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A comparative study of the effects of clofibrate, ciprofibrate, WY-14,643, and di-(2-ethylhexyl)-phthalate on liver protein expression in mice

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Summary

A comparative study of the effects of clofibrate, ciprofibrate, WY-14,643, and di-(2-ethylhexyl)-phthalate on liver protein expression in mice. Giometti, C.S., Taylor, J., Gemmell, M. A., Tollaksen, S. L., Lalwani, N. D., and Reddy, J. K. (1991). *Fundam. Appl. Toxicol.* . Two-dimensional gel electrophoresis (2DE) coupled with computer-assisted data analysis was used to examine liver protein expression in untreated mice and in mice exposed to 0.25% clofibrate, 0.025% ciprofibrate, 0.1% [4-chloro-6(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643), or 2.0% di-(2-ethylhexyl)-phthalate (DEHP). To determine the subcellular distribution of the proteins seen in 2DE patterns of liver, preparations of mitochondria, microsomes, peroxisomes, and cytosol were also analyzed by 2DE. At the doses administered, all of these chemicals are known to cause comparable levels of peroxisome proliferation accompanied by an increase in beta-oxidation of lipids, and, upon prolonged administration, hepatocellular carcinoma. The similarities in biological effects suggest a pleiotropic biochemical response to this group of chemicals. Analysis of 2DE patterns of liver proteins showed that 260 proteins out of a total of approximately 400 were changed in abundance ($P \leq 0.001$) by at least one of the four treatments used. Out of these 260 quantitative protein changes, 45 were comparable in magnitude and direction for all four treatments while the remainder showed treatment-related variability in either magnitude or direction of response. These results indicate, therefore, that against a background of protein changes that are characteristic of the individual chemicals, there is a set of pleiotropic protein changes common to the peroxisome proliferators. The subcellular location of the proteins involved in this pleiotropic response does not appear to be restricted to the peroxisome fraction.

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A number of plasticizers and hypolipidemic drugs have been shown to cause peroxisome proliferation in rat and mouse liver (Reddy and Lalwani, 1983). An increase in the activity and synthesis of enzymes in the peroxisomal lipid catabolism pathways has been observed, beginning a few days after administration of peroxisome proliferators and increasing steadily through approximately 14 days of treatment, when the increase levels off (Reddy and Lalwani, 1983). If treatment is discontinued, the level of these enzymes returns to normal, as does the peroxisome content of the liver. Prolonged administration of these peroxisome proliferators, however, has been shown to induce hepatocellular carcinoma. The similar biological responses observed for the members of this group of chemicals have led to the suggestion that a pleiotropic response is induced at the molecular level which causes the end effects of peroxisome proliferation and subsequent carcinoma (Watanabe *et al.*, 1985).

To explore the possibility of a pleiotropic response at the level of protein expression, we used two-dimensional electrophoresis to study the effects of clofibrate, ciprofibrate, DEHP, and Wy-14,643 on liver protein expression in mice. Doses of chemical known to cause comparable levels of peroxisome proliferation (Reddy and Lalwani, 1983) were given to provide a common effect with which the protein differences observed could be correlated. Quantitative results were obtained by using computer-assisted image and data analysis (Anderson *et al.*, 1981; Giometti and Taylor, 1990). With this approach, changes in individual protein abundances could be measured and compared among the treatment groups.

Materials and Methods

Treatment of animals. Male CBF₁ mice (offspring of the hybrid cross of BALB/cJANL females and C57BL/6JANL males) were given regular rodent chow or chow mixed with the following peroxisome proliferators *ad libitum* for 15 days: 0.25% clofibrate, 0.025% ciprofibrate, 0.10% Wy-14,643, 2.0% DEHP (all w/w). These doses were chosen because they produce comparable levels of peroxisome proliferation in both the rat and mouse liver systems (Reddy and Lalwani, 1983). There were 10 individuals in each treatment group in two identical experiments, for a total of 100 animals to be analyzed.

Tissue preparation for electrophoresis. The right apical lobe of the liver was removed from each animal after cervical dislocation. The tissue was homogenized in NP40-urea mix containing 9 M urea, 4% Nonidet P40, 2% 2-

mercaptoethanol, and 2% ampholytes (LKB pH 9-11). One mL of NP40-urea mix was used per 125 mg wet tissue weight. The homogenate was centrifuged at approximately $435,000 \times g$ for 5 min; the supernatant was saved and stored at -70°C until electrophoresis. The average protein content of the liver preparations (according to the Ramagli modification of the Bradford assay [Ramagli and Rodriguez, 1985]) was $29.2 \mu\text{g}$ per μL .

