Comparisons of protein changes in human and rodent hepatocytes induced by the rat-specific carcinogen, methapyrilene

There is a growing concern that the rodent biossay may not always serve as an appropriate model to assess the carcinogenic risk for humans exposed to certain compounds. Mechanistic research that examines the effects of a compound in rodent and man could help in the interpretation of bioassay results. This paper reports a novel use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) technology to assess similarities and differences in the response of rodents and humans to the rat-specific hepatocarcinogen, methapyrilene (MP). A sequential examination of rodent and human hepatic proteins was conducted following in vivo exposure of rats and mice and in vitro exposure of rat, mouse, and human hepatocytes to MP. Results showed that covalent modifications observed in rats and mice in vivo were duplicated both qualitatively and quantitatively in the corresponding in vitro systems and that these modifications correlated with carcinogenic susceptibility. Covalent modifications in human hepatocytes were minimal despite exposure to concentrations of MP that were 6-fold higher than those used in rodent hepatocytes. These studies suggest that in the case of MP the rat is not the most appropriate model for assessing the human situation. Furthermore, these data show that in vitro-in vivo comparisons based on 2-D PAGE may provide adjunctive information for extrapolating rodent toxicity/bioassay results to human risk assessment.

1 Introduction

In 1976, the chronic rodent bioassay was established as the standard method to establish the carcinogenic potential of a compound [1]. Today, there is a growing concern that for many compounds the rodent bioassay and/or the means by which it is conducted, using maximum tolerated doses, may not serve as an appropriate model to assess the carcinogenic risk to humans [2-6]. This is especially true for compounds that do not appear to interact directly with DNA. However, since it is not likely that the rodent bioassay will be replaced in the foreseeable future, it will continue to be necessary to establish the relevance of bioassay results to humans through additional mechanistic and comparative studies.

We report here a novel application of two-dimensional polyacrylamide gel technology (2-D PAGE) to studies aimed at elucidating and comparing the effects of the carcinogen methapyrilene (MP) and rodent and human hepatic proteins in vitro. The refinement of 2-D PAGE coupled with computerized data analysis has allowed simultaneous resolution, cataloguing and quantification of over 1000 major hepatic proteins. This can be compared to standard experimental protocols where only one or a few endpoints are examined at a time. Applied to toxicology, 2-D PAGE provides a broad view of the effects of a compound on the molecular anatomy of the cell or tissue of interest [7] and when proteins can be identified. 2-D PAGE can provide mechanistic insight into the changes induced by xenobiotics.

2-D PAGE has been used to analyze changes in hepatic proteins caused by carbon tetrachloride, cyclohexamide, ibuprofen [8] alcohol [9] and Aroclor 1254 [10] and has been used in in vitro systems to determine compound-induced changes in protein-profiles [11-13]. More recently, changes in protein-profiles during hepatocarcinogenesis [14] and liver regeneration [15] have been characterized. Using protein analysis by 2-D PAGE to make cross-species and in vivo-in vitro comparisons has remained largely unexplored.

Methapyrilene is an antihistamine (H1 antagonist) that was used in various medications for over 20 years. In 1980, MP was shown to cause a 100% incidence of hepatocellular carcinomas in F-344 rats [16] and it was subsequently withdrawn from the market. Despite a strong response in the rat, no carcinogenic response has been observed in the mouse [17], hamster or guinea pig [18]. In addition, morphologic changes observed in the rat, including mitochondrial alterations [19], were not observed in other species [20]. These results suggest that MP is a rat-specific carcinogen. MP is not genotoxic in routine genetic toxicity tests and does not appear to bind to DNA [21,22]. Despite positive responses in some specialized tests [23,24] and a weak response in the Ames assay [25], MP is considered a nongenotoxic carcinogen [26] that may operate by increasing cell replication [27] and/or by other mechanisms that do not involve direct interaction with DNA.

