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Alterations in Hepatic Kinase Activity Following Whole Body γ-Irradiation of Mice

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ABSTRACT

The chronological activation of the signaling molecules following whole body γ-irradiation was investigated in mouse liver. The activity of two kinases, tyrosine kinase and protein kinase C (PKC), was found to respond differently to γ-irradiation. Tyrosine kinase was found to respond to much lower doses of irradiation (10 cGy), whereas PKC was found to be activated at comparatively higher doses (3 Gy). Tyrosine kinase showed a sharp activation at 30 min and then a decline to normal values at 1 h. Activation of PKC was apparent at as early as 15 min of irradiation and showed a maximal increase at 30 min. This was followed by a decline to normal values at 1 h. The response of the whole organ was found to be different from that of reported effects on a single cell. These results suggest that the data obtained from the single-cell studies would have limited application in the experiments involving the whole animal. Interruption of these signals at various steps is currently being used to manipulate tumor response to radiotherapy. In such cases, the difference in response of a single cell and a whole animal must be considered. Antioxid. Redox Signal. 3, 483-492.

INTRODUCTION

IONIZING RADIATION initiates a cascade of signaling events in the cell. These include the activation of tyrosine kinase (9, 20, 41), protein kinase (11, 12, 21, 28), nuclear factor-κB (NFκB) (3, 44, 45), and DNA-dependent protein kinase (4) and the expression of many immediate early genes (31). Some of these signaling molecules have also been implicated in the development of radioresistance (1, 7, 8, 30, 36, 42, 43), which is a major concern in radiotherapy. The earliest report of such a possible link was that by FitzGerald *et al.*, who found that transfection of NIH 3T3 cells with a human H-ras oncogene was able to increase the resistance level of the recipient cell line (10). Following this, many other signaling molecules have been added to the list, and these include protein kinase C (PKC), the activation of which inhibits the ceramide pathway, which in turn inhibits apoptosis and leads to radioresistance (8). NF-κB, one of the transcription factors that is activated by ionizing radiation in eukaryotes (3, 45), has recently been associated with the development of radioresistance (42). Many attempts have been made to discover the modulators of these signaling molecules with a view to inhibit the development of radioresistance. Inhibitors at all stages of signaling are being looked for. Wortmanin, an inhibitor of phosphatidylinositol 3-kinase DNA-dependent protein kinase by the virtue of the homology in their sequence (7), and alkyllysophospholipids (34) have been reported to sensitize mammalian cells to radiation. UCN-O1 hydroxystaurosporine, an analogue of staurosporine, has potent and selective inhibitory action against PKC (37, 38) and

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has been found to work as an indirect sensitizer (40). This agent is under phase 1 clinical trial at the National Cancer Research Institute, NIH, Bethesda, MD, U.S.A. (24). However, all the above studies except for a few are based on single cells. There is a paucity of data where signaling following whole body irradiation is concerned. It has been demonstrated that whole body irradiation leads to activation of various oncogenes like c-*fos*, c-*myc*, c-*src*, H-ras (2, 17), and recently NF-κB (46). To modulate these signaling molecules for clinical advantage, it is essential that the appearance of these signaling molecules following whole body γ-irradiation and the time course of their appearance are understood. Moreover, the response of the signaling molecule following whole body irradiation may also vary from organ to organ. This has been well documented in the case of NF-κB where following irradiation, activation of NF-κB had been observed in many tissues in culture (5, 13, 41) but variations *in vivo* have subsequently been reported (45). In the latter study, radiation was found to induce tissue-specific activation of NF-κB, which was contrary to some of the *in vitro* findings (3, 13, 31, 33, 45). Activation of NF-κB following irradiation *in vivo* was reported to occur only in the peripheral lymphoid tissues and was absent in other nonlymphoid tissues (46).

