Table 2. Expression of LIFR, CD36, kinogen 1, IGFBP1, calbD9K and Cyp2C13 in the liver of E2 treated rats

		mRNA levels (fg/pg βactin)					
		LIFR	CD36	kinogen 1	IGFBP1	calbD9K	Cyp2C13
♂	C	0.42 ± 0.034	5.0 ± 0.70**	0.41 ± 0.063	43.1 ± 10.1	20.8 ± 4.5	12.9 ± 1.11
	Cast	0.34 ± 0.031	21.0 ± 0.82	0.37 ± 0.041	59.6 ± 28.2	15.8 ± 2.9	9.44 ± 1.15
	Cast+E2	0.79 ± 0.049**	32.8 ± 1.38**	0.55 ± 0.087**	71.3 ± 9.4	24.4 ± 2.9**	6.93 ± 0.66
♀	C	24.60 ± 2.18**	59.3 ± 1.43**	1.00 ± 0.096	294 ± 60.6*	119 ± 6.2*	1.25 ± 0.21
	Ovx	1.18 ± 0.24	36.8 ± 1.63	0.81 ± 0.081	186 ± 23.5	72.4 ± 7.8	0.75 ± 0.33
	Ovx+E2	5.38 ± 0.98*	55.1 ± 9.77	1.17 ± 0.089**	249 ± 38.1*	89.8 ± 2.6*	1.13 ± 0.04

Mean ± S.E.M. (n = 4). *P < 0.05, **P < 0.01 vs. castration (male) or ovx (female).

es were confirmed with a capillary DNA sequencer (ABI 310; PerkinElmer Life Sciences). The extracted fragments were used as standards for quantification. The PCR conditions were 30 sec initial denaturing followed by 40 cycles for 5 sec per cycle at 95°C and 34 sec per cycle at 60°C. All mRNA contents were normalized with reference to β-actin mRNA.

Statistical analysis

Statistical comparisons were made by Dunnett's multiple comparison test.

RESULTS

Estrogen-responsive genes in the liver of 10-week-old rats

Candidate estrogen-responsive genes identified from the literature (Diel *et al.*, 1995; Singhal *et al.*, 2009) were examined for their response to estrogen in liver tissue. Candidates were insulin-like growth factor binding protein 1 (IGFBP1), calbindin (calb) D9k, kinogen 1, leukemia inhibitory factor receptor (LIFR), CD36 and CYP2C13. LIFR was found to be a significant estrogen-responsive gene, while CD36 also displayed weak responses (Table 2).

Estrogen and androgen receptor mRNA expression

Detectable amounts of ERα mRNA were noted in liver tissue with expression level at 17.0 ± 2.2 fg/pg β-actin while ERβ mRNA was not detected (< 0.01 fg/pg β-actin) in 10-week-old rats. The level of AR mRNA was also determined as 4.65 ± 0.74 fg/pg β-actin.

ERα mRNA level was relatively low at the beginning of experiment (at 6 weeks of age) and increased to constant levels at week 4 (at 10 weeks of age) in the control group (Fig. 1). When animals were treated with test chemicals, PB administration did not alter the ERα mRNA levels. In CF and PBO groups, on the other hand, the expression of ERα mRNA was significantly reduced at weeks 4 and 13.

The control AR mRNA level was also relatively low at day 3 (at 6 weeks of age) and increased to constant levels at week 4 (Fig. 1). PB administration strongly suppressed the AR mRNA expression at week 4 and it remained low at week 13. In CF and PBO groups, AR mRNA levels were also lower than that of control but increased afterward.

Expression of LIFR and CD36

The control level of LIFR expression decreased from day 3 (at 6 weeks of age) to week 13 (at 19 weeks of age) (Fig. 2). PB administration increased LIFR expression at week 4 and it remained higher than the control level at week 13. PBO strongly suppressed LIFR expression at week 4 but returned the control level at week 13, while CF had little effect on LIFR expression. The control level of CD36 increased from day3 to week 13. CD36 expression was suppressed by PB but elevated by CF and PBO.

DISCUSSION

Our previous study showed that well known non-genotoxic hepato-tumorigenetic agents including PB, CF (a peroxisome proliferator), and PBO (a pesticide synergist), induce hepatic hypertrophy (Nemoto *et al.*, 2011). To varying degrees, the increase in liver weight was evident even at day 3 in all of the groups. PBO induced severe diffuse hypertrophy with doubling of the liver weight, while less severe pathological features were noted in the CF and PB groups. The present study demonstrated that the expression of ERα and an estrogen responsive gene, LIFR, was dramatically changed after administration of these chemicals.

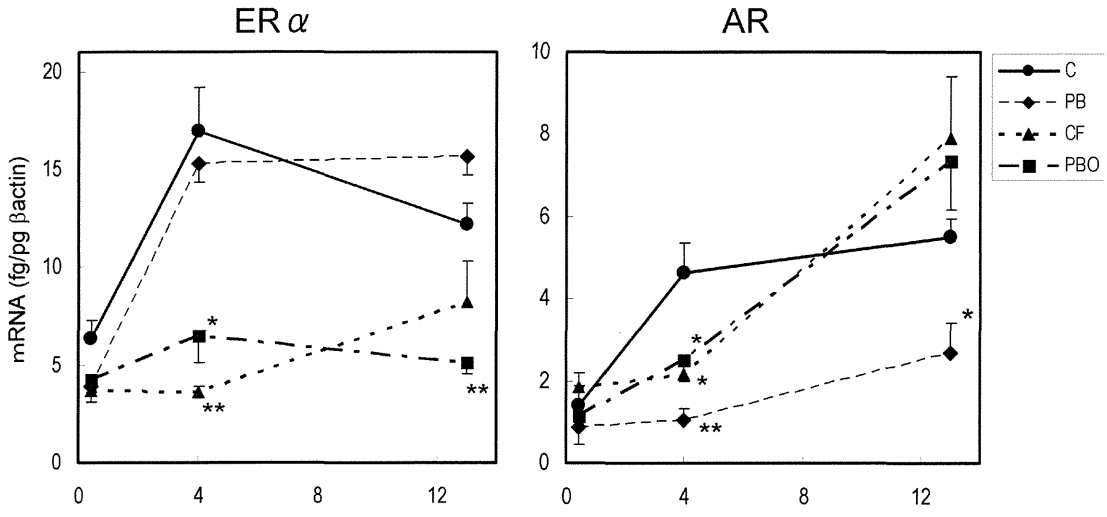

Fig. 1. ERα and AR mRNA levels in the liver. F344 rats (6 weeks old) were fed diets with PB, CF, or PBO, or a basal diet (C) for 3 days, 4 and 13 weeks. All mRNA levels were normalized to the expression of β-actin. Bars indicate SEM.

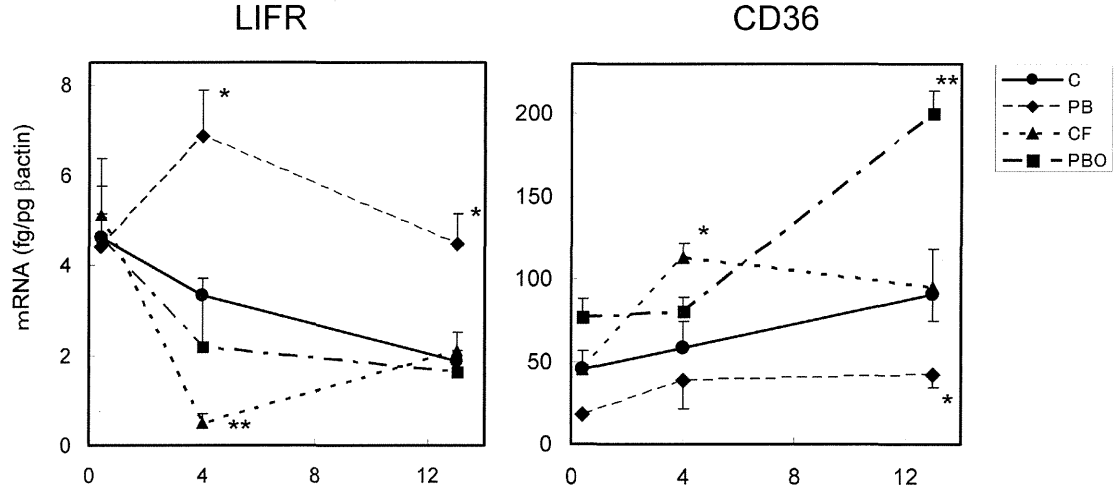

Fig. 2. LIFR and CD36 mRNA levels in the liver. F344 rats (6 weeks old) were fed diets with PB, CF, or PBO, or a basal diet (C) for 3 days, 4 and 13 weeks. All mRNA levels were normalized to the expression of β-actin. Bars indicate SEM.

