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Membrane-derived second messenger regulates x-ray-mediated tumor necrosis factor α gene induction

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ABSTRACT **Cells adapt to adverse environmental conditions through a wide range of responses that are conserved throughout evolution. Physical agents such as ionizing radiation are known to initiate a stress response that is triggered by the recognition of DNA damage. We have identified a signaling pathway involving the activation of phospholipase A_2 and protein kinase C in human cells that confers x-ray induction of the tumor necrosis factor α gene. Treatment of human cells with ionizing radiation or H_2O_2 was associated with the production of arachidonic acid. Inhibition of phospholipase A_2 abolished radiation-mediated arachidonate production as well as the subsequent activation of protein kinase C and tumor necrosis factor α gene expression. These findings demonstrate that ionizing radiation-mediated gene expression in human cells is regulated in part by extranuclear signal transduction. One practical application of phospholipase A_2 inhibitors is to ameliorate the adverse effects of radiotherapy associated with tumor necrosis factor α production.**

Signaling pathways activated by DNA damage contribute to survival of prokaryotes and eukaryotic cells following exposure to x-rays or UV light. In irradiated *Escherichia coli*, damaged DNA forms a complex with the Rec A protease, resulting in the transcriptional induction of various genes including those encoding DNA repair enzymes (1). In yeast, UV light and x-rays result in the induction of genes that participate in the repair of damaged DNA (2, 3). Genes whose products are proposed to recognize damaged or unreplicated DNA and to participate in intracellular signaling that regulates cell cycle progression and DNA repair have been identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (4, 5). The complexity of this signaling pathway is demonstrated by the number of genes involved in sensing DNA damage and transmitting the signal (5). DNA damage is presumed to be the initiating event in mammalian cell induction of stress response genes following x-ray or UV exposure (6, 7). However, the mechanisms of DNA damage recognition have not been identified in mammalian cells.

Signal transduction pathways activated by ionizing radiation include increased phosphotransferase activity of cytoplasmic protein kinases (8–10). Moreover, inhibition of protein kinases blocks radiation-mediated gene induction and effects diverse biological endpoints such as apoptosis (10), radiation survival (11), and induction of the cytokine tumor necrosis factor α (TNF-α) (9). The calcium/phospholipid-dependent protein kinase (protein kinase C; PKC) is activated within 15 sec of ionizing radiation exposure and is extinguished by 90 sec in human leukemia HL-60 cells (9). Second messengers that participate in PKC activation following exposure to external stimuli include free fatty acids such as arachidonic acid (12). Previous studies have suggested that phospholipase A_2-mediated hydrolysis of oxidized membrane phospholipids is a primary means of bioreduction following oxidative injury (13, 14) (reviewed in ref. 15). For example, arachidonic acid release is increased following treatment with H_2O_2 due to hydrolysis of oxidized membrane phospholipids (16, 17). To determine whether arachadonic acid production was associated with radiation-mediated signal transduction, we quantified arachidonic acid production in irradiated HL-60 cells and found an increase in arachidonate within 30 min following irradiation.

Since phospholipase A_2 hydrolyzes phosphatidylcholine to arachidonic acid, we studied the effects of the phospholipase A_2 inhibitors mepacrine (18) and bromphenylbromide (BPB) (19). In addition, we studied the effects of dexamethasone and pentoxifylline on radiation-induced fatty acid hydrolysis, as these agents have been shown to inhibit phospholipase A_2, reduce the production of cellular mediators of inflammation and tissue injury, and inhibit lipopolysaccharide-induced TNF-α production in monocytes (20, 21). Moreover, glucocorticoids and pentoxifylline are employed clinically to prevent some acute toxicities of radiotherapy (22, 23). We found that each agent attenuated arachidonic acid release into the medium of cells treated with x-rays or H_2O_2. Thus, extranuclear second messengers are in part responsible for radiation-mediated signal transduction, and inhibition of this pathway may provide a means of attenuating the inflammatory-like response observed in irradiated tissues through the inhibition of TNF-α gene induction.

