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Protein kinase C mediates x-ray inducibility of nuclear signal transducers EGR1 and JUN

(protein kinase inhibitors/immediate early genes/radiation injury)

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ABSTRACT **The cellular response to ionizing radiation includes growth arrest and DNA repair followed by proliferation. Induction of immediate early response genes may participate in signal transduction preceding these phenotypic responses. We analyzed mRNA expression for different classes of immediate early genes (*JUN*, *EGR1*, and *FOS*) after cellular x-irradiation. Increased expression of the *EGR1* and *JUN* genes was observed within 0.5–3 hr following x-ray exposure. Preincubation with cycloheximide was associated with superinduction of *JUN* and *EGR1* in x-irradiated cells. Inhibition of protein kinase C activity by prolonged stimulation with phorbol 12-myristate 13-acetate or the protein kinase inhibitor H7 prior to irradiation attenuated the increase in *EGR1* and *JUN* transcripts. *FOS* expression was not coregulated with that of *EGR1* following x-irradiation, suggesting a distinct regulatory pathway of this gene as compared with its regulation following serum and phorbol ester. These data implicate the EGR1 and JUN proteins as signal transducers during the cellular response to radiation injury and suggest that this effect is mediated in part by a protein kinase C-dependent pathway.**

Exposure to ionizing radiation results in pleiotropic biological responses such as cell cycle-specific growth arrest and repair of damaged DNA. Proliferation subsequent to irradiation results in repopulation of injured tissues and tumors (1, 2). In addition, certain cells produce growth factors or cytokines in response to x-rays (3, 4). To effect these delayed responses to injury, x-rays may induce nuclear signal-transducer genes whose products couple early biochemical second-messenger signals to long-term phenotypic changes. Several of these genes encode transcription factors that initiate a cascade of molecular events. Immediate early response genes that encode transcription factors include the *JUN* family (*JUN*, *JUNB*, and *JUND*; refs. 5–9), the *EGR* family (*EGR1*, *-2*, *-3*, and *-4*; refs. 10–12), and the *FOS* family (*FOS*, *FRA1*, and *FOSB*) (13–16). JUN homodimers and JUN/FOS heterodimers regulate transcription by binding to AP-1 sites in certain promoter regions (17). The recent demonstration that x-rays induce the *JUN* and *FOS* genes in human myeloid leukemia cells suggests that nuclear signal transducers participate in the cellular response to ionizing radiation (18).

EGR1 (also known as *zif/268*, NGFI-A, *Krox-24*, TIS-8; refs. 19–23) encodes a nuclear phosphoprotein with a Cys_2–His_2 zinc-finger motif that is partially homologous to the corresponding domain in the Wilms tumor-susceptibility gene (24). The EGR1 protein binds with high affinity to the DNA sequence CGCCCCCGC in a zinc-dependent manner (25, 26). The rapid and transient induction of *EGR1* expression in the transition of quiescent cells from the G_0 to G_1 phases of the cell cycle is regulated by efficient cis- and trans-regulatory mechanisms (23, 27). Both serum and phorbol ester inducibility of *EGR1* are mediated through multiple CArG-box [CC(A+T-rich)$_6$GG] domains in the 5′ promoter region of *EGR1* (19, 28). Moreover, FOS binding down-regulates *EGR1* via these CArG-box domains (29). In addition, ischemic injury to the kidney results in *EGR1* induction (30). Thus, *EGR1* is an immediate early gene that is induced during tissue injury and participates in signal transduction during cellular proliferation and differentiation.

The biochemical processes that precede immediate early gene induction after mitogenic stimulation include signal transduction via kinase activation (31, 32). Protein kinase C (PKC)-dependent and -independent pathways participate in *EGR1* gene induction by serum and growth factors (33). Prolonged exposure to phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) results in down-regulation of PKC activity (31, 32). Moreover, inhibition of PKC by the isoquinoline sulfonamide H7 results in reduced enzyme activity, whereas HA1004, a selective inhibitor of cyclic nucleotide protein kinases and calmodulin, has little effect on PKC activity (34, 35). These inhibitors can thus be used to determine whether kinase activation is required for immediate early gene induction.

