THROMBOXANE INCREASE IN IRRADIATED ANIMALS IS CAUSED BY STIMULATION OF CYCLOOXYGENASE ACTIVITY

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The irradiation of whole body of rabbits with a dose of 6.0 Gy causes an increase in thromboxane synthesis from exogenous arachidonic acid. The uptake of $[^{14}\text{C}]$arachidonic acid and the total amount of radioactivity released during collagen stimulated aggregation of platelets are not changed following the exposure of animals. The irradiation changes the relation between released arachidonic acid and synthesized thromboxane. The amount of 12-hydroxyeicosatetraenoic acid remains unchanged.

The results indicate that the increase in thromboxane synthesis is not associated with the activation of phospholipase but is caused by stimulation of cyclooxygenase activity.

Eicosanoids, prostacyclin (PGI$_2$) and thromboxane (TXA$_2$), play an essential role in the platelet-vessel wall interaction. TXA$_2$ and PGI$_2$ are antagonists: TXA$_2$ has a proaggregating [1] and PGI$_2$ an antiaggregating effect on platelets [2]. Radiation injury is associated with significant disturbances in PGI$_2$ and TXA$_2$ synthesis. Production of PGI$_2$ in blood vessels
increases during the first two days after exposure of animals to sublethal doses of ionizing radiation [3, 4]; thereafter its synthesis decreases. The whole body irradiation causes an increase in the synthesis of TXB₂, which is the stable degradation product of TXA₂. A level of TXB₂ is increased in plasma and serum of irradiated animals and in platelets washed and stimulated by thrombin, as well as in the medium of incubated thoracic aorta fragments that were isolated from these animals [5].

The contrasting properties of PGI₂ and TXA₂ and their different reaction to irradiation complicates the interpretation of the role played by these metabolites of arachidonic acid in radiation injury. We have previously demonstrated that irradiation of animals abolishes the effect of H₂O₂ on PGI₂ and TXB₂ synthesis from endogenous arachidonic acid; this indicates that in the radiation caused disturbances in PGI₂ and TXB₂ synthesis a peroxidative factor is involved [4, 5]. Since cyclooxygenase is activated or inhibited by H₂O₂ and lipid peroxides, depending on their concentration [6, 7, 8], the increase in TXB₂ synthesis following irradiation might be due to increased activity of the cyclooxygenase stimulated by H₂O₂ and other peroxides [5], the formation of which is elevated in irradiated tissue. The increase in TXB₂ synthesis following irradiation can also be due to the increased release of arachidonic acid.

To shed more light on the mechanism of the increase in TXB₂ synthesis in whole body irradiated animals, studies on (i) formation of TXB₂ in platelets from exogenous arachidonic acid (ii) incorporation of arachidonic acid into platelets, and (iii) release of arachidonic acid from platelets and subsequent formation of TXB₂ were undertaken; ¹⁴C-labelled arachidonic acid was used in all three kinds of experiments.

MATERIALS AND METHODS

Arachidonic acid [¹⁴C] (spec. activity 2.02 GBq/mmol) was purchased from the Radiochemical Centre, Amersham, England.

Collagen was prepared in our laboratory according to Bornstein & Piez [9]. Thromboxane B₂ was purchased from Calbiochem AG, arachidonic acid and 12-hydroxyeicosatetraenoic acid (12-HETE) were purchased from Sigma Chemical Co. Thin-layer chromatography plates precoated with silica gel, 60F₂₅₄ were from E. Merck (Darmstadt, F.R.G.). All solvents were of analytical reagent grade.
Three-month-old Belgian breed rabbits were irradiated with X-rays generated with a Stabiliran-250 apparatus (Siemens, F.R.G.), operated at 180 kV, 20 mA and additionally filtered through 1 mm Cu. Each rabbit was irradiated separately by exposing its either side to half of the total dose. The target to source distance was 60 cm and the dose-rate (measured within the phantom body using a dosemeter type VAK-253 and spherical ionizing chamber manufactured by Otto Schoen Messelektronik, F.R.G.) was 17 cGy/min. On the basis of exposure time the total dose delivered was 6.0 Gy. The radiation dose output was simultaneously controlled with a dosemeter. Fourteen rabbits were irradiated and 5 of them survived 28 days.

