Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
1398362_at	Notch gene homolog 2 Drosophila	Notch2	−3.5	−5.5	−1.2
1370243_a_at	Prothymosin alpha	Ptma	−1.5	−2.5	−1.0
Cell adhesion					
1386947_at	cadherin 1	Cdh1	−2.9	−2.1	−1.2
1369224_at	cadherin 17	Cdh17	−4.5	−10.1	−1.5
1368642_at	cadherin 2	Cdh2	−2.2	−3.3	−1.2
1387259_at	Cadherin 2	Cdh2	−2.2	−2.3	−1.1
1369854_a_at	CEA-related cell adhesion molecule 1	Ceacam1	−2.0	−2.8	−1.4
1370371_a_at	CEA-related cell adhesion molecule 10	Ceacam10	−1.7	−2.2	−1.2
1370234_at	Fibronectin 1	Fn1	−2.7	−3.0	−1.1
1382027_at	Integrin beta 3 Cd61	Itgb3	−2.3	−3.5	−1.7
1372002_at	Gap junction membrane channel protein alpha 1	Gja1	−1.5	−2.2	−1.1
1367849_at	syndecan 1	Sdc1	−1.6	−2.1	−1.1
1370043_at	activated leukocyte cell adhesion molecule	Alcam	−2.4	−3.4	−1.3
1374432_at	Activated leukocyte cell adhesion molecule	Alcam	−2.1	−5.0	−1.6
1370108_a_at	Lin-7 homolog a C. elegans	Veli1	−2.5	−3.9	−3.7
1373027_at	Afadin	Af6	−1.9	−2.3	−1.2
Cellular morphogenesis					
1388459_at	Collagen type XVIII alpha 1	Col18a1	−3.0	−3.6	−1.2
1370959_at	Collagen type III alpha 1	Col3a1	−1.9	−2.6	−1.6
1376099_at	Collagen type V alpha 1	Col5a1	−2.2	−3.2	−1.4
1370895_at	Collagen type V alpha 2	Col5a2	−1.5	−2.1	−1.2
1371725_at	Myosin heavy polypeptide 9	Myh9	−2.7	−2.9	−1.2
1387402_at	myosin, heavy polypeptide 9	Myh9	−2.4	−3.3	−1.1
1369720_at	myosin Ib	Myo1b	−2.1	−2.9	−1.1
1386941_at	plectin	Plec1	−2.6	−2.7	−1.2
1370993_at	Laminin gamma 1	Lamc1	−2.2	−2.7	−1.2
1386956_at	scavenger receptor class B, member 1	Scarb1	−1.4	−3.3	−1.2
Immune response					
1371926_at	Interleukin 6 signal transducer	Il6st	−2.2	−1.9	−1.1
1368280_at	cathepsin C	Ctsc	−2.1	−3.2	−1.3
1387005_at	cathepsin S	Ctss	−1.3	−2.4	−1.1
1387893_at	Complement component 1 s subcomponent	C1s	−2.3	−13.6	−1.3
1370892_at	Complement component 4a	C4a	−2.3	−3.6	−1.1
1368558_s_at	allograft inflammatory factor 1	Aif1	−1.7	−3.3	−1.7
1370479_x_at	Alpha-2u globulin PGCL4	Obp3	−2.9	−81.0	−1.2
1387985_a_at	Alpha-2u globulin PGCL4	Obp3	−2.6	−207.5	−1.1
Coagulation					
1374320_at	Coagulation factor 5	F5	−2.5	−3.8	−1.6
1387351_at	fibrillin-1	Fbn1	−1.9	−3.2	−1.4
1371258_at	Fibrinogen alpha polypeptide	Fga	−2.1	−1.8	−1.5
1387323_at	kallikrein B, plasma 1	Klk3	−2.6	−2.7	−2.4
1369225_at	kininogen 1	Kng1	−1.8	−2.5	−1.1

Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
Drug and xenobiotic methabolism					
1387243_at	Cytochrome P450 family 1 subfamily a polypeptide 2	Cyp1a2	−2.2	−8.1	−1.2
1387913_at	Cytochrome P450 family 2 subfamily d polypeptide 22	Cyp2d22	−2.6	−4.0	−1.6
1368608_at	cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	−2.0	−3.0	−1.3
1368265_at	cytochrome P450 monooxygenase CYP2T1	Cyp2t1	−2.2	−3.7	−1.3
1370387_at	Cytochrome P450 family 3 subfamily a polypeptide 13	Cyp3a13	−3.6	−50.2	−1.7
1368467_at	cytochrome P450, family 4, subfamily F, polypeptide 2	Cyp4f2	−1.7	−2.3	−1.4
1367979_s_at	cytochrome P450, subfamily 51	Cyp51	−2.1	−1.4	−1.0
1389218_at	UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	−2.9	−2.1	−1.3
1367938_at	UDP-glucose dehydrogenase	Ugdh	−2.4	−1.9	−1.2
1388410_at	UDP-glucose pyrophosphorylase 2	Ugp2	−3.0	−4.4	−1.2
Transport					
1370465_at	ATP-binding cassette sub-family B MDR TAP member 4	Abcb1	−5.5	−10.8	−1.3
1368497_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Abcc2	−4.4	−2.5	−1.4
1369455_at	ATP-binding cassette, sub-family G (WHITE), member 5	Abcg5	−1.6	−3.1	−1.9
1369440_at	ATP-binding cassette, sub-family G (WHITE), member 8	Abcg8	−2.4	−5.6	−3.9
1398862_at	ATPase Ca transporting cardiac muscle slow twitch 2	Atp2a2	−2.6	−3.9	−1.2
1368698_at	ATPase Ca transporting plasma membrane 2	Atp2b2	−2.3	−3.4	−1.9
1387285_at	ATPase, Ca++ transporting, plasma membrane 2	Atp2b2	−2.1	−2.1	−1.7
1368621_at	aquaporin 9	Aqp9	−1.9	−8.4	−1.4
1390591_at	Na Pi cotransporter 4	Slc17a3	−1.8	−4.9	−1.5
1369746_a_at	Solute carrier family 21 member 10	Slc21a10	−1.8	−3.5	−1.6
1368461_at	solute carrier family 22 (organic anion transporter), member 8	Slc22a8	−2.0	−3.1	−1.2
1369169_at	solute carrier family 23 (nucleobase transporters), member 1	Slc23a1	−1.9	−2.6	−1.3
1368600_at	solute carrier family 26 (sulfate transporter), member 1	Slc26a1	−2.0	−2.5	−1.3
1369099_at	solute carrier family 30 (zinc transporter), member 1	Slc30a1	−1.6	−4.4	−1.3
1386960_at	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	−2.2	−2.3	−1.2
1368296_at	Solute carrier organic anion transporter family member 2b1	Slco2b1	−2.1	−2.4	−1.3
Stress response					
1371442_at	Hypoxia up-regulated 1	Hyou1	−2.9	−3.9	−1.6
1370665_at	Hypoxia up-regulated 1	Hyou1	−2.2	−3.3	−1.2

Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
Regulation of blood pressure					
1387811_at	angiotensinogen	Agt	−2.2	−2.1	−1.1
1369664_at	arginine vasopressin receptor 1A	Avpr1a	−3.0	−2.7	−1.4
1367801_at	endothelin converting enzyme 1	Ece1	−1.9	−2.2	−1.2
1386953_at	hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	−2.2	−4.6	−1.1
1368102_at	hydroxysteroid 11-beta dehydrogenase 2	Hsd11b2	−1.8	−3.6	−1.9
1387994_at	Hydroxysteroid 17-beta dehydrogenase 9	Hsd17b9	−1.6	−1.8	−1.3
Others					
1368490_at	CD14 antigen	Cd14	−2.1	−5.1	−1.3
1370891_at	CD48 antigen	Cd48	−1.3	−2.1	−1.2
1367709_at	CD63 antigen	Cd63	−1.4	−2.9	−1.1

[a]: The smallest ratio to control value observed in single-dose studies of CFB, WY and GFZ is shown. Negative figure means the reciprocal number of ratio; e.g., −3.0 means that one of the drugs reduced the gene expression to 1/3 of corresponding control. [b]: The smallest ratio to control value observed in repeated dose studies of CFB, WY and GFZ is shown. [c]: The smallest ratio to control value observed *in vitro* studies of CFB, WY and GFZ is shown. The columns are shaded when the corresponding probe sets appear in Fig. 2.