Here we demonstrate that *EGR1* and *JUN*, but not *FOS*, are rapidly and transiently expressed in the absence of *de novo* protein synthesis after exposure to ionizing radiation. These data implicate *EGR1* and *JUN* in signal transduction following x-irradiation. In addition, we report that down-regulation of PKC by TPA and H7 is associated with attenuation of *EGR1* and *JUN* gene induction by ionizing radiation. These data implicate activation of PKC and subsequent induction of *EGR1* and *JUN* as signaling events that initiate the mammalian cell phenotypic response to ionizing radiation injury.

METHODS

Cell Cultures. Methods of establishment of human epithelial cell lines have been described (36). Culture medium for epithelial tumor cells was 95% Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 [DMEM/F-12 (3:1)] with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Culture medium for normal fibroblasts (AG1522) and cell line 293 was DMEM/F-12 (3:1) with 10% fetal bovine serum, penicillin, and streptomycin. Cycloheximide (CHI, 5 μg/ml) or actinomycin D (5 μg/ml) was added to cell lines SQ-20B and 293 30 min prior to irradiation.

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Abbreviations: CHI, cycloheximide; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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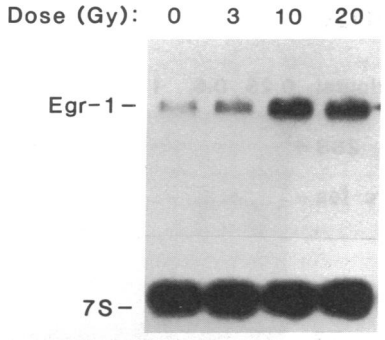

FIG. 1. SQ-20B cells were exposed to the indicated doses of x-rays and RNA was isolated after 1 hr. Hybridization of the probe for 7S RNA demonstrates equivalent loading of lanes. *EGR1* expression increased with the x-ray dose.

RNA Analysis. Cells were maintained at confluency for 4 days followed by serum deprivation (0.1% fetal bovine serum) for 24 hr. Cells were then x-irradiated (GE Maxitron x-ray generator) with 3, 10, or 20 Gy. RNA was extracted using the single-step guanidinium thiocyanate/phenol/chloroform method (37) at intervals ranging from 15 min to 6 hr. RNA was size-fractionated by 1% agarose/formaldehyde electrophoresis and transferred to a nylon membrane (GeneScreen*Plus*, New England Nuclear). These Northern blots were then hybridized to ^{32}P-labeled DNA probes containing *JUN* (38), *FOS* (39), or *EGR1* (10, 11) cDNA inserts followed by autoradiography for 3–14 days at −85°C with intensifying screens. Northern blots were hybridized to a probe specific for 7S RNA to normalize for variations in RNA loading.

Kinase Inhibitors. Cell line SQ-20B was pretreated with 1 μM TPA for 40 hr to down-regulate PKC and then stimulated with TPA, serum, or x-rays (20 Gy). Controls included x-irradiation without TPA pretreatment, TPA (50 nM) without TPA pretreatment, and untreated cells. RNA was isolated after 1 hr and hybridized to *EGR1*. SQ-20B cells were preincubated with 100 μM H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine] or 100 μM HA1004 [*N*-(2-methylaminoethyl)-5-isoquinolinesulfonamide] (Seikagaku America, St. Petersburg, FL) for 30 min or with 1 μM TPA for 40 hr and then exposed to 20 Gy of x-irradiation. RNA was extracted 1 hr after irradiation. Positive control cells treated under the same conditions but in the absence of inhibitor also received 20 Gy, while negative control cells received neither H7 nor x-rays. RNA was extracted 1 hr after 20 Gy without inhibitor. Northern blots were hybridized to *EGR1* or 7S probes as described above. When 293 cells pretreated with the above inhibitors were irradiated, RNA was extracted after 3 hr and the Northern blot was hybridized to *JUN* and 7S probes.