Utilizing 2-D PAGE technology, studies from these laboratories have shown that MP treatment results in the covalent modification of four specific mitochondrial proteins that include the -subunit of F, ATPase (Mitcon: 1), two mitochondrial matrix proteins (Mitcons: 2 and 3) and carbamoyl phosphate synthetase [28]. While it is not clear whether this modification results from MP or a metabolite or from a cellular process such as glycosylation or acetylation, etc., modifying the subunit of F, ATPase does not appear to alter its function, since the oxidative function of mi-
tochondria from treated rats is not impaired [29]. Compari-
sions between 2-D gel patterns obtained from rats and mice
with MP in vivo revealed a correlation between the
susceptibility of each species to the carcinogenic effects of
MP and the extent of covalent modification of mitochon-
drial proteins: 1-3 [28]. Using these endpoints, we have ex-
tended these studies to examine the effects of MP on rod-
ent hepatocytes to determine if and to what degree these
modifications occurred in vitro.

2 Materials and methods

2.1 Hepatocyte cultures and exposures

Primary hepatocytes were obtained from male F-344 rats,
6 weeks of age, and male CD-1 mice, 6 weeks of age, using
collagenase perfusion methods previously described [30].
Rodent hepatocytes were plated on 60 mm collagen-coated
plastic culture dishes at a density of ~2.2 X 10^6 cells/plate
in plating medium that consisted of Leibovitz's L-15 med-
ium, 28 mm N-(2-hydroxyethyl) piperazine-N'(2-ethane-
sulfonic acid) (HEPES), 10 μM hydrocortisone, 10% fetal
bovine serum (heat inactivated), 50 μg/mL gentamicin sul-
fate, 100 μg/mL kanamycin sulfate, 0.02 units/mL insulin,
and 8.3% tryptose phosphate broth. Cells were incubated at
37°C without CO2. The rodent hepatocytes were allowed to
attach for 4 h and then washed with HEPES buffer (2.4 mm
HEPES buffer, 140 mm NaCl, 6.7 mm KCl, 1.2 mm CaCl2, X
H2O, 10 mg/mL Phenol Red, pH 7.4) + 4 μL/mL DMSO ± 90 μg/mL MP.
After 6 or 48 h the cells were solubilized in 0.5 mL 2-D PAGE
solubilizing solution, which consisted of 9 M urea, 2% Nonidet P-40 (NP-40), 0.5% dithiothreitol, 2% pH 9-11
carrier ampholytes (LK), then centrifuged at 100,000 X g
for 30 min (TL-100 Beckman, TLA-100.3 rotor, 45,000 rpm)
[28] and stored at -70°C until 2-D PAGE analysis could be
conducted.

2.2 2-D PAGE analysis

Acidic and neutral soluble proteins were resolved based on
molecular weight and isoelectric point using sodium dode-
cyl sulfate-denaturing polyacrylamide gels as previously de-
scribed [28]. Proteins were separated using the 20 X 25 cm

![Figure 1](image-url)

*Figure 1.* Photograph of Coomasie Blue-stained 2-D PAGE pat-
terns of hepatic proteins from (a) methapyriline-treated (1000
ppm for 1 week) and (b) control rat. Molecular weights are in kilo-
daltons (kDa). The isoelectric point has been estimated on stand-
ards of carbamylated creatinine phosphokinase (CPK pI) [34] or p/
calculated (pI Calc) from CPK charge standard positions. Coval-
ent modifications present in treated samples extend from right
to left with unmodified protein on the right. Data analyses were per-
formed using a Kepler® software system (Large Scale Biology.
Corp.).
ISO-DALT system [33], then fixed and stained using colloidal Coomassie Brilliant Blue G-250 as previously described [28]. Following staining, each stained gel was digitized in red light at 120 micron resolution using either a Molecular Dynamics laser scanner or a Eikonix 78/99 charge coupled device (CCD) scanner. Images were processed using the Kepler software system (Large Scale Biology Corp). The extent of modification of mitochondrial proteins, previously identified using cell fractionation techniques [34], was determined based on the relative abundance of the various charge-modified forms of the parent protein (Fig. 1) and was expressed as a charge modification index (CMI) (Fig. 2). Mitcons:1–3, previously identified as F$_{1}$ ATPase, and two mitochondrial matrix proteins were selected as marker proteins with which to calculate the CMI. Overall changes in gene expression caused by MP in the various test systems were also determined by comparing the overall percentage change in protein abundance in each treatment group. Proteins that: (i) had a coefficient of variation of <20% within a group, (ii) were present in at least all but one of the gel patterns, and (iii) showed an abundance difference compared to controls that was significant at $p < 0.001$ were summed and normalized to the total number of proteins analyzed in the gel. Due to the limited number of samples in each group, comparisons on individual proteins were not considered statistically reliable.

