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Prevention of thromboxane B_2-induced hepatocyte plasma membrane bleb formation by certain prostaglandins and a proteinase inhibitor

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Isolated hepatocytes incubated in the presence of thromboxane B_2 developed many plasma membrane blebs which are a characteristic feature of toxic or ischaemic cell injury. When hepatocytes were incubated in the presence of both thromboxane B_2 and prostacyclin, bleb formation was strongly inhibited. 16,16-Dimethyl prostaglandin E_2 had a similar effect. Hepatocytes incubated in the presence of both thromboxane B_2 and the non-lysosomal proteinase inhibitor, leupeptin, were also well protected from the formation of blebs. This implies that thromboxane B_2 is able to activate non-lysosomal proteinases which appear to attack certain cytoskeletal proteins. The data presented are consistent with thromboxane B_2 acting as an intermediary in a proposed mechanism of cell injury and death in which elevated cytosolic free Ca^{2+} levels activate phospholipase A_2 and the arachidonic acid cascade.

The formation of plasma membrane blebs appears to be a consequence of toxic or ischaemic cell injury [1,2] and has been observed not only in intact isolated cells, but also in cells in culture [3] and in tissue sections [4]. The development of plasma membrane blebs may be related to modification of the cytoskeleton because similar cell surface changes have been observed after treatment of isolated hepatocytes with agents such as cytochalasin B or D and phalloidin which are thought to act directly on the cytoskeletal proteins [5,6]. Although the molecular mechanism of bleb formation remains obscure, there is evidence that Ca^{2+} ions play an important role in controlling the assembly of the major constituent proteins of the cytoskeleton [7]. The administration of any one of a substantial number of drugs and chemicals (e.g., paracetamol [8,9], bromobenzene [10], CCl_4 [11], cystamine [12], *tert*-butyl hydroperoxide [13] and some benzoquinones [14]) causes disruption of Ca^{2+} homeostasis. Some of these substances have been shown to stimulate the formation of plasma membrane blebs [14–16] which predictably are formed in cells treated with Ca^{2+} ionophores in appropriate conditions [16].

In addition to the formation of blebs and its effect on cytoskeletal proteins, an increase in free cytosolic Ca^{2+} stimulates the activities of a number of enzymes including phospholipase A_2 (PLA_2) and non-lysosomal proteinases [12,16,17]. It is noteworthy that inhibitors of these proteinases also prevent bleb formation in hepatocytes treated with agents causing an increase in cytosolic free Ca^{2+} [16].

Activation of PLA_2 releases arachidonic acid from membrane phospholipids which in turn stimulates eicosanoid synthesis. Although enhanced eicosanoid production does not necessarily imply a role in toxicity, tissues do respond to chemical disturbance by generating eicosanoids and it is only during injury that high levels can be measured [18]. Recently it was shown that liver homogenates from mice that had received an hepatotoxic dose of paracetamol have enhanced production of thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2). Toxicity could be prevented by administration of prostacyclin (PGI_2) or a thromboxane synthetase inhibitor [19]. Work in this laboratory showed that enhanced production of TXB_2 and PGE_2 (assayed as 11-deoxy-13,14-dihydro-15-keto-11β,16ϵ-cycloprosta-

Abbreviations: TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; PGE_2, prostaglandin E_2; dmPGE_2, 16,16-dimethyl PGE_2; PGI_2, prostacyclin. NDGA, nordihydroguaiaretic acid.