Subcellular fractionation. The whole livers from ten control CBF₁ mice were combined and homogenized in ice-cold 0.25 M sucrose containing 10 mM EDTA (pH 7.5) as described previously for rat liver (Watanabe *et al.*, 1985). Centrifugation in a Beckman J-21C centrifuge at 4°C according to the procedure outlined by Baudhuin *et al.* (Baudhuin *et al.*, 1964) isolated heavy mitochondria ($5500 \times g$ for 10 min) and light mitochondria ($15,000 \times g$ for 15 min). The microsomal fraction was recovered by centrifuging the resulting supernatant at $100,000 \times g$ for 2 h. The supernatant from this step is referred to as the cytosol fraction. Peroxisomes were isolated from the light mitochondrial fraction as described by Hajra and Bishop (Hajra and Bishop, 1982) using a gradient of metrizamide. All particulate fractions were solubilized in a small volume of NP40-urea mix; the cytosol was mixed 1:1 with NP40-urea mix. The protein concentrations (based on the Ramagli modification of the Bradford assay (Ramagli and Rodriguez, 1985)) of the fractions were cytosol, $3.5 \mu\text{g}/\mu\text{L}$; light mitochondria, $4.0 \mu\text{g}/\mu\text{L}$; heavy mitochondria, $5.2 \mu\text{g}/\mu\text{L}$; microsome, $6.9 \mu\text{g}/\mu\text{L}$; and peroxisome, $2.1 \mu\text{g}/\mu\text{L}$.

Two-dimensional Electrophoresis. For the first dimension, aliquots (10 μL) of mouse liver samples were separated in 10-inch rods by isoelectric focusing (IEF) as described by Anderson and Anderson (1978a). Pharmalytes pH 3-10 (50%) and Biolytes pH 5-7 (50%) were used to generate the pH gradient for IEF. The second-dimension separation was performed as described by O'Farrell (1975) on 9-17% slab gels prepared as described by Anderson and Anderson (1978b). Gels were fixed and stained in 0.125% (w/v) Serva Blue R (Coomassie Blue R250) in 2.5% H_3PO_4 and 50% ethanol and destained in 20% ethanol.

Data analysis. Destained 2DE gels in water were digitized using an Eikonix 785 scanner, and spot models were generated as previously described (Anderson *et al.*, 1981). A total of 97 out of the 100 2DE gels done were scanned (three gels were damaged prior to scanning), each representing a single mouse. Figure 1 shows the spot model used as the master pattern for this investigation. This model, generated from a 2DE pattern in the ciprofibrate group, was chosen to serve as master pattern because it contains all of the protein spots found in

2DE patterns from either control or treatment samples. Each of the sample (object) patterns was matched to this master, with an average of 400 protein spots matched per pattern. The matching procedure assigns the appropriate master spot number (MSN) to each spot matched in an object pattern within the experiment (Giometti and Taylor, 1990; Taylor *et al.*, 1981).

The staining intensity of the proteins used in this study is proportional to the amount of protein present in the spot (Giometti *et al.*, 1987). Therefore, the quantity of protein in each spot in the spot models is reported as an integrated density (spot volume). Patterns were compared using the multigroup analysis feature of the GR42 software (Giometti and Taylor, 1990). A search for proteins present in the one or more of the treatment groups but absent from the control group and for proteins present in the control group but absent from any of the treatment groups was done. To identify significant quantitative changes in proteins, Student's *t*-test (two-tailed) was used to identify a set of spots (the highlighted spots in Fig. 1) which exhibited a significant change ($P \leq 0.001$) in mean integrated density for at least one treatment group when compared with the control group.

Results

The search of the 2DE patterns for proteins either gained or lost as a result of treatment with the peroxisome proliferators resulted in the discovery of one protein (MSN 9508) that was not detectable in any of the control patterns, but was present in varying amounts in all patterns from the four treatment groups (Figure 2). None of the proteins consistently detected in the control patterns were completely missing from any of the treatment group pattern.

A search of the 2DE patterns from the treatment groups for proteins that differed in abundance from the control group with a statistical significance of $P \leq 0.001$ revealed 259 proteins (65% of the approximately 400 matched protein spots found in the mouse liver patterns) that were altered in abundance (either increased or decreased) by one or more of the treatments (highlighted in orange in Figure 1). The amount of protein loaded onto 2DE gels was comparable for each sample analyzed, averaging 290 μg with a standard deviation of 32 μg . Thus, the differences in protein abundance observed were not a function of variability in the amount of protein sample loaded onto individual gels.

