A p53-independent Pathway for Activation of WAF1/CIP1 Expression Following Oxidative Stress*

(Received for publication, June 6, 1995, and in revised form, August 17, 1995)

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Incubating human cells in diethylmaleate (DEM) depletes the intracellular pool of reduced glutathione (GSH) and increases the concentration of oxidative free radicals. We found that DEM-induced oxidative stress reduced the ability of p53 to bind its consensus recognition sequence and to activate transcription of a p53-specific reporter gene. Nevertheless, DEM treatment induced expression of WAF1/C1P1 but not GADD45 mRNA. The fact that N-acetylcysteine, a precursor of GSH that blocks oxidative stress, prevented WAF1/CIP1 induction by DEM suggests that WAF1/CIP1 induction probably was a consequence of the ability of DEM to reduce intracellular GSH levels. DEM induced WAF1/CIP1 expression in Saos-2 and T98G cells, both of which lack functional p53 protein. DEM treatment did not produce an increase in membrane-associated protein kinase C, but ERK2, a mitogen-activated protein kinase, was phosphorylated in a manner consistent with ERK2 activation. DEM treatment also produced a dose-dependent delay in cell cycle progression, which at low concentrations (0.25 mM) consisted of a G2/M arrest and at higher concentrations (1 mM) also involved G1 and S phase delays. Our results indicate that oxidative stress induces WAF1/CIP1 expression and arrests cell cycle progression through a mechanism that is independent of p53. This mechanism may provide for cell cycle checkpoint control under conditions that inactive p53.

The accumulation of oxidative damage is believed to be an important cause of aging and may contribute to the increased incidence of cancer in older individuals (1). Reactive oxygen radicals are capable of damaging many cellular components including DNA (2, 3). A wide variety of DNA damages result in the activation of mechanisms that arrest cell cycle progression at specific checkpoints, presumably to allow time for the damage to be repaired. Activation of the G1 checkpoint mechanism by DNA damage requires the function of the p53 tumor suppressor protein, which transiently accumulates in cells after exposure to several agents that damage DNA (4, 5). p53 is a transcription factor that binds to specific DNA sequence elements and activates transcription; among the genes whose transcription is activated by p53 are WAF1/CIP1 and GADD45 (6–8). P53 also suppresses transcription from other genes that do not have p53-specific binding elements (9, 10). WAF1/CIP1 encodes a potent 21-kDa (p21) inhibitor of cyclin-dependent kinase activities that are required for progression from the G1 to the S phase of the cell cycle (11, 12). In vitro experiments have shown that p53 is sensitive to oxidation and that the oxidized form of p53 is unable to bind its cognate DNA cis-element (13, 14).

If the p53 protein is similarly sensitive to oxidation in vivo, then inactivation would render it incapable of protecting a cell from the DNA damages provoked by oxidizing radicals. To address this possibility, we asked whether p53 DNA binding and transactivation are affected by treatment with diethylmaleate (DEM), an agent that increases the intracellular concentration of free radicals by depleting the cellular store of reduced glutathione (GSH).

MATERIALS AND METHODS

Cell Culture and Transfections—Hep3B, HeLa, COS-2, Saos-2, and T98G cells were cultured in Dulbecco’s modified Eagle’s medium (Flow Laboratories) containing 10% fetal calf serum (HyClone), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Flow Laboratories). COS-2 and Saos-2 cell lines were transfected by the calcium phosphate procedure (13) in 60-mm dishes with 30 μg of the pcMV-neo expression vector (16) containing a human wild-type p53 cDNA (pcMV-p53) (17) and 10 μg of a multimeric p53 responsive element (PG13) (18) driving the transcription of the luciferase reporter gene in the pGL2-promoter vector (Promega). Control transfections with the parental vector were also carried out for determination of basal luciferase activity. 24 h after the transfection, DEM (Sigma) was added to the culture at a final concentration of 0.5 or 1 mM (19, 20), and 6 h later extracts were prepared for luciferase activity determination (Promega) according to the manufacturer’s instructions. Control experiments were performed in cells transfected as described above and not exposed to DEM or transfected with pRSV-luciferase reporter plasmid to evaluate the effect of DEM on a p53-independent promoter.