Various lines of evidence suggest the involvement of sex steroid hormones in hepatocellular carcinogenesis in humans as well as in rodents. In a previous study in which rats were exposed to the peroxisome proliferator agents CF and BR931, total ER binding decreased while nuclear ER increased (Eagon *et al.,* 1996). Interestingly, in hepatic tumors induced by 9-10 month administration of these agents, ER activity was markedly decreased. Conversely, AR binding activity did not change in the short term and increased in tumor tissue. Studies of human HCC cases have indicated significant losses of ER and retention or increase of AR (Nagasue *et al.,* 1989; Boix *et al.*, 1993), which is consistent with the findings in our rat model. The reduction of ER expression may be involved in the development of HCC, and the eventual loss of expression was clearly related to HCC. Then, the decrease in ER expression and altering estrogen signaling in CF- and PBO-treated rats may be indicative to hepatocarcinogenesis. Hepatocarcinogenic chemicals may potentially change steroid hormone metabolism and serum hormone levels.

Previous studies, however, have indicated no change in serum estradiol levels by PB (Mesia-Vela *et al.*, 2006) or CF (Eagon *et al.*, 1996).

In rat liver, the ER concentration is low before puberty and subsequently increases (Rochman *et al.*, 1985). In the present experiment in male rats, the expression of ERα mRNA was low in 6-week old rats (experimental day 3) and increased at week 4 and beyond. Similarly, AR expression increased from day 3 to week 4 and beyond, a finding consistent with a previous study showing lower expression of AR mRNA before puberty (Song *et al.*, 1991).

In the present study, we examined the expression of several estrogen- and androgen-responsive gene candidates to determine the possible markers of estrogen- and androgen signaling. There are a limited number of reports regarding sex steroid hormone-dependent genes in the liver, although the liver is considered to be a target organ for sex steroids. IGFBP1, calb D9k and kinogen 1 were reported to be up-regulated by E2 in the rat Fe33 hepatic tumor cell line (Diel *et al.*, 1995). A cDNA microarray analysis of hepatic genes in E2-treated Sprague-Dawley rats showed up-regulation of LIFR, CD36, and CYP2C13 (Singhal *et al.*, 2009). Despite the reported E2-responsiveness of all of these genes, only LIFR was confirmed to be regulated in our study, while weak E2 regulation of CD36 was noted. Candidates for androgen-responsive genes were A2MG and carbonic anhydrase 3 (Carter *et al.*, 1984; Chatterjee *et al.*, 1990). Since neither of the genes displayed significant androgen responsiveness in our system (data not shown), no further examinations were conducted.

Recently, LIFR has been identified as a specifically down-regulated gene in human HCC cases based on cDNA microarray analysis (Okamura *et al.*, 2010). Promoter hypermethylation of the LIFR gene was observed in 48% of tumor tissues, and this was clearly related to the suppression of gene expression. LIFR is an integral component of the glycoprotein 130-LIFR signaling complex, which acts as a signal receptor for cytokines such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor. PTEN (phosphatase and tensin homolog deleted on chromosome 10) was found to act as a general tumor suppressor of somatic malignancies (Li *et al.*, 1997). Since PTEN-Akt-FOXO signaling regulates the LIFR-Stat3 pathway, which plays a critical role in suppressing malignant transformation, loss of PTEN results in tumorigenesis correlating with low expression of LIFR (de la Iglesia *et al.*, 2008). LIFR itself may therefore act as a tumor suppressor in HCC. The present study demonstrated that LIFR mRNA expression was regulated by estrogen. CF and PBO suppressed ERα mRNA expression and as a consequence, the expression of LIFR was reduced. Therefore LIFR may be a key mediator of the ability of CF and PBO to promote HCC. On the other hand, change in expression of CD36, a weak responder to estradiol, did not correlate to ER levels. The expression of CD36 probably does not primarily depend on estrogen.

PB has been studied extensively but the mechanism of its hepatocarcinogenic action in rodents is unclear. In 2-stage carcinogenesis models, the tumor-promoting activity of PB is apparent (Farinati *et al.*, 2002). PB promotes focal hepatic lesion growth both by increasing DNA synthesis and decreasing the rate of apoptosis (Kolaja *et al.*, 1996). Peroxisome proliferators including CF are another group of non-genotoxic chemicals that induce hepatocarcinogenesis (Rao *et al.*, 1991). Chronic administration of these types of chemicals results in altered areas of liver, followed by neoplastic nodules, and finally HCC. The mechanism of carcinogenesis is unclear, although there appears to be a strong association with the potency of peroxisome proliferation, and increased oxidative stress may also play a role. PBO, a pesticide synergist, was not considered as a carcinogen in humans until a strong positive result was reported in a 2-year study of F344 rats (Takahashi *et al.*, 1994). It was suggested that the carcinogenic mechanisms of PBO are similar to those of PB in terms of induction of CYP2B and inhibition of gap junctional intercellular communication (Okamiya *et al.*, 1998). However, the present study showed that the ER-expression and estrogen-signaling profiles of PBO were similar to those of CF but differed from those of PB.

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Original Article

Altered expression of *GADD45* genes during the development of chemical-mediated liver hypertrophy and liver tumor promotion in rats

Shogo Ozawa[1,*], Toshie Gamou[1,*], Wataru Habano[1], Kaoru Inoue[2], Midori Yoshida[2], Akiyoshi Nishikawa[2], Kiyomitsu Nemoto[3] and Masakuni Degawa[3]

[1]*Department of Pharmacodynamics and Molecular Genetics, School of Pharmacy, Iwate Medical University, 2-1-1, Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan*
[2]*Division of Pathology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[3]*Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1, Yada, Suruga-ku, Shizuoka 422-8526, Japan*

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ABSTRACT — The purpose of our study was to examine the altered gene expression associated with nongenotoxic chemical-mediated liver hypertrophy and successive liver tumor promotion. Five-week-old male rats were fed a basal diet or a diet containing phenobarbital (PB) or clofibrate (CF) for 3 days, 4 weeks, and 13 weeks. Hepatic expression profiling of cell growth- and stress-related genes, as well as those involved in xenobiotic metabolism, was performed by DNA microarray and/or real time quantitative reverse transcription-polymerase chain reaction. The induction of liver hypertrophy and hepatic cytochrome P450 (CYP) isoforms (CYP2B1/2B2 for PB and CYP4A1 for CF) by PB and CF were clearly observed at all the treatment periods examined. Genes encoding DNA damage-inducible 45 (GADD45) family proteins, in particular *GADD45g* (GADD45 gamma) were down-regulated by treatment with either PB or CF for 4 and 13 weeks. The chemical-mediated development of liver hypertrophy, induction of hepatic CYPs, and suppression of hepatic *GADD45g* gene at week 13 disappeared at 4 weeks following cessation of the chemical treatment. Additionally, DNA microarray data indicated that cell cycle-related genes such as cyclins *CCNB1* and *CCNA2* and cyclin-dependent kinase inhibitor *CDKN3* were also down-regulated by treatment with either PB or CF at 13 weeks. Since GADD45 functions as a chemical and radiation stress sensor by interacting with cyclins and cyclin-dependent kinase inhibitors, the decrease in the gene expression of *GADD45g* mRNA observed in this study, may be associated with nongenotoxic chemical-induced tumor promotion of hepatocarcinogenesis rather than liver hypertrophy.

Key words: Liver tumor promotion, Liver hypertrophy, Growth arrest and DNA damage-inducible gene 45, Constitutive androstane receptor, Phenobarbital, Clofibrate

INTRODUCTION

A number of xenobiotics that cause hypertrophy and altered foci in the liver are often reported to induce cytochrome P450s (CYPs) in rodents (Chen and Eaton, 1993; Huang *et al.*, 2005; Deguchi *et al.*, 2009). Phenobarbital (PB), an inducer of the CYP2B subfamily enzymes, has been characterized as a typical nongenotoxic carcinogen, which, through its agonistic effect on the constitutive androstane receptor (CAR), causes liver hypertrophy and successive promotion of liver tumors (Feldman *et al.*, 1981; Yamamoto *et al.*, 2004). Clofibrate (CF), a typical peroxisome proliferator-activated receptor α (PPARα) agonist, is also a nongenotoxic hepatocarcinogen and shows an ability to induce hypertrophy, hyperplasia, and tumor formation in the rodent liver (Holden and

Correspondence: Shogo Ozawa (E-mail: sozawa@iwate-med.ac.jp)
*These authors equally contributed to this work.

Tugwood, 1999; Reddy *et al.*, 1979). Peroxisome proliferator-mediated activation of PPARα results in induction of various enzymes, including CYP4A1, fatty acid β-oxidation enzyme, acyl-CoA oxidase, apolipoproteins, fatty acid transporters, lipoprotein lipase, and thioesterases (Peters *et al.*, 2005).

Toxicogenomic studies conducted to identify molecular markers of hepatotoxicity and hepatocarcinogenicity in rodents reveal that hepatotoxicants may be classified into 3 types of chemicals: CYP inducers, hepatocellular necrosis inducers, and hepatocellular cholestasis inducers (Hamadeh *et al.*, 2002a, 2002b; de Longueville *et al.*, 2003; Jessen *et al.*, 2003; Kramer *et al.*, 2004; Elrick *et al.*, 2005; Stierum *et al.*, 2005). PB and CF are classified as CYP inducer-type hepatotoxicants (de Longueville *et al.*, 2003); however, little is known regarding the key molecular events involved in the process of chemical-mediated development of liver hypertrophy and hepatocarcinogenesis.

