Perturbations in phosphoinositide metabolism and protein kinase C activity in mouse liver following whole body irradiation

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The involvement of the signal transduction pathway in mouse liver following whole body irradiation was investigated. Mice were exposed to 100Co gamma rays (3 Gy) and sacrificed after different time intervals. Various elements of phosphatidyl inositol signal transduction pathway were investigated. Alterations could be seen as early as 15 min of irradiation. These changes are reflected in elevation in DAG levels and increased activation of PKC, an enzyme which is involved in tumorigenesis. The chronological appearance of various transducers following whole body irradiation is of significance since these early effects may set the stage for radiation-induced tumorigenesis and hence may be used to manipulate tumor response to radiotherapy.

The signalling cascade in the cell has been known to respond to various stimuli including mitogens, chemicals and irradiation. Carcinogens like aflatoxin B1 (AFB1) and N-nitrosodiethylamine (NDEA) have been reported to alter the phosphoinositide turnover1 2 protein kinase C (PKC) activity modifying phosphorylation of various nuclear enzymes3 and the activity of GAP leading to an overexpression of ras oncogene3. Alterations in the signal transduction pathway and the expression of various oncogenes are also among the multiple cellular responses associated with ionizing radiation. Exposure to low dose γ-radiation results in the induction of various genes including c-fos, PKC, -tubulin and interleukin 1 in Syrian hamster embryo cells5 6. Following whole body exposure of mice to γ-rays (3 Gy) expression of oncogenes c-src and c-H-ras in gut and liver has been reported7. Increased expression of these genes may play a significant role in the development and progression of transformed cells. Since oncogene expression is interlinked with signal transduction pathways, certain observations on signalling after irradiation have also been reported, such as activation of PKC in HL-60 cells following ionizing radiation8 activation of phospholipase D (PLD) transphosphatidylation activity indicating involvement of PLD in response to gamma radiation in SQ 20B cell line9 and an alteration of tyrosine phosphorylation in human B lymphocyte precursors10. However, all these studies were confined to single cell and are inconclusive. Information on signal transduction following whole body irradiation is scanty although the expression of oncogenes following whole body gamma ray exposure was found to be altered7. We have attempted to provide the link between such expression of oncogenes and changes, if any, in the phosphatidylinositol (PI) signal transduction pathway following whole body irradiation of mice. The present study is also important since no direct link has been established between radiation-stimulated protein tyrosine phosphorylation and downstream signals10.

Materials and Methods

Irradiation and subcellular fractionation

Male Swiss inbred mice (8-10 week old) were fed stock laboratory diet and subjected to whole body irradiation from a 100Co Theratron Junior Teletherapy unit at the rate of 35 cGy/min. Groups of mice were exposed in a specially designed well ventilated acrylic box. The area of exposure was kept constant and the total dose was 3 Gy. Animals were sacrificed at 15, 30, and 60 min after whole body irradiation. Livers were removed, washed and homogenized in 0.25 M sucrose in 0.025 M Tris-HCl (pH 7.4) containing 1 mM PMSF and 10 μg/ml leupeptin. All operations were carried at 4°C unless otherwise specified. Nuclei and unbroken cells were removed by centrifuging at 10,000 g for 15 min. Membranes were collected by sedimentation at 100,000 g for 45 min. In another set of experiments involving PKC, the liver was homogenized in cold 0.25 M sucrose in 15 mM Tris-
HCl (pH 7.5) containing 50 μg/ml PMSF, 10 μg/ml leupeptin, 5 mM EDTA and 0.3 % w/v β-mercaptoethanol. The homogenate was filtered through 2 layers of cheese cloth and crude nuclei were pelleted at 800 g for 10 min at 4°C. Subsequent centrifugations were also carried out at 4°C. Crude nuclear suspension was layered over a cushion of 2.2 M sucrose and purified by centrifuging at high speed. The purified nuclear fraction was suspended in homogenizing buffer. The supernatant from 800 g centrifugation was further centrifuged at 100,000 g for 1 hr. The supernatant from this constituted the cytosol fraction. The pellet comprising the particulate fraction was suspended in a medium containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA and 0.5 % Triton X-100 and left on ice (with intermittent shaking) for 1 hr, after which it was centrifuged at 100,000 g for 10 min. The resulting supernatant constituted the solubilized particulate fraction.