Electrophoretic Mobility Shift Assays—COS-2, Hep3B, and HeLa cells were transfected with 10 μg of the pcMV-p53 construct per 100-mm dish; the cells were left untreated or treated with 1 mM DEM for 3 h and then harvested for the preparation of nuclear extracts according to Ref. 21. Electrophoretic mobility shift assays were performed with approximately 1 ng of end-labeled double-stranded oligonucleotide by using 10 μg of nuclear proteins in a volume of 12 μl in 20

* Supported by grants from Associazione Italiana per la Ricerca sul Cancro (to T. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Oxidative Stress-induced WAF1/CIP1 Expression

**RESULTS**

Oxidative Stress Inhibits Sites-specific p53 DNA Binding in Vivo—We evaluated the possibility that oxidizing conditions might interfere with the ability of p53 to activate transcription in vivo first by examining the ability of p53 from oxidatively stressed cells to bind DNA in a sequence-specific manner (Fig. 1). Oxidative stress was induced by treating cells in culture with DEM, a weak electrophile that induces a rapid and transient depletion of GSH by forming DEM-GSH conjugates in a reaction catalyzed by the enzyme glutathione S-transferase (25). Exposure to 1 mM DEM reduces the levels of GSH in cells by about 90% within 30 min, and this reduction impairs the cell’s ability to scavenge oxidative radicals produced as a normal consequence of oxidative metabolism (Ref. 19 and data not shown). To provide adequate amounts of wild-type p53, COS-2, Hep3B, and HeLa cells were transfected before treatment with pCMV-p53, a plasmid containing the wild-type human p53 cDNA under expression control of the strong, constitutive cytomegalovirus promoter (17). 36 h after transfection and 3 h before harvesting, cells were exposed to 1 mM DEM. The nuclear extracts were then analyzed for p53 binding ability by electrophoretic mobility shift assays with a 32P-labeled double-stranded oligonucleotide probe containing the p53 DNA binding sequence from the RGC promoter (22). A retarded band was observed with extracts from COS-2 and Hep3B cells that had been transfected with the pCMV-p53 expression vector (Fig. 1, lanes c and g). This band was specific for wild-type p53 as shown by the fact that excess cold competitor RGC oligonucleotide caused a dramatic reduction in its intensity (lane d), but an excess of an oligonucleotide unrelated to the p53 binding motif did not (lane e). The position of the complex also was dependent on the presence of the PAb421 antibody, which was added to reactions to enhance sequence specific binding. The p53-specific band was decreased significantly in intensity in nuclear extracts from COS-2 and Hep3B cells that had been exposed to 1 mM DEM (lanes f and h); however, adding DEM directly to extracts had no effect on the ability of p53 to bind DNA (data not shown). Similar results were observed with nuclear extracts from treated and untreated HeLa cells (data not shown). Thus, exposing cells to DEM significantly reduced the ability of the intracellular p53 to bind a DNA recognition site.

To determine if treatment of cells with DEM also impaired the ability of wild-type p53 to activate transcription in vivo, we examined the effect of DEM treatment on the transcription of a reporter gene whose transcription was dependent on wild-type p53 function (Fig. 2). Saos-2 cells, a human cell line that does not express an endogenous p53, and COS-2 cells were co-transfected with two plasmids: pCMV-p53 and PG13-luciferase, a reporter plasmid in which luciferase expression is driven by 13 direct repeats of the p53-binding sequence 5'-CCTGCTGAGTTCGCGG-3' placed upstream.
of a basal SV40 early promoter (see "Materials and Methods"). Transfection with both plasmids resulted in high levels of luciferase expression, whereas after transfection with the PG13-luciferase reporter alone, very little luciferase was produced in Saos-2 cells, and expression in COS-2 cells also was modest. In cells co-transfected with both plasmids, luciferase activity was significantly decreased by exposing cells for 6 h beginning 24 h after transfection to either 0.5 mM DEM or 1 mM DEM. Expression from two p53-independent promoters, pSV2-CAT and RSV-luciferase, was not affected by DEM treatment (Fig. 2); thus, DEM does not act as a general inhibitor of transcription, nor is it an inhibitor of luciferase activity. We conclude that oxidative stress impairs the ability of p53 to function as a transcriptional activator in vivo in a dose-dependent fashion. Together with studies showing that exposing p53 to oxidizing conditions in vitro impairs its sequence-specific DNA binding ability (13, 14), this experiment and the gel shift experiments described above suggest oxidative stress may block the ability of p53 to activate transcription in vivo by inactivating its ability to bind specific DNA sequences.

