EFFECT OF PARACETAMOL ON MITOCHONDRIAL MEMBRANE FUNCTION IN RAT LIVER SLICES

WILHERMINA M. A. NAZARETH, JASWINDER K. SETHI and ANDRE E. M. MCLEAN*
Laboratory of Toxicology, Department of Clinical Pharmacology, University College London, 5 University Street, London WC1E 6JJ, U.K.

(Received 29 October 1990; accepted 1 May 1991)

Paracetamol (N-acetyl-p-aminophenol, acetaminophen) in overdosage leads to liver damage in man and many species of animals [1-4]. Paracetamol levels in the peripheral blood can reach a concentration of more than 3 mM for 4 hr after an overdose of 20 g or more [1]. The portal blood will presumably reach near saturation, especially in the first few hours. The initial phases of paracetamol metabolism involve the formation of glucuronide and sulphate conjugates, which are safe excretable products [5]. A small fraction of the paracetamol is also oxidized in the cytochrome P450 system to form a reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) which combines with glutathione. However, as the glutathione levels fall, further reactive metabolite attacks cell components which leads to final cell destruction [6, 7].

The mechanism of the late stages of cell toxicity remains unestablished, but several ideas have been proposed. They involve covalent binding of the reactive metabolite to essential hepatic proteins [8, 9], oxidation of macromolecules especially cation pumping ATPases [10] and alteration in the electron transport chain of the cell [11]. Histochemical and ultrastructural studies have also shown that the late stages of liver necrosis are associated with structural damage to subcellular components including the mitochondria [12, 13]. Although the exact mechanism is not known, there is general agreement that failure of intracellular calcium ion regulation is a common late and perhaps reversible final stage in the process of cell necrosis [14]. Mitochondria have been implicated as being a potentially sensitive target for damage and their integrity can be followed by a mitochondrial membrane probe, such as TPMP+ (triphenylmethylphosphonium) [15, 16].

TPMP is a penetrating cation which accumulates predominately in the mitochondrial matrix because this has a high negative potential relative to the cytoplasm [15].

Loss of potential causes the release of TPMP+ from the organelles and subsequently from the cells. Using a similar approach, the plasma membrane potential can be monitored by measuring the distribution of a permeant anion SCN-, which is excluded from cells maintaining an intact potential [15, 16]. A fall in the plasma membrane potential can therefore be followed by an increase in the uptake of SCN- by the cell.

The aim of this study was to investigate the involvement of the plasma and mitochondrial membrane potential in paracetamol toxicity using liver slices as the in-vitro model.

The model developed in this laboratory allows investigation of the two phases, paracetamol exposure and subsequent development of cell injury in paracetamol toxicity [11, 17]. Phenobarbital pretreated rats were used because they show a great sensitivity to paracetamol poisoning.

In many respects, liver slices behave in a similar fashion to the liver in the whole animal. The slice cells are not permeable to molecules such as EDTA, which are excluded in vivo but which enter freely into suspended hepatocytes. Slices maintain the inter-cell adhesion and communication in contrast to isolated hepatocytes which have their surfaces stripped by exposure to proteases and environmental changes which force major change in morphology and activity. Liver slices from phenobarbital pretreated rats show a marked loss of potassium, gain in water content and leakage of soluble enzymes such as lactate dehydrogenase in the 4 hr following 2 hr of exposure to paracetamol. The slices from rats
Table 1. Effect of Valinomycin and CCCP on the intracellular retention levels of the membrane potential indicators, TPMP⁺ and SCN⁻ in rat liver slices

<table>
<thead>
<tr>
<th>Additions</th>
<th>[³H]TPMP⁺ dpm/mg slice wt</th>
<th>([% retention relative to control] relative control)</th>
<th>[¹⁴C]SCN⁻ dpm/mg slice wt</th>
<th>([% retention relative to control] relative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium (Low K⁺) Control</td>
<td>4933 ± 266</td>
<td>100</td>
<td>862 ± 68</td>
<td>100</td>
</tr>
<tr>
<td>+ CCCP (80 μM)</td>
<td>1297 ± 51*</td>
<td>26</td>
<td>879 ± 76</td>
<td>102</td>
</tr>
<tr>
<td>+ Valinomycin (10 μg/mL)</td>
<td>1485 ± 64*</td>
<td>30</td>
<td>940 ± 81</td>
<td>109</td>
</tr>
<tr>
<td>High K⁺ medium Control</td>
<td>4341 ± 174</td>
<td>88</td>
<td>1402 ± 73*</td>
<td>162</td>
</tr>
<tr>
<td>+ Valinomycin (10 μg/mL)</td>
<td>1365 ± 48*</td>
<td>72</td>
<td>1630 ± 84*</td>
<td>198</td>
</tr>
</tbody>
</table>