RESULTS

***EGR1* and *JUN* Expression After X-Irradiation.** We investigated expression of mRNA encoding the nuclear signal transducers EGR1, JUN, and FOS during the cellular response to ionizing radiation in the human epithelial tumor cell line (SQ-20B) and two normal tissue human cell lines derived from fibroblasts (AG1522) and kidney epithelium (293). Control RNA from unirradiated cells demonstrated low but detectable levels of *EGR1* and *JUN* transcripts. In contrast, *EGR1* expression increased in a dose-dependent manner in irradiated cells (Fig. 1). Levels were low but detectable after 3 Gy and increased in a dose-dependent manner following 10 and 20 Gy. A dose of 20 Gy was used in experiments examining the time course of gene expression, so that transcripts were easily detectable. Cells remained viable as determined by trypan dye exclusion during this time course (data not shown). A time-dependent increase in *EGR1* and *JUN* mRNAs was observed (Fig. 2). SQ-20B cells demonstrated coordinate increases in *EGR1* and *JUN* expression by 30 min after irradiation that declined to baseline within 3 hr. In contrast, in AG1522 cells the *EGR1* transcript level was increased over basal at 3 hr whereas *JUN* mRNA was increased at 1 hr and returned to basal by 3 hr. In the 293 cell line, the level of *JUN* mRNA was increased at 6 hr whereas that of *EGR1* mRNA was increased at 3 hr and returned to basal by 6 hr (Fig. 2).

Effects of Protein Synthesis Inhibition on *EGR1* and *JUN* Induction. To determine whether *EGR1* and *JUN* participate as immediate early genes after x-irradiation, the effects of protein synthesis inhibition by CHI were studied in cell lines

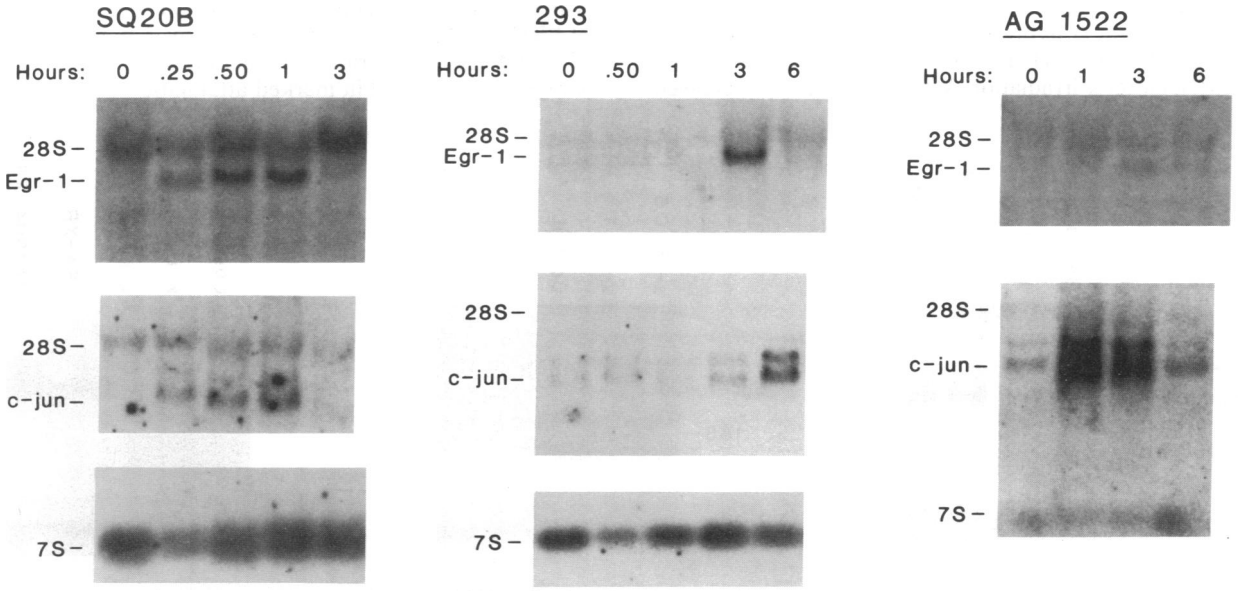

FIG. 2. Confluent, serum-deprived cells were x-irradiated with 20 Gy and total cellular RNA was extracted at the indicated times. RNA (10 μg per lane) was size-fractionated and Northern blots were hybridized to *EGR1* or *JUN* probes. The 7S probe was hybridized to demonstrate relative loading of lanes. Expression of *EGR1* and *JUN* increased after 15 min and peaked at 1 hr in cell line SQ-20B. In cell line 293, *EGR1* expression peaked at 3 hr whereas the *JUN* mRNA level increased at 6 hr. Cell line AG1522 demonstrated an increase in *EGR1* mRNA at 3 hr whereas *JUN* was expressed at 1 hr.