Given the common response of peroxisome proliferation induced by all four

treatments, a set of common protein effects was expected as a reflection of that cellular response. Examination of the quantitative data showed that of the 259 proteins with significant changes, 45 exhibited similar responses, both in the direction and magnitude of the induced changes for all four treatments (represented by the profiles of MSN 5, 39, 281, and 512 in Figure 3). For 132 other proteins, however, the magnitude of the induced changes, although in the same direction, varied with the treatment (e.g., MSN 78, 99, 296, and 400 in Figure 3), and, in some cases, was not statistically significant for one or two of the treatments. The magnitude of the increase in MSN 9508 expression (Fig. 2) also differed significantly between treatment groups, with the mean integrated density for MSN 9508 in the clofibrate group 3149 ± 2034 , in the ciprofibrate group 15706 ± 6188 , in the Wy-14,643 group 30711 ± 6634 , and in the DEHP group 19257 ± 3909 .

In contrast to the proteins which showed a uniform response to all four treatments, 16 proteins were found to vary in both the direction and magnitude of the quantitative alterations depending on the treatment used (e.g., MSN 18, 80, 147, and 370 in Fig.3). Finally, 66 proteins were significantly altered in abundance by only one treatment (e.g., MSN 53, 88, 112, and 274 in Fig.3). Of these 66 treatment-specific effects, 38 were found in the clofibrate group, 5 in the ciprofibrate group, 14 in the Wy-14,643 group, and 9 in the DEHP group.

Thus, of the 260 alterations in the liver protein profile observed, only 17% were similar among the four treatment groups, i.e., in parallel with the peroxisome proliferation response. The quantitative data for those 45 proteins are shown in Table I. When the subcellular distribution of these proteins was examined in normal tissue, 28 of the 45 proteins were found predominantly in one of the five fractions isolated (see Methods), while the remainder were either evenly distributed among two or more fractions or were undetectable in the fractions analyzed. Of the 28 proteins found, one (MSN 136) was found predominantly in the light mitochondria fraction (minus peroxisomes), two were enriched in the microsomal fraction (MSN 281 and MSN 345), and two were enriched in the peroxisome fraction (MSN 39 and MSN 293). The remainder were found most abundant in the heavy mitochondria and cytosol fractions (Figure 4). Although assays for marker enzymes indicative of a particular fraction (e.g., catalase as a marker for peroxisomes or cytochrome C oxidase as a marker for mitochondria) were not done in this study, liver proteins known to be associated with a specific organelle have been identified for each of the fractions shown in Figure

4. Catalase is enriched in the peroxisome fraction relative to the others, while ornithine aminotransferase is enriched in the mitochondria fraction, and serum albumin is enriched in the cytosol fraction. The corresponding enrichment of unidentified proteins with these markers is assumed to indicate their association with those organelles. These results indicate that the subset of proteins showing a common response to peroxisome proliferators are not restricted to a single subcellular compartment.

Discussion

Watanabe *et al.* (1985) reported that a set of 12 proteins in rat liver showed quantitative changes after the animals were treated with ciprofibrate, Wy-14,643, or DEHP. This finding, together with the common biological responses of peroxisome proliferation, activation of enzymes in the lipid oxidation metabolic pathway and eventual hepatocellular carcinoma, prompted the authors of that study to suggest a pleiotropic response to chemicals regarded as peroxisome proliferators. Our results in the mouse system support the occurrence of a common protein response in the liver of animals treated with clofibrate, ciprofibrate, Wy-14,643, or DEHP. In addition, unlike the study done by Watanabe *et al.* (1985), a large number of protein changes was observed that were treatment dependent. The largest group of these treatment-dependent changes includes the 132 proteins that showed commonality in the direction of the change but treatment specificity in the magnitude of the change. The most obvious example of this variable response is seen in MSN 9508 where clofibrate caused a subtle induction of this protein relative to Wy-14,643 while ciprofibrate and DEHP both caused a comparable intermediate degree of induction. These proteins may also be part of the pleiotropic response since the same response is elicited but to varying degrees. The variability suggests, however, that the response of these proteins is uncoupled from the peroxisome proliferation event.

The set of 45 proteins found to respond in a similar manner after treatment with peroxisome proliferators accounts for 17% of the 260 protein changes observed and correlates most closely with the equal peroxisome proliferative response observed in previous studies (Reddy and Lalwani, 1983). The proteins in this set are not localized to a particular subcellular compartment according to the patterns shown in Figure 4, i.e., they are not all associated exclusively with the peroxisome fraction. The possibility exists that fragments of peroxisomes could

have been present in other fractions depending on their density (i.e., proteins normally located inside peroxisomes could leak out into the cytosol fraction during fractionation, or large peroxisomes could be isolated with the mitochondria fraction based on similar densities). However, the enrichment of these proteins in a particular fraction suggests that they are truly an integral part of that fraction rather than a contaminant. These results suggest, therefore, that a portion of the pleiotropic changes observed are related to phenomena other than peroxisome proliferation induced by the treatments used.