Elrick *et al.* (2005) reported that PB activates several stress response genes, including growth arrest and DNA damage-inducible 45β gene (*GADD45b*) in rats. GADD45 was originally identified to be associated with p53 in a radiation-induced DNA damage response (Zhan *et al.*, 1993); however, it has since been confirmed that GADD45 family members GADD45α, GADD45β, and GADD45γ interact with cyclins and cyclin-dependent kinase inhibitors, and are implicated in cell cycle arrest as a cellular stress response. Therefore, GADD45α, β, and γ play an important role in the stress signaling pathways controlling cell cycle arrest, DNA repair, cell survival, and/or apoptosis (Liebermann and Hoffman, 2008).

GADD45α is involved in the DNA repair and survival of damaged cells (Hollander *et al.*, 2001; Gupta *et al.*, 2006). GADD45β is an antiapoptotic factor, inhibiting c-Jun N-terminal kinase (JNK) signaling in cooperation with NF-κB (Papa *et al.*, 2004, 2007), and GADD45γ inhibits the cell cycle progression from S to G1 phase through interactions with proliferating cell nuclear antigen and p21 (Zhao *et al.*, 2000; Azam *et al.*, 2001). Therefore, the next logical step is to examine the expression of GADD45 family members during the process of chemical-mediated development of liver hypertrophy and hepatocarcinogenesis.

In the present study, we administered the CYP inducer-type liver hypertrophic chemicals PB and CF to Fischer 344 (F344) rats for 3 days, 4 weeks, and 13 weeks. Subsequent hepatic gene expression profiling of rats treated with PB or CF for 13 weeks revealed down-regulation of some cyclins, cyclin-dependent kinase inhibitors, and *GADD45g*. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis indicated that liver hypertrophy and induction of CYP(s) (CYP2B1/2B2 for PB and CYP4A1 for CF) resulted from treatment with either PB or CF for all periods examined and, notably, suppression of the *GADD45g* gene was observed from 4 to 13 weeks after dietary treatment with either chemical.

MATERIALS AND METHODS

Chemicals

PB and CF were purchased from Wako Pure Chemicals Co. Ltd. (Tokyo, Japan). All other chemicals used were of the highest grade available.

Animals and treatments

Sixty male F344 rats (4-week-old), weighing 80 ± 5 g, were purchased from Charles River Co. Ltd. (Tokyo, Japan) and were housed 5 rats per cage with access to tap water and a commercial basal diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) during the acclimatization period (one week). During the 1-week acclimatization period, daily clinical signs and body weights were checked. At 5 weeks of age, healthy animals were randomly assigned into 3 groups (20 rats per group) to avoid significant differences of group mean body weights among control (untreated), PB-treated, and CF-treated groups at the beginning of experiment. The rats were fed a basal diet (CRF-1; Oriental Yeast Co. Ltd.) or CRF-1 containing 500 ppm of PB or 2,500 ppm of CF for up to 13 weeks, as illustrated in Figure 1. Both doses are reported to cause liver neoplasms after long-term treatment (Feldman *et al.*, 1981; Reddy *et al.*, 1979), and therefore were selected as liver hypertrophy and tumor-inducible doses for this experiment. After 3 days, 4 weeks, and 13 weeks of administration of PB or CF, the livers of 5 rats from each treatment group were excised. At 13 weeks, 5 rats from each treatment group entered a 4-week recovery period, during which they were fed a basal diet (CRF-1), and subsequently, at 17 weeks after the start of the treatment, the livers of the 5 rats were excised. After sacrifice, absolute and relative weights of the liver were measured; the livers were cut into small pieces and stored at -80°C until use. All experiments were performed in accordance with the guidelines for animal experiments set forth by the National Institute of Health Sciences.

Histopathology and morphology

The rat livers from the control and chemical-treated groups were examined for histopathology at day 3, and weeks 4, 13, and 17. After fixation in 10% neutral buff-

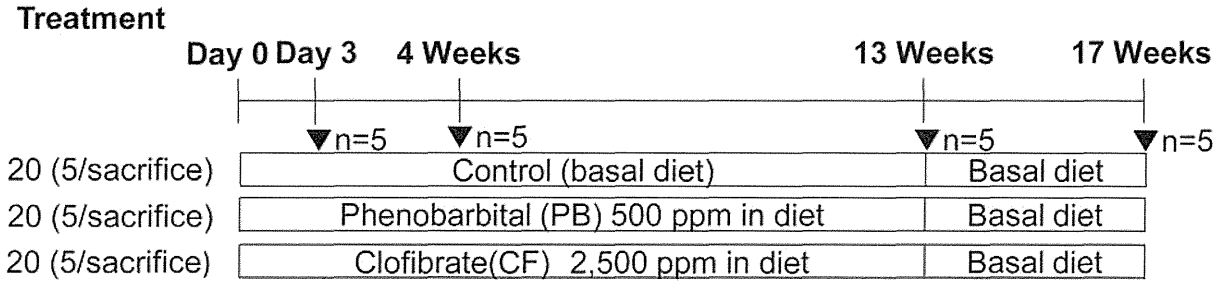

Fig. 1. Experimental study design. Schedules for the administration of phenobarbital and clofibrate are illustrated. A total of 20 animals were used for each chemical, 5 animals were used for each treatment group and period, and were sacrificed at 3 days, 4 weeks, 13 weeks, and 17 weeks (13 weeks plus an additional 4-week chemical-free period).

ered formalin, the livers were embedded in paraffin wax, cut into 3 μm thick sections, routinely processed, and stained with hematoxylin and eosin (H & E).

DNA microarray and RT-PCR analyses

Hepatic gene expression was analyzed by DNA microarray (Agilent Technologies) and qRT-PCR. Total RNA was extracted from a frozen rat liver (approximately 100 mg), using TriPure reagent (Roche) according to the manufacturer's instructions; total concentration and quality of the purified RNA were measured by spectrophotometry. RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan) as well as ratio between Absorbance unit at 260 nm and that at 280 nm, which should be higher than 1.9.

For DNA microarray analysis, total RNA (500 ng), which comprised total hepatic RNA (100 ng per rat) from 5 individual rats in each experimental group, was labeled with cyanine-3 using a Quick-Amp Labeling Kit (Agilent Technologies). The labeled targets were hybridized to Whole Rat Genome 4x44K oligo DNA microarrays (Agilent Technologies) and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Image analysis and raw array data generation were processed using Feature Extraction software (Agilent Technologies). Normalization of the raw array data was performed using Genespring software Ver. 11.

For qRT-PCR analysis, cDNA was prepared from 2 μg of total RNA by reverse transcription using an RT-PCR kit (Invitrogen) according to the manufacturer's instructions.

qRT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was performed using the ABI PRISM 7500 Real-time PCR System (Applied Biosystems) following the manufacturer's protocol supplied with the SYBR Green PCR Master Mix.

The mRNA levels of *GADD45a*, *GADD45b*, *GADD45g*, *CYP2B1*, *CYP2B2*, and *CYP4A1* were determined, using *GAPDH* as an internal control. PCR primers were designed using the Primer3Plus software and the primer sequences are listed in Table 1; the PCR efficiency for all target genes was almost equal. Target mRNA levels were determined according to the $2\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). C_t values for the specific target gene ($C_{t\,target}$) and *GAPDH* ($C_{t\,GAPDH}$) were obtained for the chemical-treated and control (chemical-untreated) rats. Assuming these values as $C_{t\,GAPDH}$,

Using A for chemical-treated group = $(C_{t\,target} - C_{t\,GAPDH})_{treatment}$.

Using B for control group = $(C_{t\,target} - C_{t\,GAPDH})_{control}$.

We took the average (B_{mean}) of the control group (n = 5) for each target gene and mRNA levels of the target gene relative to the control group were calculated as $2^{(A-Bmean)}$. The mRNA levels of each chemical-treated group (n = 5) were expressed as mean (S.D.).

In the present qRT-PCR analysis, we examined differences in Ct values for GAPDH among the untreated, PB- and CF-administered study groups. As a result, we did not observe any significant difference in Ct values for GAPDH. Therefore, GAPDH was considered as an appropriate internal control for the present $2\Delta\Delta Ct$ analysis to evaluate effects of PB and CF on various gene expressions.

Statistical analysis

The data on the body weights, organ weights, and hepatic mRNA levels were expressed as mean (S.D.) (n = 5). Statistically significant difference between the control and chemical-treated groups was evaluated by one-way ANOVA with a post hoc test (Dunnett's test).

RESULTS

Increases in the absolute and relative liver weights

The chemical treatments did not result in a remarkable decrease in the body weights of rats using the experimental conditions illustrated in Fig. 1. Changes in the absolute and relative liver weights (g liver weight/100 g body weight) are illustrated in Figs. 2A and B for each treatment period. The absolute liver weights of rats fed 500 ppm PB and 2,500 ppm CF significantly increased at day 3, week 4, and week 13 as compared with controls. Percent increases in PB-fed rats were 25%, 24%, and 35% at day 3, week 4, and week 13, respectively; those in CF-fed rats were 24%, 36%, and 31%, respectively. Statistically significant increases in relative liver weights were observed for up to 13 weeks after the PB and CF treatments. Percent increases at day 3, week 4, and week 13 were 24%, 24%, and 33% in PB-treated rats, and 26%, 49%, and 35% in CF-treated rats, respectively. After the 4-week recovery period, the absolute and relative liver weights had returned to those of the control rats.