Liver slices were incubated as described in Materials and Methods and exposed to the inhibitors for 30 min. Three flasks were used per condition in each experiment. Results are means ± SD for three separate experiments. “Low K⁺ medium” (ordinary Ringer medium, 7 mM K⁺), “High K⁺ medium” (Ringer medium containing all potassium salts instead of sodium salts, 150 mM K⁺). * Indicates significant difference from control values (P < 0.05).

not given phenobarbital are far less sensitive to paracetamol and can be used as controls in which paracetamol exposure does not lead to cell injury.

MATERIALS AND METHODS

Animals

Male Wistar rats (OLAC, Bicester, U.K.) weighing 120–150 g were fed stock pellets (SDS, Witham, U.K.) and given sodium phenobarbitone as a solution containing 1 mg/mL as a sole source of drinking water for at least 5 days, where increased cytochrome P450 levels were required [18]. Rats were killed by exsanguination under fentanyl citrate (0.105 mg/kg, i.m.) and diazepam (2.5 mg/kg, i.p.) anaesthesia (Janssen, Wantage, U.K.). The liver was rapidly removed and the liver slices of 0.3 mm thickness or less were cut by hand on a Stadie-Riggs stage with a long razor blade (A. H. Thomas Co., Philadelphia, PA, U.S.A.).

Slices weighing about 100–120 mg were put into 25 mL Erlenmeyer flasks containing 4 mL of Ringer solution with the following composition: 125 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM NaH₂PO₄, 1 mM CaCl₂, 10 mM glucose and 15 mM Hepes buffer, pH 7.4 at 37° as described previously [17]. The slices were put into the Ringer solution at room temperature and the experiment started by placing the flasks into an incubator bath at 37° under oxygen with shaking (90 strokes/min).

Chemicals

Paracetamol, carbonyl cyanide m-chlorophenylhydrazone (CCCP), valinomycin, cysteine and potassium [¹⁴C]thiocyanate (3.9 mCi/mmol) were purchased from the Sigma Chemical Co. (Poole, U.K.). [methyl-³H]Methyltriphenylphosphonium iodide (52.8 Ci/mmol) was purchased from Du-Pont Ltd, New England Nuclear Products Division (Stevenage, U.K.). All other reagents were of analytical grade and were bought from Sigma or BDH Ltd (Poole, U.K.). SKF-525A was a gift from Smith Kline and French (Welwyn Garden City, U.K.).

Measurement of changes in mitochondrial membrane potential

Changes in the mitochondrial potential were assessed by determining the intracellular [³H]TPMP⁺ remaining in the slice after treatment [19]. A known mitochondrial uncoupler CCCP and valinomycin a K⁺ ionophore were used to check the validity of the system [15, 20]. High K⁺ medium was prepared by replacing all the sodium salts of the Ringer medium with potassium salts [15].

Method I. The slices were initially loaded with TPMP⁺ by incubating in Ringer medium containing 0.25 μCi/mL [³H]TPMP⁺ for 45 min. These loaded slices were then transferred to fresh Ringer medium containing, where appropriate, 10 mM paracetamol. In experiments where the time course was greater than 2 hr, the slices were taken out of the flasks and re-incubated in fresh Ringer medium. At the end of the incubations, the slices were digested in 1 M NaOH 2 mL at 37° for 2 hr. The intracellular [³H]-TPMP⁺ was measured in 200 μL aliquots neutralized with 100 μL of glacial acetic acid by liquid scintillation counting (LSC). The quantity of cell protein was determined by the method of Lowry et al. [21], using crystalline bovine serum albumin (fraction V) as standard. Control and paracetamol loaded treated slices were taken at each time point, and radioactive (dpm/mg protein) compared.

