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Dose-responses in rat hepatic protein modification and expression following exposure to the rat hepatocarcinogen methapyrilene

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Dose-related effects of methapyrilene (MP) on protein modification and expression were examined using two-dimensional gel electrophoresis (2-D PAGE) coupled with computer analysis. Methapyrilene was administered *ad libitum* at doses of 0, 62.5, 125, 250 and 1000 p.p.m. to male F-344 rats for 12 weeks beginning at 8 weeks of age. Following treatment, livers were removed and frozen for 2-D PAGE analysis. Separation of hepatic proteins was conducted using ISO-DALT® technology. Changes in abundance and modification of hepatic proteins were determined using the Kepler™ software package. Covalent modifications of three specific mitochondrial proteins were quantified using a charge modification index. Dose-response relationships were analyzed using Tukey's trend test. Results demonstrated that covalent modification of the three mitochondrial proteins was linearly related to dose and that a dose effect could be found at all dose levels in 2 out of 3 proteins. Two forms of change in protein expression were observed: (i) a dose-dependent change with effects at all doses and (ii) a change only at the toxic dose of 1000 p.p.m. MP. These results demonstrate a molecular effect of MP at doses that do not produce cellular responses including toxicity or increases in cell replication suggesting that these specific mitochondrial modifications are molecular dosimeters but are probably not direct factors and/or sufficient factors in carcinogenesis. This study also demonstrates the potential use of 2-D PAGE electrophoresis to delineate the effect of dose on expression of specific proteins.

Introduction

Methapyrilene (MP*) is an antihistamine that was used in various medications for over 20 years. In 1980, MP was reported to induce liver tumors in rats (1) and was subsequently withdrawn from the market. Since that time, numerous studies have been conducted to elucidate its specificity and mechanism of action. MP causes liver cancer in rats, but not mice (2), hamsters or guinea pigs (3). MP is negative in most routine genetic toxicology tests (4,5) but positive results have been reported in more specialized tests (6,7,8). MP has been reported to bind to DNA *in vitro* (9) but binding to DNA *in vivo* was not found (10,11). However, studies have shown that MP or a metabolite covalently binds to hepatic proteins (12) and is localized to the mitochondria (13). MP does not appear to be an initiator of carcinogenesis but also does not appear to be a strict promoter. Instead MP appears to operate synergistically with the initiator DEN (14). MP may induce liver cancer by increasing hepatocyte proliferation over an extended period of time (15,16).

*Abbreviations: MP, methyapyrilene; 2-D PAGE, two-dimensional gel electrophoresis; Mitcon, mitochondrial protein; BrdU, bromodeoxyuridine.

Two-dimensional gel electrophoresis (2-D PAGE) has been used to characterize the effects of various xenobiotics (17). MP, in particular, has been shown to affect the expression of numerous proteins and cause covalent modification of four specific mitochondrial proteins (18); the β-subunit of F1-ATPase, (Mitcon:1) (19), an inner mitochondrial matrix protein inducible by heat shock (Mitcon:2) (19-20,21), a mitochondrial matrix protein which is a member of a family of mammalian stress inducible proteins (Mitcon:3) (19,20,22), and carbamoyl phosphate synthase (Mitcon:4). Previous studies demonstrated that modification of these proteins assessed using a charge shift index (17) correlated with species suspectibility to the carcinogenic effects of MP (23). The work reported here extends these studies to examine the dose-response relationships between protein expression and MP dose and between protein modification and MP dose.

Specifically, rats were administered MP at doses previously used in the MP carcinogenicity study (24). Changes in hepatic proteins were studied using 2-D PAGE followed by computer analysis which allows the simultaneous cataloguing and quantification of up to 1500 proteins. The extent of charge modification was then compared across dose groups using Tukey's trend test (25). In addition, specific proteins were monitored that showed dose-related changes in expression.

Materials and methods

Animal dosing and tissue preparation

In order to conserve the use of animals, animal tissues previously used in cell replication studies (16) were also used for these 2-D PAGE studies. Therefore, the methodology for dosing of animals includes all techniques used for both studies, although results from only the 2-D PAGE analysis are listed in this article.