DISCUSSION

In the present study, analysis of gene expression in rat liver was done with three peroxisome proliferators, clofibrate, WY-14643 and gemfibrozil, stored in our database. The changes of gene expression by these compounds observed *in vivo* (single and repeated) were largely in accordance with the report by Kramer *et al.* (2003), in which the effect of clofibrate on the gene expression profile in rat liver was analyzed. Among the genes whose expression was affected, a large number of genes were overlapped between *in vivo* and *in vitro*, both in up- and down-regulated ones. Between *in vivo* and *in vitro* experiments, however, there were many common genes in up-regulated ones but none in down-regulated ones.

A large number of genes related to b-oxidation were up-regulated by a single dose, and similar changes were also noted for *in vitro* experiments. The genes that possess PPRE sequence in their promoter regions, e.g., acyl-CoA oxidase (Tugwood *et al.*,1992), carnitine palmitoyl transferase I (Brandt *et al.*, 1998), carnitine palmitoyl transferase II (Barrero *et al.*, 2003) and fatty acid desaturase 2 (Tang *et al.*, 2003) were found to be up-regulated both *in vivo* and *in vitro*. An exception was that malic enzyme (Castelein *et al.*, 1994), whose promoter region contains PPRE, was induced *in vivo* but not *in vitro*. On the other hand, there were many genes whose promoter regions had no PPRE sequence showing common induction for *in vivo* and *in vitro*. For example, CD36, a fatty acid transporter, and CYP4A14, involved in fatty acid hydroxylation, were up-regulated both *in vivo* and *in vitro*, but there has been no report that their promoter regions contain functional PPRE. Apart from their mechanism, the genes that show common changes *in vivo* and *in vitro* (as listed by the present study) are considered to be useful to assess pharmacological and toxicological effects *in vivo* from *in vitro* experiments. This will be discussed later.

There were also data suggesting the limitations of *in vitro* experiments. Among the genes modulated by administration of fibrates *in vivo*, those related to the functions of proliferation, apoptosis, immune response, transcription activation and repression, transporter, cell adhesion, blood coagulation and regulation of blood pressure, did not show any changes *in vitro*. It is well known that peroxisome proliferators are non-genotoxic carcinogens for rodents and their most convincing mechanism is presently considered to be the activation of proliferation in addition to attenuation of apoptosis (Michalik *et al.*, 2004; Boitier *et al.*, 2003). In the present study, many of the genes related to proliferation and apoptosis were mobilized by fibrates *in vivo* but not at all *in vitro*. There have been many reports describing the fact that stimulation of proliferation by peroxisome proliferators requires Kupffer cell or TNFα produced by the cell. Rose *et al.* (1997)

reported that acceleration of proliferation in rat hepatocytes by WY-14643 was attenuated by the reduction of TNFα via inactivation of Kupffer cells. It was also reported that WY-14643 failed to cause cell proliferation in hepatic parenchymal cells cultured under the condition where non-parenchymal cells had been eliminated, and that its proliferative effect was recovered when Kupffer cells were added to the culture (Parzefall *et al.*, 2001). As for the attenuation of apoptosis, it was reported that a high concentration of TNFα inhibited spontaneous TGFβ1-induced apoptosis in primary cultured hepatocytes (Rolfe *et al.*, 1997). These reports