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glandin E_2 (bicyclic PGE_2)) may be measured in the plasma of rats as a consequence of paracetamol-induced hepatotoxicity. The TXB_2 reached maximum concentration about 14 h after the administration of paracetamol whereas serum glutamate-pyruvate transaminase activity, which has been correlated with liver cell necrosis, showed little increase until after 20–24 h suggesting that TXB_2 formation precedes cell death. Both eicosanoid generation and hepatotoxicity are prevented by administration (i.p.) of inhibitors of phospholipase A_2, cyclooxygenase and thromboxane synthetase [20]. The same inhibitors of the arachidonic acid cascade also prevent Ca^{2+}-ionophore-induced plasma membrane blebs in isolated hepatocytes but nordihydroguaiaretic acid (NDGA), a potent and specific inhibitor of 5-lipoxygenase [21] does not protect at its ID_{50} dose level [22]. The above data obtained in this laboratory coupled with the implication of thromboxane in a number of disease states, e.g., the pathogenesis of certain ulcerative disorders of the stomach [23] indicated that some eicosanoids and thromboxane in particular may have a cytotoxic role. Recent work in this laboratory showed that incubation of isolated hepatocytes with TXB_2 results in the formation of many plasma membrane blebs, PGE_2 forms far fewer blebs but PGI_2 does not enhance bleb formation [22].

This paper describes the effects of some known cytoprotective agents (e.g., 16,16-dimethyl PGE_2, PGI_2 and a proteinase inhibitor) on TXB_2-induced bleb formation in isolated hepatocytes.

Materials and Methods

Sodium pentobarbitone (Nembutal) was supplied by Ceva Ltd., Watford, and collagenase (*Clostridium histolyticum* EC 3.4.24.3) was obtained from Boehringer Mannheim, Lewes, Sussex. All other biochemicals were purchased from Sigma Chemical Co. Ltd. (U.K.) and other reagents were of the highest grade available from Fisons, U.K.

Male CFHB rats (230–280 g) from our own colony (obtained originally from Interfauna Ltd.) were used for the isolation of hepatocytes by means of a two-stage in situ collagenase perfusion technique based on the methods of Berry and Friend [24] and of Seglen [25] as described previously [22]. Hepatocyte viability was determined by means of Trypan blue exclusion [26]. The mean ($\pm$ S.E.) viability was 93% $\pm$ 1.2 ($n = 10$). The cells were quantified using a Neubauer haemocytometer.

The extent of contamination of the hepatocyte preparations with non-parenchymal cells was quantified by staining the cells (Giemse) in permanent preparations [27]. The mean ($\pm$ S.E.) percentage of non-parenchymal cells in the preparations was 4.03% $\pm$ 0.64 ($n = 10$).

Hepatocytes ($1 \cdot 10^6$ cells/ml of Ca^{2+}-Mg^{2+} HBSS (pH 7.4) were incubated for 30 min (Figs. 1–4) in a final volume of 1 ml in a water bath at 37 °C with gentle shaking and equilibrated with 95% O_2/5% CO_2. For some experiments the time of incubation was extended for up to 4 h (Table I).

TXB_2, prostacyclin (PGI_2) and 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$) were dissolved in ethanol in amounts which ensured that the final ethanol concentration in the incubation did not exceed 1%. The solutions were stored at −20 °C for a maximum period of six weeks. Solutions of leupeptin in incubation medium were prepared immediately before use.

TXB_2, PGI_2, $dmPGE_2$ and leupeptin were added to the incubations at the times and concentrations given in the legends to the figures. After incubation 0.25 ml of the cell suspension was removed and placed in 0.75 ml of fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)) for 1 h. Another 0.25 ml of the cell suspension was removed to assess the viability of cells bearing blebs by means of Trypan blue exclusion.

The percentage of hepatocytes developing plasma membrane blebs as a result of a particular treatment was assessed after glutaraldehyde fixation by the combined use of a Neubauer haemocytometer and a Zeiss GFL phase contrast microscope with a magnification of $\times 200$ as described previously [22].

All values in the text and figures are the mean $\pm$ S.E. of n determinations. Statistical analysis of the data was performed using Student's t-test by finding the standard error of the difference between the two means and testing the size of the difference by this standard error [28]. Differences between values were considered significant if $P < 0.01$.