Twenty-five male F-344 rats 8 weeks of age (Charles River Laboratories, Raleigh, NC) were placed into one of 5 groups of 5 rats each. Groups 1-5 were administered 0, 62.5, 125, 250 or 1000 p.p.m. MP (Sigma Chem. Co., St Louis, MO) in the diet (Purina #5002) *ad libitum* for 12 weeks (Figure 1). Animals were housed in shoe box cages mounted in ventilated racks, kept in temperature (70-72°F), humidity (30-80%) and light (12 h light/dark) controlled rooms. During the final week of exposure to MP, osmotic minipumps (Alza Corp., Palo Alto, CA) containing 50 mg/ml bromodeoxyuridine (BrdU) (CalBiochem, La Jolla, CA) in 0.5 N $NaHCO_3$ (26) were implanted dorsally midway between the left and right scapulae. After 12 weeks, animals were killed by CO_2 asphyxiation and livers were removed. Portions of liver were snap frozen in liquid N_2 and stored at -70°C until 2-D PAGE analysis could be conducted.

Prior to protein separation, frozen liver sections were homogenized using a 7 ml Dounce homogenizer at a ratio of 280 mg tissue per 3 ml solubilizing solution [9 M urea (BDH, Poole, UK), 2% NP-40 detergent (Sigma Chemical Co.), 0.5% dithiothreitol (Sigma Chemical Co.), 2% pH 9-11 ampholytes (LKB, Piscataway, NJ)] as described (18). The homogenate was then centrifuged at 100 000 *g* (48 000 r.p.m.) in Beckman TL-100 (Beckman Instruments Inc., Fullerton, CA) for 30 min at 20°C to remove non-soluble proteins. The supernatant fluid was frozen for subsequent 2-D PAGE analysis.

A separate experiment was conducted to determine the effect of BrdU on protein expression so that its effects on hepatic proteins could be differentiated from those of MP. Six 7 day Alzet minipumps, three containing 50 mg/ml BrdU in 0.5 $NaHCO_3$ and three containing 0.5 $NaHCO_3$ were implanted into 6 male Fisher 344 rats 6 weeks of age. Animals were housed as described and fed Purina #5002 chow *ad libitum*. After 1 week the rats were killed with CO_2 asphyxiation and livers were removed and homogenized for 2-D PAGE as described above.

2-D gel electrophoresis

Acidic and neutral proteins were separated based on isoelectric point and mol. wt using 20 × 25 cm ISO-DALT® 2-D PAGE system (Hoeffer Scientific, San Francisco, CA) following procedures previously described (27–29). Briefly, 10–15 μl of sample were loaded onto first dimension isoelectric focusing gels consisting of a single standardized batch of ampholytes (pH 4–8) BDH and electrophoresed for 33 000–34 000 volt-hours using an increasing voltage protocol. First dimension tube gels were then loaded onto second dimension gradient acrylamide slab gels and held in place by polyester fabric wedges (Large Scale Biology Corp., Rockville, MD). The top 5% of the slab gel was 11% acrylamide and the lower 95% of the gel varied linearly from 11 to 19%. This gradient gel was generated using the computer controlled gradient casting system Angelique™ (Large Scale Biology Corp.) The second dimension electrophoresis gels were run in groups of 20 gels at 10°C in cooled DALT® tanks (Hoeffer Scientific) until the bromophenol blue marker reached the bottom of the gel.

Following electrophoresis, gels (10 per batch) were fixed in 1.5 l of 50% ethanol/2% phosphoric acid for 2 h, followed by 3 × 30 min washes in cold tap water. Gels were then immersed in 1.5 l 34% methanol/17% ammonium sulfate/2% phosphoric acid for 1 h followed by the addition of a gram of powdered Colloidal Coomassie Blue G-250 stain (Serva, Paramus, NJ). Gels were stained with shaking for 4 days to reach equilibrium intensity.

