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The Effects of Peroxisome Proliferators on Protein Abundances in Mouse Liver

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We have investigated the effects of five peroxisome proliferators (PPs: clofibric acid, DEHP, WY14,643, nafenopin, and LY171883) on the abundances of a large number of proteins in the livers of treated mice at 5- and 35-day time points. LY171883 was investigated at a range of doses, and one of its close structural analogs that is not a peroxisome proliferator (LY163443) was included as a negative control compound. Liver samples were analyzed by quantitative 2-D electrophoresis. Data for a selected set of 107 liver protein spots that respond strongly to at least one of the test compounds was subjected to principal component analysis to search for global protein pattern changes. The first component (PC1) accounted for 51% of the total data variance and was identified as a global measure of peroxisome proliferation by its correlation with enzymatic peroxisomal β-oxidation. Component PC2 (7%) separated 5- and 35-day exposures, and PC3 (5%) separated groups treated with LY163443 from the rest. We used PC1 as a surrogate for equivalent dose in order to examine the effects of diverse compounds, with widely differing potencies, on a common scale. Analyzed in this way, the data indicate that all the peroxisome proliferators tested produce effects over wide time and dose ranges that fall on or near a single curve. Examination of specific protein responses showed that many proteins individually show a unified response curve, but that curves for different proteins were different. In particular, it appears that some constitutive proteins showing modest inductions with a high dose plateau (such as cytosolic epoxide hydrolase) are inducible at lower doses than some proteins showing very strong, nonplateaued inductions (such as the 80-kDa peroxisomal bifunctional enzyme). The results provide support for a unified receptor-based mechanism controlling the main PP response, but demonstrate that individual responsive genes can show quite different dose–response curves.

Peroxisome proliferation occurs in the livers of rodents in response to the administration of a range of compounds, including hypolipidemics, plasticizers (Reddy and Lalwani, 1983), and leukotriene receptor antagonists (Eacho *et al.*, 1986). While the associated short-term effects are reversible, chronic treatment with peroxisome proliferators (PPs) induces liver tumors (Reddy and Lalwani, 1983; Bendele *et al.*, 1990), leading to the classification of such compounds as nongenotoxic carcinogens.

The nature of the effect is complex. It involves changes in the abundance of large sets of liver proteins observable using two-dimensional electrophoresis (Watanabe *et al.*, 1985; Giometti *et al.*, 1991a,b; Witzmann *et al.*, 1994) and thus must involve the differential regulation of many genes. While only a few of the affected proteins have been identified, at least some, and probably a majority, are nonperoxisomal. This result in turn suggests that the phenomenon called peroxisome proliferation involves a diverse series of metabolic changes in liver cells.

Abundant evidence now exists that the main trigger for peroxisome proliferation involves binding of PP to one of several peroxisome proliferator-activated receptors (PPARs), ligand-activated transcription factors of the steroid hormone receptor superfamily. In the mouse, at least three such receptors have been sequenced: mPPAR α (Isseman and Green, 1990; Gearing *et al.*, 1994), mPPAR β (Amri *et al.*, 1994), and mPPAR γ (Chen *et al.*, 1993; Zhu *et al.*, 1993), though comparative evidence on xenopus, human, and rodent PPAR genes suggests that at least five subfamily members exist (Chen *et al.*, 1993). PPARs appear to form dimers with the retinoid X receptor (Gearing *et al.*, 1993; Isseman *et al.*, 1993), and the activated complex binds to specific peroxisome proliferator response elements (PPREs) located upstream of a series of genes including the first two enzymes of the peroxisomal system: fatty acyl-CoA oxidase (ACO; Tugwood *et al.*, 1992) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE; Bardot *et al.*, 1993). Evidence is accumulating that fatty acids, as well as PP, bind to these receptors (Isseman *et al.*, 1993b; Banner *et al.*, 1993), suggesting that the PPAR regulatory system normally functions to control fatty acid metabolism. At least one similar human receptor (hPPAR) has been shown to be capable of transactivating PPRE-containing reporter constructs (Sher *et al.*, 1993), raising the possibility that PP may produce gene regulation effects in human liver.

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While PPAR regulation offers a very attractive mechanism for coordinate control of the PP response, its general validity must rest on an examination of the regulatory behavior of a broad range of liver proteins. Indeed earlier 2-D electrophoretic studies by Giometti (Giometti *et al.*, 1991a,b) were interpreted as suggesting that different PP produced substantially different effects, which would limit the applicability of a unified PPAR regulation system and point to distinct regulatory pathways influenced by individual PP. Hence we were particularly concerned in this investigation to test the validity of this view using a larger series of PP and what we believe to be an improved statistical approach. Do most PP-affected proteins show regulation consistent with a single, unified receptor-based mechanism? Do structurally varied PPs produce effects consistent with such a mechanism? We believe the answer to be affirmative in both cases, but in the course of the investigation have demonstrated that individual proteins can show quite varied dose–reponse curves within the overall unified PP response.

MATERIALS AND METHODS

Animal dosing, tissue preparation, and enzyme measurements. In the first experiment, 14 groups of 6 male B6C3F1 mice received either control diet or diet incorporating the following PPs for either 5 or 35 days before necropsy: 0.30% LY171883, 0.30% LY163443, 0.50% clofibric acid, 0.01% WY14,643, 0.05% Nafenopin, or 0.60% di(2-ethylhexyl)phthalate (DEHP). In a second experiment, male B6C3F1 mice received LY171883 at dietary concentrations of 0, 0.003, 0.01, 0.03, 0.10, 0.30, and 0.60% for 5 days (5 animals in each of the 7 groups). At necropsy, a portion of the liver was homogenized in an eightfold excess (w:v) of 9 M urea, 2% NP-40, 2% ampholytes (pH 9–11, LKB Inc.), and 0.5% dithiothreitol. A second portion was collected and the 150-g supernatant assayed for peroxisomal β-oxidation as the cyanide insensitive reduction of NAD^+ using 50 mM palmitoyl CoA as substrate (Lazarow, 1981).

Two-dimensional electrophoresis. Sample proteins were resolved by 2-D electrophoresis using the 20 × 25-cm ISO-DALT 2-D gel system (Anderson and Anderson, 1978a,b; Anderson *et al.*, 1994; Hoefer Instruments, Inc.). Isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (BASO) first dimensions were used to resolve acidic–neutral and basic proteins, respectively. All IEF gels were prepared using a single standardized batch of ampholytes (BDH 4-8A) and the gels were run for 33,000 to 34,500 V-hr. First dimension BASO gels use wide-range Servalyte and a focusing time of 5000 V-hr. Ten to 20 μl of solubilized protein was applied to each gel.

Second dimension gradient SDS slab gels were prepared using an Angelique computer-controlled gradient casting system (Large Scale Biology Corp.). Each gel was identified by a computer-printed filter paper label polymerized into the gel. First dimension IEF tube gels were loaded directly onto the slab gels without equilibration and held in place by polyester fabric wedges. Second dimension slab gels were run in groups of 20 in DALT tanks thermostatted at 10°C.

Following SDS electrophoresis, slab gels were stained for protein using a colloidal Coomassie blue G-250 procedure in covered plastic boxes, with 10 gels per box. This procedure involves fixation in 1.5 liters of 50% ethanol/2% phosphoric acid overnight, three 30-min washes in 2 liters of cold deionized water, and transfer to 1.5 liters of 34% methanol/17% ammonium sulfate/2% phosphoric acid for 1 hr followed by addition of 1 g of powdered Coomassie blue G-250 stain. Staining required approximately 4 days to reach equilibrium intensity.

Data analysis. Stained 2-D gels were digitized in red light at 133-μm resolution using an Eikonix 1412 scanner, and the resulting images processed using procedure PROC008b within the Kepler 2-D software system (Large Scale Biology Corp.). This procedure makes use of digital filtering, mathematical morphology techniques, and digital masking to remove background and uses full two-dimensional least-squares optimization to refine the parameters of a 2-D Gaussian shape for each protein spot, yielding a spotlist giving position, shape, and density information for the detected spots. Spot volumes, measurements of integrated Coommassie blue binding, and hence of protein abundance were calculated from these parameters and expressed in units of pixel-gray levels.

Two experiment packages (PEROX1–435 and PEROX–DOSES1) were constructed using the Kepler experiment definition database to assemble the 14 and 7 groups of IEF/SDS 2-D patterns corresponding to the groups of treated and control animals in the first and second experiments, respectively. All groups of the multicompound experiment consisted of six animals except the control groups (23 and 15 gels in the 5- and 35-day control groups, respectively), while all groups of the LY171883 dose response consisted of five animals except for the 0.03% dose group (4 gels, as a result of the death of one animal). Experiment packages (PEROX2–435 and PEROX–DOSES2) were constructed for the BASO/SDS 2-D pattern showing the basic proteins. Each 2-D pattern was matched to the appropriate "master" 2-D pattern (pattern B6C3F1MST2 in the case of the IEF/SDS gels of mouse liver and B6C3F1BASOMST1 for BASO/SDS gels), thereby providing linkage to the existing rodent protein 2-D databases. In this matching, a series of about 50 proteins was matched by an experienced operator working with a montage of all the 2-D patterns in the experiment. Subsequently, an automatic program matched additional spots to the master pattern using as a basis the manual landmark data entered by the operator. The operator subsequently inspected matching for spots considered important to the experiment.

The groups of gels making up an experiment were scaled together (to eliminate quantitative differences due to gel loading or staining differences) by a linear procedure based on a selected set of spots. These had spot volumes between 500 and 15,000 pixel-gray levels, and nonelongated shapes (ratio of major to minor axis <2), were present on at least 33 or 35 gels and did not appear to vary in abundance with drug dose. In PEROX–DOSES1, for example, scaling was based on 106 spots, and scale factors ranged between 0.75 and 1.78. After scaling, the number of spots showing intragroup CV < 0.15 ranged from 152 to 206 spots over the five groups.

A set of protein spots was selected on the IEF/SDS gels of the multicompound study as representing the proteins most strongly affected in the experiment. This set fulfilled the following criteria, implemented in the Kepler vector system: group average abundance changed away from the appropriate control value in the same sense (increase or decrease) in all treated groups; at least one PP-treated group showed a $p < 0.001$ difference from appropriate controls at each time point; and each spot was present on most gels (present in all but one gel of all but two of the experimental groups and present in all but two gels of all groups). To this set of 100 proteins were added seven spots representing proteins either induced from undetectable levels and hence excluded from the automatically selected group or showing large changes that did not achieve the general level of statistical significance required. The resulting 107 protein spots can be taken as showing some demonstrable abundance change with respect to controls, though they are by no means all of the proteins affected.

Multivariate statistical analysis. Principal components analysis was undertaken using PROC FACTOR (method = principal) within the SAS software system (SAS Institute) using data for all 107 selected IEF/SDS gel spots in the 110-gel multidrug experiment, where any missing data were filled with the group average value. The resulting scoring coefficients were saved and applied to the gel data from the second, dose–response experiment so as to obtain comparable estimates of principal component scores for gels from both experiments.

