells were fixed, and nuclear morphology was visualized by staining the cells with DAPI (Fig. 5). A slightly lower concentration of 2ME₂ was used in these experiments as compared with our immunoblot analysis (Fig. 1, A and B) to increase the number of cells retained on the coverslip, however, similar results were obtained with 2 µM 2ME₂. In the treated population, there is a clear decrease in the number of cells and an increase in the number of mitotic figures and condensed nuclei. Cells stained with anti-DR5 antibody followed by a rhodamine-labeled secondary antibody (Fig. 5) showed brighter staining of the treated population overall consistent with the increase in DR5 protein expression observed by immunoblot analysis (Fig. 1A). Staining with an antibody that recognizes the activated cleavage fragment of caspase-3 followed by a FITC-labeled secondary antibody shows that despite occasional staining of cells in the untreated population, there are significantly more activated caspase-3-positive cells in the treated cells. Because in this assay only the cells that are still attached were evaluated, this assessment underestimates the total number of cells that have activated caspase-3. Costaining with anti-DR5 and antiactivated caspase-3 antibodies shows that 2ME₂ treatment results in active caspase-3 only in cells that have up-regulated DR5 as indicated by the yellow cells (Fig. 5), consistent with DR5-dependent caspase-3 activation.

DN-FADD Inhibits 2ME₂-induced Apoptosis. Several DRs signal apoptosis by recruitment of the adaptor protein FADD to the oligomerized DR complex, where FADD facilitates the binding and activation of procaspase-8. To further test the hypothesis that DR5 mediates 2ME₂-induced apoptosis, DN-FADD was stably transfected into MDA-MB-231 cells. As shown in Fig. 6A, Fas, TNF, and TRAIL-mediated apoptosis were markedly reduced by stable overexpression of DN-FADD protein as compared with the treated cells transfected with vector alone. These death-inducing cytokines have been shown to mediate apoptosis through a DR-mediated pathway that activates caspase-8 through a FADD-dependent pathway (22, 23). Similarly, 2ME₂ treatment of two independent clones of cells transfected with DN-FADD resulted in a marked reduction in the amount of apoptosis observed as compared with the cells expressing the vector alone (Fig. 6B). These data indicate that a DR-mediated pathway is used in the mechanism of 2ME₂-induced apoptosis. Although these data do not exclude a role of other DRs, only DR5 is up-regulated in these cells in response to 2ME₂ treatment.

p.o. Administered 2ME₂ Inhibits Primary Tumor Growth and Up-Regulates DR5 in Vivo. 2ME₂ has been shown to be effective at inhibiting both primary tumors and metastatic disease in a variety of
tumor models through induction of apoptosis (12, 13). Oral administration of 2ME₂ in an orthotopic MDA-MB-231 breast cancer model in nude mice resulted in a reduction of tumor volume (Fig. 7A). At the termination of study the tumor burden was reduced ~65% in the animals treated with 150 mg/kg 2ME₂. Consistent with data using cultured cells, immunohistochemistry analysis of MDA-MB-231 tumors demonstrated that 2ME₂ treatment results in up-regulation of DR5 protein expression (Fig. 7B) but not DR4 protein expression (data not shown). Similar results were obtained using U87-MG in a xenograft tumor model (24). These data show that treatment of tumors with doses of 2ME₂ that produce a reduction in tumor burden in two animal models results in the up-regulation of DR5 protein.

**DISCUSSION**

2ME₂ is an endogenous metabolite of estradiol, yet it is distinct among estradiol metabolites because of its inability to engage ERs as an agonist. In addition, its unique antiproliferative and apoptotic activities are mediated independently of the ERs (3). 2ME₂ is also unique among the many antiangiogenic agents currently in clinical trials because it targets both the endothelial cell and the tumor cell compartment of a growing tumor. The physiological function and molecular mechanism of this compound are not clearly defined. To further understand the mechanism of action of 2ME₂, we investigated the pathways of activation of the apoptotic response. In this study, we
show that 2ME₂ treatment results in an increase in DR5 protein expression in cultured cells at doses that induce apoptosis and in tumor models at doses that produce a significant decrease in tumor burden. 2ME₂ treatment of cells results in the activation of caspase-8 followed by caspase-9 and caspase-3; furthermore, caspase-3 activation was identified only in 2ME₂-treated cells that have up-regulated DR5 protein. Finally, 2ME₂-induced apoptosis is attenuated by overexpression of DN-FADD. Collectively, these data show that 2ME₂ mediates apoptosis through engagement of the extrinsic pathway.