Effect of DEM on the Expression of p53-regulated Genes—p53 activates the expression of several endogenous genes including WAF1/CIP1, GADD45, and HMD2 (human mdm-2) in response to a variety of DNA damage-inducing agents (26–28). Before asking if DEM treatment blocked activation of these endogenous genes by DNA damage-inducing agents, we first asked if exposing cells to DEM alone affected their expression in HeLa cells. HeLa cells that had been left alone or exposed for 3 h to 1 mM DEM were examined for WAF1/CIP1 and GADD45 expression by Northern blot analysis (Fig. 3A). DEM treatment produced a substantial increase in WAF1/CIP1 mRNA, but the amount of GADD45 mRNA was not appreciably affected by this treatment. Pretreating the cells with N-acetylcysteine, which increases the intracellular GSH concentration, largely prevented the DEM-induced increase in WAF1/CIP1 mRNA (Fig. 3B); this result supports the notion that DEM acts by decreasing the intracellular concentration of GSH. HeLa cells are impaired in their ability to express p53; thus, the finding that WAF1/CIP1 mRNA levels increased in HeLa cells exposed to DEM suggested that another mechanism was responsible for activating the expression of this gene. To show unambiguously that the DEM-mediated increase in WAF1/CIP1 mRNA was not dependent on p53, we prepared RNA from Saos-2 and T98G glioblastoma cells, both of which lack functional p53 genes. As was the case for HeLa cells, the levels of GADD45 mRNA were unchanged in response to DEM concentrations between 0.25 and 1 mM; however, the levels of WAF1/CIP1 mRNA were increased in both p53 defective cell lines. Furthermore, the observed increases in WAF1/CIP1 mRNA were proportional to the DEM concentrations to which the cells were exposed (Fig. 4). Western blot analysis of the T98G cells also showed that DEM treatment produced a dose-dependent increase in the levels of Waf1 protein (Fig. 5). Thus, we conclude that cells have a p53-independent mechanism for activating WAF1/CIP1 expression in response to oxidative stress.

DEM Treatment Activates the MAP kinase ERK2 but Not Protein Kinase C—Recently, several groups have shown that exposure of cells to TPA, a phorbol ester that activates protein kinase C, induces a significant increase in the concentration of WAF1/CIP1 mRNA through a p53-independent pathway (29–31). We therefore asked if DEM treatment resulted in the activation of protein kinase C. As shown in Fig. 6, TPA induced a significant increase in the fraction of protein kinase C activity that was membrane-associated in both Saos-2 and HeLa cells; a concomitant decrease was observed in cytosolic protein kinase activity.
Life Technologies, Inc. assay kit (61).

Protein kinase C activity was assayed with the cytosolic fraction (hatched bars) and a membrane preparation (white bars) and a cytosolic fraction (hatched bars). Protein kinase C activity was assayed with the Life Technologies, Inc. assay kit (61).

**FIG. 6.** Effect of DEM Treatment on protein kinase C activity. Saos-2 (A) and HeLa (B) cells were exposed to 20 ng/ml TPA or 1 mM DEM for 2 h, then harvested, and fractionated as described under "Materials and Methods" to provide a membrane preparation (white bars) and a cytosolic fraction (hatched bars). Protein kinase C activity was assayed with the Life Technologies, Inc. assay kit (61).

C activity. In contrast, DEM treatment had no effect on the amount or distribution of protein kinase C activity, suggesting that DEM does not induce WAF1/CIP1 through a protein kinase C-mediated pathway.