METHODS

Arachidonic Acid Assay. HL-60 cells in logarithmic growth were incubated for 16 hr in RPMI 1640 medium supplemented with 1.0 mg of fatty acid free bovine serum albumin per ml and 0.2 μCi of [^{3}H]arachidonic acid per ml (1 Ci = 37 GBq) as described (24, 25). Mepacrine (20 μM), BPB (10 μM), dexamethasone (1 μM), or pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine, Hoffmann–La Roche) (1 mM) was added to labeled cells 1 hr prior to irradiation with 10 Gy at 1 Gy/min. At the indicated times, an aliquot (1 ml) of supernatant was assayed by the addition of Hydrofluor and scintillation spectroscopy. Experiments were performed three or four times and results are presented as the mean ± standard error.

Diacylglycerol (DAG) Kinase Assay. DAG release from irradiated cells was quantified as described (26). Serum-deprived HL-60 cells were pelleted and irradiated (10 Gy at 2.5 Gy/sec) and 100% methanol was added immediately on ice. Extracts were dried under N_2 and resuspended into cardiolipin, *n*-octylglucoside, and bacterial DAG kinase in

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Abbreviations: TNF-α, tumor necrosis factor α; PKC, protein kinase C; BPB, bromphenylbromide; DAG, diacylglycerol.

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the presence of [^{32}P]ATP. Cellular DAG was quantified by using a known concentration of synthetic DAG that reacted with DAG kinase. Bradykinin was used as a positive control and resulted in a 3.0-fold (±0.4) increase in DAG, while x-irradiation produced DAG levels 1.2 ± 0.4 as compared to untreated controls.

Assay of PKC Activity. AT cells were grown to confluence and serum deprived for 24 hr. Medium was aspirated, cells were washed with phosphate-buffered saline (PBS), and then γ-irradiated with 10.8 Gy using a ^{60}Co source (Gammacell 220) at a dose rate of 2.7 Gy/sec. Protein was extracted on ice at 15-sec intervals following irradiation by the addition of 0.4 ml of lysis buffer [20 μM Tris·HCl, pH 7.5/0.5 mM EDTA/0.5 mM EGTA/10 mM 2-mercaptoethanol (TEM) with 0.5% Triton X-100, and leupeptin and aprotinin, each at 25 μg/ml]. Cells were homogenized and protein was partially purified as we have described (9). Protein extract (25 μl) was added to 25 μl of TEM, 5 μl of phospholipid (2.8 mg of phosphatidylserine per ml and 10 mM phorbol ester in Triton X-100 mixed micelles, GIBCO) (27, 28), and 10 μl of [^{32}P]ATP/substrate containing 5 × 10^7 cpm of [^{32}P]ATP per ml (New England Nuclear), 100 μM ATP, 250 μM synthetic peptide Gln-Lys-Arg-Pro-Ser(8)-Gln-Arg-Ser-Lys-Tyr-Leu, 5 mM $CaCl_2$, and 100 mM $MgCl_2$ (GIBCO) (28) in 20 mM Tris·HCl (pH 7.5). Following incubation for 5 min at 30°C, samples were dried on phosphocellulose and washed in 1% H_3PO_4, twice for 5 min, followed by washing in H_2O, twice for 5 min. Scintillation spectroscopy of each sample and 10 μl of unwashed [^{32}P]ATP/substrate was performed. To calculate the rate of ^{32}P incorporation into the peptide substrate, 100 μM of synthetic PKC-specific inhibitor peptide (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn) (GIBCO) (29) in 20 mM Tris (pH 7.5) was added to PKC assays prior to addition of [^{32}P]ATP/substrate and samples were incubated, washed, and counted as described above. Background ^{32}P incorporation was subtracted from that of assays without inhibitor and the rate of ^{32}P incorporated into the peptide substrate was calculated (pmol/min) as described (9, 28). Phosphorylation rates were normalized to 10^6 cells per assay.