Histopathology

Representative H & E-stained liver sections of the control and chemical-treated rats are shown in Fig. 3. Treatment with PB for 4 weeks resulted in development of centrilobular hypertrophy of hepatocytes (Fig. 3B), which occurred in a time-dependent manner from day 3 up to week 13. The hepatocytes displayed a ground-glass appearance in their cytoplasm; these histological changes in the liver resulting from PB treatment for 13 weeks disappeared 4 weeks after the cessation of the chemical treatment. The CF-dependent hepatocellular hypertrophy characterized by eosinophilic cytoplasm was also observed at week 4 (Fig. 3C) and week 13, and the hepatocellular hypertrophy displayed a diffuse distribution throughout the lobules of the liver.

Effects of PB and CF on hepatic gene expression

Altered gene expression in the liver of the rats treated with PB or CF for 13 weeks were initially examined by DNA microarray analyses and revealed changes in gene expression levels, including the *GADD45* family genes and the *CYP2B/4A* subfamily genes, which were further examined by qRT-PCR.

Altered expression of genes related to cell cycle arrest and DNA damage

Changes in the hepatic gene expression profile of genes related to cell cycle arrest and DNA damage were first examined in rats treated with PB and CF for 13

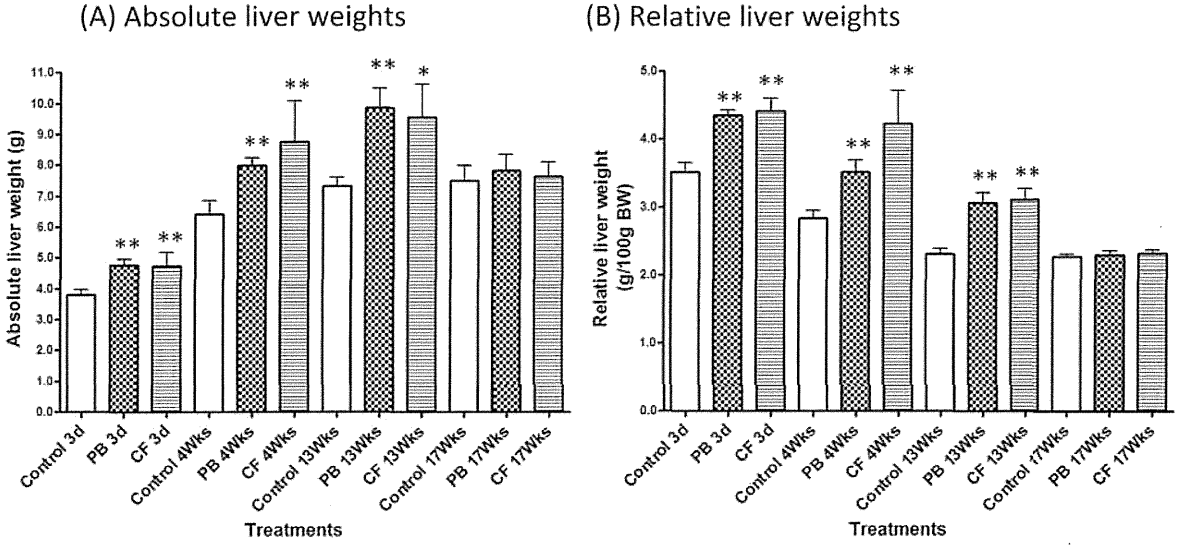

Fig. 2. Liver weights of rats administered with phenobarbital (PB) or clofibrate (CF). Absolute liver weights (A) and relative liver weights (g/100 g body weight, B) are shown as a mean (S.D.) for 5 rats from each experimental group. The difference in the values from those of the corresponding controls was evaluated by Dunnett's test. *, **Statistically significant differences from the corresponding controls: $*P < 0.05$, $**P < 0.01$.

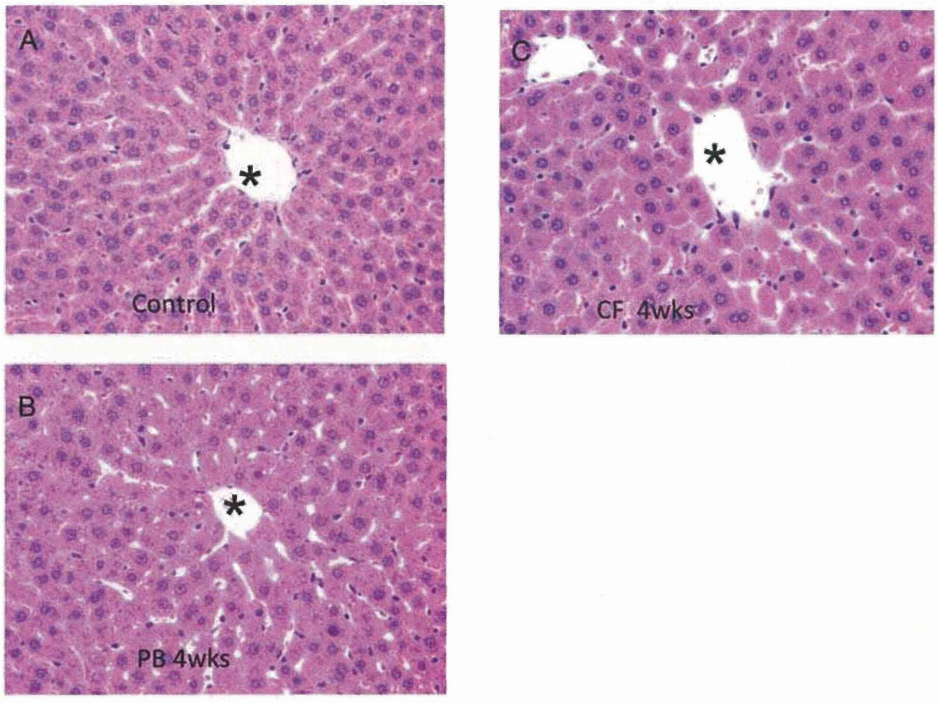

Fig. 3. Representative liver hypertrophy observed microscopically in control rats and rats treated with phenobarbital (PB) and clofibrate (CF) for 4 weeks. Representative photographs (H & E staining) of livers of control rats (A) and those administered with phenobarbital (B) or clofibrate (C) for 4 weeks are shown. Doses of chemicals are 500 ppm for phenobarbital and 2,500 ppm for clofibrate. PB induced marked hepatocellular hypertrophy with ground-glass appearance in centrilobular area (B). Diffuse hepatocellular hypertrophy with eosinophilic cytoplasm was a feature of CF treatment(C). *, Central vein.

Table 1. Nucleotide sequence of oligonucleotide primers used for determining mRNA levels in quantitative real-time PCR

Accession number	Gene symbol	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
NM_024127	*GADD45a*	TCTGTTGCGAGAACGACATC	TGTGATGAATGTGGGTTCGT
NM_001008321	*GADD45b*	GCTGGCCATAGACGAAGAAG	TGACAGTTCGTGACCAGGAG
AB020978	*GADD45g*	TCGCACAATGACTCTGGAAG	AGGGTCCACATTCAGGACT
NM_001134844	*CYP2B1*	CTTGTCCCTATTGGAGTACC	AGACAAATGCGCTTTCCTGTG
XM_341808	*CYP2B2*	CATCCCTTGATGATCGTACC	CAGACACCTTCAATCCTGAG
NM_175837	*CYP4A1*	TTGCTCTTGCTGAATGGACA	CCAGCTGTTCCCATTTGTCT
NM_017008	*GAPDH*	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

weeks. The representative results (change in > 2.0-fold or < 0.5-fold by the chemical treatments) are summarized in Table 2. The genes of cyclins (*CCNB1*and*CCNA2*), cyclin-dependent kinase inhibitor *CDKN3*, and *GADD45g* were down-regulated by both PB and CF treatments. As previously reported (Motojima *et al.*, 1998; Tamura *et al.*, 2006), the genes *LPL*, *CD36*, and *LEPR*, associated with fatty acid metabolism and energy generation (Holden and Tugwood, 1999), were up-regulated by both chemical treatments (data not shown).

Since cyclins and cyclin-dependent kinase are known to function as chemical- and radiation-induced stress sensors by interacting with GADD45 proteins, the altered gene expression of hepatic GADD45 family members, GADD45α, GADD45β, and GADD45γ, by PB and CF treatments were further examined by qRT-PCR.

Altered gene expression of GADD45α

Dietary treatment with PB for 4 and 13 weeks resulted in decreases in levels of hepatic *GADD45a* mRNA by 43% and 26%, respectively (Fig. 4). Treatment with CF for 4 and 13 weeks led to significant decreases in the *GADD45a* mRNA level (by 81% and 59%, respectively). The CF-dependent decrease in *GADD45a* mRNA level was restored after the 4-week recovery (basal diet) treatment.