Method II. In some experiments the slices were loaded using 0.25 or 0.05 μCi/mL [³H]TPMP⁺ with 0.125 μM cold carrier TPMP⁺. The concentration of the [³H]TPMP⁺ in the incubation medium and in the slices was measured and used to determine the intracellular to extracellular distribution ratio i.e. (dpm/μL slice water)/(dpm/μL incubation medium). Cell water content was assumed to be five times the protein content. The results could equally have been
Paracetamol and mitochondrial membrane

Measurement of changes in plasma membrane potential

Changes in the plasma membrane potential were monitored by the increase in the uptake of $[^{14}\text{C}]\text{SCN}^-$ by the slice. For verification of the procedure, the slices were incubated with 10 µg/mL valinomycin in low or high K$^+$ medium for 30 min, before addition of $[^{14}\text{C}]\text{SCN}^-$. Using 10 mM paracetamol, the slices were incubated for 2 hr. After exposure, the slices were transferred into fresh Ringer medium containing 0.5 µCi/mL $[^{14}\text{C}]\text{SCN}^-$ and allowed to incubate for various time periods. At the end of the incubation, the slices were digested as described previously and an aliquot measured by LSC.

Measurement of enzyme leakage (LDH) and glutathione

Injury was assessed by measuring leakage of lactate dehydrogenase from the slice into the incubation medium. Lactate dehydrogenase activity (LDH) released was expressed as a percentage of the amount of enzyme activity originally present in the flask, based on the original slice weight and LDH assays on homogenates of liver slices sampled before incubation [17, 22].

Glutathione (as acid soluble SH) was measured by the method of Ellman improved by Beutler et al. [23].

Statistical analysis

Significance of the differences were determined using Student's t-test with a P < 0.05 being taken as indicating significant difference.

RESULTS

Intracellular levels of indicators of membrane potential

The suitability of TPMP$^+$ and SCN$^-$ as indicators of changes in membrane potentials for suspensions and monolayer cultures of hepatocytes has been previously demonstrated [15, 16, 19]. Since slices were used in this study, we verified the applicability of the probes under these conditions. Uptake of TPMP$^+$ was essentially complete after 30 min when the ratio of more than 30:1, intracellular to extracellular, had been reached. Further incubation times did not lead to increased uptake and tetraphenylboron did not affect uptake.

The slices were treated with known uncouplers at concentrations stated in Table 1. After 30 min of incubation with an excess of the mitochondria uncoupler CCCP more than 70% of the accumulated TPMP$^+$ was released. Valinomycin also caused a 70% drop in TPMP$^+$. Incubation of liver slices in a high K$^+$ medium caused a relatively small decrease in the intracellular level of the mitochondrial marker (12%). Non-specific binding to the slice was demonstrated by initially freezing the slice for an hour before undertaking the experiment. Less than 4% of the marker was detectable in the intracellular fraction (data not shown). The effects of valinomycin remained similar in the high K$^+$ medium, indicating that in our model using slices the release of TPMP$^+$ mainly reflects a loss of mitochondrial membrane potential as has been previously shown for hepatocytes [15, 16, 19].

The accumulation of SCN$^-$ under the same condition was also measured. In a high K$^+$ medium, the intracellular level of SCN$^-$ increased, in agreement with the expected depolarization of the plasma membrane. A further rise was observed when valinomycin was added, probably reflecting a higher uptake of K$^+$ by the permeable high K$^+$ slices. This data indicates that the SCN$^-$ probe responds readily to a change in the plasma membrane and is also suitable in our model.

Exposure of loaded slices to 10 mM paracetamol led to a consistent decrease (28%) in TPMP$^+$ retention from 30 min of exposure onwards (Fig. 1). Slices exposed to 10 mM paracetamol for 2 hr followed by removal of paracetamol gave the same result (data not shown). For the first 3 hr of incubation there was no significant evidence of cell injury in the form of increased enzyme leakage.
Time (hr)

Fig. 3. Effect of paracetamol on the intracellular accumulation of $[^{14}C]SCN^-$ following 10 mM paracetamol exposure (2 hr). Slices were transferred to fresh medium containing $[^{14}C]SCN^-$ and accumulation monitored relative to control slices taken at each time point. Each point represents the mean ± SD of at least three experiments performed in triplicate. * P < 0.05 as compared to the control.