To determine the effects of phospholipase A_2 inhibitors on radiation-mediated PKC activation, we added mepacrine, BPB, dexamethasone, or pentoxifylline to HL-60 cell cultures 1 hr prior to γ-irradiation with 10.8 Gy using a ^{60}Co source (Gammacell 220) at a dose rate of 2.7 Gy/sec. Cells were placed on ice and lysis buffer was added at 60 sec following irradiation as described (9). Phosphotransferase activity was assayed as described above. Phosphorylation rates were normalized to 10^6 cells per assay. Experiments were performed three times and results are presented as the mean ± standard error.

RNA Analysis. Cells were grown to a density of 10^6 per ml and exposed to 10 Gy (GE Maxitron x-ray generator) as described (30). RNA was extracted using the single step guanidinium thiocyanate/phenol/chloroform method (31) at 1 hr following irradiation. Control RNA from nonirradiated cells treated with otherwise identical conditions and RNA from irradiated cells were size fractionated by 1% agarose/formaldehyde electrophoresis. Ethidium bromide staining of the RNA demonstrated equal loading of each lane. RNA gels were then transferred to a nylon membrane (GeneScreen-*Plus*, New England Nuclear). Northern blots were hybridized to the ^{32}P-labeled TNF-α cDNA probe (24) followed by autoradiography for 3 days at −85°C with intensifying screens. Equal loading of lanes was demonstrated by 7S RNA hybridization. Mepacrine, dexamethasone, BPB, or pentoxifylline was added to HL-60 cell cultures 1 hr prior to irradiation with 10 Gy (1 Gy/min) using a GE Maxitron generator.

RESULTS

Effect of the Phospholipase A_2 Inhibitors on Radiation-Induced Fatty Acid Hydrolysis. We quantified arachidonic