**Incorporation studies**

Incorporation of [γ-32P]ATP into the phospholipids PI, PIP, PIP2 was examined. The reaction mixture contained 20 mM HEPES (pH 7.5), 4mM ATP and [γ-32P]ATP 2500-3000 cpm / pmol. The reaction was carried out for 20 min at 30°C. The reaction was terminated by the addition of 1.5 ml of chloroform : methanol (1:2 v/v) and the phospholipids were separated by two phase partitioning. Inositol lipids were further purified by thin layer chromatography. The plates were autoradiographed and the spots corresponding to PIP, PIP2 and PI-3,4,5-trisphosphate (PIP3) were scraped off and their radioactivity was determined in an LKB Rackbeta 1217 Liquid Scintillation Spectrometer essentially as described by Pasupathy et al.

**Enzyme assays**

The activation of the individual enzymes PI kinase and PIP kinase were monitored as described by Lindberg et al. The reaction mixture (50μl) contained 50mM HEPES, pH 7.5, 1mM DTT, 4mM MgCl2, 0.4mM ATP, 10 μCi [γ-32P]ATP and 20 μg protein. The reaction was terminated after 1 min. For PIP kinase, 50 mM HEPES pH 7.8 and 15 μg protein were used. The rest of the ingredients were the same as in PI kinase assay. This reaction was terminated after 4 min. Inositol phospholipids were separated and purified as described above. PI-3 kinase activity was studied after the addition of the external substrate PIP2 essentially as described by Downes and Carter and modified by us. PKC activity was estimated in the particulate, cytosol and nuclear fractions of mice liver homogenate. PKC activity was estimated in the nuclear, cytosolic, and particulate fractions after an initial purification on DEAE cellulose and using a kit supplied by Amersham. The assay employed was a modification of the mixed micelle assay. Each reaction mixture (75 μl) contained 50 mM Tris-HCl (pH 7.5), 2.5 mM DTT, 12.5 μM ATP, 3.75 mM Mg2+, 1 mM Ca2+, 0.15 μg phorbol 12-myristate 13-acetate, 0.67 mol % L-phosphatidyl-L-serine, 75 μM peptide, 0.2 μCi [γ-32P]ATP and 50 μg enzyme protein (different fractions) and 0.5 % (w/v) sodium azide. An aliquot was spotted on a filter paper disc which was washed in 5 % acetic acid and the insoluble radioactivity was measured.

**Estimations**

DAG was extracted from 500 mg fresh liver and the level was estimated employing DAG kinase (DAG assay kit) which phosphorylates DAG to form [2-3P]PA in the presence of [γ-32P]ATP. The procedure was as described by Preiss et al. Inositol 1,4,5-trisphosphate (IP3) level was estimated using Amersham’s IP3 level measurement kit which is based on the competition between unlabeled IP3 and a fixed quantity of tritium labelled IP3 for a limited number of binding sites on a bovine adrenal binding protein preparation. Protein was estimated by Lowry’s procedure.

**Results**

The incorporation of radioactivity from [γ-32P]ATP into the three endogenous phospholipids, namely PI, PIP2, PIP3 was measured following whole body irradiation. The incorporation was found to be significantly elevated in PIP and PIP2 at 15 min following irradiation and remained so till 60 min post irradiation. The incorporation in PIP2 was found to decrease at 15 and 30 min followed by an increase at 60 min after irradiation. These results are shown in Table 1.

The rapid turnover of these phospholipids makes it very difficult to draw any conclusions from such incorporation studies. Hence their enzymatic synthesis was studied and the results are shown in Table 2. The individual enzyme activities followed a slightly different pattern. A significant increase PI kinase activity (as measured by PIP production) was found at 15 and 30 min following irradiation. The activity somewhat decreases at 1 hr, but yet is above...
the control. PIP kinase activity shows a marginal decrease at 15 min followed by an increase at 1 hr. A slight increase in PI3-kinase activity is seen at 1 hr period which agrees very well with the increased incorporation of \( [\gamma^{32}P]ATP \) into PIP3 depicted in Table 1.