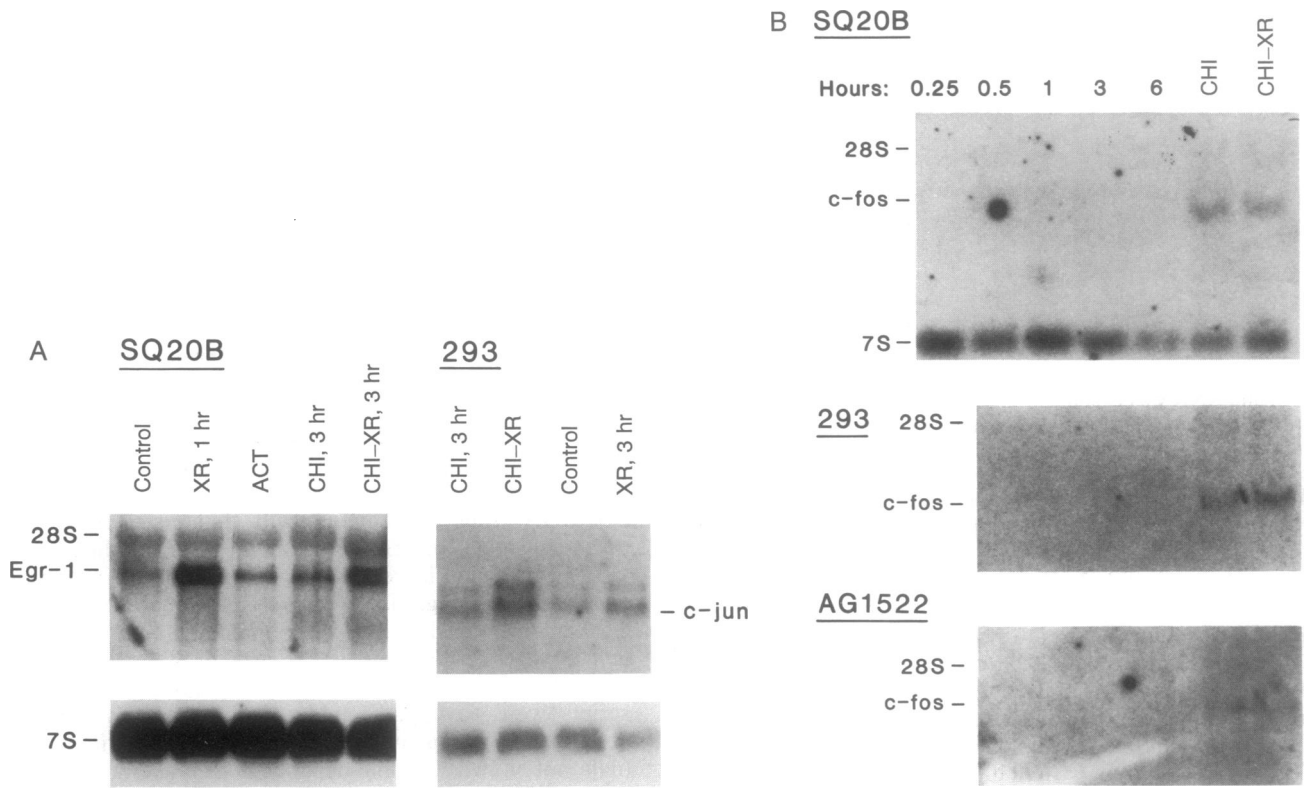

FIG. 3. Confluent cells were exposed to 20 Gy of x-rays (XR) and RNA was extracted 1 hr (SQ-20B cells) or 6 hr (293 cells) later unless stated otherwise. (*A*) Preincubation of cells with CHI (5 μg/ml) resulted in persistent elevation of *EGR1* mRNA at 3 hr and of *JUN* mRNA 6 hr after x-irradiation, whereas actinomycin D (ACT, 5 μg/ml) inhibited *EGR1* induction. Hybridization to the 7S probe demonstrates equal loading of lanes. (*B*) *FOS* cDNA was hybridized to Northern blots containing RNA extracted at the indicated times and from CHI-pretreated cells.