Another factor that is known to alter the appearance of the signaling molecules is the dose of radiation. Different doses have been reported to show a variable appearance of some immediate early genes in 244B human lymphoblastic cells (31). Here, it was demonstrated that the appearance of c-*fos*, c-*jun*, c-*myc*, and H-ras was maximum at 0.5 Gy, and c-*fos* showed maximum response at 0.25 Gy. The induction was maximum at 1 h and declined at 4 h after irradiation. The activation was demonstrated to involve the second messenger signaling pathways, and fos activation was attributed to the activation of protein tyrosine kinase. The present study was undertaken with a view to delineate the response of signaling molecules tyrosine kinase, PKC, and mitogen-activated protein (MAP) kinase to different doses of γ-irradiation and to study the time of maximal activation of these signaling molecules.

MATERIALS AND METHODS

Male Swiss inbred mice (3–4 weeks) were maintained on a stock laboratory diet with 12-h light/12-h dark cycle. These were subjected to whole body irradiation from a ^{60}Co Theratron Junior Teletherapy unit at a rate of 99.94 cGy/min. Groups of mice were exposed in a well ventilated Perspex box. The area of exposure was constant. One group of mice was irradiated at different doses, *i.e.*, 10 cGy, 1 Gy, 3 Gy, and 5 Gy, and killed at 30 min after irradiation. The other group of mice was irradiated at 3 Gy and killed at various time intervals ranging from 15 min to 24 h.

Fine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ-^{32}P]ATP (specific activity, 5,000 Ci/mmol) was obtained from Board of Radiation and Isotope Technology (Mumbai, India). All other chemicals, reagents, and materials were procured locally.

Tyrosine kinase activity was estimated in the membrane (6) and the nuclear fractions of mouse liver homogenate. The membrane fraction was isolated by homogenizing the liver in buffer containing 5 m*M* HEPES (pH 7.4), 250 m*M* sucrose, and 1 m*M* EGTA (32). The pellet was obtained by centrifuging the homogenate at 3,500 rpm for 20 min at 4°C, resuspending in the original volume of homogenizing buffer and 45% percoll sucrose mixture, and spinning at 12,000 rpm for 20 min at 4°C. For the nuclear fraction, liver was homogenized in buffer containing 50 m*M* Tris-HCl (pH 7.4), 0.32 *M* sucrose, 0.3% β-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 25 μg/ml leupeptin. The homogenate was filtered through cheese cloth, and crude nuclei were pelleted at 800 g for 10 min. Purified nuclei were obtained by density gradient centrifugation by layering over 2.2 *M* sucrose at 113,304 *g* for 70 min at 4°C. The tyrosine kinase activity was estimated in the plasma membrane (40 μg) in a reaction mixture containing 20 m*M* triethanolamine (pH 7.5), 50 m*M* NaF, 1 m*M* dithiothreitol (DTT), and 1 mg/ml Glu^{80} Tyr^{20} according to (6). The reaction was initiated by addition of [γ-^{32}P]ATP-Mg ($5–8 \times 10^6$ cpm/tube). After incubating at 37°C for 15 min, 25-μl aliquots were spotted on Whatman 3 MM

filter paper and washed with cold 10% trichloroacetic acid plus 8% pyrophosphate. The strips were dried and counted.

PKC activity was estimated in the particulate, cytosolic, and nuclear fractions of livers of mice according to the method described by Mistry *et al.* (27). The liver was excised and homogenized in a buffer containing 0.25 *M* sucrose, 50 m*M* Tris-HCl (pH 7.4), 50 μg/ml PMSF, 10 μg/ml leupeptin, 5 m*M* EDTA, and 0.3% (wt/vol) β-mercaptoethanol. Nuclei were isolated by centrifugation at 800 *g* for 15 min and purified as described above. The nuclear fraction was suspended in homogenizing buffer. The supernatant from the 800 *g* centrifugation was further centrifuged at 100,000 *g* for 1 h. The supernatant from this constituted the cytosolic fraction. The pellet comprising the particulate fraction was suspended in a medium containing 20 m*M* Tris-HCl (pH 7.5), 2 m*M* EDTA, and 0.5% Triton X-100 and was sonicated six times for 10 s each, with 1-min intervals between successive sonications. After being kept on ice for 30 min, the solution was centrifuged at 100,000 *g* for 10 min. The resulting supernatant constituted the solubilized particulate fraction. PKC activity in nuclei, cytosol, and particulate was determined by the modification of the mixed micelle assay (15), and the protocol supplied by Amersham Pharmacia Biotech (U.K.) with the assay kit was followed. Each reaction mixture (75 μl) contained 50 m*M* Tris-HCl (pH 7.5), 2.5 m*M* DTT, 12.5 μM ATP, 3.75 m*M* Mg^{2+}, 1 m*M* Ca^{2+}, 0.15 μM phorbol 12-myristate 13-acetate, 0.67 mol% 1-α-phosphatidyl-1-serine, 75 μM peptide, 0.2 μCi [γ-^{32}P] ATP, 50 μg of enzyme protein, and 0.5% (wt/vol) sodium azide. The reaction was carried out at 37°C for 15 min. An aliquot was spotted on a filter paper disc, which was washed in 75 m*M* orthophosphoric acid and counted.