Altered gene expression of GADD45β

Expression levels of *GADD45b*, a CAR-regulated gene (Yamamoto and Negishi, 2008), at 3 days and 4 weeks after PB treatment were 2.8-fold ($P < 0.01$) and 4.1-fold ($P < 0.001$) higher than corresponding controls, respectively (Fig. 5). In contrast, in CF-treated rats, no such significant increases at day 3 and week 4 were observed, while significant down-regulation of *GADD45b* occurred at week 13. By DNA microarray analysis, *GADD45b* was down-regulated at week 13 (0.46-fold of control). After the 4-week recovery (basal diet) period, slight, but significant elevation of *GADD45b* mRNA level was observed (2.1-fold, $P < 0.05$).

Altered gene expression of GADD45γ

Statistically significant and persistent down-regulation of *GADD45g* gene expression by PB and CF was observed at weeks 4 and 13 (Fig. 6). Treatment with PB for 4 and 13 weeks significantly decreased the level of *GADD45g* mRNA by 82% and 64%, respectively. Similarly, CF treatment for 4 and 13 weeks decreased the mRNA level by 80% and 85%, respectively. The CF-dependent decreases in the level of *GADD45g* mRNA were restored after the 4-week recovery (basal diet) period. In the case of PB, significant, but rather deviated, elevation in *GADD45g* mRNA level was detected after the 4-week recovery (basal diet) period.

Altered gene expression of CYP2B1 and CYP2B2

Significant increases in the level of hepatic *CYP2B1* mRNA were observed in the rats treated with PB by 82-fold at day 3, 65-fold at week 4, and 26-fold at week 13 (Fig. 7). The increased level at week 13 returned to the level of the corresponding controls after the 4-week

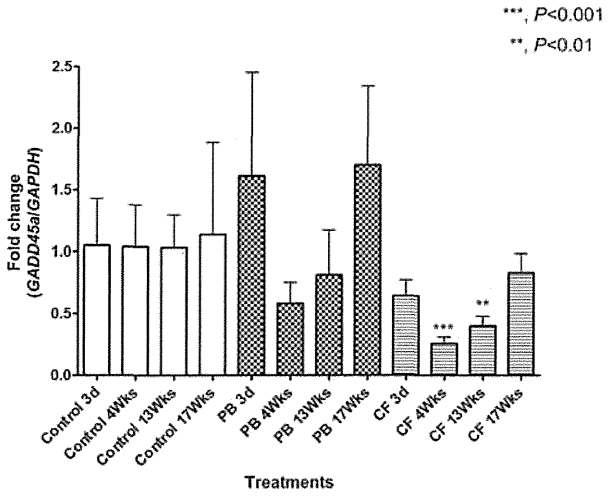

Fig. 4. *GADD45a* mRNA expression in livers of rats administered with phenobarbital (PB) or clofibrate (CF). Hepatic *GADD45a* mRNA levels were measured in rats dosed with 500 ppm phenobarbital or 2,500 ppm clofibrate. *GADD45a* mRNA levels are shown as a mean (S.D.) for 5 rats from each experimental group. The differences in the *GADD45a* mRNA levels were compared between the control and the chemical-administered groups for each time point. **, ***Statistically significant differences from the corresponding controls: ** $P < 0.01$, *** $P < 0.001$.

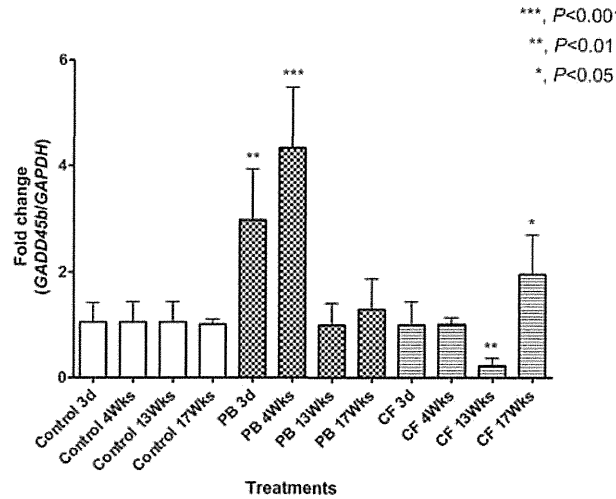

Fig. 5. *GADD45b* mRNA expression in livers of rats administered with phenobarbital (PB) or clofibrate (CF). Hepatic *GADD45b* mRNA levels were measured in rats dosed with 500 ppm phenobarbital or 2,500 ppm clofibrate. *GADD45b* mRNA levels are shown as a mean (S.D.) for 5 rats from each experimental group. The differences in the *GADD45b* mRNA levels were compared between the control and the chemical-administered groups for each time point. *, **, ***Statistically significant differences from the corresponding controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. Expression changes of cyclins and related genes (> 2.0- or < 0.5-fold) in livers of rats after administration of PB or CF for 13 weeks together with known up-regulation of metabolism genes

Gene name (Probe ID)	Accession number Systematic name	Description	Gene Ontology (biological process)	Fold change	
				PB	CF
CCNA2 (A_44_P527480)	NM_053702	Cyclin A2 (Ccna2), mRNA	GO:0000074 (regulation of progression through cell cycle)	0.36	0.26
CCNB1 (A_44_P534089)	NM_171991	Cyclin B1 (Ccnb1), mRNA	GO:0000086 (G2/M transition of mitotic cell cycle)	0.29	0.26
CDKN3 (A_44_P513360)	BC167026 NM_001106028	Cyclin-dependent kinase inhibitor 3, mRNA	GO:0000079 (regulation of cyclin-dependent protein kinase activity)	0.25	0.13
GADD45a (A_44_P216395)	NM_024127	Growth arrest and DNA- damage- inducible 45 alpha (Gadd45a), mRNA	GO:0000074 (regulation of progression through cell cycle) ;GO:0000079 (regulation of cyclin-dependent protein kinase activity)	0.95	0.64
GADD45b (A_43_P16529)	NM_001008321	Growth arrest and DNA- damage- inducible 45 beta (Gadd45b), mRNA	GO:0000074 (regulation of progression through cell cycle) ; GO:0006915 (apoptosis) ;GO:0006950 (response to stress)	1.67	0.46
GADD45g (A_44_P365379)	NM_001077640	Growth arrest and DNA- damage- inducible 45 gamma (Gadd45g), mRNA	GO:0006469 (negative regulation of protein kinase activity)	0.49	0.27
CYP2B1 (A_44_P316194)	ENSRNOT00000047540	Cytochrome P450 2B1	GO:0006118 (electron transport)	20	1.1
CYP4A1 (A_44_P1059556)	NM_175837	Cytochrome P450 4A1, mRNA	GO:0006118 (electron transport)	1.0	3.1

Fold change represents ratio of hepatic mRNA levels of chemical treated rats to those of control diet-fed rats.

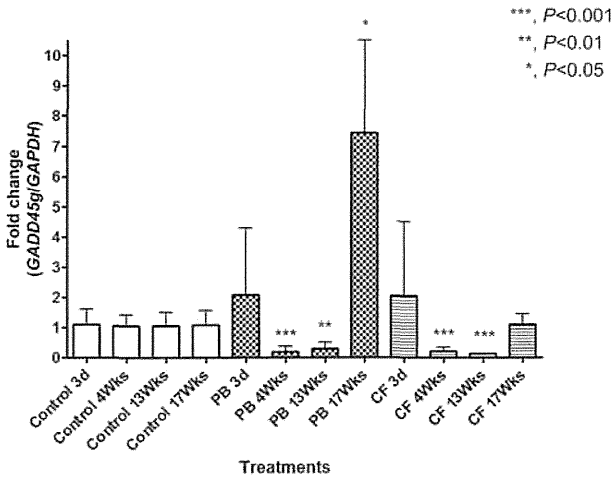

Fig. 6. *GADD45g* mRNA expression in livers of rats administered with phenobarbital (PB) or clofibrate (CF). Hepatic *GADD45g* mRNA levels were measured in rats dosed with 500 ppm phenobarbital or 2,500 ppm clofibrate. *GADD45g* mRNA levels are shown as mean (S.D.) for 5 rats from each experimental group. The differences in the *GADD45g* mRNA levels were compared between the control and chemical-administered groups for each time point. *, **, ***Statistically significant differences from the corresponding controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

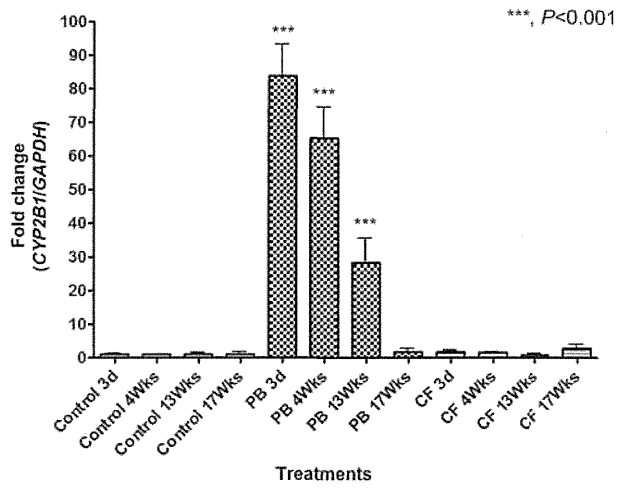

Fig. 7. *CYP2B1* mRNA expression in livers of rats administered with phenobarbital (PB) or clofibrate (CF). Hepatic *CYP2B1* mRNA levels were measured in rats dosed with 500 ppm phenobarbital or 2,500 ppm clofibrate. *CYP2B1* mRNA levels are shown as mean (S.D.) for 5 rats from each experimental group. *CYP2B1* mRNA levels were compared between the control and the chemical-administered groups for each time point. ***Statistically significant differences from the corresponding controls: *** $P < 0.001$.

recovery (basal diet) treatment. In addition, treatment with PB resulted in an increase in the level of *CYP2B2* mRNA: 10.3-fold at day 3, 6.1-fold at week 4, and 8.3-fold at week 13 (data not shown). No significant increases were observed with CF treatment.