293 and SQ-20B after x-ray exposure. CHI treatment alone resulted in a slight but detectable increase in *EGR1* and *JUN* transcripts normalized to 7S RNA (Fig. 3*A*). In the absence of CHI, *EGR1* and *JUN* expression returned to baseline. In contrast, SQ-20B cells pretreated with CHI demonstrated persistent elevation of the *EGR1* level at 3 hr and 293 cells demonstrated persistent elevation of the *JUN* level at 6 hr after irradiation, thus indicating superinduction of these transcripts. We next hybridized the same Northern blots to a *FOS* cDNA probe. There was no increase in the level of *FOS* mRNA after x-irradiation. While *FOS* mRNA increased with CHI alone, there was no further induction of *FOS* expression when CHI-pretreated cells were irradiated (Fig. 3*B*).

Effects of Protein Kinase Inhibition on *EGR1* and *JUN* Induction. Protein kinase inhibition was used to determine whether PKC is required for the induction of nuclear signal transducers following x-ray exposure. Prolonged stimulation with micromolar concentrations of TPA causes the depletion of PKC (31, 32). Hence, SQ-20B cells were incubated with 1 μM TPA for 40 hr prior to induction with serum, TPA, or x-rays. This resulted in marked attenuation of the increases

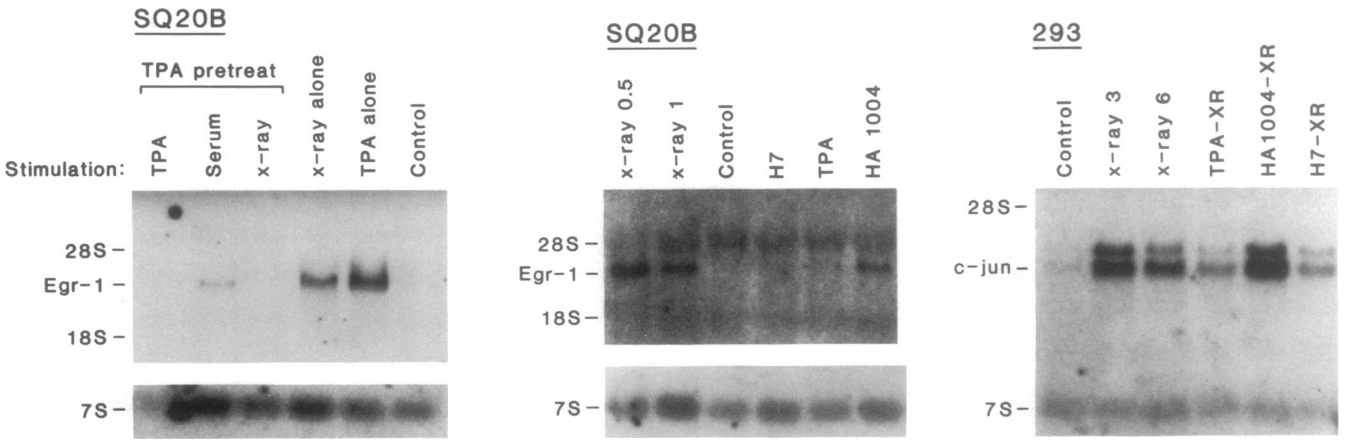

FIG. 4. Cells were pretreated with 1 μM TPA for 40 hr or with 100 μM H7 or HA1004 for 30 min before x-ray induction (20 Gy). (*Left*) TPA-pretreated SQ-20B cells were stimulated with TPA (50 nM), serum (20%), or x-rays (20 Gy) and RNA was extracted 1 hr later. TPA pretreatment attenuated x-ray induction of *EGR1*. (*Center*) Cells pretreated with H7, TPA, or HA1004 were irradiated and RNA was extracted after 1 hr. H7 and TPA attenuated *EGR1* induction as compared with RNA from x-irradiated cells extracted at 0.5 and 1 hr. (*Right*) TPA-, HA1004-, or H7-pretreated 293 cells were irradiated (XR) and RNA was extracted after 3 hr. RNA was extracted from x-ray controls at 3 and 6 hr.

in *EGR1* and *JUN* transcripts (Fig. 4). H7 is a nonspecific inhibitor of protein kinases, including PKC, whereas HA1004 is a selective inhibitor of cyclic nucleotide-dependent protein kinases. Increasing concentrations of H7 resulted in a dose-dependent reduction in *EGR1* gene expression in SQ-20B cells, with maximal inhibition at 100 μM. H7 (100 μM) added prior to x-irradiation also resulted in marked attenuation of *EGR1* and *JUN* expression in cell lines SQ-20B and 293 (Fig. 4). A selective inhibitor of cyclic nucleotide-dependent protein kinases, HA1004, had no detectable effect on EGR1 or JUN expression after x-irradiation (Fig. 4).