MAP kinase activity was estimated in the nuclear and cytosolic fractions. For isolating the nuclear fraction, the liver was homogenized in buffer containing 10 m*M* Tris, 150 m*M* NaCl, 2 m*M* EGTA, 2 m*M* DTT, 1 m*M* orthovanadate, 1 m*M* PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin (pH 7.4). Pure nuclei were obtained by the procedure described above. MAP kinase activity was assayed using a kit provided by Amersham Pharmacia Biotech. This assay is highly selective for p42/p44 MAP kinase. Each reaction mixture contained 10 μl of peptide in HEPES, sodium orthovanadate, and 0.005% sodium azide (pH 7.4), 5 μl of MgATP, 10 μg of enzyme protein, and 1 μCi of [γ-^{32}P]ATP. The reaction was carried out for 30 min at 30°C. An aliquot was spotted on a filter paper disc, washed with 75 m*M* orthophosphoric acid, and counted. For all the above assays radioactivity was counted in an LKB Rackbeta 1217 liquid scintillation spectrometer. Protein estimation was done according to the method of Lowry *et al.* (23).

RESULTS

The tyrosine kinases are a composite group of enzymes composed of growth factors, other membrane-bound tyrosine kinases, and cytosolic tyrosine kinases (26). An increase in activity of the enzyme was observed in the membrane fraction of the liver of irradiated mice at as early as 15 min of irradiation at 3 Gy. The activity was found to increase maximally at 30 min and then drop to normal values at 4 h (Fig. 1A). The activity was assayed until 24 h, but was found to be unchanged (data not shown). The nuclear tyrosine kinase showed a significant increase at 15 min and remained high until 30 min, whereas the membrane-bound tyrosine kinase showed an activation only at 30 min of irradiation with a sharp return to normal values at 1 h (Fig. 1B). The response of the enzyme to different doses has also been looked at. Both the membrane-bound and the nuclear kinase were found to respond to very low doses of γ-irradiation, *i.e.,* 10 cGy (Table 1), and the activity was found to increase with increasing doses.

PKC, a very crucial enzyme in signaling, was assayed in three fractions, particulate, cytosolic, and nuclear. The activity in the three fractions showed a different time course of activation. As with the tyrosine kinases, the nuclei and the particulate showed an activation at 15 min with maximal increase in activity at 30 min. At 15 min, the cytosolic PKC showed a marginal drop, followed by a significant increase in activity at 30 min of irradiation. The