Further biochemical characterization, including identification where possible, of the 45 proteins observed in this study to be altered in a common manner by all peroxisome proliferators should increase our understanding of the mechanisms involved in the common biological changes induced by these chemicals. Thus far the peroxisome proliferation phenomenon has been observed reproducibly only in rodents. Elucidation of the mechanism of the rodent response will be useful for isolation of the reason for this species specificity. In addition, further investigation of the treatment-dependent changes observed in this study, i.e., those that are uncoupled from peroxisome proliferation, may lead to the detection of protein changes that occur in nonrodent systems and represent chemical effects that are relevant to human health.

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Table 1. The treatment group mean integrated densities for mouse liver proteins showing a pleiotrophic response to peroxisome proliferators. MSN, master spot number; Group 1, control; Group 2, clofibrate; Group 3, ciprofibrate; Group 4, Wy-14,643; Group 5, DEHP. The numbers shown in parentheses represent the coefficient of variation (CV; standard deviation divided by the mean times 100) within each treatment group

MSN	Group 1	Group 2	Group 3	Group 4	Group 5
2	48338 (16.9)	66753 (11.9)	67874 (8.8)	63098 (16.4)	74817 (15.2)
5	24815 (11.4)	32870 (10.9)	32636 (13.8)	34511 (14.7)	40329 (10.3)
9	53157 (12.5)	77357 (10.9)	85996 (9.8)	77432 (14.4)	91578 (10.5)
35	9389 (12.5)	11770 (11.2)	15583 (8.4)	11613 (13.4)	14631 (10.6)
39	14932 (19.8)	29355 (13.4)	29072 (13.4)	32517 (10.5)	31335 (13.8)
79	7533 (16.0)	12385 (9.9)	12276 (13.7)	12618 (14.5)	13175 (10.7)
105	4993 (18.8)	9022 (12.4)	8605 (12.5)	8194 (11.9)	8916 (13.7)
128	12639 (14.8)	17417 (10.0)	16241 (9.7)	19821 (10.6)	16434 (10.5)
136	8093 (17.5)	11073 (7.9)	9828 (12.8)	10748 (14.2)	11147 (12.0)
146	7285 (31.0)	13266 (15.8)	13174 (9.7)	13387 (26.2)	12763 (10.3)
169	4507 (15.5)	5845 (9.6)	5908 (11.5)	5966 (11.9)	6404 (11.1)
181	2194 (19.6)	2963 (13.9)	3102 (12.0)	3187 (15.3)	3851 (16.1)
183	1548 (29.7)	2329 (12.4)	2727 (21.1)	2777 (21.3)	2906 (17.5)
187	7240 (22.8)	11403 (11.7)	10923 (7.9)	10709 (10.4)	13372 (13.2)
199	2091 (21.3)	3263 (19.1)	3038 (20.8)	3259 (19.4)	3345 (21.6)
206	3433 (21.6)	4504 (13.5)	4935 (13.3)	4918 (13.7)	4644 (16.1)
214	3676 (20.8)	5197 (13.2)	4958 (12.1)	5573 (11.3)	5788 (12.9)
224	3407 (40.1)	6646 (16.4)	6728 (18.5)	8384 (26.9)	6695 (29.1)
226	2875 (18.9)	4331 (11.3)	3680 (11.6)	3538 (15.1)	4534 (12.8)
246	3152 (18.7)	4453 (21.8)	4502 (17.9)	5745 (14.3)	5216 (27.6)
265	3750 (27.2)	5866 (11.6)	5370 (11.8)	6723 (10.4)	5898 (16.9)
277	3686 (23.4)	5258 (11.5)	5311 (9.2)	5817 (9.4)	6634 (14.0)
281	2529 (20.8)	4026 (14.2)	3712 (13.0)	4017 (15.1)	4454 (18.7)
293	1527 (33.4)	2249 (15.8)	2184 (17.4)	2787 (23.9)	2608 (27.1)
295	2128 (20.2)	3146 (9.2)	3014 (21.8)	2855 (12.1)	2767 (12.3)
317	2365 (18.0)	3294 (11.9)	2936 (8.1)	2851 (8.6)	2939 (14.7)
338	979 (25.9)	2126 (43.5)	2047 (44.6)	1609 (18.0)	1751 (17.7)
343	2325 (25.4)	4354 (17.0)	3626 (16.0)	3887 (23.9)	4637 (18.0)
345	1231 (21.8)	2260 (28.6)	1743 (22.9)	1984 (24.8)	2072 (26.4)
376	826 (21.3)	1284 (16.0)	1170 (11.7)	1280 (23.4)	1102 (17.5)
406	1219 (29.4)	2281 (21.8)	2550 (17.3)	2169 (22.2)	2000 (30.9)
414	829 (23.9)	1425 (13.6)	1262 (25.6)	1712 (22.3)	1393 (20.6)
425	1872 (29.2)	2926 (13.3)	2453 (14.1)	2589 (15.8)	3090 (15.0)
429	1431 (26.7)	1956 (7.3)	1852 (12.2)	1898 (15.8)	2042 (23.7)
512	888 (21.7)	1284 (16.5)	1275 (14.6)	1137 (17.3)	1188 (20.0)
645	640 (46.9)	1073 (22.2)	1187 (21.3)	1338 (19.3)	980 (23.9)
648	596 (39.6)	1234 (37.5)	1057 (37.8)	1231 (27.9)	1211 (33.4)
2007	937 (37.9)	1836 (18.1)	1611 (27.7)	1965 (29.8)	2124 (32.5)
2065	507 (18.2)	1105 (21.0)	990 (25.4)	1230 (22.7)	1041 (21.4)
2095	623 (16.1)	1019 (15.7)	996 (36.4)	1458 (38.3)	1343 (32.1)
2233	1481 (26.6)	2540 (17.7)	2347 (16.5)	2623 (12.7)	2971 (16.3)
2433	1264 (23.2)	2260 (16.7)	2215 (26.6)	2429 (19.1)	2687 (16.9)
2670	4712 (39.5)	9443 (33.6)	10392 (32.7)	8657 (36.2)	8522 (38.5)
9501	2442 (22.9)	3496 (22.8)	3533 (24.2)	3836 (28.8)	4571 (15.8)
9523	2073 (24.3)	3386 (28.3)	3325 (28.3)	3148 (20.8)	3869 (21.0)