An increase in PIP2 can in turn increase the level of DAG and IP3 which are two important second messengers. Indeed the level of DAG was found to increase significantly at 15 min and continued to remain so till 60 min following irradiation, while IP3 level increased at 15 and 30 min and decreased at 60 min. following wholebody irradiation as seen in Table 3.

Once the level of DAG increases it can activate PKC, a crucial enzyme in signal transduction. PKC activity was found to be increased at 15 min post irradiation in both the particulate as well as the cytosolic fractions (Table 4). Activities in both the fractions decrease by 1 hr.

<table>
<thead>
<tr>
<th>Time After Irradiation (min)</th>
<th>PIP</th>
<th>PIP2</th>
<th>PIP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.34±0.11</td>
<td>0.46±0.04</td>
<td>0.86±0.03</td>
</tr>
<tr>
<td>15</td>
<td>0.80±0.14</td>
<td>0.64±0.04</td>
<td>0.52±0.07</td>
</tr>
<tr>
<td>30</td>
<td>0.83±0.08</td>
<td>0.72±0.05</td>
<td>0.57±0.07</td>
</tr>
<tr>
<td>60</td>
<td>0.87±0.08</td>
<td>0.84±0.02</td>
<td>1.10±0.04</td>
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</tbody>
</table>

Mice were subjected to whole body irradiation and the isolated plasma membrane (400 μg) from their livers was incubated with \([\gamma^{32}P]ATP\) for 1 hr followed by separation of phospholipids for radioactivity measurement as described in the text.

<table>
<thead>
<tr>
<th>Time After Irradiation (min)</th>
<th>PIP</th>
<th>PIP2</th>
<th>PIP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.1±3.5</td>
<td>9.3±1.1</td>
<td>1.11±0.04</td>
</tr>
<tr>
<td>15</td>
<td>41.7±4.17</td>
<td>8.3±0.2</td>
<td>1.30±0.03</td>
</tr>
<tr>
<td>30</td>
<td>50.6±3.1</td>
<td>8.1±1.8</td>
<td>1.12±0.01</td>
</tr>
<tr>
<td>60</td>
<td>26.3±1.8</td>
<td>14.0±1.2</td>
<td>1.50±0.08</td>
</tr>
</tbody>
</table>

Membrane fraction from livers of mice following whole body irradiation was used for enzymatic assay using \([\gamma^{32}P]ATP\). The radioactivity was measured in PIP (PI kinase assay for 1 min) and PIP2 (PI2 kinase assay for 4 min) respectively. For PI3-kinase, PIP was used as the exogenous substrate. Details are given in the text.

<table>
<thead>
<tr>
<th>Time after irradiation (min)</th>
<th>PIP</th>
<th>PIP2</th>
<th>PIP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.07±0.20</td>
<td>40.1±0.9</td>
<td>40.1±0.9</td>
</tr>
<tr>
<td>15</td>
<td>6.96±0.30</td>
<td>59.6±1.5</td>
<td>59.6±1.5</td>
</tr>
<tr>
<td>30</td>
<td>12.40±0.50</td>
<td>78.3±1.7</td>
<td>78.3±1.7</td>
</tr>
<tr>
<td>60</td>
<td>17.50±0.43</td>
<td>51.5±2.0</td>
<td>51.5±2.0</td>
</tr>
</tbody>
</table>

Levels were measured in mice livers following whole body irradiation as described in the text.