In our evaluation of which DRs were induced in response to 2ME₂ treatment, we found that DR5 was consistently up-regulated. DR4 protein up-regulation was observed in treated PC3 cells in vitro; however, there was no significant increase in DR4 protein expression in response to 2ME₂ treatment in vivo. Consistent with previous reports, 2ME₂ treatment resulted in up-regulation of Fas in HUVEC, but this treatment did not result in up-regulation of Fas in tumor cell lines (data not shown). Similarly, the ligand for DR5 TRAIL was up-regulated in response to treatment of HUVEC but not of tumor cell lines. Our in vivo analysis showed a mixed response in that only some of the treated animals showed a slight increase of TRAIL protein expression. Thus, whereas DR4, Fas, and TRAIL up-regulation varied as a function of cell type, DR5 was consistently up-regulated in response to 2ME₂ treatment. Alternatively, TRAIL produced in the tumor vasculature could exert its effects in the surrounding tumor cells. Currently, this cannot be assessed in these models because antibodies recognizing murine TRAIL are not available.

The cell type-specific Fas and TRAIL up-regulation suggest that some aspects of the mechanisms by which 2ME₂ induces apoptosis in tumor and endothelial cells may be distinct. Two antiangiogenic proteins, pigment epithelium-derived factor and thrombospondin 1, have recently been reported to selectively induce apoptosis in proliferating endothelial cells by up-regulating FAS ligand. Fas was shown to be up-regulated in only proliferating endothelial cells and, thus, allows these antiangiogenics agents to selectively inhibit the growing
or remodeling endothelium (25). Whether these endogenous angiogenesis inhibitors also up-regulate DR5 is unknown. The finding that several 2ME₂ analogs show preferential inhibition of endothelial cells over tumor cells (26, 27) additionally suggests that the mechanisms of 2ME₂-induced apoptosis in endothelial cells versus tumor cells may be different.

There are several possible mechanisms by which 2ME₂-induced apoptosis in tumor cell lines in vitro could be mediated through DR5. TRAIL protein is present and it is possible, as has been suggested for other agents that up-regulate DR5 without up-regulating TRAIL, that the newly generated DR is able to use the constitutively expressed ligand. However, we were unable to affect 2ME₂-induced apoptosis with a soluble DR5 alone or in combination with an antagonist antibody against Fas and soluble TNF receptor (data not shown). These data suggest either that the interaction with the endogenous ligand is not readily accessible to exogenously added inhibitors or that the up-regulated DR5 does not require additional activation. This has been shown in transfected cell lines in which overexpression of DR5 can induce apoptosis in a ligand-independent manner (23). The up-regulation of DR5, the kinetics of caspase activation, the coexpression of activated caspase-3 only in cells expressing DR5, and the attenuation of apoptosis mediated by expression of DN-FADD are all consistent with DR5 involvement in 2ME₂-mediated cell death, irrespective of the mechanism underlying this process.

The inhibitory effects of 2ME₂ have been attributed to several mechanisms, including disruption of microtubule function (4–6), inhibition of superoxide dismutase (9), inhibition of angiogenesis (28), and induction of apoptosis (16, 29). 2ME₂ has been demonstrated to cause G₂–M arrest, to bind to tubulin and microtubules inhibiting both polymerization and depolymerization (4, 5), and to cause abnormal spindle formation in cultured cells (30). In contrast, it has been noted that treatment of cells with atiproliferative concentrations of 2ME₂ cause microtubule stabilization in some cases (6) and does not result in inhibition of mitotic spindles in others (31). We have only observed destabilization of microtubules on cells in culture at concentrations of 2ME₂ significantly higher than those used in this study to see effects on DR5 up-regulation. However, effects on microtubule dynamics occur at concentrations that do not affect stability (32). We found that treatment of tumor-bearing animals with vinblastine, a drug that destabilizes microtubules, also resulted in up-regulation of DR5 protein (data not shown). Paclitaxel and docetaxel, two agents that stabilize microtubules, have been shown to up-regulate DR4 and DR5 protein expression and sensitize prostate cell lines to the cytotoxic effects of TRAIL (33). However, these effects may not represent a general cellular observation because paclitaxel did not up-regulate DR4 and DR5 protein or enhance the activity of TRAIL in non-small cell lung cancer or breast cancer cell lines (34–36). In the case of 2ME₂ it is still uncertain how up-regulation of DR5 is related to its effects on microtubule stability and this requires additional investigation.

In the current study, we demonstrate that 2ME₂ up-regulates DR5, uses the extrinsic apoptotic pathway, and makes cells more sensitive to TRAIL. Similar to 2ME₂, TRAIL exerts potent antitumor activity in vivo without affecting normal tissue, suggesting that a combination of 2ME₂ and TRAIL may be an effective antitumor therapy. Furthermore, in the absence of dose-limiting toxicity for 2ME₂, DR5 expression may serve as a surrogate marker for biological response in the clinic, DR5 may also serve as a marker for assessing and discriminating 2ME₂-like molecules from other related antiproliferative agents because structurally related molecules could induce antiproliferative responses through a variety of mechanisms.

ACKNOWLEDGMENTS

We thank Sauda Ayub for help in preparing the figures. We also thank Drs. Don Bottaro and Anthony Treston for critical reading of the manuscript.

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