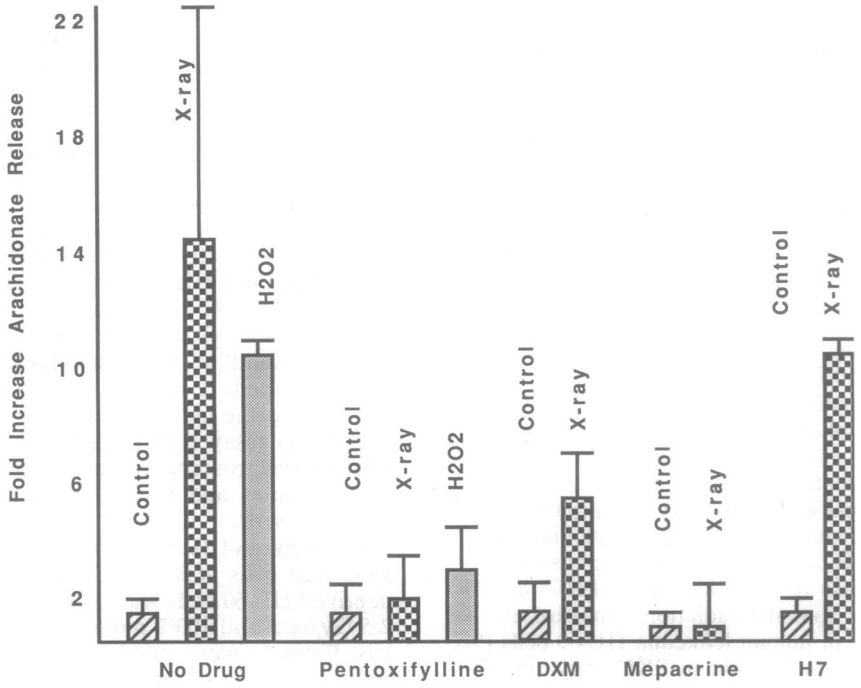

FIG. 1. Arachidonic acid release following treatment of HL-60 cells with x-rays and H_2O_2. HL-60 cells in logarithmic growth were incubated for 16 hr in RPMI 640 medium supplemented with 1.0 mg of fatty acid-free bovine serum albumin per ml and 0.2 μCi of [^{3}H]arachidonic acid per ml as described (24, 25). Mepacrine (20 μM), BPB (10 μM), dexamethasone (DXM, 1 μM), or pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine, Hoffmann–La Roche) (1 mM) was added to labeled cells 1 hr prior to irradiation with 10 Gy at 1 Gy/min. At the indicated times, an aliquot (1 ml) of supernatant was assayed by the addition of Hydrofluor and scintillation spectroscopy. Experiments were performed three or four times and results are presented as the mean ± standard error.

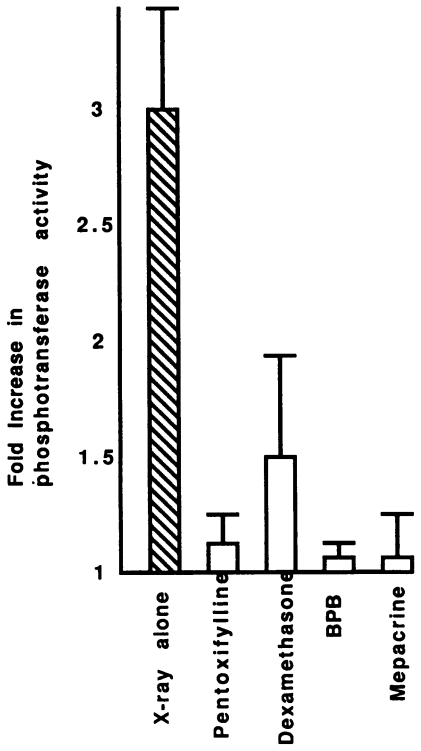

FIG. 2. X-ray-induced PKC activation. Mepacrine (20 μM), BPB, dexamethasone, or pentoxifylline was added to HL-60 cell cultures 1 hr prior to γ-irradiation with 10.8 Gy using a ^{60}Co source (Gammacell 220) at a dose rate of 2.7 Gy/sec. Cells were placed on ice and lysis buffer was added at 60 sec following irradiation as described (9). Phosphotransferase activity was assayed as described (9, 28) using [^{32}P]ATP/substrate containing 5×10^7 cpm of [^{32}P]ATP per ml (New England Nuclear) and synthetic peptide Gln-Lys-Arg-Pro-Ser(8)-Gln-Arg-Ser-Lys-Tyr-Leu. Phosphorylation rates were normalized to 10^6 cells per assay. Experiments were performed three times and results are presented as the mean ± standard error.

acid production in irradiated HL-60 cells, which have served as a model for the study of radiation-mediated TNF-α gene induction and PKC-dependent signal transduction (9). HL-60 cells were incubated with [^{3}H]arachidonic acid for 3 hr, washed, and irradiated with 10 Gy at 1 Gy/min. Fatty acid release into the medium was significantly increased following irradiation (Fig. 1). Previous work has also shown that arachidonic acid release is increased following treatment with H_2O_2 (16, 17), which served as a positive control. To confirm that arachidonate was produced, we performed gas chromatographic analysis of lipids extracted from irradiated HL-60 cells. Using this approach, an increase in arachidonate was detectable at 30 min following irradiation. Conversely, DAG levels did not change following irradiation as determined by the DAG kinase assay.

We studied the effects of the phospholipase A_2 inhibitors mepacrine, BPB, dexamethasone, and pentoxifylline on radiation-induced fatty acid hydrolysis. Each attenuated arachidonic acid release into the medium of cells treated with x-rays or H_2O_2 (Fig. 1). Since PKC has been shown to activate phospholipase-mediated hydrolysis of membrane phospholipids (32, 33), we added the PKC inhibitor H7 to determine whether PKC activation contributes to lipid hydrolysis following irradiation. H7 pretreatment had no detectable effect on arachidonic acid release following irradiation of HL-60 cells (Fig. 1). This is consistent with the finding that PKC inhibition produced no reduction in arachidonic acid release following H_2O_2 treatment (33).