Altered gene expression of CYP4A1

Up-regulation of the *CYP4A1* gene is reported in male rat livers following exposure to CF (Baker *et al.*, 2004). Therefore, we examined the effect of dietary CF on expression of *CYP4A1*, a PPAR-α target gene, and confirmed a significant increase in the level of hepatic *CYP4A1* mRNA by CF (Fig. 8). The *CYP4A1* mRNA levels at day 3, week 4, and week 13 were 2.1-, 2.5-, and 2.8-fold higher than the corresponding controls, respectively. No significant increase in the *CYP4A1* mRNA level was observed in any PB-treated rats.

DISCUSSION

The ultimate goal of our study was to determine signature gene expression for liver hypertrophy and successive liver tumor promotion induced by nongenotoxic chemicals. Liver hypertrophy developed in rats treated with either PB or CF for 3 days, 4 weeks, and 13 weeks. Similarly, the induction of hepatic *CYP2B1/2B2* and *CYP4A1* was observed in PB- and CF-treated rats, respectively, for all the treatment periods examined. These chemical-induced increases in liver weight and liver hypertrophy and levels of hepatic CYPs (*CYP2B1/2B2* or *CYP4A1*) were returned to those of the control rats at 4 weeks after the cessation of the chemical treatment. Thus, the nongenotoxic chemicals displayed reversible effects on induction of liver hypertrophy and hepatic CYPs, which is consistent with a known good correlation between the development of liver hypertrophy and the induction of hepatic CYPs, such as PB (CAR activation)-dependent CYP2B induction and CF (PPARα activation)-dependent CYP4A1 induction (Chen and Eaton, 1993; Honkakoski *et al.*, 1998a, 1998b; Wei *et al.*, 2000; Sueyoshi and Negishi, 2001; Yoshinari *et al.*, 2001; Yamamoto *et al.*, 2004; Huang *et al.*, 2005).

Gene expression profiling in the liver of the rats treated with either PB or CF for 13 weeks revealed the down-regulation of genes associated with cell cycle regulation and DNA damage repair: cyclins *CCNB1*and *CCNA2*, cyc-

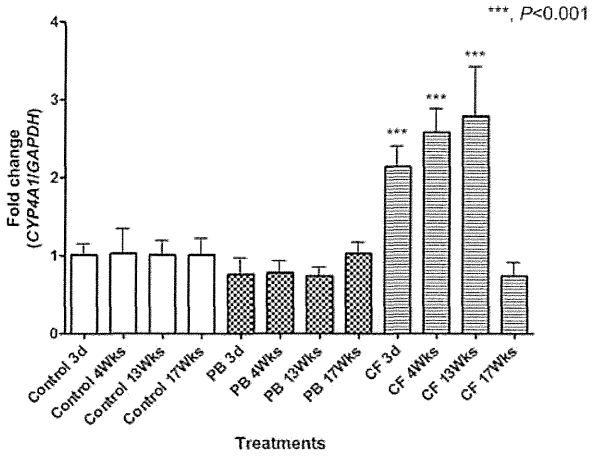

Fig. 8. *CYP4A1* mRNA expression in livers of rats administered with phenobarbital (PB) or clofibrate (CF). Hepatic *CYP4A1* mRNA levels were measured in rats dosed with 500 ppm phenobarbital or 2,500 ppm clofibrate. *CYP4A1* mRNA levels are shown as mean (S.D.) for 5 rats from each experimental group. *CYP4A1* mRNA levels were compared between the control and the chemical-administered groups for each time point. ***Statistically significant differences from the corresponding controls: *** $P < 0.001$.

lin-dependent kinase inhibitor*CDKN3*, and *GADD45g*. In particular, significant decreases in the expression levels of *GADD45g* were persistently observed by treatment with either PB or CF for 4 and 13 weeks. It is noteworthy that these chemicals showed different characteristics in terms of the activation of CYP genes (*CYP2B1/2B2* and *CYP4A1*) and their transcription factors (CAR and PPARα). *GADD45g* has an inhibitory effect on cell cycle progression to adapt to cellular stress (Zhao *et al.*, 2000; Azam *et al.*, 2001; Vairapandi *et al.*, 2002; Togo *et al.*, 2004), and also functions as a tumor suppressor (Ying *et al.*, 2005). Genotoxic or cytokine-causing stress was loaded in many studies as variety of "cellular stress" where *GADD45g* was involved. In contrast, PB and CF are nongenotoxic carcinogens. Thus PB- and CF-mediated suppression of *GADD45g* gene may be key events that occur during the promotion of hepatocarcinogenesis. No induction of liver cell growth-associated marker (i.e., Ki-67) was observed in rats treated with either chemical for up to 13 weeks (Inoue, Yoshida, Nishikawa, unpublished), suggesting that the liver tumor-associated cell proliferation may occur after longer-term treatment with nongenotoxic carcinogens. After the 4-week recovery (normal diet) treatment, the elevation of hepatic *GADD45g* mRNA level was detected in the rats of PB-administered group. Those rats would probably be destined to liver malignancy after longer-term exposure. We currently speculate that repair process from any possible PB-caused toxicity might have started in those rats during the recovery treatment to result in the elevation of *GADD45g* mRNA level. The elevation varied depending on stochastic PB-damage repair process occurred in the individual animals. Further studies are needed to give an answer to this speculation.

Furthermore, in our preliminary experiments, administration of the nongenotoxic hepatocarcinogen piperonyl butoxide (PBO) to rats resulted in a marked decrease in gene expression of *GADD45g* together with an increase in *CYP2B1* expression (Gamou *et al.*, unpublished). Studies have shown that the hepatocarcinogenicity of PB, CF, and PBO may involve oxidative stress (Elrick *et al.*, 2005; Muguruma *et al.*, 2007; de Longueville *et al.*, 2003). This reported oxidative stress by PB, CF and PBO, and our present results on the sustained down-regulation of *GADD45g* may constitute a common cellular stress response pathway. We further consider that the down-regulation of Gadd45γ may be associated with the process involved in triggering liver tumor-associated cell growth. Further investigation is needed to prove these hypotheses by investigating influence of oxidative stress and *GADD45g* expression on liver tumor promotion.

Thus we raise hypothesis that cell growth proliferation toward malignancy might be triggered by or associated with the down-regulation of Gadd45γ. Besides Gadd45γ, down-regulation of Gadd45α and Gadd45β should also be considered with respect to tumor promotion. Down-regulation of Gadd45α, which is involved in cell survival in response to DNA damage (Hollander *et al.*, 2001; Liebermann and Hoffman, 2008), was observed in rats treated with CF for 4 weeks and 13 weeks. *GADD45a* and *GADD45b* have also been shown to interact with cdc2-cyclin B1 complex to exert an inhibitory effect on cell cycle progression to adapt cellular stress (Vairapandi *et al.*, 2002). These findings also raise possibility that down-regulation of Gadd45α has some role in directing hepatocytes to malignancy during the liver tumor promotion process in CF-treated rats. Up-regulation of Gadd45β, which is activated along with CYP2B subfamily genes through activation of CAR by PB (Yamamoto and Negishi, 2008), occurred in rats treated with PB for 3 days and 4 weeks, but not for 13 weeks. Significant increases in the expression levels of hepatic *CYP2B1/2B2* were observed in the rats treated with PB for 13 weeks; however, no significant up-regulation of *GADD45b* was observed, indicating that up-regulation of

GADD45b might not occur solely through activation of CAR. *GADD45b* might have been down-regulated through currently unknown mechanisms other than CAR around the period of 13 week-administration of PB to return its expressed level to that of untreated rats. We hereby point out a possible role of the Gadd45β down-regulation in liver tumor promotion in rats treated either with PB (cancellation of the observed up-regulation at 4week) or CF (down-regulation) for 13 week, as Vairapandi *et al.* showed interaction of Gadd45β with cdc2-cyclin B1 complex to exert an inhibitory effect on cell cycle progression (Vairapandi *et al.*, 2002). Accordingly, the altered gene expression of *GADD45a* and *GADD45b* by PB and CF might not necessarily be related to the development of liver hypertrophy. Nor that of *GADD45g* might be, as *GADD45g* down-regulation did not occur by treatment with either PB or CF for 3 days.