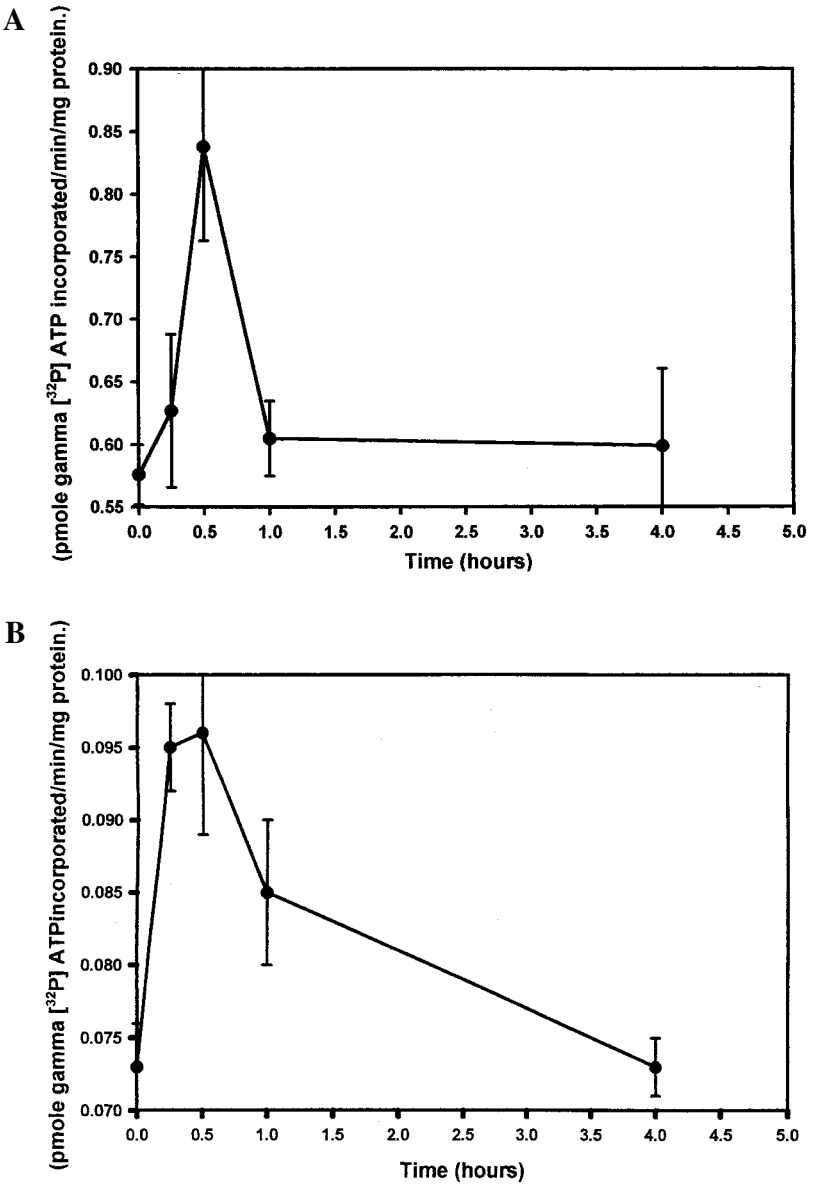

FIG. 1. Tyrosine kinase activity in membrane (A) and nuclei (B) isolated from mouse liver at different time intervals following the whole body γ-irradiation at 3 Gy. The results shown are only up to 4 h of irradiation. The values do not change until 24 h. Mice were killed at 30 min after irradiation, and the membrane and nuclei were isolated as described in Materials and Methods. The results are averages of three independent experiments ± SD.

nuclear and particulate PKC activities dropped at 1 h, whereas the cytosolic activity remained high at 1 h of irradiation (Fig. 2). All three fractions showed a return to normal values at 4 h and later time periods (data not shown). Among these fractions, the particulate and cytosolic PKC showed a maximal increase in activity, whereas the nuclear was marginally increased (Fig. 2). The enzyme was found to respond to relatively high doses of γ-irradiation. There was no increase in activity at 10 cGy. Only at 1, 3, and 5 Gy was the activity found to increase (Table 2).

Although both the tyrosine kinase and PKC activities were found to alter following whole body γ-irradiation, there were significant differences in their responses. The tyrosine kinase of the membrane responded with a 17% increase in activity at 10 cGy, whereas PKC did not increase at this dose (Tables 1 and 2). The

TABLE 1. EFFECT OF DIFFERENT DOSES OF WHOLE BODY γ-IRRADIATION ON TYROSINE KINASE ACTIVITY IN MOUSE LIVER

	Tyrosine kinase activity	
Dose (Gy)	*pmol of [γ-^{32}P]ATP incorporated/ min/mg of protein*	*% of control*
0	0.603 ± 0.038	
0.10	0.705 ± 0.023*	117
1	0.738 ± 0.040*	122
3	0.856 ± 0.066†	142

Tyrosine kinase activity was estimated in the membrane fraction of the liver of mice irradiated at different doses. The mice were killed at 30 min after irradiation. The membrane was isolated as described in Materials and Methods. The values are averages of three independent experiments ± SD. The results were statistically evaluated using the Student's *t* test.