3 Results

In rodent hepatocytes each Mitcon protein was modified to approximately the same degree at each time point (Table 1 and Fig. 3). After 6 h of exposure to MP, modifications were observed in the same 4 mitochondrial proteins modified during in vivo exposure [28]. Similar to in vivo exposures, modifications generated in vitro were more extensive in the rat as compared to the mouse, with overall charge indices of $-0.3$ and $-0.07$, respectively. By 48 h, overall charge modification indices for Mitcons:1–3 had risen to $-0.81$ in the rat and $-0.33$ in the mouse. In comparison, treatment with MP at 1000 ppm in the diet for 10 weeks resulted in a charge index of $-1.1$ in the rat and $-0.14$ in the mouse. In vitro exposure to 15 $\mu$g/mL MP for 48 h also generated covalent modifications in several extramitochondrial proteins (unpublished observations). Unlike rodent hepatocytes, where 25 $\mu$g/mL MP has been shown to be toxic [35], human hepatocytes were still $85\%$ viable after 48 h of exposure to 90 $\mu$g/mL MP, demonstrated by Trypan Blue exclusion. Concentrations of 90 $\mu$g/mL MP, were therefore used in the human hepatocyte studies. Although MP treatment caused similar changes in Mitcons:1–3 within rodent species, changes induced in human Mitcons:1–3 were more heterogeneous.

<table>
<thead>
<tr>
<th>Charge (A)</th>
<th>-6</th>
<th>-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (B)</td>
<td>450</td>
<td>800</td>
<td>1580</td>
<td>2560</td>
<td>4040</td>
<td>7900</td>
<td>12,000</td>
</tr>
<tr>
<td>A - B</td>
<td>-2700</td>
<td>4000</td>
<td>-6320</td>
<td>-7680</td>
<td>-8080</td>
<td>7900</td>
<td>0</td>
</tr>
</tbody>
</table>

Charge Index = Total Charges / Total Protein

$= 36,680 / 29,330$

$= 1.25$

Figure 2. An example of the calculation of the charge modification index (CMI). Spots in the charge train of covalent modification of a specific protein are assigned a relative charge number (row 2). The parent protein has a relative charge of 0. The quantity of protein with each relative charge number, determined by spot size and density, is listed in row 3. Protein x charge (row 4) for each spot is summed and then normalized to the total protein (sum of row 3) to obtain the charge modification index. Successive covalent modifications caused by MP make the protein more acidic and cause an integral change in charge number [28].

Figure 3. Graphic representations of 2-D PAGE profiles of rat, mouse, and human hepatocytes demonstrating covalent modifications in Mitcon:1 (lower darkened spot) and Mitcon:2 (upper darkened spots) following in vitro and in vivo exposure to MP. In vivo results were previously published [28].
Table 1. Individual and collective CMIs of Mitcons:1–3