DISCUSSION

We demonstrate that mRNA levels of transcription factors *EGR1* and *JUN* increase in a time- and dose-dependent manner following exposure of cells to ionizing radiation. The potential importance of the induction of *EGR1* and *JUN* by ionizing radiation is illustrated by the recent finding that x-ray induction of the platelet-derived growth factor (PDGF) α chain stimulates proliferation of vascular endothelial cells (4), whereas x-ray induction of tumor necrosis factor (TNF) has been postulated to produce autocrine and paracrine tumor cell killing (3). PDGF and TNF may contribute to the physiological effects of x-rays on normal tissues and tumors. PDGF has AP-1- and EGR1-binding domains whereas TNF has elements similar to AP-1 and EGR1 target sequences (40, 41). We speculate that x-ray induction of PDGF and TNF may be regulated by EGR1 and JUN.

When *EGR1* is induced by serum or TPA, *FOS* is induced and has been shown to down-regulate *EGR1* (29). Therefore, x-ray regulation of *EGR1* may be distinct from serum and TPA induction in the sense that *FOS* is not expressed after x-irradiation. Down-regulation of *EGR1* may occur through a different mechanism than serum or TPA. *FOS* transcripts are undetectable following x-irradiation in several human sarcoma, epithelial tumor, and normal tissue cell lines (D.E.H., unpublished observation). JUN homodimers recognize the AP-1-binding domain with less affinity than the FOS/JUN heterodimer (17). Radiation-induced JUN may also bind to a preexisting member of the FOS family before binding to the AP-1 site. We have recently found that *FOS* as well as *JUN* are induced by x-rays in HL-60 promyelocytic leukemia cells (18). In view of the above findings, this pattern of induction may be specific for HL-60 cells and implicates participation of nuclear signal transducers in the pleiotropic phenotypic response of various human cell types to radiation injury. We speculate that induction of transcription factor genes may be cell-type-specific.

Prolonged exposure to TPA causes down-regulation of PKC by a feedback inhibition pathway (31, 32). This depletion is specific for PKC. H7 is a nonspecific inhibitor of PKC (34). The isoquinoline sulfonamide HA1004 demonstrates more selective inhibition of cAMP-dependent protein kinase (protein kinase A), cGMP-dependent protein kinase, and calmodulin and relatively less PKC inhibition (35). When added to cells prior to x-irradiation, HA1004 did not attenuate gene expression. These findings suggested that *EGR1* and *JUN* are regulated through a PKC-dependent pathway following ionizing radiation exposure and that the protein kinase A and calmodulin pathways are not required for the induction of these genes. The finding that PKC is activated during the cellular response to radiation injury is supported by the observation that a 2-fold increase in transcriptional activity of the long terminal repeat of Moloney murine sarcoma virus induced by x-rays is attenuated by PKC inhibition (42). We have not ruled out the possibility that other protein kinase pathways are involved in signal transduction following irradiation.

Data presented here show that *EGR1* and *JUN*, but not *FOS*, are rapidly and transiently expressed in the absence of *de novo* protein synthesis after ionizing radiation exposure. Induction of growth arrest, DNA repair, and radical-scavenging proteins have been described following DNA damage in bacteria, yeast, and mammalian cells (43–46). These events are commonly the result of a molecular cascade following activation of transcriptional activators and repressors. Inductions of *EGR1* and *JUN* are implicated as signaling events that initiate the mammalian cell phenotypic response to ionizing radiation injury. Posttranslational events, such as kinase activation, precede activation of nuclear signal transducers that subsequently participate in transactivation of late genes resulting in phenotypic responses to x-irradiation. PKC may be one of a number of important regulatory enzymes in the response of cells to x-rays. Protein kinase inhibitors may represent a new class of pharmacologic agents to manipulate the tumor response to radiotherapy.

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