Results and Discussion

TXB_2, which has a physiological half life of 20–30 min, is formed by the rapid hydrolysis of the extremely labile TXA_2, which has a physiological half-life of about 30 s. TXA_2 is formed from prostaglandin hydroperoxides [29]. In the past, TXB_2 has been generally regarded as a relatively biologically inactive substance [30] but the data in Fig. 1 show that it is able to cause marked cell injury manifested as plasma membrane blebs, when incubated with isolated hepatocytes for 30 min. The effect is dose-dependent. At a concentration of 10 μM TXB_2 more than half the cells develop plasma membrane protusions. Even at nM concentrations of TXB_2 substantial bleb formation occurs and this concentration is similar to the levels of TXB_2 measured in the plasma taken from rats 16 h after the administration of an hepatotoxic dose of paracetamol [20]. At physiological concentrations, TXB_2 does not cause plasma membrane bleb formation (Fig. 1). Incubation of iso-

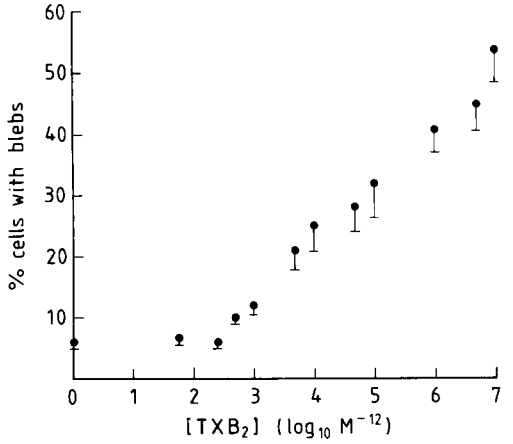

Fig. 1. Effect of different concentrations of TXB_2 on the development of plasma membrane blebs in isolated hepatocytes. Isolated hepatocytes were incubated for 30 min. either with no addition or addition of TXB_2. The mean values (± S.E.) are of data from three experiments. The probability of there being no significant difference between cells treated with TXB_2 (5.0 nM to 10 μM) and untreated cells is very small ($0.01 < P > 0.001$). The percentage viability of cells bearing blebs is expressed below as the mean ± S.E. in order of increasing concentrations of TXB_2 the first figure being that for zero TXB_2: 76 ± 3.1; 77 ± 2.7; 74 ± 2.7; 71 ± 3.5; 68 ± 4.1; 65 ± 4.0; 61 ± 3.9; 59 ± 3.8; 52 ± 3.2; 50 ± 3.8.

lated hepatocytes with higher concentrations of TXB_2 (50 μM, 100 μM) for up to 4 h shows that its effects are both time and concentration dependent (Table 1). 50 μM TXB_2 causes bleb formation in about 75% of the cells after 4 h but 100 μM TXB_2 causes the rupture of most cells after incubation for 3 h. Liver cells in vivo may be subjected to toxic levels of TXB_2 for considerable periods of time following the administration of an hepatotoxin that inactivates the cellular Ca^{2+} pumps because the cytosolic free Ca^{2+} level will remain high resulting in the persistent activation of phospholipase A_2 and the arachidonic acid cascade.

Although certain properties of TXA_2 (e.g., platelet pro-aggregating activity and vasoconstrictor activity) have been studied intensively, the possible cytotoxic role of the thromboxanes has received much less attention. However, there are reports in the literature which provide indications that the thromboxanes may have such a role. For example, thromboxane has been implicated in the pathogenesis of certain ulcerative disorders in the stomach [23] and a carbocyclic thromboxane analogue has been shown to induce the release of lysosomal enzymes from large granule fractions of liver incubates [31]. Furthermore, several diseases have now been related to an imbalance in the PGI_2-TXA_2 system. For example, platelets from patients with arterial thrombosis, deep vein thrombosis or recurrent venous thrombosis produce more prostaglandin endoperoxides and TXA_2 than normal and have a shortened survival time [32]. Platelets from rabbits made atherosclerotic by dietary manipulation [33] and from patients who have survived myocardial infarction [34] are abnormally sensitive to aggregating agents and generate more TXA_2 than controls. Blood of patients with Prinzmetal's angina [35] has been shown to have elevated levels of TXB_2 and measurement of TXB_2 levels in coronary sinus blood of patients with unstable angina indicated that TXA_2 release is associated with recent episodes of angina [36] although it was not possible to distinguish whether release is cause or effect. A number of other conditions have been described in which there is a tendency for thrombosis to develop where the protective PGI_2 level is decreased relative to the amount of thromboxane [37].