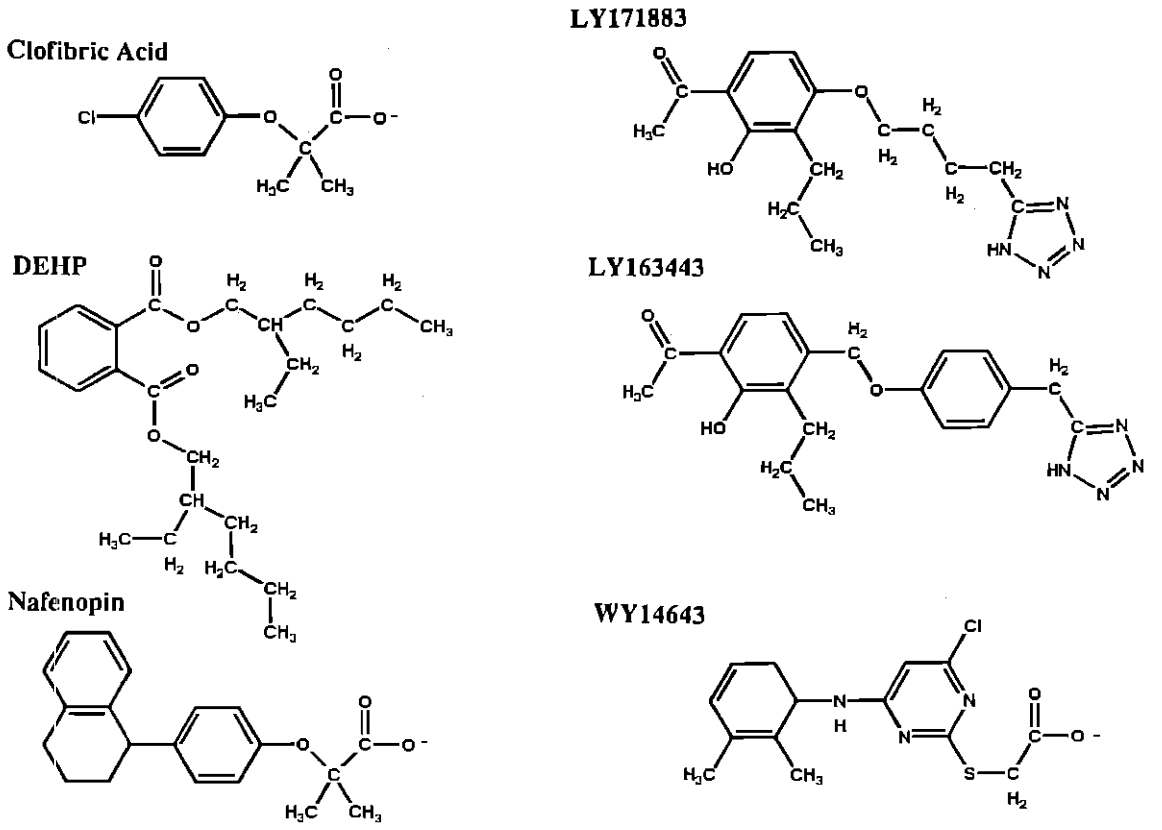

FIG. 1. Schematic chemical structures of the compounds used.

Protein sequencing. Protein spots were cut from wet, Coomassie blue-stained 2-D gels and submitted to internal tryptic digestion (Rosenfeld *et al.*, 1992). Individual peptides separated by HPLC were sequenced using a Perkin-Elmer 477A sequenator.

RESULTS

Protein Changes in Liver following Peroxisome Proliferator Treatment

Five PP were investigated: clofibric acid, DEHP, WY14643, nafenopin, and LY171883 (Fig. 1). LY163443, an analog of LY171883 that is not a PP (Eacho *et al.*, 1989), was included as a negative control. Treatment effects were measured in six animals per group at 5- and 35-day time points using two-dimensional electrophoresis, and quantitative abundance data were collected for several hundred proteins (Table 1). Among the proteins measured, 102 met criteria for reliably detected significant quantitative change (t test $p < 0.001$ for at least one compound with protein detected on almost all gels). Five additional spots that showed strong effects just beyond the limits used for automatic selection were added. The resulting set of 107 proteins are indicated in Fig. 2 on a standard 2-D protein pattern of mouse liver. Nine additional very basic proteins were selected from among those showing treatment related changes on BASO-type 2-D gels, including the 80-kDa bifunctional enzyme (PBE).

Multivariate Statistical Analysis

Given the complexity of these gene expression changes, we attempted first to determine whether one overall pattern of change (the same for all compounds, doses, and times of treatment, apart from a simple scale factor) could account for a significant proportion of the variation observed. This was accomplished by means of a principal component analysis (PCA) applied to abundance data on the set of 107 protein spots selected from the IEF/SDS 2-D gels. PCA is a multivariate statistical technique that automatically extracts a series of mutually independent patterns from a table of many variables (here the protein spot abundances) measured on many samples (here representing the livers of individual animals). For simplicity, each protein abundance is expressed in terms of its difference from the average value over all samples and the magnitude of the difference is normalized by dividing it by the standard deviation (SD), again over all samples. Hence each sample is characterized by a