<table>
<thead>
<tr>
<th>Time</th>
<th>[MP]</th>
<th>Mitcon:1</th>
<th>Mitcon:2</th>
<th>Mitcon:3</th>
<th>Average Treated-control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1W</td>
<td>Control</td>
<td>-0.19</td>
<td>-0.29</td>
<td>-0.23</td>
<td>-0.24</td>
</tr>
<tr>
<td>1W</td>
<td>1000 ppm</td>
<td>-0.04</td>
<td>-0.12</td>
<td>-0.16</td>
<td>-0.20</td>
</tr>
<tr>
<td>1W + 4 WR</td>
<td>1000 ppm</td>
<td>-0.13</td>
<td>-0.25</td>
<td>-0.27</td>
<td>-0.22</td>
</tr>
<tr>
<td>1W + 4 WR</td>
<td>Control</td>
<td>-0.15</td>
<td>-0.42</td>
<td>-0.45</td>
<td>-0.34</td>
</tr>
<tr>
<td>10W</td>
<td>Control</td>
<td>-0.15</td>
<td>-0.42</td>
<td>-0.45</td>
<td>-0.34</td>
</tr>
<tr>
<td>10W</td>
<td>1000 ppm</td>
<td>-1.43</td>
<td>-1.52</td>
<td>-1.37</td>
<td>-1.10</td>
</tr>
<tr>
<td>10W + 4 WR</td>
<td>Control</td>
<td>-0.14</td>
<td>-0.32</td>
<td>-0.30</td>
<td>-0.25</td>
</tr>
<tr>
<td>10W + 4 WR</td>
<td>1000 ppm</td>
<td>-0.17</td>
<td>-0.32</td>
<td>-0.34</td>
<td>-0.28</td>
</tr>
<tr>
<td>Mouse in vivo</td>
<td>10W</td>
<td>Control</td>
<td>-0.32</td>
<td>-0.32</td>
<td>★ -0.34</td>
</tr>
<tr>
<td>Mouse in vivo</td>
<td>10W</td>
<td>1000 ppm</td>
<td>-0.37</td>
<td>-0.46</td>
<td>★ -0.48</td>
</tr>
<tr>
<td>Rat in vitro</td>
<td>6 HRS</td>
<td>Control</td>
<td>-0.14</td>
<td>-0.33</td>
<td>-0.24</td>
</tr>
<tr>
<td>Rat in vitro</td>
<td>6 HRS</td>
<td>15 ug/mL</td>
<td>-0.39</td>
<td>-0.53</td>
<td>-0.80</td>
</tr>
<tr>
<td>48 HRS</td>
<td>Control</td>
<td>-0.16</td>
<td>-0.32</td>
<td>-0.40</td>
<td>-0.29</td>
</tr>
<tr>
<td>Mouse in vitro</td>
<td>6 HRS</td>
<td>15 ug/mL</td>
<td>-1.01</td>
<td>-1.18</td>
<td>-1.11</td>
</tr>
<tr>
<td>48 HRS</td>
<td>15 ug/mL</td>
<td>-0.26</td>
<td>-0.19</td>
<td>★ -0.22</td>
<td></td>
</tr>
<tr>
<td>48 HRS</td>
<td>15 ug/mL</td>
<td>-0.36</td>
<td>-0.22</td>
<td>★ -0.29</td>
<td>-0.07</td>
</tr>
<tr>
<td>Human in vitro</td>
<td>6 HRS</td>
<td>Control</td>
<td>-0.20</td>
<td>-0.25</td>
<td>-0.23</td>
</tr>
<tr>
<td>Human in vitro</td>
<td>48 HRS</td>
<td>90 ug/mL</td>
<td>-0.22</td>
<td>-0.29</td>
<td>-0.30</td>
</tr>
<tr>
<td>Human in vitro</td>
<td>48 HRS</td>
<td>90 ug/mL</td>
<td>-0.23</td>
<td>-0.48</td>
<td>-0.34</td>
</tr>
</tbody>
</table>

a) CMI was derived as depicted in Fig. 2. In vivo results were previously reported [28]. WR = weeks on MP and HRS = weeks off MP prior to sample collection. In vitro rodent data is the average of 2–3 animals. Human hepatocyte data is the average of three experiments using hepatocytes from the 3 individuals described in Section 2.1.