Imaging and computer analysis

Each stained slab gel was digitized in red light at 133 μm resolution using an Eikonix 1412 scanner. Images were processed using the Kepler™ software system with procedure PROC008b which provided spot lists of spot size, position and density. Processed 2-D PAGE patterns were grouped according to treatment. Each pattern was matched to a master pattern, F344MST3, for F344 male rat liver from which a detailed listing of proteins has previously been published (30). The master pattern F344MST3 is a computer composite of protein patterns from numerous control F344 male rat livers. It serves as a baseline to which all patterns from treated and untreated animals are initially matched to allow comparison of individual protein spots across all 2-D gels in the experiment. Subsequently, the treated and untreated groups are compared to each other based on spot numbers assigned from the master pattern. During initial analysis, ~50 large protein spots (i.e. albumin) are selected by the researcher and tagged on each gel. The software then automatically matches 500–700 additional spots per gel. Differences in total protein loaded on individual gels are compensated for by normalizing the summed abundances of a large number of spots (scaling); a coordinated decrease or increase in the magnitude of several hundred proteins compared to the master would mostly be caused by a difference in total protein applied to the gel. The computer thus multiples the spot abundances for each protein pattern by a gel specific scaling factor to account for loading differences. This allows for valid comparisons of protein expression among gels and groups of gels. After appropriate scaling has been completed proteins showing significant changes in abundance among groups are identified using statistical and logical operator functions within the Kepler™ vector system. These functions allow the user to display all proteins whose abundance is significantly different from a level of significance selected by the user. To be considered for quantitative statistical comparison, a protein must appear in all or all but one of the animals in one of the groups (i.e. be reliably detectable). If a protein spot disappears in a treated group or animal, it cannot be stated that protein expression has been reduced to zero, only that its expression is below levels of detection or that it has been chemically modified in such a way as to shift its position on the 2-D gel. Similarly, if a protein not originally observed in the control group appears in a treated group, one cannot state that a new protein is expressed. The appearance of the spot could be caused by an increase in expression to levels that are detectable or by modification of protein that has resulted in a change in its mol. wt or charge resulting in a new spot. The creation of new spots caused by a change in charge is depicted in Figure 1.

The charge modification index (18) was used to determine the average shift in charge caused by MP treatment for each of three mitochondrial proteins Mitcon:1, Mitcon:2 and Mitcon:3 and in addition for two cytosolic proteins Cytosol:1 and Cytosol:2. The charge modification index is calculated as follows:

$$\frac{\sum_{i=0}^{n} [(\text{charge})_i(\text{abundance})_i]}{\sum_{i=0}^{n} (\text{abundance})_i} = \text{Charge modification index}$$

where i is one in a series of spots from 0 to n (i.e. right to left for Mitcon:2 in Figure 1) generated by the accumulation of covalent modifications to the protein; charge is the integral charge arbitrarily assigned to the spot (from 0 to $-n$, negative in this case because modifications caused by MP are negative), and abundance is the integrated density (abundance) of spot i as determined by the Kepler™ software. For example, increasing covalent modification of one Mitcon:2 protein molecule by MP treatment results in a greater negative charge and an intergal shift of the protein to the left (Figure 1). The parent spot is assigned a relative charge of 0 and each successive spot is assigned a relative charge of −1, −2 and so on. Since Mitcon:2 is a population of molecules, the average charge of the population of molecules shifts to the left (becomes more negative) with increasing modification. The charge modification index represents a relative average charge for a molecule in the population. The charge modification index for each treatment group was calculated and then compared amongst treatment groups to determine the effects of MP dose on covalent modification.

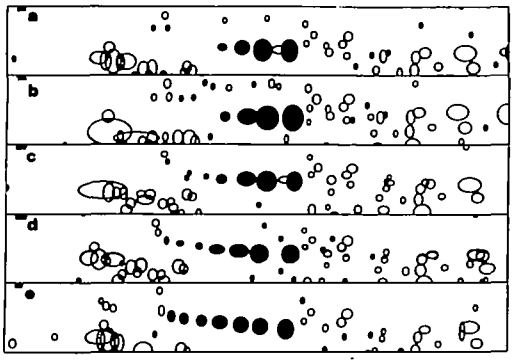

Fig. 1. Computer graphic section of 2-D gel containing Mitcon:2 (spots darkened by computer enhancement) following 12 weeks of treatment with MP at (a) 0 p.p.m., (b) 62.5 p.p.m. (c) 125 p.p.m., (d) 250 p.p.m. and (e) 1000 p.p.m. in the diet. This figure demonstrates that the increased dose causes an integral shift of Mitcon:2 spots to the left. In calculating the charge modification index the darkened spot to the right in (e) would be assigned a charge of 0 and the darkened spot to the left in (e) would be assigned a charge of −7. Open circles are graphic representations of other proteins in the area.