TABLE 1
Relative Change Data on 116 Protein Spots over All Experimental Groups

Treatment group	a	b	c	d	e	f	g	A	B	C	D	E	F	G	1	2	3	4	5	6	7
β Oxidation	0.8	1.1	5.8	4.7	8.0	10.0	8.0	1.1	1.3	7.0	9.6	10.6	14.4	14.3	0.4	0.5	0.8	0.9	2.3	6.6	6.8
PC1 Score	-1.2	-0.6	0.1	0.2	0.6	1.1	1.0	-1.0	-0.5	0.3	0.8	0.8	1.5	1.7	-1.2	-1.2	-1.0	-0.8	-0.5	0.0	0.5
Spot IEF:7 - HSC70	39.8 (6)	1.2	1.2	1.3	1.8	1.6	1.9	46.6 (8)	0.9	1.3	1.3	1.3	1.3	1.4	24.6 (6)	1.0	1.3	1.4	1.7	1.9	2.3
Spot IEF:13	22.1 (4)	1.0	1.0	0.8	1.0	0.8	0.7	25.8 (3)	0.9	0.8	1.0	0.7	0.7	0.7	15.3 (2)	1.2	1.2	1.1	1.1	1.0	1.1
Spot IEF:14 - HSP60	49.2 (6)	1.0	1.1	1.2	1.5	1.5	1.7	56.0 (5)	0.8	1.3	1.2	1.2	1.3	1.4	54.1 (5)	1.0	1.1	1.1	1.1	1.3	1.4
Spot IEF:19 - grp75	18.3 (2)	1.0	1.1	1.2	1.2	1.3	1.1	18.8 (1)	0.9	1.1	1.2	1.2	1.3	1.4	17.6 (3)	1.2	1.2	1.1	1.1	1.1	1.2
Spot IEF:22 - cEH	28.3 (3)	1.1	1.7	1.7	2.1	1.7	2.1	31.2 (5)	0.9	1.5	1.9	1.7	2.1	2.0	17.2 (5)	1.5	1.3	1.4	2.2	2.9	3.2
Spot IEF:23 - HSP60	20.7 (2)	0.9	1.0	1.3	1.3	1.5	1.5	19.7 (2)	0.9	1.4	1.2	1.3	1.4	1.6	26.4 (4)	1.0	1.0	1.0	1.1	1.1	1.2
Spot IEF:26 - grp75	14.4 (1)	1.4	1.0	1.2	1.2	1.1	1.2	16.4 (1)	0.8	1.0	1.2	1.1	1.2	1.4	13.9 (5)	1.2	1.2	1.1	1.1	1.1	1.3
Spot IEF:29	8.3 (2)	1.1	0.7	0.5	0.6	0.7	0.7	11.2 (2)	0.8	0.6	0.5	0.4	0.4	0.4	9.8 (6)	0.7	0.7	0.6	0.6	0.6	0.6
Spot IEF:31 - proAlbumin	10.1 (1)	1.0	1.4	1.2	1.6	1.6	1.4	10.5 (1)	1.1	1.1	1.2	1.3	1.3	1.5	10.7 (2)	0.9	1.1	1.2	1.2	1.4	1.9
Spot IEF:33	45.6 (9)	0.8	0.6	0.5	0.5	0.4	0.4	53.8 (13)	0.6	0.7	0.5	0.4	0.3	0.2	40.9 (10)	1.1	0.7	0.7	0.9	0.7	0.5
Spot IEF:34 -β Actin	40.3 (6)	0.9	0.8	0.8	1.0	0.8	0.8	42.2 (6)	0.7	0.9	0.7	0.7	0.5	0.7	35.5 (8)	0.8	0.8	0.9	0.9	0.7	0.9
Spot IEF:36	14.0 (3)	0.9	0.8	0.8	0.7	0.6	0.5	14.6 (2)	1.0	0.7	0.8	0.6	0.5	0.5	13.9 (1)	1.2	1.1	1.0	0.9	0.7	0.7
Spot IEF:40 - HSP90	4.2 (1)	1.9	2.1	2.0	2.1	3.1	2.7	5.9 (1)	1.1	1.2	1.9	1.2	1.9	1.8	1.9 (0)	1.8	1.6	1.7	2.1	2.2	3.6
Spot IEF:47 - P450Red.	3.8 (1)	2.1	1.7	1.7	2.4	2.9	2.4	4.2 (1)	1.8	1.9	1.8	1.6	1.5	2.8	1.5 (0)	0.8	1.8	1.9	2.6	2.9	5.8
Spot IEF:51 - MUP	98.6 (16)	1.2	0.8	0.9	0.7	0.5	0.7	113.9 (28)	1.2	1.0	0.7	0.6	0.4	0.2	89.0 (42)	1.3	1.1	1.1	1.1	1.2	0.8
Spot IEF:61 - grp75	7.3 (1)	1.0	1.1	1.4	1.3	1.5	1.2	7.1 (1)	1.0	1.1	1.1	1.3	1.4	1.5	7.9 (2)	1.3	1.2	1.1	0.7	0.9	1.0
Spot IEF:62	12.5 (3)	0.9	1.1	1.2	1.7	1.8	1.5	13.6 (2)	0.7	1.1	1.4	1.2	1.3	1.8	13.3 (5)	0.7	0.9	0.8	1.1	0.8	1.4
Spot IEF:65	17.9 (2)	0.8	0.8	0.9	0.9	0.7	0.7	17.3 (1)	1.0	0.9	0.8	0.9	0.7	0.6	15.6 (1)	1.1	1.0	0.9	0.9	0.9	0.8
Spot IEF:73	38.4 (7)	1.0	0.9	0.8	0.9	0.5	0.8	38.8 (3)	1.0	0.8	0.8	0.7	0.7	0.5	33.5 (3)	1.1	1.0	0.9	0.9	0.9	0.7
Spot IEF:76	13.5 (1)	1.0	1.2	1.3	1.4	1.2	1.1	14.3 (1)	1.1	1.3	1.6	1.4	1.5	1.3	16.3 (2)	0.9	1.0	0.9	0.9	1.0	1.2
Spot IEF:77	26.8 (2)	1.0	1.0	1.0	0.8	0.8	0.9	26.3 (2)	1.1	1.0	0.8	0.9	0.7	0.6	25.6 (3)	1.0	0.8	0.8	0.9	0.9	0.8
Spot IEF:79	40.7 (4)	0.7	0.8	0.8	0.6	0.6	0.6	38.2 (2)	0.8	0.7	0.7	0.6	0.6	0.6	34.8 (7)	0.9	1.0	0.9	1.0	0.7	0.7
Spot IEF:85	29.4 (7)	1.2	1.5	1.1	1.4	1.1	1.1	30.2 (5)	1.1	1.3	1.5	1.5	1.5	1.4	24.1 (8)	1.0	1.5	1.3	1.2	1.5	1.8
Spot IEF:91	24.7 (5)	1.0	1.4	1.2	1.3	1.3	1.4	27.7 (2)	0.9	1.0	1.5	1.4	1.4	1.3	24.0 (5)	1.0	1.1	1.1	1.1	1.2	1.2
Spot IEF:94	43.7 (5)	0.9	0.9	0.8	0.8	0.6	0.7	44.0 (4)	0.8	0.8	0.9	0.9	0.8	0.7	42.8 (9)	1.1	1.0	0.9	0.9	0.8	0.7
Spot IEF:103	13.0 (3)	1.5	1.2	1.4	1.6	1.4	1.7	12.8 (2)	1.4	1.4	1.4	1.7	1.6	1.8	11.9 (2)	1.1	1.1	1.1	1.1	1.4	1.4
Spot IEF:105	30.5 (3)	0.4	0.9	0.8	0.8	0.7	0.8	28.9 (2)	0.4	0.9	0.9	0.8	0.8	0.8	36.0 (1)	0.9	0.8	0.8	0.6	0.7	0.6
Spot IEF:111	33.8 (4)	0.9	1.0	0.7	0.9	0.7	0.7	32.2 (3)	0.8	0.9	0.9	1.0	0.9	0.8	44.1 (7)	0.8	0.9	1.0	0.7	0.8	0.8
Spot IEF:114	8.7 (1)	1.0	1.2	1.3	1.6	1.3	1.4	9.3 (1)	1.1	1.5	1.6	1.5	1.5	1.9	8.4 (1)	1.0	1.2	1.1	1.3	1.4	1.5
Spot IEF:124	5.4 (1)	1.1	1.8	1.6	1.9	2.2	1.8	5.5 (1)	1.2	1.8	1.9	1.8	2.2	2.4	4.2 (1)	0.9	1.0	1.2	1.5	1.5	2.2
Spot IEF:127	26.5 (4)	0.8	0.9	0.8	0.9	0.6	0.5	24.8 (3)	0.8	0.9	0.9	1.0	0.7	0.6	27.3 (3)	0.9	0.9	0.8	0.9	0.7	0.6
Spot IEF:129	71.8 (10)	1.0	0.8	0.7	0.8	0.4	0.5	70.5 (16)	1.2	0.7	0.7	0.8	0.4	0.4	84.8 (9)	1.1	1.3	1.0	0.9	0.5	0.5
Spot IEF:134	4.9 (2)	1.4	2.4	2.0	2.7	2.4	2.3	6.0 (1)	1.0	1.7	2.1	1.9	1.6	1.6	1.9 (1)	2.8	1.1	1.8	3.1	4.0	4.7
Spot IEF:136	9.9 (1)	0.8	0.9	0.8	0.9	0.8	0.6	10.2 (1)	0.8	0.8	0.7	0.7	0.6	0.6	6.0 (2)	1.1	1.1	1.1	1.3	1.1	1.3
Spot IEF:137	2.1 (0)	0.9	2.7	3.3	5.2	4.9	4.0	1.8 (0)	1.3	2.4	4.0	4.7	5.0	4.3	2.0 (0)	0.0	0.8	1.5	0.9	1.3	2.7
Spot IEF:140	3.5 (1)	1.6	2.9	2.1	3.1	2.4	2.9	4.0 (1)	1.4	2.3	2.9	2.9	3.4	3.4	2.4 (1)	1.2	1.3	1.4	1.6	2.8	3.1
Spot IEF:141	12.0 (1)	0.9	0.8	0.9	0.8	0.9	0.9	11.1 (1)	0.9	0.9	0.8	0.8	0.7	0.7	14.9 (3)	0.8	0.9	0.9	0.8	0.7	0.7
Spot IEF:144	6.9 (1)	0.9	1.0	0.9	0.8	0.8	0.8	7.5 (1)	1.0	0.9	0.9	0.9	0.8	0.7	6.5 (1)	1.1	1.1	0.9	1.0	0.9	0.8
Spot IEF:145	9.8 (2)	0.9	1.2	1.4	1.7	1.7	1.7	11.3 (1)	1.0	1.5	1.5	1.4	1.8	1.8	8.8 (1)	1.1	1.1	1.1	1.0	1.4	1.4
Spot IEF:150	6.6 (1)	1.8	1.3	1.5	1.6	1.6	1.4	8.6 (1)	2.8	1.5	1.6	1.7	2.0	1.8	7.2 (2)	1.1	1.0	1.0	1.3	1.6	1.7
Spot IEF:151	15.1 (2)	0.9	1.4	1.4	1.2	1.7	1.6	14.8 (1)	1.0	1.3	1.5	1.5	1.9	1.6	16.7 (4)	1.2	1.3	1.3	1.2	1.4	1.3
Spot IEF:152	2.5 (0)	1.3	2.7	2.4	2.2	3.3	1.9	2.8 (1)	1.1	1.9	3.2	2.7	4.0	3.9	1.5 (0)	1.4	1.5	1.6	2.4	3.1	4.1
Spot IEF:153	2.6 (1)	1.6	1.9	1.5	1.9	1.8	1.7	3.5 (1)	1.2	1.3	1.8	1.6	2.2	2.1	1.4 (0)	0.9	1.4	1.2	2.0	2.3	3.7
Spot IEF:159	17.8 (2)	1.0	0.8	0.7	0.8	0.6	0.5	16.4 (1)	1.1	0.7	0.6	0.7	0.6	0.5	17.3 (1)	1.0	1.0	1.0	0.8	0.7	0.7
Spot IEF:162	88.7 (20)	0.8	0.8	0.7	0.5	0.3	0.8	73.3 (32)	0.7	0.6	0.3	0.2	0.2	0.2	70.8 (10)	1.5	1.2	1.1	1.2	0.9	0.8
Spot IEF:163	0.5 (0)	1.1	18.1	25.0	50.8	61.8	63.8	0.9 (0)	0.6	13.8	21.7	34.0	56.3	56.9	0.0 (0)	0.0	0.0	0.7	2.7	13	27
Spot IEF:167	5.6 (1)	1.2	1.4	1.2	1.3	1.6	1.6	5.8 (1)	1.0	1.2	1.6	1.4	1.7	1.6	5.3 (1)	1.1	1.0	1.1	1.1	1.3	1.3
Spot IEF:168	9.1 (1)	1.1	1.9	1.9	2.0	2.4	2.1	9.1 (1)	0.9	2.0	2.5	2.4	3.0	2.9	6.4 (1)	1.2	1.3	1.4	2.1	2.8	2.8
Spot IEF:178	13.2 (1)	0.6	0.8	0.9	0.7	0.8	0.8	12.4 (1)	0.5	0.9	0.8	0.9	0.8	0.8	12.7 (2)	1.1	1.1	1.1	1.0	0.8	0.9
Spot IEF:183	1.9 (0)	1.9	2.2	2.9	2.9	4.2	3.4	2.2 (1)	1.4	1.4	2.9	2.6	2.5	2.9	1.0 (0)	1.1	1.2	1.5	2.0	3.4	3.6
Spot IEF:184 -P-450Red	2.0 (0)	2.3	1.5	1.6	2.1	2.9	2.2	1.9 (0)	2.4	2.2	1.9	1.9	1.7	3.3	3.6 (3)	0.4	0.6	0.8	0.8	0.7	1.4
Spot IEF:189	11.1 (1)	0.9	1.0	0.8	0.8	0.9	1.0	10.8 (1)	0.7	1.0	0.8	0.8	1.0	0.9	12.2 (1)	0.9	0.9	0.9	0.9	0.9	0.8
Spot IEF:191	11.0 (1)	0.9	1.2	1.2	1.4	1.2	1.2	10.1 (1)	1.1	1.2	1.2	1.3	1.3	1.2	9.8 (0)	1.0	1.0	1.1	1.1	1.2	1.2
Spot IEF:195	10.0 (1)	0.7	0.8	0.8	0.6	0.6	0.6	8.4 (1)	0.8	0.7	0.6	0.6	0.6	0.6	8.3 (1)	1.0	1.1	1.0	0.8	0.7	0.7
Spot IEF:196	9.8 (3)	1.2	1.9	1.5	2.0	1.9	1.7	11.4 (1)	0.9	1.3	1.5	1.5	1.7	1.6	8.7 (1)	1.3	1.9	1.6	1.8	2.4	2.3
Spot IEF:214 - HSP60	5.2 (1)	0.9	1.2	1.5	1.1	1.4	2.0	5.1 (2)	0.8	1.6	1.1	1.4	1.3	2.0	7.4 (0)	1.4	1.3	1.3	1.0	0.0	0.0
Spot IEF:217	23.6 (3)	1.0	1.0	0.9	1.0	0.8	1.0	21.6 (3)	0.9	0.9	0.8	0.8	0.9	0.7	26.5 (2)	1.1	1.0	1.0	0.9	0.9	0.8
Spot IEF:218	3.1 (1)	0.9	1.3	1.5	1.1	1.9	1.4	3.2 (1)	0.7	1.3	1.4	1.3	1.4	1.6	2.6 (0)	0.9	1.2	1.0	0.7	1.1	1.7
Spot IEF:219	26.6 (5)	0.8	0.8	0.8	0.6	0.5	0.7	19.7 (4)	0.7	0.6	0.3	0.4	0.3	0.2	27.6 (6)	1.3	0.9	0.8	0.8	0.8	0.5