Platelet-rich plasma (PRP) was prepared from whole blood samples withdrawn (10 ml) from rabbit into a plastic tube containing ethylene-diaminetetraacetate (EDTA, 0.1 ml, 15%) and centrifuged at 200 × g for 15 min at room temperature. The washed platelets were obtained from a sample of PRP containing about 3 × 10^8 platelets by centrifuging it at 12000 × g for 1 min at room temperature. The plasma was removed and the platelet pellet was resuspended in 1 ml of solution composed of 0.03 M phosphate buffer, pH 6.5, 0.11 M NaCl, glucose, 0.1 mg/ml and bovine serum albumin, 1 mg/ml.

This suspension was supplemented with [14C]arachidonic acid (to 4 μM) and formation of TXB2 during incubation (10 min) at 37°C was estimated. The reaction was stopped by cooling in an ice bath and adding 20 μl of 1 N HCl and 150 μl of saturated NaCl solution. The samples were then extracted three times with 1 ml portions of chloroform. The organic phases were pooled and dried under a stream of nitrogen. The residue was redissolved in chloroform and subjected to thin-layer chromatography [10]. The plates were developed with a chloroform:methanol:acetic acid:water mixture (90:8:1:0.8, by vol.). The components were visualized by exposing the developed plates to iodine vapour. Thromboxane containing fractions were scraped off and their radioactivity was measured in a Beckman LS 8100 scintillation counter.

Incorporation of [14C]arachidonic acid into isolated platelets was carried out by incubating 1 ml mixture of PRP and PPP containing 5 × 10^8 platelets per ml with 2 μM [14C]arachidonic acid at 37°C for 1 h. The platelets were then separated from the plasma by centrifugation (15 min, 2500 × g) and washed twice with Tyrode solution, pH 6.5 (NaCl – 8 g, KCl – 200 mg, MgCl2 – 100 mg, Na2HPO4 – 50 mg, NaHCO3 – 1 g, glucose – 1 g, per 1000 ml).
Release of [\(^{14}\)C]arachidonic acid and its metabolites from the labelled platelets was studied by incubating them with collagen (400 \(\mu\)g/0.5 ml) for 10 min under conditions which caused aggregation of platelets. Blanks were incubated without collagen. To determine the total radioactivity released without separating the various metabolites the incubation was terminated by adding formalin (final concentration 1.5%) according to Costa & Murphy [11]. The platelets were then pelleted (1 min, 12,000 \(\times\) g), the supernatant was drawn off and its radioactivity measured. The percentage of the released radioactivity was calculated as indicated in Table 3.

For separation and radioactivity measurement of arachidonic acid metabolites the incubation was terminated by centrifugation and the supernatant was subjected to extraction and thin-layer chromatography as described above.

The data were subjected to the analysis of variance. The significance of group differences was tested using Student’s \(t\)-test for small samples with unequal variances [12].

RESULTS

Whole body irradiation of rabbits with a dose of 6.0 Gy markedly increased conversion of arachidonic acid to thromboxane by their platelets. Synthesis of the stable thromboxane metabolite (TXB\(_2\)) was increased as early as within 1 day after irradiation and remained at an elevated level up to the end of observation (28th day) (Table 1). This ability of platelets from irradiated animals to increase the metabolism of arachidonic acid was observed both in platelets which survived irradiation (1 - 14 days after irradiation) and in those which were formed \textit{de novo} (14 - 28 days after exposure).

When platelets from unirradiated rabbits were washed and incubated with arachidonic acid in the presence of H\(_2\)O\(_2\), the TXB\(_2\) formation was increased about twice at H\(_2\)O\(_2\) concentrations from 1 to 100 \(\mu\)M and decreased to below the original level when the concentration of H\(_2\)O\(_2\) was raised to \(1 \times 10^{-3}\) M. Platelets isolated from irradiated rabbits 34 days after exposure to 6.0 Gy were not sensitive to H\(_2\)O\(_2\) stimulation of arachidonic acid metabolism (conversion to thromboxane), their metabolic activity in this respect was already elevated and to some extent comparable to that of control platelets stimulated with H\(_2\)O\(_2\). However, \(1 \times 10^{-3}\) M H\(_2\)O\(_2\)
Table 1

*The effect of whole body irradiation of rabbits on thromboxane B₂ synthesis from [²¹⁴C]arachidonic acid by platelets.*

The mean ± S.D. values for n (number of) animals are given

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>n</th>
<th>Tromboxane B₂ pmol/10⁸ platelets</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Non-irradiated</td>
<td>10</td>
<td>32.2 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>40.1 ± 8.6</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>41.4 ± 6.7</td>
<td>0.01</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>42.7 ± 7.1</td>
<td>0.01</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>53.8 ± 9.5</td>
<td>0.001</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>56.9 ± 1.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

decreased the metabolic activity of platelets from irradiated rabbits, similarly as of those from unirradiated ones (Fig. 1).