Effect of the Phospholipase A_2 Inhibitors on Radiation-Induced PKC Activation. PKC phosphotransferase activity is

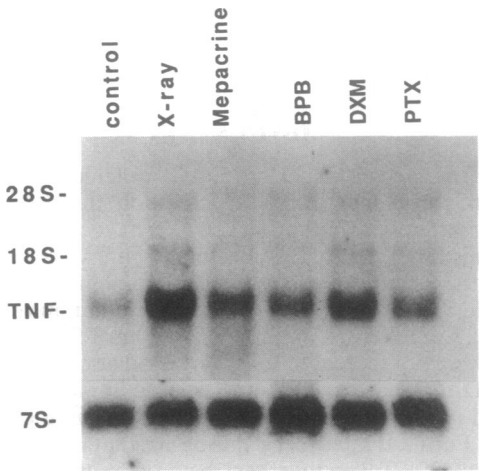

FIG. 3. X-ray-induced TNF-α gene expression. Mepacrine (20 μM), dexamethasone (DXM), BPB, or pentoxifylline (PTX) was added to HL-60 cell cultures 1 hr prior to irradiation with 10 Gy (1 Gy/min) using a GE Maxitron generator. RNA was extracted at 1 hr after irradiation as described (30). Control RNA from nonirradiated cells treated under otherwise identical conditions and RNA from irradiated cells were size fractionated by 1% agarose/formaldehyde electrophoresis and hybridized to a ^{32}P-labeled TNF-α cDNA probe (24). Equal loading of lanes was demonstrated by 7S RNA hybridization.

increased following the addition of arachidonate (34–36). Taken together with the findings that PKC is activated rapidly and transiently following ionizing radiation exposure and that PKC activity is required for radiation-induced TNF-α gene induction in HL-60 cells (9), these data suggested that arachidonate activation of PKC might be the signaling pathway that confers TNF-α induction. To determine whether radiation-induced arachidonate production is associated with PKC activation, we quantified the phosphotransferase activity of PKC in irradiated HL-60 cells pretreated with mepacrine, BPB, pentoxifylline, or dexamethasone. Protein was extracted at 60 sec following irradiation, and phosphotransferase activity was quantified *in vitro*. The PKC-specific peptide substrate from myelin basic protein (28) and the PKC inhibitor peptide from the PKC regulatory domain (29) were employed to quantify PKC activity following irradiation. A 3-fold increase in phosphotransferase activity was found at 45 sec following irradiation as compared to untreated control cells. Mepacrine, BPB, pentoxifylline, and dexamethasone, added 1 hr prior to irradiation, reduced the x-ray-induced increase in PKC phosphotransferase activity (Fig. 2). These data are in concordance with a previously undescribed x-ray-induced signaling pathway following x-ray exposure, whereby oxidized membrane phospholipids are hydrolyzed to arachidonate, which in turn activates PKC.

Effect of the Phospholipase A_2 Inhibitors on Radiation-Induced TNF-α Gene Expression. Although transcription of certain radiation-inducible genes occurs through both PKC-dependent and independent signaling pathways (37), TNF-α induction is dependent upon PKC activation (9) and thus represents a radiation-mediated gene that can be studied to determine the significance of phospholipase inhibition on radiation-mediated gene induction. Mepacrine, BPB, pentoxifylline, or dexamethasone was added to HL-60 cells 1 hr prior to x-irradiation. RNA was isolated 1 hr following irradiation at the time of peak TNF-α expression (38). The finding that each of these agents blocked radiation-induced TNF-α gene expression (Fig. 3) indicated that radiation-induced TNF-α expression is dependent on signaling through phospholipase A_2. Moreover, attenuation of radiation-mediated gene induction by these phospholipase A_2 inhibi-

tors suggests that signal transduction activated by ionizing radiation is in part initiated through hydrolysis of oxidized membrane lipids.