Growth factors and DNA damage-inducing agents activate the MAP kinase cascade (32-34), which, in turn, activates the transcription of specific genes through phosphorylation of immediate early transcription factors including c-jun. Activation of the MAP kinase ERK2 is accomplished by its phosphorylation by a MAP kinase kinase, and this phosphorylation decreases the mobility of the ERK2 polypeptide during SDS-polyacrylamide gel electrophoresis (32). Thus, ERK2 activation can be accessed by monitoring ERK2 mobility by Western blot analysis. Fig. 7 shows that exposing HeLa cells to 1 mM DEM produced a significant increase in the phosphorylated (active) form of ERK2. The increase in mobility was similar to that induced by TPA; furthermore, the change was prevented by the pretreatment of cells with N-acetylcyesteine. We conclude that the DEM-induced reduction in GSH concentration activates the MAP kinase cascade in a manner similar to the way it is activated after exposing cells to DNA damaging agents such as UV light.

**FIG. 7.** Effect of DEM Treatment on ERK2 activity. HeLa cells were exposed to TPA, DEM, NAC, or NAC and DEM, and the cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting for phosphorylated (upper band) and unphosphorylated ERK2 as described under "Materials and Methods." Values shown are the percentage of cells in the G1, S, or G2/M phases from a representative experiment in which at least 15,000 cells were analyzed for each point.

**DISCUSSION**

The p21 protein product of WAF1/CIP1 is a potent inhibitor of cyclin-dependent kinase activities that are required for progression from the G1 phase of the cell cycle into S phase (11, 12). Transcription of the p21 gene is induced following DNA damage as a consequence of the accumulation of wild-type p53, and the elevated concentration of p21 protein mediates, at least in part, p53-induced growth arrest in response to DNA damage (7, 12). These recent findings have evoked considerable interest because the p53 gene is mutated in many human tumors, and alternate methods of inducing p21 expression could provide approaches for controlling tumor growth and influencing the survival of tumor cells that have lost functional p53.

In this paper we show that p21 is rapidly induced in response to DEM, an agent that increases intracellular oxidative damage by decreasing the intracellular pool of glutathione. p21 induction by DEM was prevented by pretreatment with N-acetylcyesteine, a precursor of reduced glutathione, indicating that the DEM-mediated induction of p21 is a consequence of oxidative damage. Induction of p21 by DEM was independent of p53 because it occurs in cell lines that do not express p53 protein (e.g., Saos-2) and that express mutant p53 proteins (e.g.
T98G) that cannot activate transcription in response to DNA damage or overexpression.

The induction of p21 following DEM exposure was associated with arrest of T98G cells in the G, S and G phases of the cell cycle (Fig. 8). Arrest at these points in the cell cycle corresponds well with the proposed actions of p21 on components of the cell cycle machinery. Arrest in G phase is probably related to inhibition of cyclin/Dbk kinase activity, which is required for progression of cells through the G phase arrest point (35). Arrest in the S phase following DEM treatment probably reflects the ability of p21 to inhibit DNA synthesis by binding to and blocking PCNA function (37). Indeed, overexpression of the PCNA binding domain of p21 is sufficient to inhibit DNA synthesis when transplanted into mammalian cells (38).

Recently, p21 expression was shown to be induced in several cell lines by agents that cause terminal differentiation through a p53-independent mechanism. p21 mRNA expression was elevated after exposure to TPA, butyrate, trans-retinoic acid, and 6-thioguanosine in HL-60, K562, and U937 cells, human hematopoietic or hepatoma-derived lines that express no mutant p53 (29–31). p21 expression also is induced by serum, PDGF, EGF, TPA, and okadaic acid in quiescent fibroblastic cell lines derived from transgenic mice lacking a functional p53 gene (26, 29). The mechanism by which these agents induce p21 expression is unknown, but induction is insensitive to cycloheximide, suggesting that it is a consequence of the activation of pre-existing transcription factors. The ability of mitogenic growth factors and TPA to induce p21 expression suggests the involvement of protein kinase C in modulating its expression. Consistent with this suggestion, TPA-resistant variants of HL-60 cells exhibit altered responses in immediate early gene expression including c-fos and c-jun (39), and these cells display an altered protein kinase C isozyme profile, are incapable of translocating protein kinase C from the cytosol to the membrane fraction, and exhibit altered protein phosphorylation after TPA treatment (39, 40). Further support for the involvement of protein kinase C comes from the observation that adriamycin, which activates protein kinase C (41), is a potent inducer of p21 in cells containing wild-type p53 (35), and, at higher concentrations, induces WAF1/CIP1 transcription in p53 null cells (26). In contrast to these results, exposing HeLa and Saos-2 cells to DEM had no effect on the amount or the distribution of protein kinase C activity (Fig. 6), indicating that the major forms of protein kinase C are not activated by DEM. Instead, DEM treatment produced an increase in the phosphorylated form of ERK2 (Fig. 7), a member of the mitogen-activated kinase family; furthermore, ERK2 phosphorylation, which is required for its activation, was prevented by pretreating cells with NAC.