In conclusion, the present study showed changes in the mRNA levels of the *GADD45* gene family members in the livers of rats treated with nongenotoxic chemicals, PB and CF. Decreases in the expression of *GADD45g* were commonly observed by the treatment with PB and CF for 4 and 13 weeks, and *GADD45a* and *GADD45b* were also down-regulated especially after the treatment with CF for 13 weeks, despite that PB and CF show different characteristics in terms of induction of hepatic CYP isoforms. We herein propose that down-regulation of *GADD45g* and, possibly, *GADD45a* and *GADD45b* might be one of the causes of development of liver tumor promotion mediated by some kind of nongenotoxic chemicals rather than liver hypertrophy, which should be elucidated through further study. It is very important to determine what kind of nongenotoxic chemical carcinogens show similar changes in expression of genes including *GADD45a*, *GADD45b*, and *GADD45g*.

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Original Article

Super-induced gene expression of the *N*-methyl-D-aspartate receptor 2C subunit in chemical-induced hypertrophic liver in rats

Kiyomitsu Nemoto[1], Takahiro Tanaka[1], Ayaka Ikeda[1], Sei Ito[1], Masanori Mizukami[1], Tokihiro Hikida[1], Toshie Gamou[2], Wataru Habano[2], Shogo Ozawa[2], Kaoru Inoue[3], Midori Yoshida[3], Akiyoshi Nishikawa[3] and Masakuni Degawa[1,4]

[1]Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[2]Department of Pharmacodynamics and Molecular Genetics, School of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan
[3]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[4]Global Center of Excellence (COE) Program, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

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ABSTRACT — To identify gene expression that can be closely involved in chemical-induced hepatocellular hypertrophy, the hepatic gene expression profile was assessed by cDNA microarray analysis in male F344 rats fed for 3 days, 4 weeks, and 13 weeks a diet containing a hepatocellular hypertrophy inducer, either phenobarbital (500 ppm), clofibrate (2,500 ppm), or piperonyl butoxide (20,000 ppm). The results showed that, in all treatment groups, the increased expressional rate of the *Grin2c* gene, which encodes the *N*-methyl-D-aspartate receptor 2C subunit (NR2C), was the highest among those of all the genes tested, as compared with the corresponding gene expression in rats fed a normal diet. Moreover, real-time RT-PCR analysis showed that the expression levels of the *Grin2c* gene in rats fed with each chemical clearly increased in a chemical treatment period-dependent fashion, and that the increased rate was closely correlated with the grade of hypertrophy of hepatocytes rather than with the increased rate in liver weight. These results suggest the possibility that chemical-induced NR2C expression relates to the development of hepatocellular hypertrophy.

Key words: Hepatocellular hypertrophy, Non-genotoxic hepatocarcinogen, Liver tumor promoter, NMDA receptor

INTRODUCTION

Many chemicals induce liver enlargement accompanied by hepatocellular hypertrophy in experimental animals, and some of them exert hepatic carcinogenicity. Therefore, when evaluating chemicals for safety, especially non-genotoxic chemicals, it is important to clarify the toxicological significance of hepatocellular hypertrophy.

To identify gene expression that is closely involved in chemical-induced hepatocellular hypertrophy, we used cDNA microarray analysis to assess the gene expression profiles of mRNA from livers of rats treated with a non-genotoxic chemical, either phenobarbital (PB) (Crampton *et al.*, 1977), clofibrate (CF) (Hess *et al.*, 1965), or piperonyl butoxide (PBO) (Fujitani *et al.*, 1992), inducing hepatocellular hypertrophy. In the process, we found that the expression of *Grin2c* gene, which encodes the *N*-methyl-D-aspartate receptor 2C subunit (NR2C), was prominently increased in all treatment groups. This report shows those results.

MATERIALS AND METHODS

Animals and Treatments

Five-week-old male F344/DuCrj rats were purchased

Correspondence: Kiyomitsu Nemoto (E-mail: nemoto@u-shizuoka-ken.ac.jp)

from Charles River Co. Ltd. (Kanagawa, Japan) and used at 6 weeks of age. Chemical-treated rats were fed a basal diet mixed with 500 ppm of PB, 2,500 ppm of CF, or 20,000 ppm of PBO for 3 days, 4 weeks, or 13 weeks. The given doses were determined in reference to reports of studies in which liver hypertrophy was induced (Crampton *et al.*, 1977; Hess *et al.*, 1965; Fujitani *et al.*, 1992). The control rats were fed the basal diet (CRF-1, Oriental Yeast Industries Co. Ltd., Tokyo, Japan) during these periods. Each group consisted of five rats. All rats were fasted overnight and then sacrificed by decapitation. The decapitation was carried out under an unanesthetized condition at the above-mentioned time points. The livers were removed from individual rats, weighed, cut into small pieces, and then frozen with liquid nitrogen and stored at -80°C. Experimental protocols were approved by the Animal Experimentation Ethical Committee at the National Institute of Health Science.

Histopathological examination

Liver samples were fixed in 4% buffered paraformaldehyde or 10% neutral buffered formalin and then routinely processed for wax embedding and sectioning. Sections were stained with haematoxylin-eosin for histopathological assessment.

Microarray analysis

Total RNAs were extracted from liver samples in each 13-week-treatment group by the use of TriPure reagent (Roche Applied Science, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. Total RNA (500 ng), which included an equal amount of total RNAs derived from five rats per treatment group, was labeled with Cyanine-3 using a Quick-Amp Labeling Kit (Agilent Technologies, Palo Alto, CA, USA). Fluorescently labeled targets were hybridized to Whole Rat 4x44K oligo DNA microarrays (Agilent Technologies). Hybridization and wash processes were performed according to the manufacturer's instructions and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for the image analysis and data extraction processes.

RT-PCR analysis

Total RNAs extracted from the liver samples of each treatment group using TriPure reagent (Roche Applied Science) were also applied to RT-PCR for determination of the expression levels of *Grin2c* and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) genes. A portion (8 μg) of the total RNA was converted into cDNA using polyd$(N)_6$ random primer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in an RT-reaction mixture (250 μl). For analysis of RT-PCR by gel electrophoresis, PCR was performed using 5 μl of the RT-reaction mixture, 0.5 μM of each primer (forward and reverse primers), and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a total reaction volume (25 μl). For analysis by real-time PCR, the reaction was performed with a GeneAmp with SYBR Green PCR Core Reagents (Applied Biosystems) in 25 μl of total reaction mixture containing 5 μl of the RT-reaction mixture, 0.5 μM of each primer (forward and reverse primers), and AmpliTaq Gold DNA polymerase (Applied Biosystems). The amplification protocol consisted of 35 cycles for gel electrophoresis analysis or 40 cycles for real-time PCR of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C for *Gapdh* mRNA, or of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C, and extension for 1 min at 72°C for *Grin2c* mRNA. For gel electrophoresis analysis, the PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel. For real-time PCR, the level of each cDNA was assessed by the relative standard curve method, as described in the PE Applied Biosystems User Bulletin 2, 1997. The expression levels of *Grin2c* cDNA were normalized to those of the *Gapdh* gene. The primers used were designed as shown: *Gapdh* (forward: 5'-TTCAACGGCACAGTCAAGG-3' and reverse: 5'-CATGGACTGTGGTCATGAG-3'); *Grin2c* (Bullock *et al.*, 2008) (forward: 5'-GGATCTGCCAGAACGAGAAGA-3' and reverse: 5'-TTGTTGCCCCAGTTCTCGA-3').

Statistical analyses

Values are expressed as the means ± S.D. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Dunnett's test. All statistical analyses used the JMP version 7 software (SAS Institute Japan, Inc., Tokyo, Japan).

RESULTS

Change in liver weight

Significant liver weight gain was observed from day 3 after treatment with PB, CF, or PBO (Fig. 1). In PBO-treated rats, the liver weight increased in a chemical treatment period-dependent fashion up to 4 weeks, and the increased rate was maintained for at least up to 13 weeks. On the other hand, no such treatment period-depend-

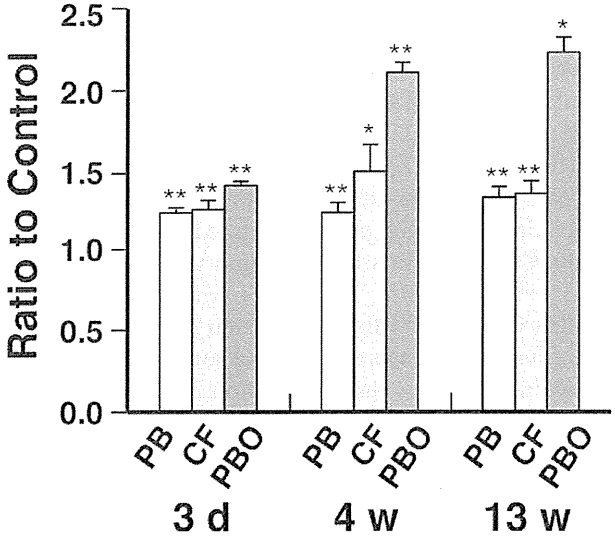

Fig. 1. Changes in the liver weight in rats treated with PB, CF, or PBO. Liver weight is represented as relative liver weight (g/100 g body weight) and then normalized to that of control rats at the same time point. Values are expressed as the means ± S.D (n = 5 in each experimental group). Significant differences from the corresponding age-matched control groups: $*p < 0.05$, $**p < 0.01$.

ent increase was observed in either PB- or CF-treated rats, although significant increases in liver weight were observed at not only 3 days but also 4 and 13 weeks after treatment with either chemical. The increased levels at 4 and 13 weeks were almost the same as those of rats treated with the corresponding chemicals for 3 days.