Movement of the balance in the opposite direction, i.e., a decrease in thromboxane and an increase in PGI_2 should render cells less susceptible to damage. PGI_2 has been shown to exert a cytoprotective effect against certain hepatotoxins, e.g., paracetamol [19], CCl_4 [38] and this would also be consistent with the idea that TXB_2 acts as an intermediary in Ca^{2+}-induced cell injury and death. The effect of PGI_2 on TXB_2-induced

TABLE I

Effects of time of exposure of isolated hepatocytes to high concentrations of TXB_2

Incubation conditions as described in Materials and Methods. The mean values (± S.E.) are of data from three experiments. The probability of there being no significant difference between cells treated with TXB_2 (50 or 100 μM) and untreated cells is very small ($0.1 > P > 0.001$).

[TXB_2] (μM)	Time of incubation (h)									
	0.5		1		2		3		4	
	viability [a]	blebs [b]	viability [a]	blebs [b]	viability [a]	blebs [b]	viability [a]	blebs [b]	viability [a]	blebs [b]
0	76.0 ± 3.1	6.0 ± 1.2	69.0 ± 3.1	18.7 ± 3.3	65.0 ± 5.0	29.3 ± 1.5	57.7 ± 2.3	33.7 ± 0.9	52.0 ± 1.5	36.0 ± 1.0
50	43.0 ± 2.3	57.3 ± 2.7	31.7 ± 1.2	63.3 ± 2.3	25.7 ± 2.9	70.3 ± 3.0	22.3 ± 1.5	73.0 ± 2.5	21.7 ± 2.0	74.3 ± 1.9
100	40.0 ± 2.0	61.0 ± 1.7	26.7 ± 1.8	68.0 ± 1.2	16.3 ± 3.4	74.3 ± 2.0	14.3 ± 0.9	14.0 ± 0.6 (Other cells ruptured)	11.0 ± 1.7	10.0 ± 0.6 (Other cells ruptured)

[a] % of cells with blebs which exclude Trypan blue.
[b] % of cells with blebs.

bleb formation in isolated hepatocytes has been investigated in this laboratory and the results (Fig. 2) show a strong cytoprotective effect. The precise mechanism of this effect remains obscure.

$dmPGE_2$ has also been shown to protect animals against the effects of a number of hepatotoxins which cause elevation of the cytosolic free Ca^{2+} level, e.g., CCl_4 [38,39], bromobenzene [40] and paracetamol [41]. The site and mechanism of action of $dmPGE_2$ has not been described. The effect of $dmPGE_2$ on TXB_2-induced plasma membrane bleb formation in isolated hepatocytes has been investigated and shown to provide cytoprotection (Fig. 3). Cytoprotection is not afforded by all prostaglandins, however, for example, PGD_2 fails to prevent TXB_2 (10 μM)-induced plasma membrane bleb formation in isolated hepatocytes (data not shown).