Statistical analysis

Charge modification indices for Mitcons:1–3 over the five dose levels of MP were analyzed using a Tukey's trend test (25), contained in the software program JMP® (SAS Institute, Cary, NC), which tests for a monotonically increasing (or decreasing) response to increasing doses of the compound. Statistically significant changes in the expression of specific proteins were determined using group-wise statistical parameters (Student's t-test) calculated within the KPL42 module in Kepler™ by comparing each treatment group with the appropriate controls.

Results

A dose-response relationship was observed between the average charge modification index of a group and the dose of MP administered to the group (Figure 1, Table I). All three mitochondrial proteins displayed a net negative shift in average charge at 1000 p.p.m. MP as expected (18). Tukey's trend analysis demonstrated that there was a significant ($P < 0.05$) shift in average charge for Mitcons:1 and 2 at all dose groups when compared to control. The average charge of Mitcon:3 was shifted significantly only at doses of 125, 250 and 1000 p.p.m. A dose-dependent charge shift in the two cytosolic proteins was also observed (Figure 3); however, the net shift was caused by a decrease in the number of spots in the charge train, was much smaller in magnitude, and was only significant at the 250 and 1000 p.p.m. (based on Tukey's trend test).

In order to assess the consistency of the effects of MP treatment on specific proteins, 76 proteins that increased and 115 proteins that decreased after 10 weeks of 1000 p.p.m. MP in previous studies (18) were selectively examined. Of these proteins 70 out of 76 were found to be increased at 12 weeks and 89 out of 115 were found to be decreased after 12 weeks of exposure.

Freezing of the tissue for 9 months prior to analysis appeared to have little effect on the protein profiles and resulted in only minor degradation of some proteins. Analysis of a few high mol. wt proteins known to be labile showed little if any breakdown

Table I. Changes in protein expression associated with methapyrilene dose

Spot #	0 p.p.m.[a]	62.5 p.p.m.	125 p.p.m.	250 p.p.m.	1000 p.p.m.
66	8.15 ± 0.31[b]	6.44 ± 0.36	5.45 ± 0.37	4.42 ± 0.46	0.78 ± 0.14
125	18.90 ± 1.21	18.20 ± 1.21	20.30 ± 1.06	18.97 ± 0.03	7.48 ± 0.89
128	6.48 ± 0.31	4.76 ± 0.26	3.03 ± 0.63	2.64 ± 0.03	0.37 ± 0.01
172	7.14 ± 0.26	7.43 ± 0.04	6.30 ± 0.24	6.76 ± 0.49	10.27 ± 4.20
226	3.55 ± 0.15	4.10 ± 0.13	4.58 ± 0.30	6.54 ± 0.57	12.98 ± 5.30
357	3.63 ± 0.16	4.21 ± 0.24	3.74 ± 0.22	4.11 ± 0.25	9.15 ± 0.28
362	5.19 ± 1.02	5.24 ± 0.30	5.95 ± 0.13	5.11 ± 0.22	1.55 ± 0.26
390	4.61 ± 0.19	5.11 ± 0.27	5.12 ± 0.07	4.79 ± 0.15	5.03 ± 0.12
571	1.74 ± 0.22	1.65 ± 0.15	1.47 ± 0.14	1.33 ± 0.13	0.66 ± 0.01

[a]Dose of MP in the diet for 12 weeks *ad libitum*. Conversion to mg/kg is ~0.01 × (p.p.m.).
[b]Values are average spot area ± SEM × 10^{-4} for 3–6 rats.

(data not shown). A side by side comparison of the profile of proteins from the control livers and the master spot pattern showed only small changes in the relative abundance of a large set of proteins and did not show a shift in the representation of large versus small proteins (analysis not shown). This analysis further indicated little if any protein breakdown in protein structure occurred during storage for <9 months and thus that freezing for this period of time did not significantly alter the interpretation of the data.