*$p < 0.1$, †$p < 0.05$ when compared with control values.

time courses of activation of the two enzymes following whole body γ-irradiation were also different. The membrane-bound tyrosine kinases showed a sharp increase in activity at 30 min and a return to normal values at 4 h, whereas the three fractions of PKC showed a varied response with time and were activated for a longer time after irradiation, *i.e.*, 15 min-to 1 h (Figs. 1 and 2).

MAP kinase is the enzyme that is fed by a number of pathways during signaling, and one of these pathways is via PKC. The activity of MAP kinase was estimated in the cytosol and the nuclear fraction. The activity of this kinase

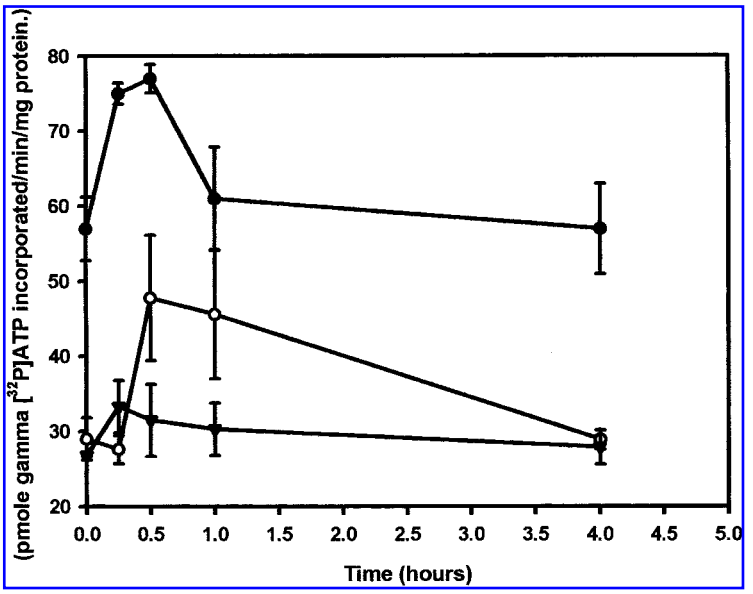

FIG. 2. PKC activity in particulate (●), cytosol (○), and nuclei (▼) isolated from mouse liver at different time intervals following the whole body γ-irradiation at 3 Gy. Mice were killed at 30 min after irradiation and the particulate, cytosolic, and nuclear fractions were isolated as described in Materials and Methods. The results are averages of three independent experiments ± SD. The values shown are up to 4 h of irradiation. They do not change at later time periods.

Table 2. Effect of Different Doses of Whole Body γ-Irradiation on PKC Activity in Mouse Liver

	PKC activity (pmol of [γ-^{32}P]ATP incorporated/min/mg of protein)		
Dose (Gy)	*Particulate*	*Cytosol*	*Nuclear*
0	51.79 ± 4.9	36.28 ± 1.3	22.95 ± 2.7
0.10	54.62 ± 0.4 (105)	36.20 ± 1.2 (98)	21.23 ± 1.1 (95)
1	71.82 ± 4.8 (139)*	45.80 ± 2.7 (126)§	26.38 ± 1.7 (115)
3	96.38 ± 7.5 (185)†	45.70 ± 1.9 (125)†	29.38 ± 2.5 (128)
5	100.14 ± 1.24 (193)‡	45.09 ± 4.4 (124)†	28.47 ± 2.3 (125)

PKC activity was estimated in the particulate, cytosolic, and nuclear fractions of the liver of mice irradiated at different doses. The mice were killed at 30 min after irradiation. The fractions were isolated as described in Materials and Methods, and PKC activity was estimated using a kit supplied by M/S Amersham. The values in parentheses are shown as % of control. The values are average of three independent experiments ± SD. The results were statistically evaluated using the Student's *t* test.