Recently, it has become clear that DNA damage-inducing agents, including oxidizing agents, may activate immediate early gene expression through pathways that do not directly involve DNA damage (32, 33, 42, 43). Treatment of cells with UV light, ionizing radiation, and H2O2 cause a rapid increase in membrane-bound tyrosine kinase activity, protein kinase C activity, and MAP kinase activity (32, 44, 45). These kinase activities activate several transcription factors including c-jun, EGR1, and NF-kappaB, and transcription factor and MAP kinase activation were prevented by pretreating cells with NAC (46–49), suggesting that in each case activation is mediated through oxidative damage. Although other mechanisms could account for the accumulation of WAF1/CIP1 mRNA in DEM treated cells, our finding that DEM treatment activates ERK2 and that activation is prevented by pretreatment with NAC is consistent with the hypothesis that oxidative damage induces transcription of the WAF1/CIP1 gene through the activation of a transcription factor(s) other than p53. A serum response element that overlaps the proximal p53 recognition element in the murine WAF1/CIP1 promoter recently was identified (50). However, serum-mediated activation of MAP kinase is not prevented by NAC (49); thus, the DEM-induced MAP kinase activation seems to take place downstream of the membrane receptor growth signal initiating machinery. A possibility to be explored is the DEM-induced inhibition of the MAP kinase phosphatase, which is inhibited by oxidants as are other tyrosine phosphatases (51). This possibility is consistent with the observed induction of MAP kinase (52) and of WAF1 (26) as a consequence of okadaic acid treatment.

Other important findings of this study are that the DEM treatment was incapable of inducing transcription of the endogenous GADD45 gene and that the transcription of an exogenous p53-regulated reporter gene was impaired in cells treated with DEM. Furthermore, the p53 from DEM-treated cells exhibited a decreased ability to bind a consensus recognition sequence. Previous studies had shown that the specific DNA binding ability of p53 is sensitive to oxidation in vitro (13, 14); this effect can be traced to the oxidation of cysteines that are critical for DNA binding (55). A similar sensitivity to oxidative damage is exhibited by transcription factor Sp1 (56) and by the glucocorticoid receptor (20). Our study extends these observations and suggests that because of its sensitivity to oxidation, p53 may be incapable of activating a response to oxidative stress in vivo. Oxidants generated through normal aerobic metabolism and by inflammatory reactions appear to be a major cause of damage to DNA and other cellular components. Exposure to oxidative stress can cause neoplastic transformation as well as a loss of proliferative capacity that resembles cellular senescence, and it was suggested that the accumulation of oxidative damage with age might lead to senescence through a p53-mediated activation of p21 (57). Recent studies indicate, however, that p53 levels are elevated only modestly in senescent cells (58). Our results with those of others suggest that p21 induction in response to oxidative stress is mediated through another mechanism, possibly involving activation of the MAP kinase cascade.

Interestingly, low concentrations of DEM also induced a G,, arrest in T98G cells. Arrest at this junction in the cell cycle does not appear to require p21 (36, 59) and consistent with these earlier studies we found no appreciable induction of p21 with low doses of DEM (Fig. 6). G2 arrest following DNA damage has been linked to a failure to remove inhibitory phosphorylations from the ATP-binding domain of the Cdc2 kinase (59, 60), suggesting that DEM could block the G2/M transition by a mechanism independent of p21 induction. Eludication of the G2 arrest mechanism evoked by DEM could provide a useful insight into additional mechanisms governing cell cycle progression during periods of oxidative stress.

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J. Biol. Chem. 1995, 270:29386-29391. doi: 10.1074/jbc.270.49.29386

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