Numerous proteins demonstrated either decreases in abundance or increases in abundances that were dose related. A representative number of proteins were selected for analysis for dose related changes in protein expression (Table I). Trend analysis showed significant and dose-related reductions in protein 66 at all doses and protein 128 at 125, 250 and 1000 p.p.m. when compared to control. Protein 226 showed a dose-related increase in abundance with significance at 250 and 1000 p.p.m. MP. In contrast, proteins 125, 362, 571 decreased and protein 357 increased only at 1000 p.p.m. Protein 390 showed no change in expression over all doses.

Treatment with BrdU did not result in the generation of any charge trains. However, 50 mg/ml BrdU administered as described did result in a net change in protein expression of 14 proteins by a factor of 1.5–2.0 (Table II). BrdU increased the abundance of protein 379, a microsomal enzyme, and decreased the abundance of 13 proteins one of which, 475, has been localized to the cytosol.

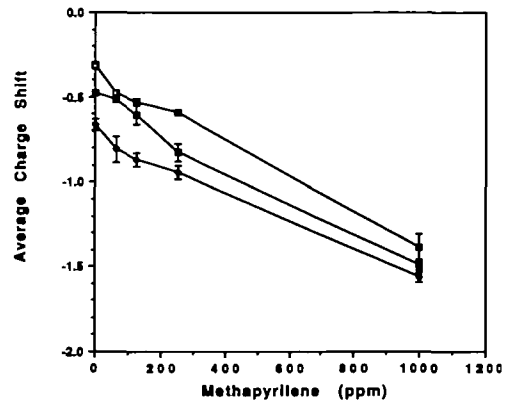

Fig. 2. Relationship between MP dose and covalent modification of Mitcon:1 (−⊡−), Mitcon:2 (−◆−), and Mitcon:3 (−■−). Averages represent mean ± SEM for 3–5 rats, with the exception of Mitcon:1 at 62.5 p.p.m. MP, which is the average of 2 rats.

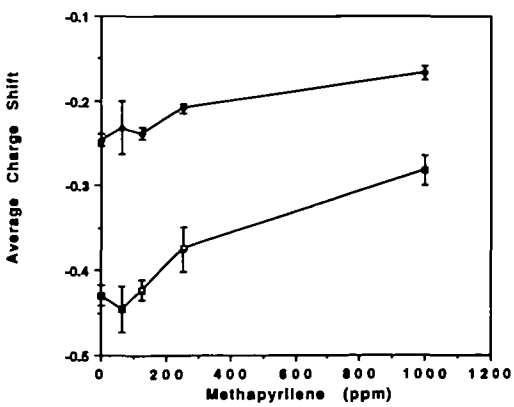

Fig. 3. Relationship between MP dose and covalent modification of Cytosol:1 (−⊡−) and Cytosol:2 (−◆−). Averages represent mean ± SEM for 3–5 rats.

Discussion

Previous studies have demonstrated that MP treatment induces covalent modifications in specific mitochondrial proteins (18) and that the extent of these modifications in rats and mice correlates with the susceptibility of the species of MP-induced hepatocellular carcinoma (23). These studies were conducted to further characterize the relationship between MP dose and charge modification and between MP dose and protein expression.

Continuous administration of MP for 12 weeks resulted in a linear dose-dependent increase in covalent modification of Mitcons:1,2, and 3. The increase in covalent modification was significant and linear at all dose levels for Mitcons:1 and 2, indicating that a no-effect level was not present over the doses examined. These results are not unexpected since a number of documented studies have demonstrated linear relationships between globin or albumin adducts and the dose of the agent inducing them (as reviewed in ref. 31). Despite the linear relationship in covalent modification, the relationship between MP dose and the biological responses of DNA synthesis (16), foci induction (data not shown), toxicity (16) and tumor induction (24) is not linear. Similar relationships were observed when comparing a series of studies that measured dose responses for DNA adducts (33), cell replication (34) and tumor induction by the genotoxic carcinogen DEN (35) and also in studies comparing tumor formation and protein adduct accumulation caused by ethylene oxide (36). The lack of a linear correlation between the extent of covalent modification and toxicity is in contrast to results reported with acetaminophen (37). Thus, these studies demonstrated that MP caused covalent changes at doses that do not affect cell replication, foci formation or toxicity, and suggest