TABLE 1—*Continued*

Treatment group	a	b	c	d	e	f	g	A	B	C	D	E	F	G	1	2	3	4	5	6	7
Spot IEF:222	3.8 (1)	1.2	1.5	1.5	1.4	1.7	1.5	4.3 (1)	1.0	1.6	1.4	1.4	1.6	1.9	2.5 (0)	1.5	1.4	1.3	1.6	1.5	2.5
Spot IEF:226	3.1 (1)	1.2	1.6	1.4	1.5	1.2	1.5	3.4 (1)	1.0	1.2	1.4	1.3	1.5	1.5	3.4 (0)	0.9	1.0	1.0	1.2	1.3	1.4
Spot IEF:227	12.5 (2)	0.7	0.5	0.6	0.4	0.4	0.3	10.8 (1)	0.8	0.8	0.7	0.6	0.3	0.4	11.3 (2)	1.1	0.8	0.7	0.8	0.7	0.4
Spot IEF:237	4.0 (1)	0.9	0.9	0.8	0.7	0.7	0.5	4.0 (1)	1.1	0.7	0.8	0.5	0.6	0.5	2.9 (1)	1.7	1.3	1.2	1.0	0.9	0.8
Spot IEF:239	2.8 (0)	1.0	1.4	1.6	2.0	2.4	1.9	3.2 (0)	1.0	1.4	1.7	1.9	2.4	2.4	2.7 (1)	1.1	1.2	1.3	1.5	1.8	1.9
Spot IEF:243	6.8 (1)	0.8	0.8	0.8	0.8	0.8	0.7	5.9 (1)	0.7	0.8	0.5	0.6	0.4	0.6	6.6 (2)	1.0	0.9	0.8	1.0	0.6	0.7
Spot IEF:246	1.6 (1)	1.5	2.4	1.5	2.3	3.0	2.7	2.7 (1)	0.7	1.3	1.8	1.3	2.5	2.9	0.9 (0)	1.0	1.2	1.1	2.0	2.8	3.5
Spot IEF:247	6.5 (1)	1.1	1.1	1.3	1.1	1.2	1.1	6.4 (0)	1.1	1.2	1.2	1.1	1.1	1.1	6.9 (0)	1.0	1.0	1.0	1.0	0.9	1.1
Spot IEF:259	2.1 (1)	1.4	3.5	2.6	3.6	3.4	3.2	2.5 (0)	1.1	2.4	2.5	2.6	2.1	1.8	0.0 (0)	2.6	0.0	0.0	5.4	7.7	10
Spot IEF:262	24.7 (5)	0.8	0.7	0.7	0.6	0.5	0.6	19.1 (4)	0.4	0.5	0.2	0.2	0.1	0.1	29.3 (9)	1.4	0.9	0.9	0.8	0.8	0.5
Spot IEF:267	7.6 (1)	0.9	0.9	0.9	0.9	0.8	0.8	7.4 (1)	0.9	0.9	0.8	0.9	0.6	0.6	7.6 (1)	1.1	1.1	1.0	0.9	0.9	0.8
Spot IEF:274	8.0 (1)	0.9	0.8	0.7	0.7	0.5	0.7	7.7 (1)	0.7	0.6	0.6	0.3	0.4	0.6	10.6 (2)	0.7	1.0	1.0	0.8	0.7	0.7
Spot IEF:277	5.4 (1)	0.9	1.3	1.6	2.0	1.9	1.7	3.3 (1)	1.2	1.4	1.7	2.2	2.1	1.8	4.3 (1)	0.8	1.1	1.0	1.0	0.8	1.5
Spot IEF:281	6.9 (1)	0.8	0.8	0.8	0.7	0.7	0.7	8.2 (1)	0.8	0.8	0.9	0.9	0.7	0.7	10.5 (3)	0.8	0.8	0.8	0.8	0.5	0.6
Spot IEF:283	9.4 (1)	0.5	0.7	0.8	0.6	0.6	0.7	9.4 (1)	0.6	1.0	0.8	0.8	0.5	0.6	9.5 (2)	1.2	1.1	0.9	1.0	0.9	0.9
Spot IEF:287	9.4 (2)	0.9	0.8	0.6	0.8	0.5	0.5	8.8 (2)	0.9	0.8	0.7	0.7	0.6	0.6	9.1 (1)	0.8	1.1	1.0	0.9	0.9	0.9
Spot IEF:288	3.2 (0)	1.1	1.2	1.5	1.7	1.7	1.6	3.3 (0)	1.1	1.1	1.2	1.3	1.3	1.3	3.9 (1)	1.0	1.1	1.1	0.9	1.0	1.2
Spot IEF:294	5.5 (0)	0.9	0.8	0.7	0.6	0.6	0.5	5.1 (0)	1.0	0.7	0.6	0.7	0.5	0.5	4.9 (1)	1.0	1.0	1.0	0.9	0.8	0.6
Spot IEF:298	3.6 (1)	1.0	0.9	1.0	0.9	0.9	0.7	3.1 (0)	0.9	0.8	0.8	0.9	0.7	0.9	3.3 (1)	0.9	1.0	0.9	1.1	1.0	1.1
Spot IEF:307	3.7 (0)	1.0	1.2	1.4	1.3	1.3	1.2	3.5 (0)	1.1	1.1	1.3	1.3	1.2	1.3	3.7 (0)	1.0	1.0	1.1	1.1	1.2	1.3
Spot IEF:310	5.5 (1)	1.0	1.0	0.9	0.8	0.9	0.6	6.0 (1)	1.2	0.9	1.0	0.9	0.9	0.8	4.5 (1)	1.2	1.1	1.0	1.1	1.1	1.1
Spot IEF:312	5.1 (1)	1.1	1.3	1.4	1.5	1.5	1.6	4.8 (0)	1.2	1.2	1.3	1.4	1.5	1.4	4.9 (0)	1.1	1.1	1.1	1.1	1.3	1.3
Spot IEF:313	3.5 (1)	0.9	0.6	0.6	0.5	0.3	0.3	3.2 (1)	0.8	0.7	0.6	0.5	0.3	0.4	2.1 (0)	1.0	1.1	0.9	1.2	1.0	1.0
Spot IEF:318	3.2 (1)	1.0	1.0	0.8	0.7	0.5	0.5	4.0 (1)	0.9	0.6	0.8	0.5	0.5	0.4	3.2 (1)	1.0	1.1	1.0	0.7	0.6	0.7
Spot IEF:332	4.1 (1)	1.1	1.2	1.1	1.3	1.3	1.3	5.0 (1)	1.1	1.0	1.3	1.4	1.3	1.2	5.0 (1)	0.9	1.1	1.0	0.9	0.9	1.1
Spot IEF:344	1.1 (0)	1.2	2.2	2.4	2.1	3.1	1.7	0.9 (0)	1.4	2.5	4.1	3.2	5.6	5.3	0.8 (0)	0.9	1.5	1.4	2.0	2.2	2.8
Spot IEF:349	4.8 (1)	1.0	1.2	1.3	1.5	1.4	1.4	4.5 (0)	1.1	1.1	1.3	1.3	1.4	1.5	4.5 (2)	1.2	1.1	1.1	1.1	1.1	1.5
Spot IEF:363	1.7 (0)	0.9	1.3	1.4	1.2	1.6	1.6	1.8 (1)	1.1	1.4	1.8	1.7	1.8	2.1	1.2 (0)	1.2	1.2	1.3	1.6	1.5	1.5
Spot IEF:370	3.5 (1)	1.1	1.9	1.6	1.9	1.5	1.8	3.7 (1)	1.2	1.7	1.9	1.9	2.0	1.9	2.0 (1)	1.5	1.6	1.5	1.4	1.8	2.6
Spot IEF:372	9.5 (1)	1.0	0.8	0.7	0.7	0.4	0.4	8.3 (1)	1.2	0.9	0.9	0.9	0.6	0.5	10.9 (1)	0.8	1.0	0.9	0.7	0.6	0.6
Spot IEF:373	5.2 (1)	1.1	1.2	1.4	1.5	1.4	1.5	5.2 (0)	1.1	1.1	1.4	1.3	1.5	1.3	5.1 (0)	1.0	1.1	1.0	1.1	1.2	1.3
Spot IEF:374	2.4 (1)	1.4	1.5	1.5	1.3	1.4	1.3	3.2 (1)	1.0	1.2	1.3	1.2	1.6	1.1	1.6 (1)	1.3	1.8	1.6	1.1	1.8	1.6
Spot IEF:381	5.3 (1)	0.9	0.8	0.8	0.7	0.6	0.6	3.9 (1)	0.8	0.7	0.6	0.7	0.5	0.4	4.9 (1)	1.0	0.6	0.8	0.7	0.6	0.5
Spot IEF:382	6.1 (1)	0.8	1.0	0.8	0.8	0.9	0.9	4.8 (1)	0.8	0.8	0.7	0.9	1.0	0.7	9.0 (1)	0.9	0.9	0.9	0.8	0.7	0.7
Spot IEF:384	4.7 (1)	0.0	0.8	1.4	1.4	1.7	1.2	4.0 (0)	1.0	1.0	1.2	1.4	1.8	1.7	0.0 (0)	0.0	0.0	0.0	0.0	3.0	7.1
Spot IEF:402	3.9 (0)	0.8	0.7	0.9	0.7	0.7	0.6	3.7 (0)	0.8	0.6	0.7	0.7	0.5	0.5	3.8 (1)	1.2	1.1	1.0	0.8	0.8	0.6
Spot IEF:424	5.4 (0)	0.9	0.8	0.8	0.9	0.7	0.7	5.4 (0)	1.3	0.8	0.8	0.9	0.8	0.7	4.7 (1)	1.2	1.0	0.9	0.9	0.9	0.8
Spot IEF:427	2.2 (0)	1.5	1.4	1.3	1.5	1.4	1.5	2.2 (0)	1.5	1.4	1.5	1.5	1.6	1.5	3.1 (0)	1.0	0.9	1.1	1.5	1.1	1.1
Spot IEF:441	5.7 (1)	0.9	0.9	0.7	0.7	0.5	0.5	4.8 (0)	1.0	0.6	0.6	0.6	0.4	0.4	4.1 (1)	1.4	1.3	1.3	1.0	0.9	0.7
Spot IEF:455	4.0 (1)	0.8	0.7	1.0	0.7	0.6	0.5	3.3 (1)	0.8	0.8	0.6	0.9	0.5	0.4	3.5 (0)	1.2	1.0	1.0	1.0	0.8	0.7
Spot IEF:456	0.9 (0)	2.0	3.7	2.5	3.2	3.8	4.4	0.9 (0)	1.8	3.1	4.2	4.4	4.7	6.0	0.6 (0)	1.7	1.6	2.1	2.6	3.4	4.4
Spot IEF:470	6.0 (1)	1.0	0.7	0.6	0.7	0.4	0.5	5.8 (1)	0.9	0.8	0.8	0.8	0.6	0.6	3.9 (2)	1.6	1.5	1.2	0.9	0.5	0.8
Spot IEF:490	1.3 (0)	1.4	2.5	2.5	3.4	3.5	3.2	0.9 (0)	3.0	3.3	4.1	4.5	6.1	5.3	1.2 (0)	0.9	0.9	0.9	1.8	2.4	3.5
Spot IEF:491	4.6 (1)	0.8	0.9	0.9	0.9	0.6	1.0	4.8 (1)	0.7	0.8	0.7	0.6	0.6	0.6	4.9 (0)	1.0	1.1	1.0	0.9	0.8	0.8
Spot IEF:501	1.4 (0)	1.4	1.3	1.2	1.5	2.2	2.0	2.0 (0)	1.0	1.0	1.4	1.0	1.2	1.5	1.3 (0)	1.2	1.2	1.1	1.4	1.5	2.0
Spot IEF:519	3.2 (0)	0.7	0.7	0.6	0.8	0.6	0.6	3.1 (0)	0.8	0.7	0.7	0.8	0.9	0.8	3.0 (0)	0.9	1.0	0.9	0.9	0.7	0.8
Spot IEF:523	2.8 (0)	0.9	0.8	0.8	0.8	0.9	0.7	2.6 (0)	0.9	0.9	0.7	0.7	0.6	0.6	2.7 (1)	1.0	0.9	0.9	0.9	0.7	0.8
Spot IEF:556	1.3 (0)	1.3	1.5	1.4	1.2	1.5	1.2	1.4 (0)	1.3	1.2	1.3	1.4	1.6	1.4	2.1 (2)	0.5	0.5	0.5	0.5	1.1	0.5
Spot BASO:21	27.7 (9)	1.0	1.7	2.2	1.4	1.3	1.6	23.4 (23)	1.1	1.7	2.2	2.3	2.2	2.6	5.3 (7)	3.3	4.5	2.5	5.1	4.7	6.5
Spot BASO:35	16.6 (7)	1.2	1.4	1.7	1.4	1.4	1.5	22.1 (22)	1.7	1.3	1.1	1.4	1.2	1.4	0.0 (0)	17	12	12	8.5	0.0	0.0
Spot BASO:40	14.8 (6)	1.0	2.6	2.7	2.9	3.1	3.1	20.8 (21)	1.2	1.8	1.6	1.6	1.9	2.6	0.7 (0)	9.3	5.6	7.9	6.0	4.5	0.7
Spot BASO:59	13.8 (4)	0.8	0.6	0.8	0.7	0.5	0.5	13.5 (13)	0.9	0.6	0.6	0.6	0.3	0.4	25.0 (9)	0.7	0.6	0.7	1.0	0.9	1.0
Spot BASO:76 - PBE	11.5 (6)	1.3	22.4	32.2	54.2	41.0	46.6	10.7 (11)	2.3	34.2	38.4	42.6	62.9	87.3	29.6 (8)	1.2	1.4	1.3	6.5	15	24
Spot BASO:84	47.1 (11)	0.8	0.7	0.8	0.6	0.5	0.7	40.9 (41)	0.6	0.5	0.3	0.1	0.1	0.1	76.9 (21)	1.0	0.8	0.7	0.9	1.1	0.8
Spot BASO:85	12.1 (5)	4.9	6.8	5.7	14.5	9.8	14.0	13.9 (14)	1.8	7.2	9.4	10.0	13.2	13.7	0.0 (0)	0.0	0.0	15	151	272	400
Spot BASO:154	18.4 (7)	1.5	1.7	2.0	2.1	2.4	2.6	19.5 (19)	1.5	1.6	2.4	2.7	3.5	2.4	35.7 (6)	0.8	0.9	1.2	1.2	1.5	1.8
Spot BASO:272	1.5 (1)	1.1	1.6	1.3	1.7	2.6	1.9	2.1 (2)	0.7	1.5	1.3	1.6	1.6	1.8	2.4 (1)	0.8	0.9	0.4	2.4	2.6	2.6
Decreased more than 2-fold		2	0	1	2	10	8		2	0	5	8	16	15		5	4	4	1	3	5
Decreased less than 2-fold		57	52	50	48	42	44		57	50	46	44	36	34		45	35	50	50	55	47
Increased less than 2-fold		53	49	50	46	41	46		53	55	50	49	43	43		61	74	57	47	36	37
Increased more than 2-fold		4	15	15	20	23	18		4	11	15	15	21	21		5	3	5	18	22	27