One further category of inhibitor of Ca^{2+}-plasma membrane bleb formation is of particular interest since its site of action appears to be the cytoskeleton. Isolated hepatocytes exposed to agents (e.g., Ca^{2+} ionophore A23187) which cause an increase in cytosolic free Ca^{2+} concentration and consequent formation of plasma membrane blebs leading to loss of cell viability also show stimulation of intracellular proteolysis [16]. Both bleb formation and cell death are prevented when inhibitors of Ca^{2+}-activated neutral proteinases such as leupeptin or antipain are included in the incubation medium [16]. Since bleb formation is stimulated by

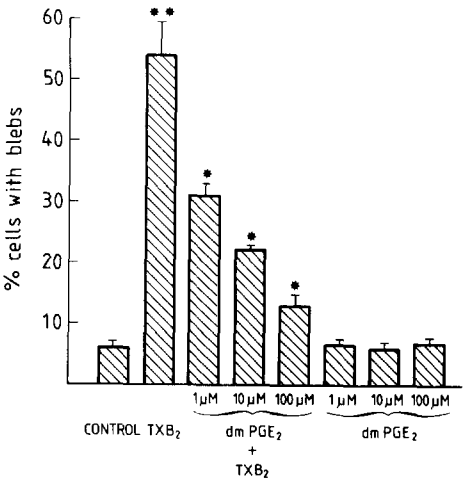

Fig. 3. Effect of $dmPGE_2$ on TXB_2-induced plasma membrane bleb formation in isolated hepatocytes. Isolated hepatocytes, preincubated with $dmPGE_2$ for 15 min, were then incubated for 30 min either with $dmPGE_3$ alone or with $dmPGE_2$ and TXB_2 (10 μM). A separate incubate contained hepatocytes and TXB_2 (10 μM) alone and a control had neither $dmPGE_2$ nor TXB_2. The mean values ($\pm$S.E.) are of data from three experiments. The probability of there being no significant difference between cells treated with TXB_2 alone (**) and cells treated with TXB_2 and $dmPGE_2$ (10 and 100 μM) (*) is very small ($0.01 > P > 0.001$). The percentage viability of cells bearing blebs is expressed below as the mean$\pm$S.E. in the order of the experiments represented by bars on the histogram from left to right: 76 ± 3.1; 50 ± 3.8; 54 ± 3.8; 58 ± 41; 63 ± 41; 69 ± 3.6; 68 ± 4.1; 67 ± 4.5).

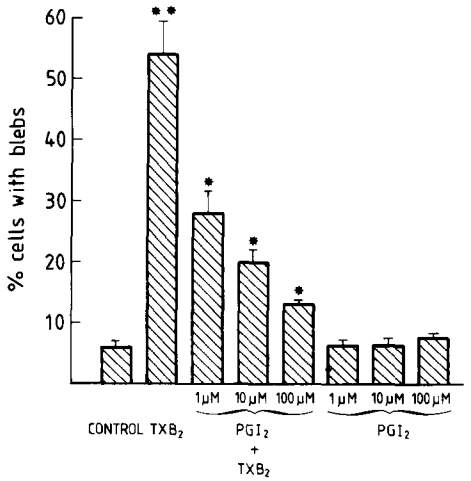

Fig. 2. Effect of PGI_2 on TXB_2-induced plasma membrane bleb formation in isolated hepatocytes. Isolated hepatocytes preincubated with PGI_2 for 15 min were then incubated for 30 min either with PGI_2 alone or with PGI_2 and TXB_2 (10 μM). A separate incubate contained hepatocytes and TXB_2 (10 μM) alone and a control had neither PGI_2 nor TXB_2. The mean values ($\pm$S.E.) are of data from three experiments. The probability of there being no significant difference between cells treated with TXB_2 alone (**) and cells treated with TXB_2 and PGI_2 (10 and 100 μM) (*) is very small ($0.01 > P > 0.001$). The percentage viability of cells bearing blebs is expressed below as the mean$\pm$S.E. in the order of the experiments represented by bars on the histogram from left to right: 76 ± 3.1; 50 ± 3.8; 55 ± 3.5; 59 ± 3.8; 63 ± 3.5; 70 ± 3.4; 70 ± 3.8; 68 ± 3.5.