Figure Legends

Figure 1. Spot model of a two-dimensional electrophoresis pattern of mouse liver proteins generated by using the Tycho software (Anderson *et al.*, 1981; Giometti and Taylor, 1990). This 2DE pattern of whole liver extract was selected as the master pattern for this experiment. The spots highlighted in orange were significantly altered in abundance by treatment of mice with peroxisome proliferators. The positions of MSN 29 (actin), MSN 5, MSN 99 (ornithine aminotransferase), MSN 39, and MSN 9508 are indicated. The pattern is oriented with the acidic side to the left and the basic side to the right, with high molecular weight at the top and low molecular weight at the bottom.

Figure 2. Montage display of the MSN 9508 region of spot models from the control and four treatment groups. A small region of each 2DE pattern is shown with spot MSN 9508 highlighted in red/orange. This figure includes spot models for approximately 50% of the mice sampled in each group. The numbers at the bottom of each box indicate the integrated density of MSN 9508 in the spot model. A, control; B, clofibrate; C, ciprofibrate; D, Wy-14,643; E, DEHP. The mean densities for each treatment group were 3149 ± 2034 for clofibrate, 15706 ± 6188 for ciprofibrate, 30711 ± 6634 for WY14,643, and 19257 ± 3909 for DEHP.

Figure 3. Response profiles of some of the proteins showing significant ($P \leq 0.001$) effects related to treatment of mice with peroxisome proliferators. These histograms summarize the mean integrated density data from control, clofibrate, ciprofibrate, Wy-14,643, and DEHP treatment groups (left to right in each panel). The MSN number in each panel indicates the protein spot measured. The proteins in the first row show effects in the same direction and of comparable magnitude induced by all treatments. The proteins in the second row show effects in the same direction with all treatments, but the magnitude of the response varies. In the third row, the proteins show a variable response both in direction and magnitude. The fourth row shows proteins that were significantly affected by only one treatment (MSN 53, DEHP; MSN 88, clofibrate; MSN 112, Wy-14,643; MSN 274, ciprofibrate).

Figure 4. Two-dimensional electrophoresis patterns of peroxisome, heavy

mitochondria, and cytosol fractions prepared from the liver of CBF₁ mice. A, peroxisomes; B, heavy mitochondria; C, cytosol. The spot numbers for proteins that showed comparable changes in abundance for all treatments are indicated, as well as the positions of known proteins: Act, actin; ALB, serum albumin; OAT, ornithine aminotransferase; CAT, catalase. The pattern orientations are as described for Figure 1.

5

39

9508

99(OAT)

(ACT) 29

9508