*$p < 0.05$, †$p < 0.01$, ‡ $p < 0.001$, § $p < 0.02$ when compared with the control values. The rest of the data was nonsignificant.

was found to be unchanged until 5 Gy of whole body γ-irradiation in both fractions (Table 3). The activity of MAP kinase was also found to be unchanged at different time intervals (Fig. 3).

DISCUSSION

The increase in activity of tyrosine kinase was observed as early as 15 min in the nuclei and 30 min in the membrane fraction (Fig. 1). The difference in the response of the two fractions to irradiation may be due to the fact that the activation in the membrane and the nuclear fraction is contributed by different species of tyrosine kinases. γ-Irradiation has been known to activate specific tyrosine kinases. These include the membrane-associated ones like epidermal growth factor receptors (26), Src-like tyrosine kinases (9). In the nuclei, there are reports on activation of lyn-like tyrosine kinases. This has been reported to be due to activation or translocation of p56/p53 lyn tyrosine kinase in the nucleus (20). Although there are various reports in the literature on the activation of tyrosine kinases, these are all *in vitro* studies. In the present study, a sharp activation of tyrosine kinases in the membrane has been observed following whole body irradiation. This may be due to the activation of membrane-bound kinases. The activation of the nuclear tyrosine kinase may be due to *in situ* activation or translocation of some specific kinases to the nucleus. The dose response of tyrosine kinase showed that, unlike PKC, it responds to very low doses of irradiation, *i.e.*, 10 cGy (Table 1). This is in agreement with the report that various immediate early genes respond differently to varying doses of irradiation. The expression of these genes has been correlated to the activation of signaling molecules (30).

The activation of PKC, a very crucial enzyme in signaling, has been reported in various cell lines following γ-irradiation (11, 12, 21, 28). In hepatocytes, irradiated at 5–50 Gy, the activation of the kinase has been reported after 30 min. This activation has been implicated to be via lipid peroxidation and due to translocation of the enzyme to the plasma membrane (28). In

Table 3. Effect of Different Doses of Whole Body γ-Irradiation on MAP Kinase Activity in Mouse Liver

	MAP kinase activity (pmol of [γ-^{32}P]ATP incorporated/min/mg of protein)	
Dose (Gy)	*Cytosolic*	*Nuclear*
0	39.12 ± 1.8	31.2 ± 0.8
0.10	33.8 ± 2.4	29.1 ± 1.3
1	34.0 ± 1.7	25.8 ± 1.6
3	36.7 ± 2.3	25.9 ± 2.0

MAP kinase activity was estimated in the cytosolic and nuclear fractions of the liver of mice irradiated at different doses. The mice were killed at 30 min after irradiation. The membrane was isolated as described in Materials and Methods. The values are averages of three independent experiments ± SD. The results were statistically evaluated using the Student's *t* test. The data were found to be not significant when compared with the control values.