(LDH). Both control and paracetamol treated slices showed the usual slight loss of enzyme activity to the medium. Only after 4 hr did major signs of cell injury appear Fig. 2) as has been described previously for enzyme leakage and potassium loss [17]. At concentrations below 10 mM paracetamol the decrease in mitochondrial membrane potential was reduced proportionally to dose, above 10 mM paracetamol there was no extra effect (data not shown).

Figure 1 shows an early time course where changes in mitochondrial membrane potential were significant after 30 min, after this the change was not time dependent.

Figure 3 shows relative change in the plasma membrane monitored by an increase in the uptake of $[^{14}C]SCN^-$ by the slice. Significant changes only occurred after 5 hr, by which time massive enzyme leakage was taking place. The slight deficit at an earlier time point was not significant.

Addition of 0.5 mM cysteine or 30 μM SKF 525A to the system did not prevent the reduced mitochondrial membrane potential (Fig. 4). When slices from an uninduced rat were used changes in the membrane potential were still detectable (Tables 2 and 3).

The addition of carrier TPMP$^+$ to the loading conditions and expression of the results as a ratio of intra- to extracellular concentrations (Method II) led to a more stable and robust system. This also showed the drop in TPMP$^+$ retention when paracetamol was added to the system, using slices from control rats not pre-induced with phenobarbital (Table 3).

DISCUSSION

Our present results demonstrate that paracetamol elicits a direct and early change in the membrane potential of the mitochondria. Studies with cadmium, mercuric chloride and cyanide using hepatocytes have all demonstrated that early mitochondria depolarization is an initial injury which is followed by ATP depletion and cell death [19, 20, 24]. Alteration in mitochondrial function was monitored through the detection of changes in the mitochondria membrane potential. The penetrating cation TPMP has been shown to be a suitable indicator in studies using hepatocytes in suspension and in monolayer cultures [15, 16, 19]. In this study we have demonstrated that TPMP is also suitable for liver slices, more than 70% of TPMP$^+$ retention

Fig. 4. Effect of (30 μM) SKF 525A and (0.5 mM) cysteine added together with 10 mM paracetamol for 30 min. Loss of intracellular $[^{3}H]TPMP^+$ determined as described in Materials and Methods. Each bar represents the mean ± SD of at least three experiments performed in triplicate. * P < 0.05 as compared to control groups.
that the first significant efflux of the marker was not due to plasma membrane potential change we observed until 5 hr after exposure, at which time there is a massive release of LDH from the slice.

Increase of GSH concentration by the addition of cysteine did not alter TPMP⁺ release indicating that the early effect was not GSH dependent. By the slice was dependent upon the mitochondrial membrane potential (Table 1). We have observed that the first significant efflux of the marker was after 30 min exposure to 10 mM paracetamol indicating that within this short period, paracetamol induced a collapse of the mitochondrial membrane potential (Tables 2 and 3). It is established that the mitochondrial membrane potential depends upon the H⁺ gradient generated by the electron-transport chain [20]. A collapse in the electrochemical gradient may occur via an alteration in the activity of the respiratory chain or in the citric acid cycle which supplies NADH.

In order to investigate the possible consequences of mitochondrial attack and to establish that the change in mitochondrial membrane potential was not due to plasma membrane potential change we monitored changes in the plasma membrane potential. After verification of the use of the penetrating probe SCN⁻ with slices, the effect of paracetamol on the uptake of SCN⁻ was evaluated. No significant alteration in SCN⁻ penetration was observed until 5 hr after exposure, at which time there is a massive release of LDH from the slice.

Increase of GSH concentration by the addition of cysteine did not alter TPMP⁺ release indicating that the early effect was not GSH dependent.