![Graph](image)

Fig. 1. The effect of H₂O₂ on [²¹⁴C]thromboxane B₂ synthesis in platelets of unirradiated (○) and irradiated (●) rabbits. Mean ± S.D. is given from 10 determinations (unirradiated) and 5 determinations (irradiated)

The whole body irradiation of rabbits with a dose of 6.0 Gy did not change the ability of their platelets to incorporate radioactive arachidonic
Table 2

Incorporation of $[^{14}C]$arachidonic acid into blood platelets of rabbits irradiated with 6.0 Gy of X-rays.

The mean ± S.D. values are given for 1 h incubation of $5 \times 10^8$ platelets with 2 µl of 1 mM radioactive arachidonic acid (2.02 GBq/mmol)

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>Incorporated radioactivity c.p.m./$5 \times 10^8$ platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46 480 ± 4 382</td>
</tr>
<tr>
<td>1</td>
<td>50 640 ± 5 242</td>
</tr>
<tr>
<td>3</td>
<td>46 889 ± 6 438</td>
</tr>
<tr>
<td>7</td>
<td>48 432 ± 9 862</td>
</tr>
<tr>
<td>14</td>
<td>50 843 ± 6 405</td>
</tr>
<tr>
<td>28</td>
<td>46 445 ± 5 906</td>
</tr>
</tbody>
</table>

acid during 1 h incubation (Table 2). Platelets prelabelled in this manner with radioactive arachidonic acid were subjected to aggregation stimulated by collagen. In this process arachidonic acid and its metabolites were released into the incubation system. The total radioactivity released from platelets derived from irradiated rabbits did not differ statistically from that released from the control ones (Table 3).

Released arachidonic acid was enzymatically converted into oxygenation products of which TXB2 and HETE were separated by thin-layer chromatography. Their radioactivity was measured and compared with that of the released arachidonic acid. It was found that irradiation changed the relation between the amounts of the released arachidonic acid and synthesized TXB2. The amount of the released arachidonic acid decreased while the amount of the synthesized TXB2 increased. Radioactivity of the HETE fractions remained unchanged (Table 4).

DISCUSSION

Whole body irradiation of rabbits with a dose of 6 Gy changed the ability of their platelets to convert arachidonic acid into thromboxane. As
Table 3

Release of radioactivity from \[^{14}C\]arachidonic acid labelled platelets of rabbits irradiated with 6.0 Gy of X-rays.

The mean percent ± S.D. of the released radioactivity is given; % of \(^{14}C\) released = 
\[
[(c.p.m. \text{ of supernatant} - c.p.m. \text{ of blank})/(c.p.m. \text{ of whole sample} - c.p.m. \text{ of blank})] \times 100
\]

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>Released radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.7 ± 2.4</td>
</tr>
<tr>
<td>1</td>
<td>29.9 ± 3.8</td>
</tr>
<tr>
<td>3</td>
<td>29.4 ± 3.0</td>
</tr>
<tr>
<td>7</td>
<td>32.3 ± 5.6</td>
</tr>
<tr>
<td>14</td>
<td>32.1 ± 5.1</td>
</tr>
<tr>
<td>28</td>
<td>29.6 ± 1.1</td>
</tr>
</tbody>
</table>

soon as on the first day after exposure synthesis of TXB\(_2\) by platelets from exogenous arachidonic acid increased (Table 1). More pronounced increases were noted 2 and 4 weeks after irradiation, when newly formed platelets entered the circulation.

This finding indicates that the increase in thromboxane synthesis in whole body irradiated animals, as also evidenced by the elevation of TXB\(_2\) in plasma and serum and by the increased release of TXB\(_2\) from vessels

Table 4

Radioactivity of arachidonic acid and its metabolites released from collagen stimulated platelets and separated by thin-layer chromatography is given as the mean value of c.p.m. ± S.D.