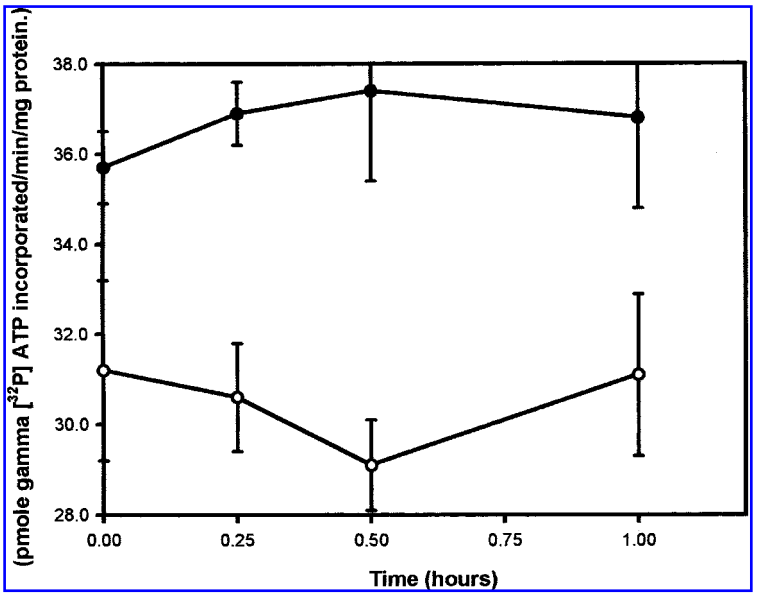

FIG. 3. MAP kinase activity in cytosol and nuclei isolated from mouse liver at different time intervals following the whole body γ-irradiation at 3 Gy. Mice were killed at 30 min after irradiation, and the cytosolic (●) and nuclear (○) fractions were isolated as described in Materials and Methods. The results are averages of three independent experiments ± SD, and were not significant when compared with control values.

the present study, following whole body γ-irradiation, all three fractions, *i.e.*, cytosolic, particulate, and nuclear, were found to respond to radiation in a different manner. There was no translocation of the enzyme to the plasma membrane either (Fig. 2). Classically, translocation of PKC to the membrane fraction would mean an increase in the activity in the particulate fraction, with a concomitant decrease in the cytosolic fraction. In this study, at 15 min there was an increase in particulate PKC and nuclear PKC. This was accompanied by only a marginal drop in the cytosolic PKC activity, which could not account for the significant increase in the particulate and the nuclear fractions. At 30 min, PKC activity in both the particulate and the cytosolic fractions was found to increase significantly accompanied by a marginal increase in the activity in the nuclei. At 1 h, the particulate PKC activity returned to normal levels, whereas the cytosolic remained activated. The activities in all three fractions were found to return to normal levels by 4 h (Fig. 2) and the subsequent time periods (results beyond 4 h not shown). Thus, it appears that PKC undergoes various fluctuations between the time of irradiation until 2 h, and it is very likely that the increase in activity in all three fractions is contributed by several isozymes of PKC. PKC is known to exist in at least 10 different isoforms, which play a crucial role in signaling, transformation, differentiation (19, 29), and the development of radioresistance (8). Activation of PKC could be partly due to the increased levels of diacylglycerol (DAG) (16). In our earlier studies, we had observed an increase in the DAG levels in livers of mice irradiated at 3 Gy. The DAG content in the control mouse liver was 3.07 nmol/g of tissue, whereas in liver of mice irradiated at 3 Gy it was 12.4 nmol/g of tissue. This increase in DAG was found to be contributed both by phosphatidylinositol and phosphatidylcholine hydrolysis (22).

The activity of the enzyme MAP kinase was found to be unaltered in cytosol as well as nuclei (Fig. 3). MAP kinase plays a central role in various signal transduction pathways, and its activation causes the phosphorylation of c-*myc* and c-*jun*. MAP kinase activation can be seen through both PKC-dependent and PKC-independent mechanisms. Activation of MAP kinase has been reported in NIH 3T3 cells by irradiation through the formation of reactive oxygen intermediates (35) and a radiation-induced translocation of the enzyme from cytosol to nucleus in Chinese hamster V79 cells (15).

Activation of MAP kinase is essential for cell growth and differentiation and may counteract apoptotic signaling (19) possibly by abrogation of Bcl-2-mediated survival (44). However, following whole body γ-irradiation until 5 Gy there was no activation of MAP kinase in the cytosol nor was there a translocation to the nuclei. The assay used is specific for p42 MAP kinase, and hence the involvement of JNK/SAPK and p38 cannot be ruled out.

Thus, in conclusion, it can be summarized that the response of these enzymes *in situ* may be very different from that in an isolated cell. In whole body γ-irradiation, as is known, the response varies from organ to organ. Moreover, the pathways that operate *in situ*, in a whole animal, where various biochemical and physiological constraints are operative, may not operate in a single irradiated cell. In recent years, these signaling molecules have been increasingly used for manipulating tumor response to radiotherapy, and UCN-O1, an inhibitor of PKC, is already under clinical trials. The above reported observations hence assume much more significance, because the knowledge of their activation at different doses and chronological order of activation would be very essential for any drug design if it is to be clinically relevant and implemented for therapy.

ABBREVIATIONS

DAG, diacylglycerol; DTT, dithiothreitol; MAP, mitogen-activated protein; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride.

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