Histopathological analysis of the liver

Histopathological analyses showed the chemical treatment period-dependent development of hepatocellular hypertrophy in PB-, CF-, and PBO-treated rats (Table 1). PB-mediated hepatocellular hypertrophy occurred in a centrilobular region, whereas the hypertrophy in response to the two other chemicals was diffusely developed in the liver tissue.

Change in hepatic gene expression

cDNA microarray analyses for the RNAs from the livers of rats treated with PB, CF, or PBO for 13 weeks were performed first. The fold change of the *Grin2c* gene expression was the largest among those of all the genes tested in each chemical treatment group (Table 2).

The expression levels of the *Grin2c* gene were therefore further analyzed by RT-PCR in the livers of rats fed with each chemical for 3 days, 4 weeks, and 13 weeks. The intensities of the RT-PCR products on gel electrophoresis were very weak in the livers of the control rats (Fig. 2A). By contrast, the dietary treatment with each chemical clearly gave rise to an induction of *Grin2c* gene expression from day 3 after the onset of feeding (Fig. 2A). Semi-quantitative analysis by real-time RT-PCR showed that the levels reached a maximum at 13 weeks, with approximately 500-, 200-, and 600-fold inductions in PB-, CF-, and PBO-treated rats, respectively (Fig. 2B).

In addition, clear increases in the expression of the *Cyp2b* subfamily genes, *Aqp3* (Aquaporin 3), and *Cyp1a1* gene were observed in PB-, CF-, and PBO- treated rats, respectively (Table 2).

DISCUSSION

The present study demonstrated the super-induction of *Grin2c* gene expression in the livers of rats treated with inducers of hepatic hypertrophy such as PB, CF, and PBO. Furthermore, super-induction of the *Grin2c* gene was found to occur in relation to the development of hepatocelluar hypertrophy rather than an increase in liver weight.

To date, there are two articles showing increased expression of *Grin2c* gene in the livers of PB- or PBO-treated rats in studies applying microarray techniques (Elrick *et al.*, 2005; Muguruma *et al.*, 2007), although its significance was not mentioned in these reports. The *Grin2c* gene encodes *N*-methyl-D-aspartate receptor (NR) 2C, which is a member of the NR2 subfamily. NR2C forms a complex with NR1, and the complex functions as an *N*-methyl-D-aspartate (NMDA) receptor (Nagy, 2008; Kalia *et al.*, 2008). It is common knowledge that NMDA receptors serve as a calcium channel, playing a critical role in neuronal functions (Nagy, 2008; Kalia *et al.*, 2008). More recently, the possible role of NMDA receptors in peripherally nonneuronal tissues has been pointed out (Hinoi *et al.*, 2004; Leung *et al.*, 2004; Katsuta *et al.*, 2009; Parisi *et al.*, 2009), although their function in the liver has not yet been clarified. Our present results have therefore led us to propose a hypothesis that increased expression of the NR2C (the *Grin2c* gene) by non-genotoxic chemicals such as PB, CF, and PBO arouses the NMDA receptors and consequently relates to the development of liver hypertrophy. Incidentally, the increases in the expression of hepatic *Grin2c* gene in each chemical-treated rats were positively correlated with the grades of chemical-induced hepatocellular hypertrophy, suggesting a possibility that the increased expression of *Grin2c* gene leads to functional changes in a hepatocyte.

Table 1. Histopathology of the liver in rats after onset of PB-, CF-, or PBO-treatments

Treatment period	Type of hepatocellular hypertrophy	Number of rats (N = 5) having hepatocellular hypertrophy (Grade of hepatocellular hypertrophy)[a]			
		Control	PB	CF	PBO
Day 3					
	Centrilobular	0	5 (mild)	0	0
	Diffuse	0	0	0	4 (mild)
Week 4					
	Centrilobular	0	5 (moderate)	0	0
	Diffuse	0	0	5 (moderate)	5 (severe)
Week 13					
	Centrilobular	0	5 (moderate)	0	0
	Diffuse	0	0	4 (moderate)	5 (moderate; 1 rat, severe; 4 rats)

[a]Grade of hepatocellular hypertrophy is represented as mild, moderate, and severe.

Treatment with PB, a liver tumor promoter in rats (Pitot, 1977; Hagiwara *et al.*, 1999), resulted in the development of centrilobular-type hepatocellular hypertrophy, while treatments with non-genotoxic hepatocarcinogens, such as CF (Rao and Reddy, 1991) and PBO (Takahashi *et al.*, 1994) led to diffuse-type hepatocellular hypertrophy. These differences between PB and the two other chemicals might be due to the distribution of the chemicals in the liver tissue, although the exact reason remains unclear. In addition, DNA microarray and real-time PCR analyses showed that the induced levels of *Grin2c* gene expression in the PBO-treated rats were higher than those in the treated time-matched CF groups. Differences between PBO- and CF-treated rats were therefore related to those in the grade of hepatocellular hypertrophy and the increase in liver weight.

As for NMDA receptors, there is accumulating evidence showing their role in the growth and development of various types of cancers and cancer cell lines (Rzeski *et al.*, 2001; Watanabe *et al.*, 2008; Stepulak *et al.*, 2009). Therefore, the relationship between the super-induction of the *Grin2c* gene expression and hepatocarcinogenesis should be considered. Incidentally, we found a slight expression of the *Grin1* gene, which encodes NR1, an essential unit for functional NMDA receptors (Nagy, 2008; Kalia *et al.*, 2008), in the livers of control and chemical-treated rats (data not shown).

PB, CF, and PBO are activators of constitutive androstane receptor (CAR), peroxisome proliferator-activator receptor α (PPAR-α), and aryl hydrocarbon receptor (AhR), respectively (Waxman, 1999; Cook and Hodgson, 1985; Ryu *et al.*, 1997). The activation of these nuclear receptors has been considered to contribute to the generation and development of liver hyperplasia and/or liver tumor (Peters *et al.*, 1997; Yamamoto *et al.*, 2004; Huang *et al.*, 2005; Shimizu *et al.*, 2000). In the present experiments, activations of CAR, PPAR-α, and AhR by PB, CF, and PBO were confirmed by the inductions of the *Cyp2b* subfamily genes, *Aqp3* (Aquaporin 3) (Patsouris *et al.*, 2004), and *Cyp1a1* gene, respectively. It would therefore be of considerable importance to examine the involvement of these nuclear receptors in the mechanism of the up-regulated *Grin2c* gene expression in response to administration of these chemicals.

In conclusion, the present study showed that the *Grin2c* gene expression is induced at an extremely high rate in the livers of rats treated with liver hypertrophy inducers, PB, CF, and PBO. These results suggest the possibility that a chemical-induced NR2C, which is encoded by the *Grin2c* gene, relates to some sort of hepatic functional changes, including the development of hepatocellular hypertrophy and/or carcinoma, in response to the expo-

Table 2. List of top ranks of genes showing high fold change of their gene expression in livers of rats 13 weeks after onset of PB-, CF-, or PBO-treatments

Treatment	Feature Num†	Probe ID‡	Accession Num	Gene	Description	Fold Change
PB-Treatment						
	18912	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	134
	18585	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	134
	25095	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	126
	10081	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	126
	6078	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	115
	7800	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	114
	38281	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	113
	17400	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	112
	56	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	97
	22477	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	75
	40077	36625	NM_080581	Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	33
	15347	10286	NM_012598	Lpl	Lipoprotein lipase	20
	24999	22930	NM_001135668	Cyp2b15	Cytochrome P450 2B1	20
	11314	10286	NM_012598	Lpl	Lipoprotein lipase	20
	5578	5026	NM_198733	Cyp2b21	Cytochrome P450, family 2, subfamily b, polypeptide 21	18
CF-Treatment						
	17400	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	466
	25095	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	463
	25811	23629	NM_031703	Aqp3	Aquaporin 3	434
	7800	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	289
	18585	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	254
	56	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	194
	10081	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	192
	31601	28946	NM_053874	Cap2	Adenylate cyclase-associated protein, 2	161
	38281	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	152
	22477	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	142
	6078	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	98
	18912	89	NM_012575	Grin2c	Glutamate [NMDA] receptor subunit epsilon-3 precursor	97
	5751	5187	TC620264	TC620264	Q2EBP1_BACCE (Q2EBP1) ErfK/YbiS/YcfS/YnhG, partial (6%)	86
	30965	28398	AW143067	AW143067	EST293362 Normalized rat muscle, Bento Soares Rattus sp. cDNA clone RGIBC65 5' end, mRNA sequence	57
	1267	1112	NM_133425	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c	54