agents such as cytochalasin B or D and phalloidin which are thought to act directly on the cytoskeletal proteins [5,6] it was suggested that cytoskeletal proteins might be the targets for the non-lysosomal proteinases [16]. More recent work on isolated human platelets exposed to menadione, which is thought to cause cell damage via oxidative and Ca^{2+}-dependent mechanisms, has demonstrated a number of changes in the cytoskeletal proteins. One of the changes observed, a decrease in the amount of actin binding protein, was prevented by including leupeptin in the incubation medium, indicating that this protein may be attacked by non-lysosomal proteinases [42]. Activation of these proteinases, as indicated by cytoskeletal changes and plasma membrane bleb formation, also occurs as a consequence of the treatment of isolated hepatocytes with oxidants or alkylators [43,44]. However, plasma membrane bleb formation in isolated hepatocytes treated solely with Ca^{2+} ionophore A23187 suggests that enhanced cytosolic levels of Ca^{2+} alone can also activate the proteinases [16,22,45]. On the basis of the proposed mechanism of Ca^{2+}-activated cytotoxicity [20,22] certain products of the arachidonic acid cascade, notably TXB_2, should also stimulate proteolysis of one or more cytoskeletal proteins and this proteolytic activity should be inhibited by leupeptin.

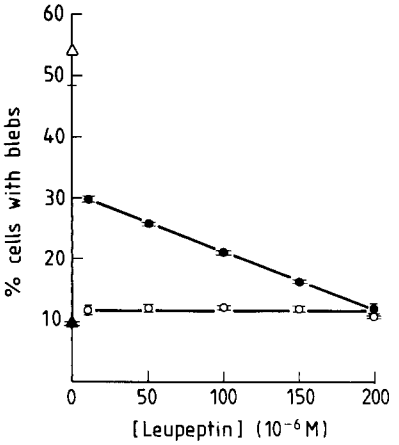

Fig. 4. Effect of leupeptin on TXB_2-induced plasma membrane bleb formation in isolated hepatocytes. Isolated hepatocytes, preincubated with leupeptin for 30 min, were then incubated for 30 min either with leupeptin alone (○) or with leupeptin and TXB_2 (10 μM) (●). A separate incubate contained hepatocytes and TXB_2 (10 μM) alone (△) and a control had neither leupeptin nor TXB_2 (▲). The mean values (±S.E.) are of data from three experiments. The probability of there being no significant difference between cells treated with TXB_2 alone and cells treated with TXB_2 (10 μM) and leupeptin (50 μM to 200 μM) is very small. ($0.01 > P > 0.001$). The percentage viabilities of cells bearing blebs are expressed below as the mean±S.E. in the following order: control (no addition), TXB_2 alone, TXB_2 and increasing concentrations of leupeptin (*) and increasing concentrations of leupeptin alone ($^+$): 75±2.3; 50±3.8; 55±2.0 *; 57±2.3 *; 59±1.8 *; 62±1.8 *; 64±2.3 *; 65±2.2 $^+$; 66±1.9 $^+$; 66±2.1 $^+$; 65±2.2 $^+$; 64±2.3 $^+$.

The effect of leupeptin on TXB_2-induced bleb formation in isolated hepatocytes has therefore been investigated and the results of these experiments (Fig. 4) show a strong cytoprotective effect. This implies that TXB_2 activates non-lysosomal proteinases but the mechanism of this process remains obscure. This observation adds further support to the proposed role for TXB_2 as an intermediary in Ca^{2+}-activated cell injury and death [20,22].

In conclusion, the ability of TXB_2 to cause plasma membrane bleb formation in isolated hepatocytes even at nM concentrations, the inhibitory effects on this process of PGI_2, $dmPGE_2$ and leupeptin and the cytodestructive properties of TXB_2 are all consistent with the proposed role for TXB_2 in a mechanism of toxicity based on Ca^{2+}-activation of phospholipase A_2 and the arachidonic acid cascade [20,22].

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