Table 3. Effect of 10 mM Paracetamol on the uptake of SCN⁻ with slices from an induced and uninduced rat

<table>
<thead>
<tr>
<th>[3H]TPMP+ concn for loading</th>
<th>Distribution ratio after incubation</th>
<th>% Change relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Paracetamol</td>
<td></td>
</tr>
<tr>
<td>0.125 µM</td>
<td>118.4 ± 5.1</td>
<td>57.3 ± 14.3*</td>
</tr>
<tr>
<td>0.25 µM</td>
<td>130.3 ± 2.8</td>
<td>64.1 ± 13.3*</td>
</tr>
</tbody>
</table>

Slices were pre-loaded with 0.05 or 0.25 µCi/mL [3H]-TPMP⁺ in the presence of 0.125 µM cold carrier for 1 hr. They were then incubated with and without paracetamol for a further 30 min in fresh medium without added TPMP⁺, under the standard conditions as described in Materials and Methods. The distribution ratio equals the amount of [3H]TPMP⁺ per unit volume of intracellular water (i.e. per mg protein X 5)/extracellular concentration. The values represent the means (±SD) of two separate experiments each performed in triplicate. * P < 0.05 as compared to control.

by the slice was dependent upon the mitochondrial membrane potential (Table 1). We have observed that the first significant efflux of the marker was after 30 min exposure to 10 mM paracetamol indicating that within this short period, paracetamol induced a collapse of the mitochondrial membrane potential (Tables 2 and 3). It is established that the mitochondrial membrane potential depends upon the H⁺ gradient generated by the electron-transport chain [20]. A collapse in the electrochemical gradient may occur via an alteration in the activity of the respiratory chain or in the citric acid cycle which supplies NADH.

In order to investigate the possible consequences of mitochondrial attack and to establish that the change in mitochondrial membrane potential was not due to plasma membrane potential change we monitored changes in the plasma membrane potential. After verification of the use of the penetrating probe SCN⁻ with slices, the effect of paracetamol on the uptake of SCN⁻ was evaluated. No significant alteration in SCN⁻ penetration was observed until 5 hr after exposure, at which time there is a massive release of LDH from the slice.

Increase of GSH concentration by the addition of cysteine did not alter TPMP⁺ release indicating that the early effect was not GSH dependent.

Table 3. Effect of 10 mM Paracetamol on the distribution of [3H]TPMP⁺ in liver slices from control rats

<table>
<thead>
<tr>
<th>[3H]TPMP⁺ concn for loading</th>
<th>Distribution ratio after incubation</th>
<th>% Change relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Paracetamol</td>
<td></td>
</tr>
<tr>
<td>0.125 µM</td>
<td>118.4 ± 5.1</td>
<td>57.3 ± 14.3*</td>
</tr>
<tr>
<td>0.25 µM</td>
<td>130.3 ± 2.8</td>
<td>64.1 ± 13.3*</td>
</tr>
</tbody>
</table>

Slices were pre-loaded with 0.05 or 0.25 µCi/mL [3H]-TPMP⁺ in the presence of 0.125 µM cold carrier for 1 hr. They were then incubated with and without paracetamol for a further 30 min in fresh medium without added TPMP⁺, under the standard conditions as described in Materials and Methods. The distribution ratio equals the amount of [3H]TPMP⁺ per unit volume of intracellular water (i.e. per mg protein X 5)/extracellular concentration. The values represent the means (±SD) of two separate experiments each performed in triplicate. * P < 0.05 as compared to control.

paracetamol on the mitochondrial membrane potential. This was confirmed by using an uninduced rat for the preparation of the slices. Rats which are not treated with any inducer are highly resistant to paracetamol toxicity in vivo or in vitro [26], since the background cytochrome P450 activity is insufficient to elicit damage readily in this species [17]. This suggests that the formation of the active metabolite NAPQI is not necessary to cause change in mitochondrial membrane potential. Equally the observation that the paracetamol causes a mitochondrial defect in liver slices from uninduced rats shows that the mitochondrial defect on its own is not sufficient to cause cell death. It is notable that further incubation with paracetamol and progression towards cell injury does not increase the mitochondrial lesion. Direct cytotoxic effects of paracetamol have previously been reported using V79 Chinese hamster cells [25, 27]. This study therefore suggests that early paracetamol damage involves mitochondria. A decline in ATP levels can be critical, a necessary, but not sufficient event for the development of cell damage [28-31]. Low ATP could lead to reduced calcium ATPase activity and thus a failure to repair any injury caused by reactive metabolites, such as an increase in intracellular calcium, so leading to final cell death.

Acknowledgements—This work was partially supported by grants from the Sir Halley Stewart and Welcome Trusts.

REFERENCES