series of protein measurements describing its difference from overall average values. In effect, the PCA procedure extracts first the predominant pattern of differences (PC1), which might involve, for example, a 1.2 SD increase in protein 1, a 0.5 SD decrease in protein 2, a 5.8 SD increase in protein 3, etc., and gives a measure of how much that pattern applies to each sample (the sample's PC1 score). Then this pattern of change is removed, and the second most important pattern, unrelated to the first, is extracted, and so on. By plotting each sample, or group of samples, on axes expressing their scores on PC1 vs PC2, for example, it is possible to summarize the similarities and differences among samples with respect to complex patterns of changes in many variables (Fig. 3). Samples that fall near one another on such plots show similar patterns of change, while those that are far apart are less similar. The procedure also yields a measure of the relative importance of each of the succeedingly smaller patterns of difference, expressed as the percentage of the total data variance explained by that component.

The result of our PC analysis of data on 107 proteins indicates that 51% of the total data variance is accounted for by a single component of change (the first principal component—PC1). This component separates the treatment groups in the experiment in a manner that might be expected for a global measure of potency as a PP (Fig. 3). Samples from animals treated with the negative control compound LY163443 (b and B in the plot) lie at positions near the control samples (a, A) with respect to the *y*-axis (PC1), while samples from animals treated with strong PPs (nafenopin and WY14643; g, G and f, F) lie far away from the controls.

This conclusion was tested by plotting the average scores on PC1 associated with the 14 treatment and control groups in the six-compound experiment against independent group-averaged enzymatic measurements of peroxisomal β-oxidation (Fig. 4). A strong relationship is observed between the global measure (PC1 score) and the specific assay of a single enzyme, although the curve shows possible divergence from a linear relationship in the low effect region. LY163443, which is not a PP, shows only a small effect using either measure. Five-day exposure groups show systematically lower levels of PP effect by both measures when compared to the 35-day exposure groups for the same compounds.

Additional, smaller independent components of change were also detected in the principal component analysis: PC2 and PC3 account for 6.7 and 5.0% of the total variation, respectively. PC2 appears to distinguish the 5- and 35-day exposure groups by characteristics independent of their differences on PC1. PC3 serves mainly to separate the groups treated with LY163443 from both the controls and the other treatment groups. A detailed examination revealed that LY163443 causes a series of protein changes unrelated to peroxisome proliferation; one that is apparently unique among compounds so far examined (to be described elsewhere). PC4 accounts for only 3.5% and PC5 an additional 2.9% of the data variance, with further components further decreased.

Dose–Response Effects

Differences between the effects of various PPs could be due to different relative potencies (and hence different levels of effect at the doses chosen), to time-varying effects, or to real differences in the nature of the biological effect produced. The issue of relative potency, in particular, must be taken into account because it is practically impossible to choose dose levels for different compounds that produce exactly the same degree of peroxisome proliferation. We therefore carried out a conventional dose–response study with a single compound (LY171883) at six doses to see whether the results would be consistent with a single pattern of change scaled by some function of dose, and thus with the assumption that the observed differences between compounds could be explained on the basis of relative potency. After a 5-day treatment, group average values of β-oxidation and PC1 score (computed using the same scoring coefficients used in the earlier six compound comparison) both showed monotonic dose–response curves (Fig. 5). Comparing these two measures against one another (as plotted in Fig. 4), the dose–response curve for LY171883 appears consistent with the curve describing the data for all the PPs examined. In Figs. 4 and 5, there is evidence that the PC1 score rises more rapidly in the low-effect range than does the β-oxidation

Note. The table presents summary data for 116 proteins over all treatment groups. Protein abundance is reported for each of the three control groups ((a, A) lower case, 5-day exposure; upper case, 35-day exposure and (1) control group for the LY171883 dose response) in units of pixel-gray levels $\times$ 10^{-3} (a measure of the total amount of Coomassie blue protein stain bound by the spot), with standard deviations in parentheses. Data for the treated groups are presented in terms of abundance ratio relative to the control (abundance in treated liver divided by abundance in control liver). Groups are identified by the same symbols used in the figures: LY163443 (b, B), LY171883 (c, C), DEHP (d, D), clofibric acid (e, E), WY14643 (f, F), Nafenopin (g, G), and a series of doses of LY171883 (0, 0.003, 0.01, 0.03, 0.10, 0.30, and 0.60% in diet; 1–7). Hence a value of 1.9 for the first protein (IEF:7:HSC70) in group "g" indicates that the protein is increased in abundance by a factor of 1.9 by Nafenopin in the 5-day exposure. Group average values for β-oxidation and PC1 score are included at the top. A tabulation of the number of proteins increased or decreased more or less than twofold in each treatment group is presented at the bottom of the table. Known proteins are identified with abbreviations: cEH is cytosolic epoxide hydrolase, HSC70 is heat shock cognate 70, HSP60 is heat shock protein 60 (Mitcon:2), grp75 is glucose regulated protein 75, MUP is the mouse major urinary protein, P450Red is the NADPH cytochrome P-450 reductase, and PBE is the peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme.

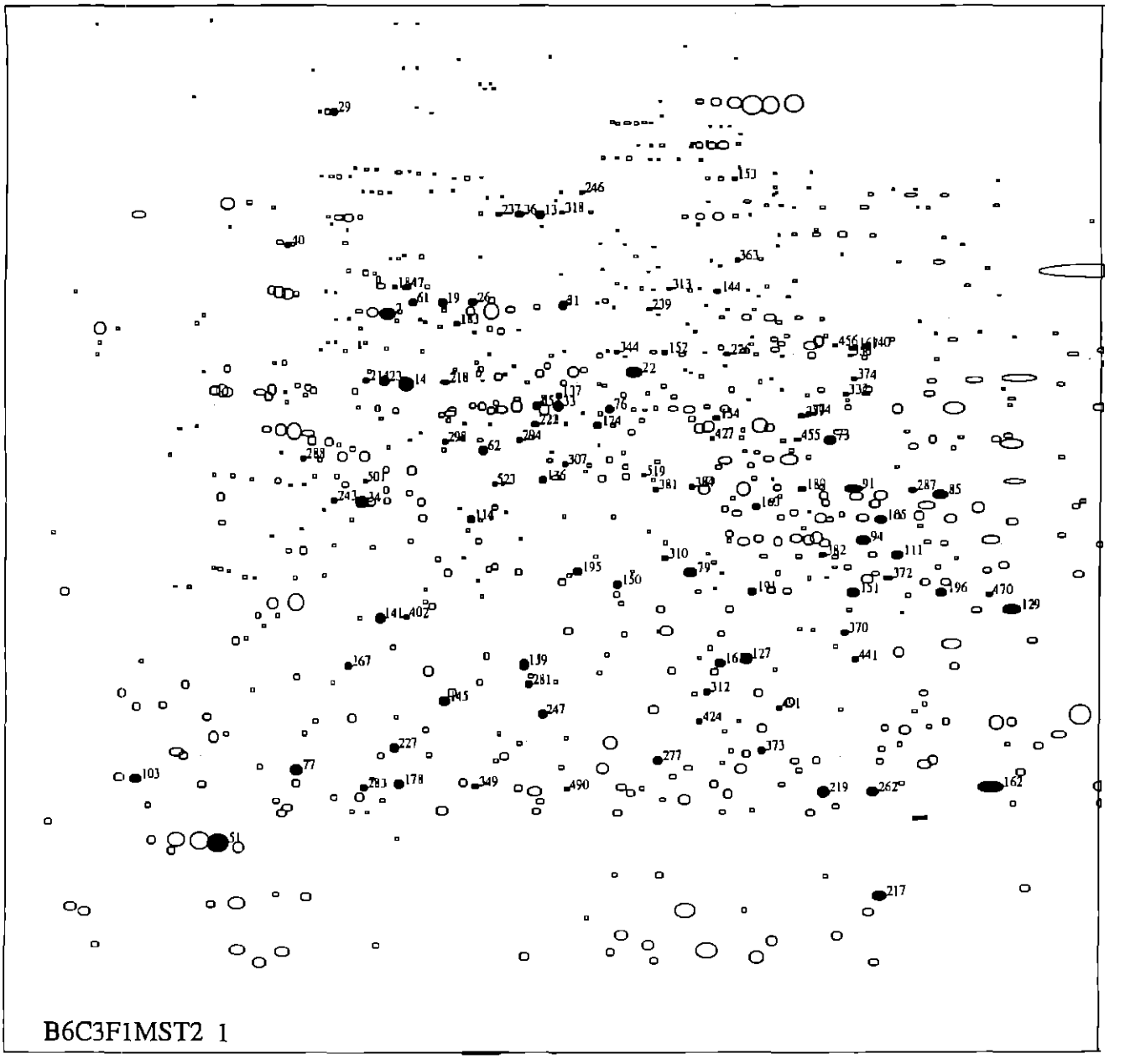

FIG. 2. Map locating proteins found to change with PP treatment. A schematic representation of the two-dimensional (IEF/SDS) protein pattern of whole mouse liver homogenate. Protein spots shown as filled ellipses comprise the set of 107 proteins described in the text. Adjacent numbers are master spot numbers in the B6C3F1MST2 master number system used in this study. Molecular mass runs from approximately 250 kDa at the top to 6 kDa at the bottom, and p*I*s run from 3.9 on the left to 6.8 on the right.

measurement. This behavior suggests that some proteins contributing to the PC1 score may show stronger effects at lower dose than do the enzymes of β-oxidation.