(Table 1). The extent of covalent modification following 6 h of exposure to 90 μg/mL MP was extremely small (−0.04). The net change—the overall modification indices after 48 h (charge index = −0.11) was due primarily to changes in Mitcon:2 and Mitcon:3 (Table 1). No covalent modification was observed in carbamoyl phosphate synthetase, the fourth mitochondrial protein modified by MP in both rats and mice. Thus it is possible that the slight changes in the charge modification indices observed in human Mitcons:1–3 may have been artifactual.

In addition to causing covalent modifications of mitochondrial proteins in the rodent, MP treatment also appeared to increase the total concentration of these proteins. Covalent modification of Mitcon:1 resulted in a net decrease in the amount of parent protein; however, a totaling of covalently modified plus parent protein revealed a net increase, consistent with an increase in mitochondrial mass [35] and respiratory function [27].

In a test for changes in overall gene expression at a significance of p < 0.001, as described in Section 2.2, 33% (45/136) of rat and 1.4% (2/143) of mouse hepatic proteins demonstrated significant changes in expression following dietary administration of 1000 ppm MP for one week. No changes were observed in human, rat, and mouse hepatocyte proteins following 6 h of treatment as described in Section 2.1. However, following 48 h of MP exposure in vitro (concentrations in Table 1), 4% (2/48), 3% (2/64) and 0% (0/26) of rat, mouse, and human hepatic proteins, respectively, showed significant changes in expression.

Comparisons among the overall charge modifications showed a ratio of 8:1.5:1 and 8:3:1 (rat:mouse:man) at 6 and 48 h, respectively. Considering that a 6-fold greater concentration of MP was used on human hepatocytes, it is concluded that MP treatment is much less effective, if effective at all, at modifying mitochondrial proteins in these cells. The in vitro to in vivo correlations observed in rodents suggest that the response of human hepatocytes reflects the response likely to occur in man. If so, modifications in humans probably occur at a very low levels, if at all. These studies showed that changes induced by MP treatment in vivo and in vitro correlate with carcinogenic susceptibility. Although the relationship between carcinogenicity and the mitochondrial modifications observed remains to be established, these data suggest that the rat may not be the most relevant model with which to assess carcinogenic potential of MP to humans.

4 Discussion

In vitro–in vivo systems offer one method to help establish the relevance of rodent bioassay results to man. Traditionally, the results from each experiment are based on one or more endpoints, including ultrastructural changes, DNA binding, nucleolar segregation [36, 37], DNA repair and peroxisomal proliferation [6]. Using 2-D PAGE, the present studies have simultaneously examined the effects of MP on a large number of endpoints comprised of proteins that make up a significant part of the molecular anatomy of hepatic tissue. These results demonstrate that mouse and rat hepatocyte cultures provide models for approximating the species-specific effects of MP on these proteins in vitro. Furthermore, the correlation between the in vitro and in vivo systems supported the use of human hepatocytes to assess the effects of MP on human hepatic proteins.

Preliminary work analyzing overall changes in protein expression was also conducted. During a one-week in vivo exposure, rats demonstrated marked changes in protein expression as compared to mice. During in vitro exposure, however, protein changes were not as great, with no detectable changes at 6 h and small changes at 48 h. This temporal
dependence indicated that continued exposure was required for protein changes to be manifest. Although MP induced equal changes in expression in rat and mouse hepatocytes, no changes were observed in human hepatocytes. While these results provide additional support for a differential effect of MP in rats as compared to humans, further work will be necessary to fully characterize changes induced in hepatocytes by MP because the power of the analysis was severely limited by the small number of samples.

In summary, these studies provide a model by which 2-D PAGE technology can be used to study and compare the effects of xenobiotics on a large number of human and rodent proteins. As more proteins are identified, these comparisons could provide even greater insight into the appropriate use of bioassay data in assessing human risk.

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5 References