Discussion

The PI cycle is the first to be altered following any genotoxic insult. Following mitogen treatment PIP2 gets hydrolyzed to yield DAG and IP3. DAG is a known internal activator of PKC and IP3 releases Ca2+ from internal stores. Perturbations in the PI cycle due to changes in PIP2 hydrolysis should be reflected in an alteration in the synthetic enzymes, the kinases (PI kinase and PI2 kinase) as well as in the incorporation of radioactivity into these lipids. In response to whole body irradiation, increased rate of incorporation into PIP and PIP2 were observed from 15 min onwards till 1 hr. However incorporation into PIP3 was found to increase only after 1 hr of irradiation. These incorporation studies represent a turnover and not the synthesizes, hence the individual kinases were assayed using endogenous substrates. As can be seen from the results depicted in Table 2, PI kinase activity was found to increase significantly at 15, 30 and 60 min after irradiation, which explains the increased incorporation into PIP. The activity of PI2 kinase which synthesizes PIP2 from PIP was also found to be unaltered at 15 and 30 min showed a significant increase at 60 min. PIP3 kinase activity observed after the addition of exogenous substrate was also found to increase at 1 hr of irradiation. Perturbations in PI cycle could be one of the earliest...
manifestations in cellular responses to any stress. Hence our studies are confined to very early periods. The increased incorporation in all the three phospholipids which seem to follow a chronological order (Table 1) suggests an involvement of PI cycle in irradiation-induced response. The increase incorporation into PIP2 (Table 1) which is spite of a decrease in PIP kinase at certain time periods (Table 2) may be due to an increased activity of PI kinase and thus increasing availability of the substrate PIP. The activity of PIP kinase does increase at 1 hr following irradiation and this could be the reason for the increased activity of PIP2 at 1 hr.

The increase in radioactivity in PIP2 as observed after 60 min is indicative of an increase in the activity of PI 3-kinase which synthesizes PIP2 from PIP3, the content of which is increased at 60 min of irradiation. Inositol lipids phosphorylated at the D-3 position by PI 3-kinase act as second messengers and are now known to activate the ser/thr kinase Akt (also called PKB) identified first as an oncogene. Akt is the major target of PI 3-kinase generated signals and mediates insulin like growth factors (IGF)-induced inhibition of apoptosis in neurons. IGF is also known to inhibit UV induced apoptosis via PI 3-kinase/Akt pathway. It is also the enzyme that converges the tyrosine kinase and PKC pathways. Thus, the enzyme PI 3-kinase possibly has a significant role to play in radiation induced effects.

An increase in PIP2 could give rise to elevated levels of DAG and IP3 which are two important second messengers. The increase in DAG with a concomitant increase in IP3 levels at 15 and 30 min can only be due to rapid hydrolysis of PIP2 by phospholipase C whose activity must be significantly increased to justify an increased DAG and IP3 levels. Although the DAG levels were high even at 1 hr after irradiation, the levels of IP3 decline indicating that some of DAG is not form PIP2 and may come from phosphatidyl choline (PC) via PLD, the involvement of which has been demonstrated in signal transduction pathways. It has been shown that the early requirements of DAG are met by PI pathway and later DAG comes from PLD pathway via hydrolysis of PC yielding PA which is then converted to DAG. An increase in IP3 level can release calcium from internal stores. Calcium levels have been shown to be increased following whole body irradiation with low dose X-rays.

Thus the elevated levels of DAG in turn activates PKC. Activation of PKC usually results in the translocation from the cytosol to the particulate fraction, where the activity in cytosol declines and that in the particulate increases. However following wholebody irradiation an increase in both cytosolic and particulate PKC was observed. Does this relate to increased expression of PKC gene? Although there is at present no supporting data is available such an observation has been made by Anderson and Woloschak. PKC once activated is known to lead to feedback inhibition and this may be the reason for the declined activity at 60 min. Once triggered by DAG, PKC can thus autoregulate itself and continued increase in DAG does not further activate PKC.

Nuclear PKC remains unaffected. The transcription factor that PKC activates, e-Jun, has also been reported to be increased following irradiation.

In conclusion, it can be said that the first signal generated following whole body irradiation is from the PI intermediates, probably PIP2, which then generates DAG. Thus, the initial requirements of DAG after mitogenic signalling are met by the PI cycle intermediates. The increase in PKC activity following irradiation is due to increased levels of DAG. Since these initial post transcriptional events, such as kinase activation (PI kinase, PI 3-kinase and PKC) precede the nuclear signal transducers (API, e-Jun etc.) interruption of the signal at the level of the kinases may be utilized to manipulate the tumour response to radiotherapy.

References