Magnitude and Polarity of PP-Induced Changes in Specific Proteins

A few proteins, including the 80-kDa bifunctional enzyme, show measured inductions of greater than 50-fold, while some others are induced from normally undetectable levels and could show even stronger relative increases. Nevertheless most of the significant protein abundance changes resulting from PP treatment are less than 2-fold (Table 1).

Of the 107 proteins selected as showing some response to at least one PP in the six-compound study, a significant number showed treatment related decreases instead of inductions. For example, nafenopin at 5 days (group "g") de-

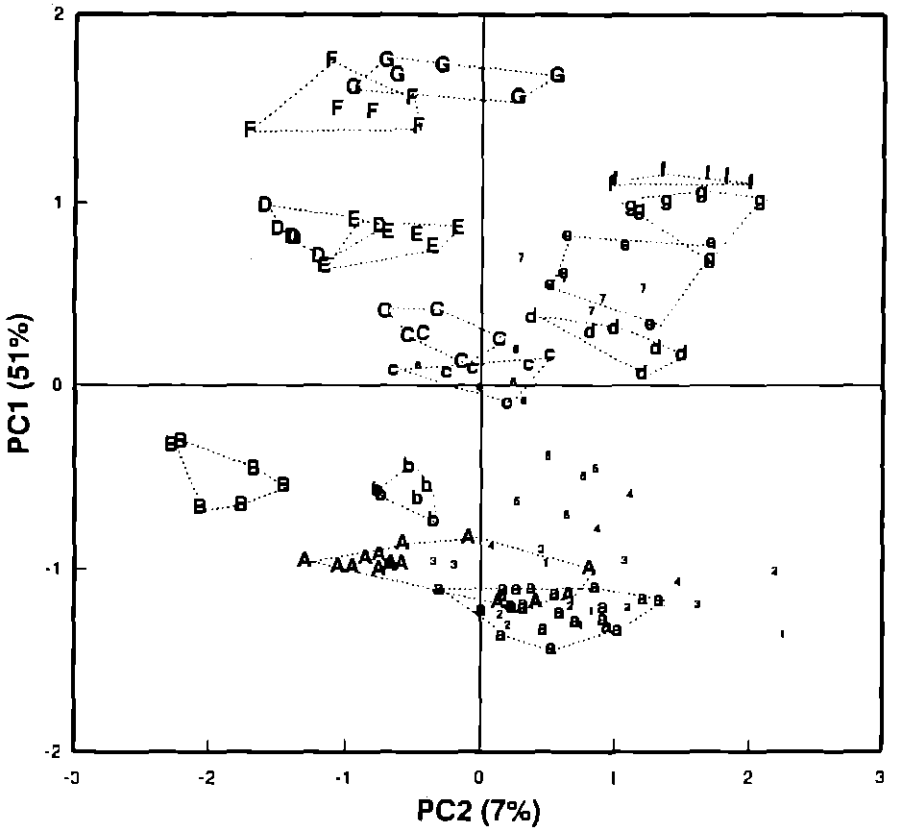

FIG. 3. Results of principal component analysis. Plots of the scores of individual 2-D gels (analyses) on the first three axes of principal component representation derived from analysis of 107 protein spots in the multicompound study. PC1, 2, and 3 accounted for 51, 7, and 5% of the total data variance, respectively. PC1 is primarily a measure of the global PP effect, PC2 separates 5- and 35-day exposures, and PC3 separates the strong PPs from LY163443, a weak proliferator with other major, non-PP-related effects. Groups are controls ((a, A) lower case, 5-day exposure; upper case, 35-day exposure), LY163443 (b, B), LY171883 (c, C), DEHP (d, D), clofibric acid (e, E), WY14643 (f, F), Nafenopin (g, G), and a series of doses of LY171883 (0, 0.003, 0.01, 0.03, 0.10, 0.30, and 0.60% in diet; 1–7). The border of each treatment group is outlined by a dotted line.

creases a total of 52 of the 116 protein spots and increases 64. Hence the numbers of proteins increased and decreased are fairly equally balanced, despite the fact that the net effect is a global increase (since 18 proteins are increased by more than twofold, while only 8 are reduced by more than twofold). Since the data from different gels was normalized together using a subset of protein spots that did not include the 107 main PP-responsive spots, the ratio of increases to decreases should be unaffected by the net effect of PP on total liver protein abundance.

In order to examine the behavior of individual proteins across the various experimental groups in the compound comparison and dose–response studies, we elected to plot each protein's group average protein abundance versus the group average score on PC1 (which we believe is likely to represent the best estimate of the level of PP response). The plot thus relates protein amount to peroxisome proliferation, with plotted symbols representing the average for a treatment group. Figure 6 shows such plots for the eight protein spots showing the largest positive contributions to the calculation of PC1 (the proteins most strongly correlated with PP) and the eight spots showing the largest negative such contributions. The purpose of these plots is to allow detection of treatments (experimental groups) whose effect departs significantly from the trend expected for peroxisome proliferation generally. For these proteins, and for most of the others less strongly associated with PC1 (and hence with peroxisome proliferation), we did not detect major departures from the trend.

A few proteins, particularly those strongly associated with principal components 2 and 3, did show outlying groups (Fig. 7). In the case of PC2, the groups representing 35-day treatments are generally clustered away from the 5-day treatment groups, while in the case of PC3, the outliers are the 5- and 35-day groups treated with LY163443.

Shapes of Response Curves

Differences were detected between the dose–response curves of individual proteins in the multidose study of

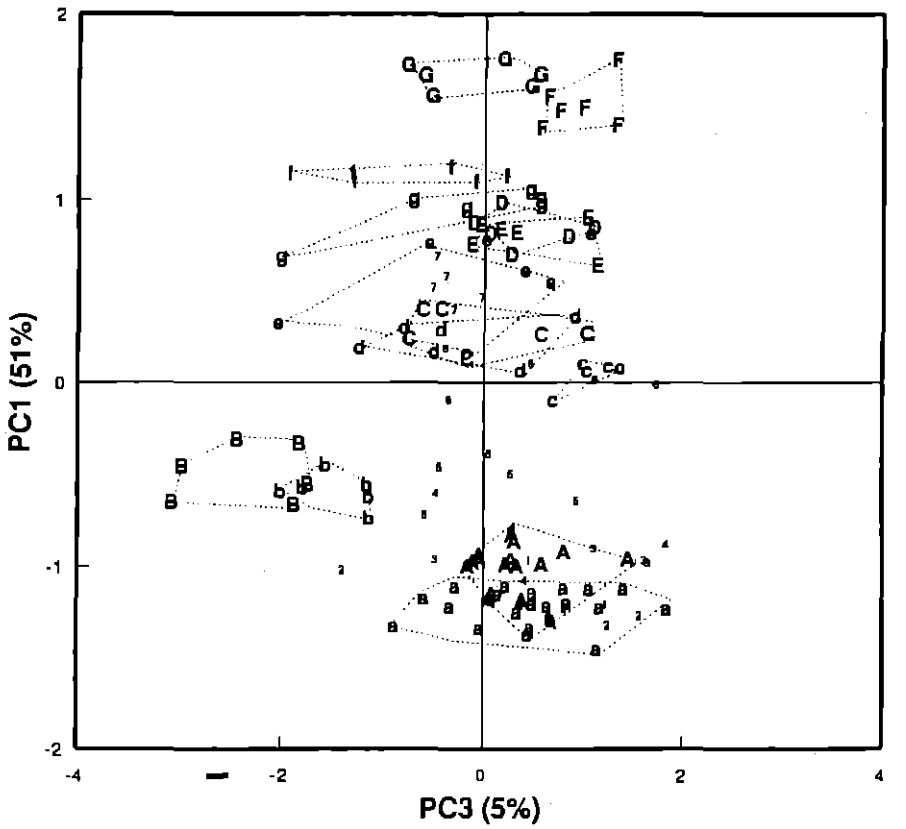

FIG. 3—*Continued*

LY171883. Some proteins, such as spots IEF:22 (cytosolic epoxide hydrolase), IEF:150, and IEF:239 show evidence for induction at low dose followed by a plateau at higher dose. This contrasts with the behavior of the peroxisomal bifunctional enzyme (spot BASO:76) and IEF:163 (Fig. 8), which are induced from almost undetectable endogenous levels and continue to increase without plateau in the dose range studied. The same effect is apparent when these proteins are compared over the larger data set covering six compounds at two time points.

Since IEF:163 is induced by PP more strongly than any other protein in the acidic to neutral p*I* range, we attempted to identify it through sequence analysis of 2-D gel-derived material. Data were obtained for two tryptic peptides having sequences DAGGELNLAR and FIPGER, but these did not match any known proteins in current sequence databases. We therefore conclude that at least one protein very strongly responsive to PP is novel.

DISCUSSION

We have examined the effects of peroxisome proliferation in mouse liver with respect to a series of variables: dose, time of exposure, PP used, and protein markers analyzed. The results support the contention that peroxisome proliferation is a complex phenomenon at the biochemical level, involving more than 100 proteins, but one that can nevertheless be largely attributed to a single coherent gene regulation pattern affecting many proteins in a coordinated way. This pattern of gene expression change characteristic of peroxisome proliferation was extracted through the use of principal component analysis, a multivariate statistical technique that uncovers a series of mutually independent patterns of change (components) that explain most of the variation occurring in the variables analyzed (here protein abundances). Each component consists of a set of coefficients for each of the proteins measured and a sum of the protein abundances weighted by these coefficients gives an aggregate measure equal to the score of the sample concerned for that component. The effect is to reduce the complexity of the data from a picture consisting of 107 separate and independent protein measurements to a picture consisting of a few components, each of which describes in a unified way changes occurring in many proteins at once. The first (and by definition largest) component in our analysis of 107 proteins measured in 14 groups of 6 animals accounted for more than 50% of all the variance in this large data set, with the second and third independent components representing no more than 7% and 5%, respectively.