DISCUSSION

Second messengers such as DAG, arachidonic acid, and calcium participate in PKC activation in response to a number of external stimuli (12). We have investigated the mechanism of PKC activation following irradiation by analyzing the second messengers DAG, arachidonic acid, and calcium, which participate in PKC activation in response to a number of external stimuli (12). We have found that DAG levels were not increased following irradiation as determined by the DAG kinase assay. Furthermore, intracellular calcium flux did not occur as determined by quantifying UV absorption in Fura-2-treated cells during irradiation with ^{90}Sr (60 cGy/sec) (39). Taken together, these data support the finding that phosphoinositol-specific phospholipase C is not activated during irradiation since the coincident increase in inositol triphosphate would mobilize intracellular Ca^{2+}. The acute effects of ionizing radiation on the lung have been shown to be associated with endothelial leakage (40). Corticosteroids prevent the acute effect when given to animals at the tie of irradiation, but this treatment did not affect lung fibrosis (41), indicating that steroids prevent the inflammatory component of radiation injury but not the fibrotic component. In the present study, arachidonic acid release was reduced when irradiated cells were pretreated with pentoxifylline, dexamethasone, or BPB. These findings are significant in that the reduction in radiation-mediated phospholipase A_2 activity in turn diminished PKC activation and TNF-α induction.

Lipid oxidation occurs in the cell membrane of irradiated cells (42). Indeed, the probable mechanism of arachidonic acid release following irradiation is phospholipase A_2-mediated hydrolysis of oxidized membrane lipids. In support of this hypothesis, whole body irradiation of animal models results in increased arachidonic acid metabolites (43, 44) (reviewed in ref. 45). Oxidative injury following H_2O_2 treatment results in phospholipase A_2-mediated arachidonic acid release in epithelial and endothelial cells (13, 14, 16). Phosphatidylcholine hydrolysis to arachidonic acid is reduced by pentoxifylline in platelets stimulated with thrombin (46). In concert with these findings and the demonstration that arachidonic acid activates PKC *in vitro* (34–36) and *in vivo* (33, 47–49) we have found that inhibition of phospholipase A_2 attenuates radiation-induced PKC activation. These results demonstrate that fatty acid hydrolysis is an early step in a signaling pathway activated by ionizing radiation that may be independent of DNA damage. On the basis of these results, we speculate that evolution of phospholipase A_2-dependent signaling pathways has provided a mechanism for higher eukaryotes to respond to reactive oxygen intermediates with cytokine production. In support of this consideration, Neta *et al.* (50) have shown that TNF-α protects hematopoietic cells from killing by ionizing radiation.

A practical application of these findings relates to reduction of radiation sequelae during the treatment of cancer. Although the effects of ionizing radiation on proliferating cell renewal systems are theorized to be due to the direct killing effects of radiation on stem cells within the injured organ, other work has suggested that TNF-α induction plays a role in the acute effects of radiation therapy (reviewed in ref. 51). For example, elevated TNF-α serum levels in patients receiving total body irradiation prior to bone marrow transplantation are associated with a greater incidence of complications such as mucositis, pneumonitis, hepatitis, and nephritis than in patients with relatively lower TNF-α serum levels (52). Because TNF-α induction is associated with acute and subacute complications of therapeutic radiation, inhibition of phospholipase A_2 represents a means of abating these sequelae. Indeed, pharmacologic agents used to ameliorate the acute and subacute sequelae of radiotherapy include glucocorticoids and pentoxifylline (22, 23, 53). For example, acute effects of radiation, such as pneumonitis and the central nervous system syndrome, have been abated by these drugs (23). The identification of a signal transduction pathway responsible for radiation-mediated arachidonic acid production, PKC activation, and TNF-α induction may allow for rational design of radioprotective drugs that do not adversely affect tumor cure rates and avoid the serious side effects of glucocorticoids. Such strategies of radioprotection offer new avenues to enhance the therapeutic ratio in clinical oncology.

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