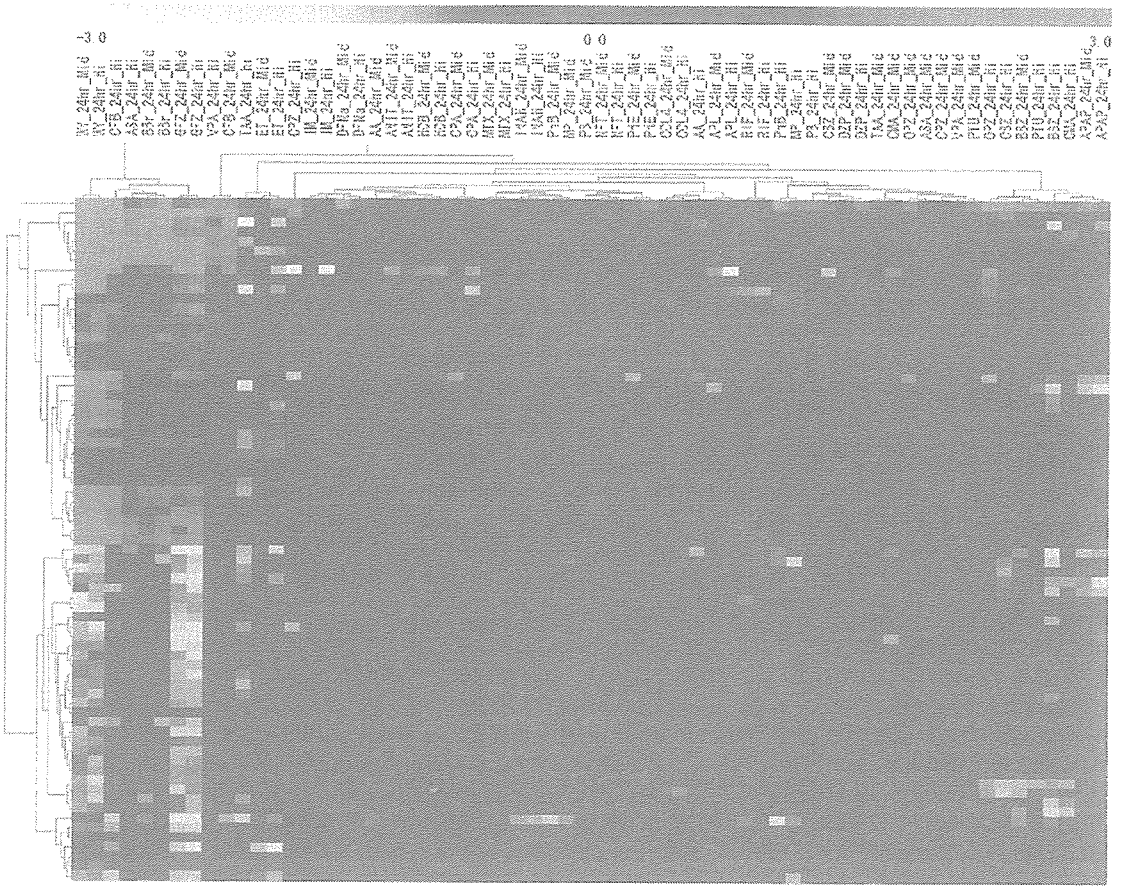


Fig. 3. A heat map view of the gene expression profile for the 32 compounds *in vivo*. Hierarchical clustering analysis of the compounds (middle and high dose, 24 hr after a single dose) was conducted using the genes that were increased (1398296_at, 1398249_at, 1390851_at, 1389551_at, 1389253_at, 1388924_at, 1388891_at, 1388756_at, 1388223_at, 1387977_at, 1387783_a_at, 1387740_at, 1387636_a_at, 1387183_at, 1387022_at, 1386927_at, 1386885_at, 1386880_at, 1379361_at, 1375845_at, 1374475_at, 1373784_at, 1373564_at, 1372134_at, 1371976_at, 1371379_at, 1370870_at, 1370818_at, 1370397_at, 1370067_at, 1368934_at, 1368283_at, 1367937_at, 1367777_at, 1367694_at, 1367659_s_at) or decreased (1398362_at, 1391485_at, 1390172_at, 1390165_at, 1390115_at, 1389218_at, 1388742_at, 1388459_at, 1387913_at, 1382944_at, 1377375_at, 1376746_at, 1376709_at, 1376593_at, 1376140_at, 1375205_at, 1374493_at, 1374320_at, 1374266_at, 1373797_at, 1372308_at, 1371368_at, 1371202_a_at, 1370043_at, 1369973_at, 1369868_at, 1368698_at, 1368642_at, 1368497_at, 1368428_at, 1368304_at, 1368036_at, 1367905_at, 1367602_at, 1367601_at) 24 hr after a single dose (complete linkage method, Euclidean distance). A cluster consisting of 3 fibrates (CFB, WY, GFZ), benzbromarone (BBr) and aspirin (ASA) was identified on the left side.

suggest that a Kupffer cell secreting TNFα, IL-1, IL-2 and IL-6 (Decker, 1990) plays an important role in hepatocyte proliferation stimulated by peroxisome proliferators. Our present results that the expression changes of genes related to proliferation or apoptosis were rarely observed for the *in vitro* system could be due to the fact that the numbers of non-parenchymal cells (including Kupffer cells) in the culture were much less than that for *in vivo* liver. Our present results also support the aforementioned concept that the Kupffer cell (or its production of TNFα) is essential for the increase of proliferation and attenuation of apoptosis caused by peroxisome proliferators.