Table II. Changes in protein expression associated with BrdU exposure[a]

Spot #	Control	BrdU-treated
359	1.85 ± 0.02	1.38 ± 0.01
379	2.86 ± 0.07	3.87 ± 0.02
411	3.66 ± 0.25	2.30 ± 0.01
436	1.36 ± 0.17	0.91 ± 0.13
475	3.70 ± 0.11	2.68 ± 0.17
617	1.48 ± 0.12	0.63 ± 0.09
626	2.58 ± 0.20	1.52 ± 0.03
721	1.37 ± 0.13	0.71 ± 0.07
820	0.70 ± 0.11	0.33 ± 0.05
831	1.45 ± 0.28	0.71 ± 0.01
837	1.49 ± 0.07	0.80 ± 0.06
875	0.96 ± 0.06	0.68 ± 0.01
1191	2.22 ± 0.32	0.11 ± 0.03
1219	0.43 ± 0.04	0.21 ± 0.02

[a]All proteins listed demonstrated significant ($P < 0.001$) differences between BrdU-treated and control animals. Numbers are mean ± SEM for 3 animals.

that covalent modifications either: (i) are not mechanistically linked to the biological phenomena or (ii) do not lead directly to the induction of these responses.

Two-dimensional gel technology allows for the screening of a large number of proteins for changes in gene expression. Applied to toxicology, the method may have use in delineating pharmacological from toxicological changes in protein expression (38). Studies conducted using single doses showed that xenobiotics can cause both increases and decreases in the expression of a large number of proteins (17). By measuring MP-induced changes in protein expression over several doses, this paper has demonstrated the ability to delineate proteins that change in expression at all dose levels from proteins that are only altered at 1000 p.p.m. MP. Proteins that changed in abundance following treatment with 1000 p.p.m. MP may be associated with the toxicological, but not the pharmacological effects of MP; since 1000 p.p.m. is the only dose examined that produced overt toxicity (16). The mechanisms behind the change in expression of some proteins only at 1000 p.p.m. MP is not clear. While the increased abundance of a specific protein may be caused by an increased expression in hepatocytes, the greater abundance could also be the result of the greater abundance of non-parenchymal cells known to be present in livers treated for 10 weeks with MP. A decrease in the abundance of a protein at 1000 p.p.m. MP, however, was most likely caused by a reduction in hepatocyte proteins. This statement is supported by the fact that a vast majority of hepatocyte proteins were not changed; indicating simple dilution was not operative. Moreover, the proliferation of non-parenchymal cells, which could have caused an apparent reduction in hepatocyte proteins, did not appear sufficient to cause a reduction of the magnitude observed.

In the interest of reducing animal use, livers obtained from cell replication studies were used. As a consequence, the livers had been exposed to BrdU administered at 50 mg/ml in 7 day Alzet minipumps. BrdU administration did not cause any detectable covalent modifications but did alter the abundance of 14 proteins. Since only one of these 14 proteins was also altered by MP treatment (data not shown) it was concluded that BrdU administration did not interfere with interpretation of the protein changes associated with MP treatment. A dose level of 65 mg/ml BrdU (38) or 50 mg/ml BrdU (unpublished observations) has been purported to be toxic as indicated by elevated rates of cell replication; however, lower doses did not appear toxic (38). Whether the changes in protein abundance induced by 50 mg/mg BrdU occur at the lower non-toxic doses remains to be determined.

These studies have utilized 2-D PAGE to further characterize changes in hepatic proteins caused by MP. While 2-D gels can be difficult to interpret because a majority of the spots have not yet been identified, the ability of 2-D PAGE to simultaneously determine changes in a large number of proteins makes possible the selection of specific proteins that may deserve further examination and identification. As work with 2-D PAGE continues, the number of spots assigned to known proteins will increase. The identification of specific spots will further enhance the ability to make mechanistic interpretations of specific spot changes and provide greater insight into the pharmacological and toxicological mechanisms associated with MP exposure.

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