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>Arachidonic acid</th>
<th>TXB(_2)</th>
<th>HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 866 ± 333</td>
<td>80 ± 26</td>
<td>1 524 ± 291</td>
</tr>
<tr>
<td>1</td>
<td>1 866 ± 173</td>
<td>111 ± 40</td>
<td>1 626 ± 180</td>
</tr>
<tr>
<td>3</td>
<td>1 369 ± 175*</td>
<td>230 ± 42*</td>
<td>1 487 ± 676</td>
</tr>
<tr>
<td>28</td>
<td>1 321 ± 352*</td>
<td>218 ± 64*</td>
<td>1 421 ± 340</td>
</tr>
</tbody>
</table>

*Significantly different from control at \(P < 0.005\)
incubated *in vitro* might be accounted for by the increased ability of platelets from irradiated rabbits to convert arachidonic acid into thromboxane (TXB$_2$). It concerns endogenous arachidonic acid [5] as well as the exogenous one, as demonstrated in this report.

An increase in TXB$_2$ synthesis due to whole body irradiation has also been reported to take place in lung [13] and kidney [14]. Increased pulmonary release of arachidonate metabolites is thought to be the cause of elevated TXB$_2$ concentration in urine upon whole body irradiation [15].

Increased synthesis of TXB$_2$ also is implicated in some pathological states such as angina pectoris [16], hypertension [17] and development of atherosclerosis [18, 19]. Radiation-induced vascular changes can also lead to atherosclerosis [20]. Radiation seems to shorten the life span by accelerating the natural processes of ageing [21]. Platelet aggregation has been found elevated in natural ageing [22, 23] and this can be caused by the increased synthesis of TXB$_2$ as has been shown in this report in platelets from irradiated rabbits.

The increase in TXB$_2$ synthesis following whole body irradiation might be due to increased activity of cyclooxygenase or increased release of arachidonic acid; the latter is believed to be mediated largely by activation of phospholipase A$_2$ [24]. Cyclooxygenase contains an activator site for lipid peroxides and the activity of this enzyme depends on the continuous low level of peroxides [8]. It has been suggested that oxygen radicals and peroxides such as hydrogen peroxide increase the activity of phospholipase A$_2$ [25]. This finding has an important implication in cell function, since any process which generates peroxides might activate cyclooxygenase and phospholipase. Since radiation is well known to generate oxidizing radicals and peroxides in the living matter, one can expect that the magnitude and direction of arachidonate metabolism might be changed depending on the extent of formation of these species. Radiation-induced free radicals have a relatively short life-time but accumulation of lipid peroxides has been observed by Nozoe & Ogata [26] even four weeks after exposure. This fact might account for the lack of the effect of H$_2$O$_2$ on the synthesis of TXB$_2$ by platelets from irradiated rabbits not only one day after exposure but also 4 weeks later (Table 1). The addition of H$_2$O$_2$ at the concentration of about 0.001 mM to platelets recovered from non-irradiated rabbits increased their ability to convert arachidonic acid to TXB$_2$ (Table 1) [26]. Control aorta also released an increased amount of TXB$_2$ upon addition of H$_2$O$_2$ [5]. In the presence of a higher concentration of H$_2$O$_2$ (about 1 mM) TXB$_2$
synthesis was inhibited (Fig. 10 in [5]). On the other hand, H2O2 did not stimulate synthesis of TXB2 in platelets or aorta derived from irradiated rabbits (Fig. 1) [5]. This might be due to the effect of endogenous peroxides generated by irradiation which activates cyclooxygenase and thus facilitates the formation of PGH2 by providing an additional amount of substrate for TXB2 synthesis.

Whole body irradiation did not change the uptake of radioactive arachidonic acid into platelets (Table 2) and it did not potentiate the release of radioactivity during collagen stimulated aggregation of platelets (Table 3). In addition, the release of radioactivity from platelets prelabelled with [14C]arachidonic acid was not stimulated by H2O2 (not shown). These results indicate that the increase in TXB2 synthesis is not associated with the activation of platelet phospholipase A2.

The data presented in this report support our earlier suggestion [4, 5] that the principal cause of disturbances of PGI2 and TXB2 synthesis in the whole body irradiated animals is the change in cyclooxygenase activity induced by peroxides formed in the irradiated tissues.

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REFERENCES