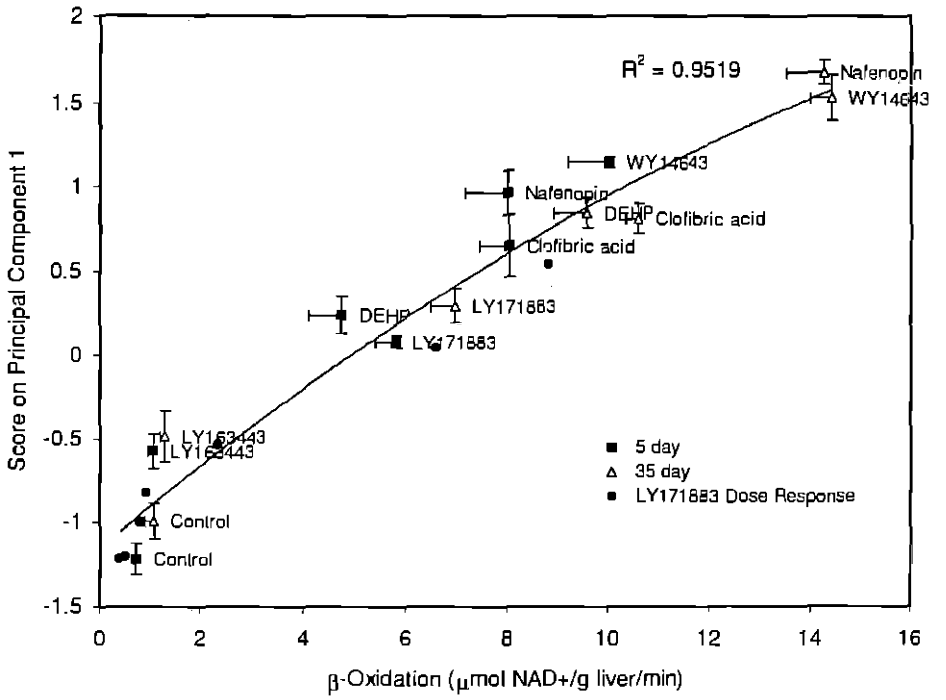

FIG. 4. Peroxisomal β-oxidation vs PC score for all groups. Group average enzymatic measurements of β-oxidation are plotted versus PC1 score for each experimental group. Error bars show 1 SD of the group values. The data are fitted to a parabolic curve and shows an approximately linear relationship except at low levels of β-oxidation, where a change in slope is evident.

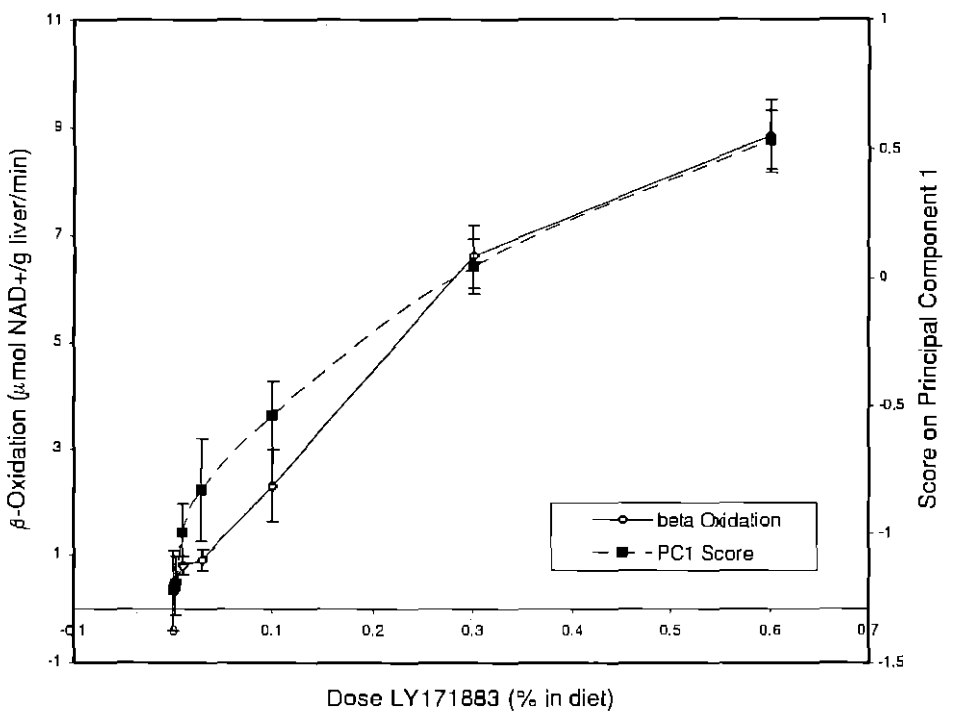

FIG. 5. Peroxisomal β-oxidation and PC1 score vs dose for LY171883. Group average values for β-oxidation (left-hand y-axis) and PC1 score (right-hand y-axis) are plotted against dose of LY171883 administered for 5 days in the diet. The scales of the two Y-axes have been adjusted so as to compare the two curves over the same span. Error bars indicate 1 SD. PC1 score rises more sharply at low dose than β-oxidation.

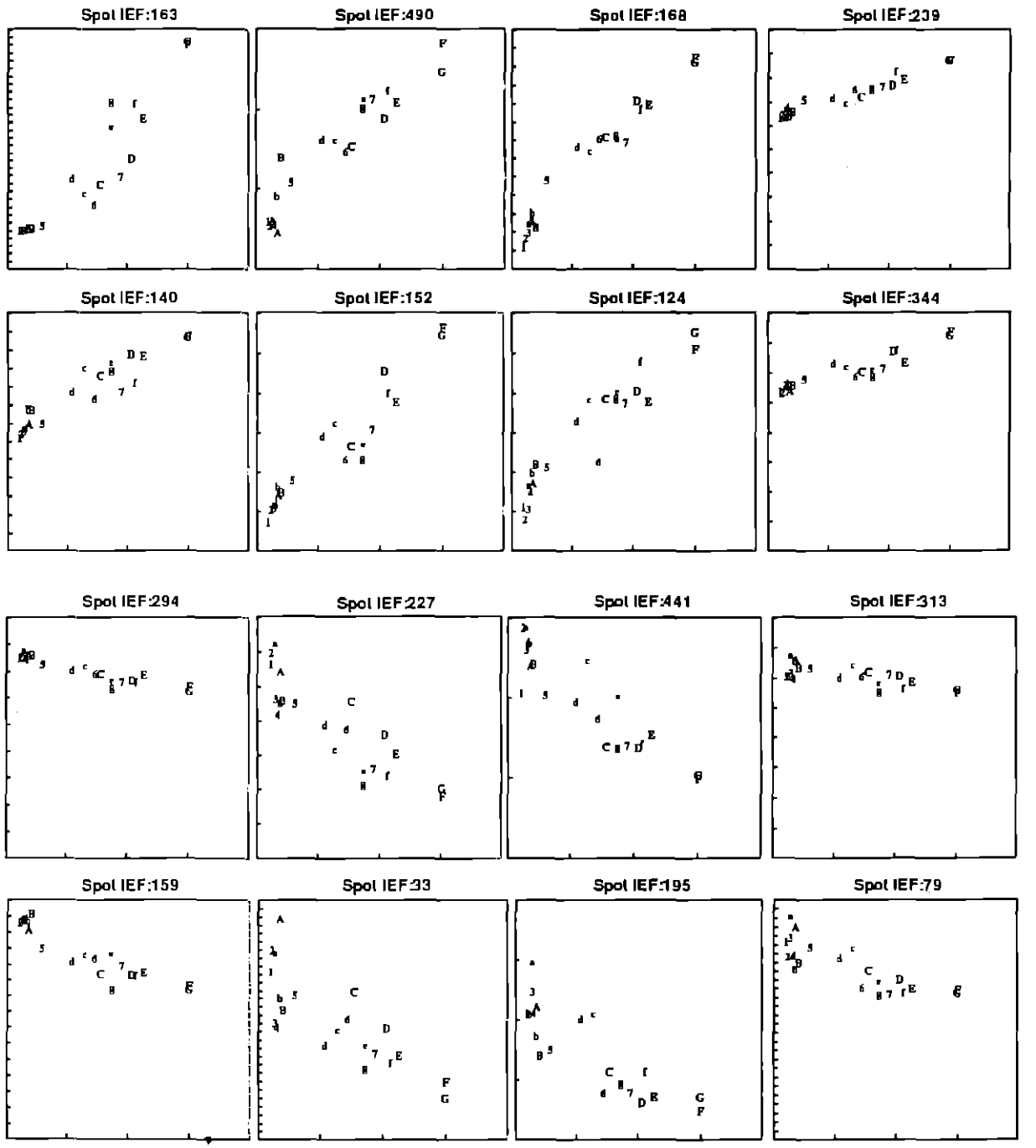

FIG. 6. Plots of abundance vs PC1 for 16 proteins associated with PC1. Plots of group average PC1 score (*x*-axis) versus group average abundance for a series of proteins (one protein per panel). The first two rows (eight panels) show the eight spots making the highest positive contributions to PC1, and the following two rows show the eight spots making the largest negative contributions to PC1. Groups are represented by the same symbols used in Fig. 3: controls ((a, A) lower case, 5-day exposure; upper case, 35-day exposure), LY163443 (b, B), LY171883 (c, C), DEHP (d, D), clofibric acid (e, E), WY14643 (f, F), Nafenopin (g, G), and a series of doses of LY171883 (0, 0.003, 0.01, 0.03, 0.10, 0.30, and 0.60% in diet; 1–7).

Because random protein measurement errors arising from the 2-D gel procedure, as well as random interanimal differences in protein levels, contribute to the variation in protein measurements, it is typical that only a few of the largest principal components relate to experimental treatments, while the remainder represent "noise" in the measurements.

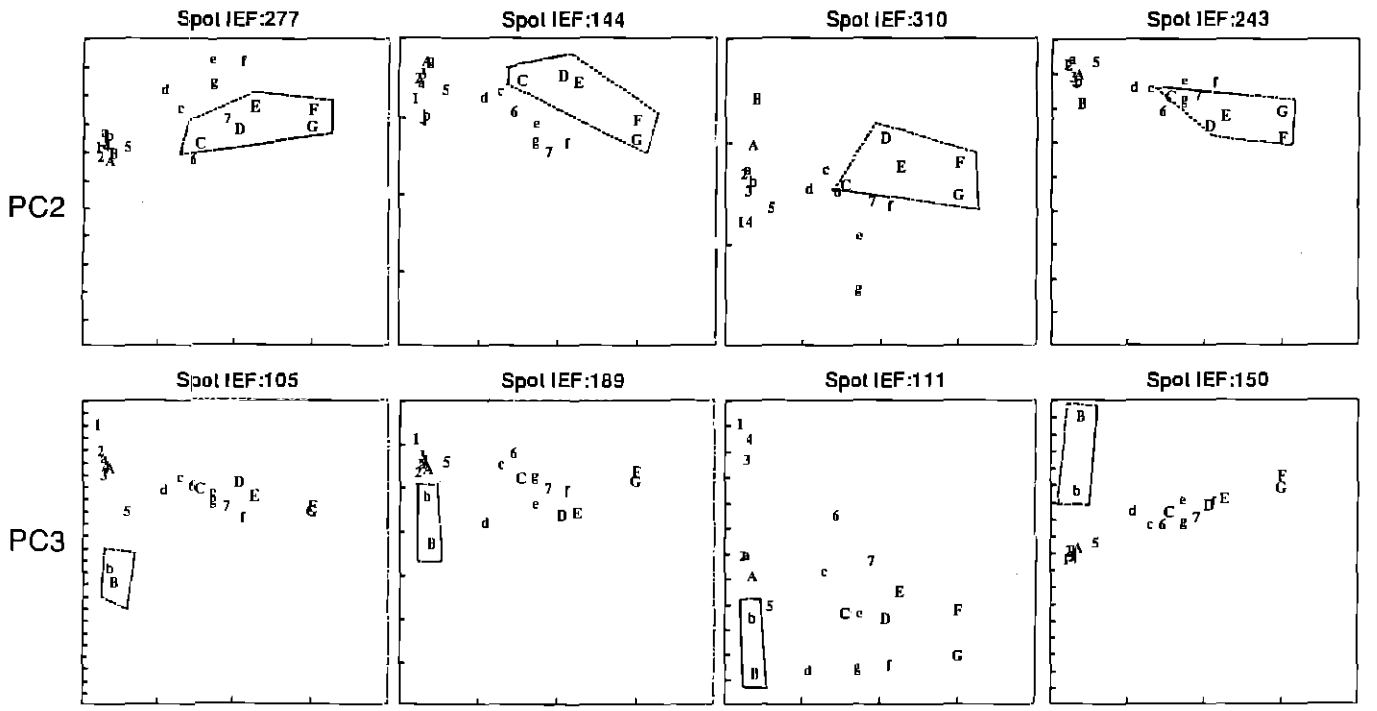

FIG. 7. Plots of abundance vs PC1 for proteins associated with PC2 and PC3. Plots similar to those shown in Fig. 6 showing, in the first row, the four spots making the largest absolute contributions to PC2 and, in the second row, the four spots making the largest absolute contributions to PC3. The former show evidence of a systematic difference between the 5-day (lower case group letters) and 35-day (upper case) treatment and control groups, while the latter show groups b and B (LY163443) as outliers.

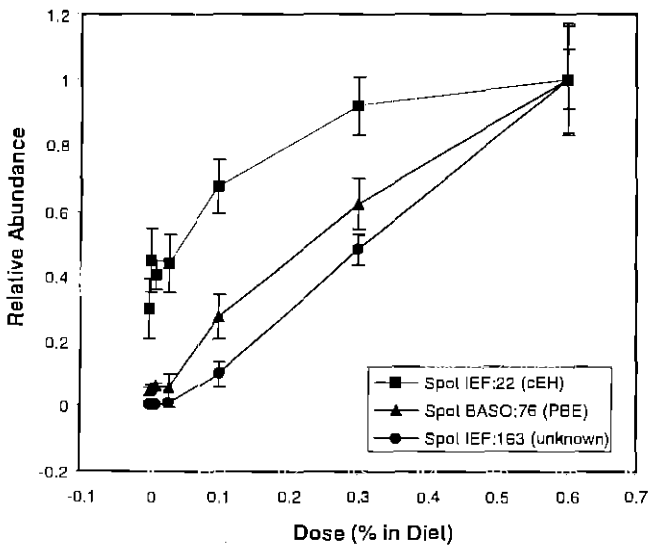

FIG. 8. Comparison of response curves for IEF22 (cytosolic epoxide hydrolase), IEF:163, and BASO:76 (80 kDa bifunctional enzyme). Relative induction plots showing fraction of maximal induction for three proteins as a function of LY171883 administered dose. cEH appears to be induced at lower doses than more inducible proteins. IEF:163, the most strongly induced protein in the acidic to neutral p*I* range, is an apparently novel protein, based on its absence from current sequence databases.

In this case, the 51% attributable to a coordinated PP effect (PC1) is a remarkably large fraction. Its size demonstrates that peroxisome proliferation was the largest influence in the study by far, leaving little room for major differences among the compounds and conditions tested.

If the first component (PC1) is indeed a global measure of peroxisome proliferation, it should correlate reasonably well with enzymatic measurements of peroxisomal β-oxidation, a characteristic inducible feature of the PP effect. A strong relationship was indeed found between these indices over all the compounds, time points, and doses tested, indicating that PC1 is a general measure of the gene expression changes caused by PP. Interestingly, the relationship between PC1 and β-oxidation is not entirely linear, especially at low dose, and this raises the possibility that the PP response includes aspects that do not follow the same dose–response curve as the enzymes of β-oxidation. Since PC1 is based on a combination of measurements of a large number of proteins, we decided to use it instead of β-oxidation as an index against which we could compare the responses of individual proteins.

When the group-averaged abundances of specific proteins were plotted against the average PC1 scores for those groups (a plot of specific vs overall protein response), we found that all the treatment groups fell on or near a consistent

curve for many proteins. Thus for these proteins, all the PP compounds produce effects at either 5 or 35 days that together approximate the effects of one compound at a range of doses. Data from the true dose–response curve of one compound, LY171883, also falls near this curve, reinforcing this view. The existence of a smooth relationship of this type, at least for some proteins, is consistent with direct gene expression control via a unified PP receptor mechanism. By selecting proteins that demonstrate such a response over a series of structurally diverse peroxisome proliferators, at different time points and doses, we have identified a core group of gene products that define the "homogeneous" PP response in mice. As it happens, some of these changes were detected earlier in a study not directed at peroxisome proliferation, where high doses of ibuprofen were found to produce a specific effect not interpretable at the time (Anderson *et al.*, 1987), but now recognizable as the core PP effect.

It is clear from the detailed behavior of these PP-responsive proteins that a simple induction picture of the effect is not adequate. While the peroxisomal enzymes, typified by the 80-kDa bifunctional enzyme (PBE; spot BASO:76), demonstrate very large inductions and thus dominate the effect in terms of protein mass, almost half of the responsive proteins show decreased abundance after treatment rather than induction. This result contrasts with the aggregate impression produced by most previously reported PP regulatory effects; these predominantly involve upregulation, although BiP/GRP78 has been reported to decrease (Motojima and Goto, 1992) under some circumstances, while increasing in others (Witzmann *et al.*, 1994). At this stage we do not know whether the downregulated genes observed here are regulated in a negative sense by PPAR (the receptor associated with many of the strong inductions) or whether there is a cascade of linked mechanisms that reduce expression of some proteins as a secondary response following major PPAR-controlled inductions. Evidence from the rat suggests that a major PP-responsive protein (rat IEF:367) also shows anti-synergistic regulation by the cholesterol-lowering treatments lovastatin and cholestyramine (Anderson *et al.*, 1991, and unpublished observations). It would thus not be surprising if PPAR regulation of enzymes of fatty acid metabolism caused secondary effects through mechanisms related to other forms of lipid.

While increased β-oxidation is often used as a biochemical indicator of the PP effect, an analysis of the dose–response and specific vs PC1 response curves for a variety of proteins indicates that some proteins showing more modest maximal inductions can be induced at lower PP doses. Chief among these is spot IEF:22 which shows low-dose induction and a plateau at higher doses. Using antibodies and pure protein, provided by Dr. Eric Dietz, and by computed p*I* and molecular weight from the known mouse sequence (Grant *et al.*, 1993), we have previously identified this spot as cytosolic epoxide hydrolase (cEH). The cEH gene is known to be induced by PP (Grant *et al.*, 1993) and the protein is partially localized in peroxisomes because of the modified peroxisome targeting sequence that it contains (Knehr *et al.*, 1993). It appears that cEH and at least two other proteins (spots IEF:150 and 239) show a plateau-limited inducibility, with initial induction at doses lower than those required to give substantial induction of the very inducible spot IEF:163 or the peroxisomal 80-kDa bifunctional enzyme (PBE). Such an effect could result from several causes: either cEH could be induced by a PPAR-independent alternative mechanism with lower inducibility threshold, cEH could be induced by a PPAR-dependent mechanism involving a PPRE with very high affinity for the receptor, or else the initial inducibility is similar to that of the other proteins, but the plateau effect limits observation of what would otherwise be a huge level of expression.

It is evident from these differences in the response curves of different proteins that specific biochemical effects of PPs could show highly nonlinear dose–response relationships. If, for example, some important biochemical event were influenced by the relative abundance of cEH and PBE, then the low-dose induction of cEH (where PBE is effectively uninduced) could lead to one outcome, while at higher doses (where cEH induction is plateaued, while PBE is rapidly increasing) an inverse outcome could occur. Thus it is possible, based on our results, that the low-dose and high-dose effects of PPs could be qualitatively different. A definitive resolution of this issue awaits a complete analysis of the biochemistry of peroxisome proliferation and identification of all the affected 2-D gel spots.

Both overall and specific protein effects on PC1 were generally larger after 35 days than after 5 days of exposure, for all PP examined. This contrasts with effects on cell replication, which are generally greater at 5 days than 35 (data not shown), and suggests that the protein changes observed on component 1 are not related directly to rates of cell division. PC2, the second largest component of change detected, separates the 5- and 35-day time points for all compounds tested, as well as for the controls. At present, the interpretation of this pattern of change is ambiguous: it could reflect adaptive gene expression changes following extended treatment, and thus may be of interest with respect to tumorigenesis. Alternatively, it could represent contributions associated with differences in animal cohorts or husbandry or differences in the 2-D analytical system over time (since the gels for the 5- and 35-day groups were not run at the same time). Finally, and we believe most likely, it could represent differences due to animal age: because the animals were relatively young at the start of the six-compound study, an additional 30 days of age in the 35-day groups could have a significant systematic effect. Earlier studies have shown evidence for significant protein changes in male mouse liver over the

range of 5 to 10 weeks of age (C. S. Giometti, personal communication).

Principal component 3 appears to be almost entirely a reflection of the effects of one compound—LY163443. This compound is a structural analog of the potent PP LY171883 (Fig. 1) and has the same pharmacologic activity (as a leukotriene receptor antagonist), but is not itself a proliferator and thus shows minimal difference from controls on PC1. The results on PC3 demonstrate, however, that LY163443 produces protein abundance alterations not shared with the PPs. These include both increases and decreases, as well as apparent charge modification of one protein (recently identified as fumarylacetoacetase by partial amino acid sequence analysis) that may be due to covalent adduct formation (details to be described elsewhere). The existence of such a component demonstrates unequivocally that the analytical approach used can not only measure the PP effect but resolve it from other, unrelated protein changes based on multiparameter protein abundance data.

The differences we observed between dose–response curves for various proteins may provide at least a partial explanation for the difference between our conclusions and those advanced by Giometti *et al.* (1991a, b) regarding the heterogeneity of liver protein responses to a series of PP. Giometti found only 19% of data variance to be explained by a component (PC2 in their case) likely to be the primary PP effect in a similar experiment and also reported that many individual proteins showed differences in levels of change caused by a series of PPs at a single dose and time point. This picture suggests that differences among PP are relatively large compared to the underlying similarities and hence that the currently accepted receptor-mediated mechanism may have a very limited power to explain the actions of structurally diverse PP. Our results suggest, on the contrary, that a large majority of the quantitative changes caused by all the PP examined result from operation of a single unified mechanism. Our results on the different dose–response curves shown by different proteins suggest an explanation for this apparent contradiction. Since Giometti used single doses and time points for each compound, and since these doses could not be set to achieve exactly equal effect levels, the different response curves we observed would predict different relative effects of the compounds on various proteins, in apparent conflict with the notion of a uniform response. However, by examining more sets of treatment conditions and using an overall measure of change (PC1) as a comparative index parameter, we observed that a unified response curve is probably a reasonable approximation for most proteins responding to PP.

At present, few of the 107 protein spots we selected as relevant to the PP effect have been identified (Table 1). We attempted to identify the most strongly induced of these (IEF:163) by sequence analysis, but found the peptide sequences we obtained to be absent from current sequence databases. This is somewhat surprising, since it suggests that one of the most strongly induced elements of the PP effect is likely to be an unknown protein. Recent progress in microanalytical methods, both chemical and mass spectrometric, give us reason to expect that most will be identified (or else found to be novel, then cloned and sequenced) within the next few years. This information will allow us to interpret the manifold effects of PP on liver gene regulation and metabolism in a comprehensive way and in particular to see whether major biochemical aspects of the pathway have escaped notice so far.

ACKNOWLEDGMENTS

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