Many genes related to cellular morphogenesis, including extracellular matrix (ECM), were down-regulated exclusively *in vivo*. Ogata *et al.* (2002, 2004) reported that the increase in mRNA of collagen type I and type III in pressure-overloaded rat heart was reduced by the administration of fenofibrate and that the proliferation of cardiac fibroblast induced by endothelin-1 was inhibited by fenofibrate. It was also reported that ETYA, a PPARα agonist, reduced the mRNA contents of elastin, tropoelastin and α-smooth muscle actin in neonatal rat lung fibroblast (McGowan

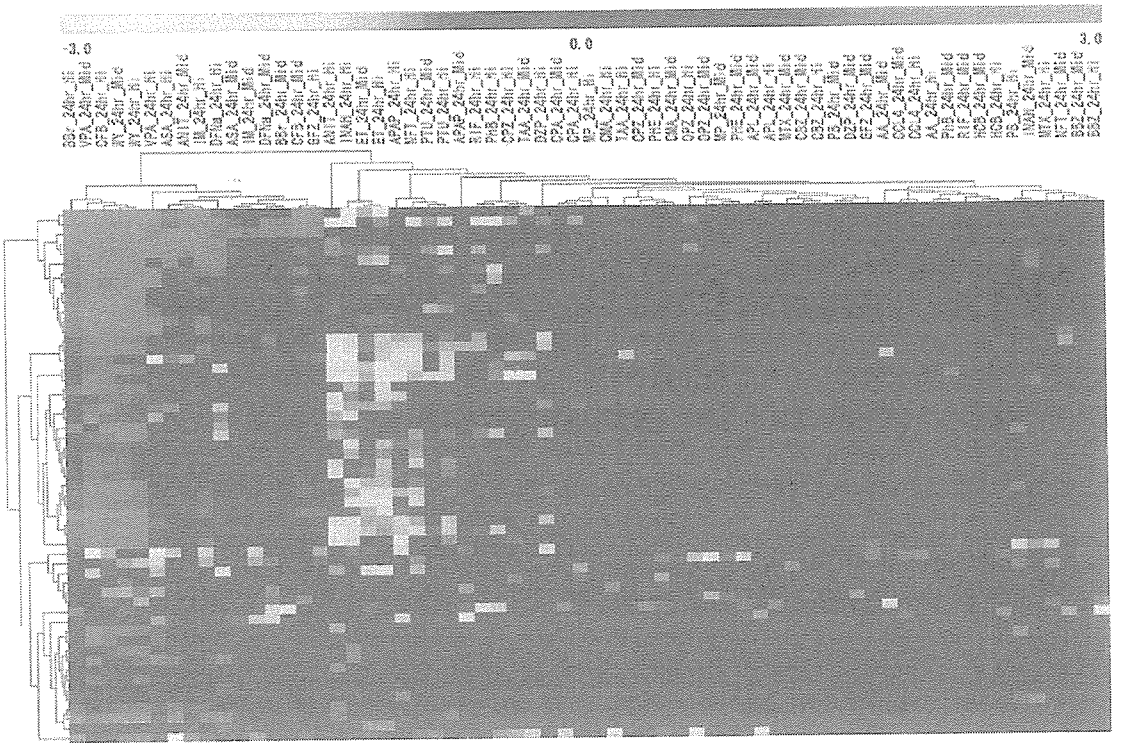
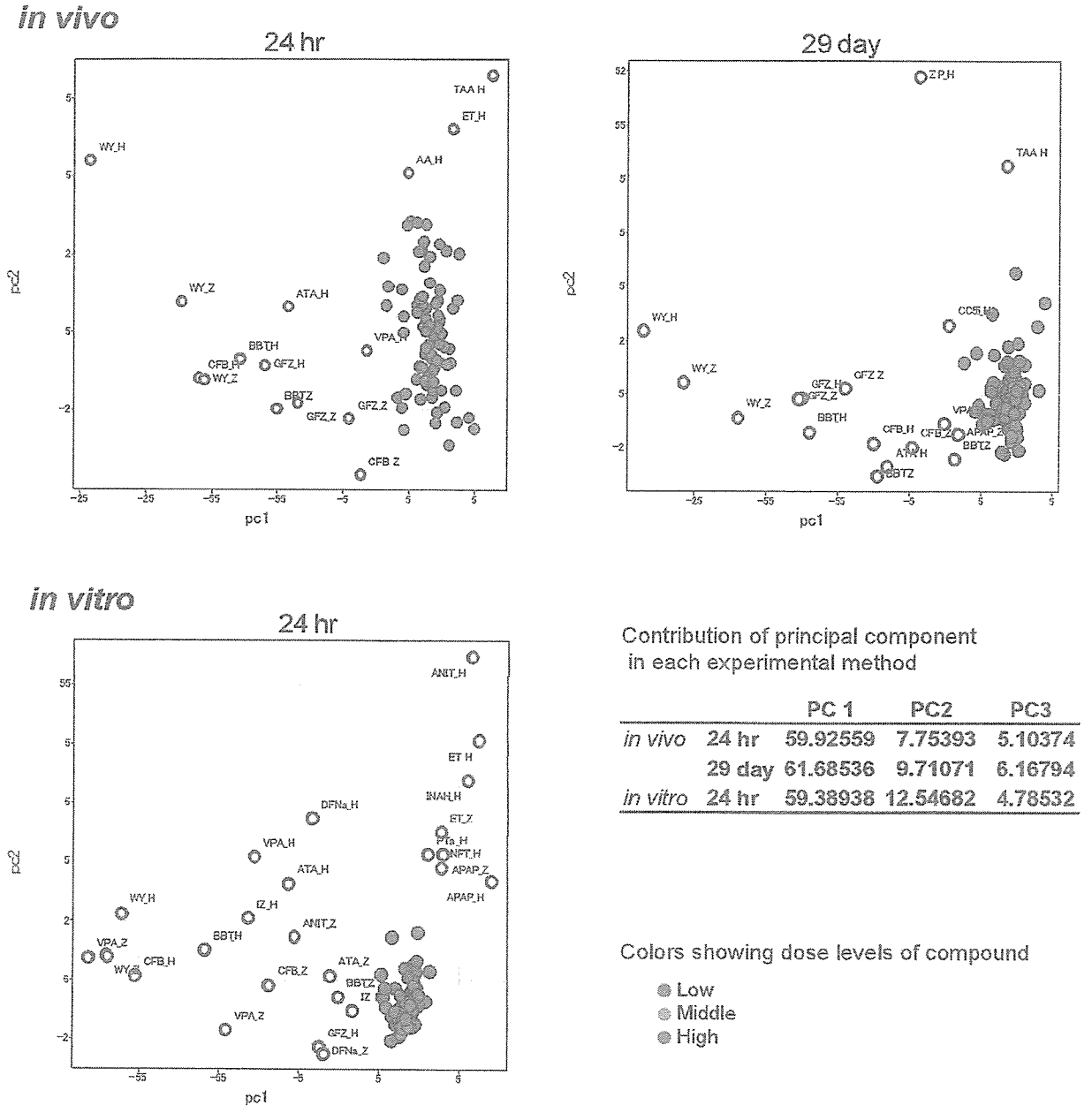


Fig. 4. A heat map view of the gene expression profile for the 32 compounds *in vitro*. Hierarchical clustering analysis of the compounds (middle & high concentrations, 24 hr of exposure) was conducted using the genes that were increased (1398310_at, 1394194_x_at, 1390383_at, 1390370_at, 1389253_at, 1388924_at, 1388756_at, 1388644_at, 1388211_s_at, 1388108_at, 1387783_a_at, 1387740_at, 1386946_at, 1386927_at, 1386901_at, 1386885_at, 1386880_at, 1383205_at, 1379361_at, 1377037_at, 1376076_at, 1374556_at, 1374478_at, 1374265_at, 1370818_at, 1370436_at, 1370397_at, 1370355_at, 1370313_at, 1370310_at, 1370237_at, 1369150_at, 1369111_at, 1368934_at, 1368797_at, 1368669_at, 1368435_at, 1368283_at, 1368150_at, 1368034_at, 1368021_at, 1367950_at, 1367897_at, 1367836_at, 1367777_at, 1367742_at, 1367689_a_at, 1367672_at, 1367659_s_at) or decreased (1387246_at, 1375791_at, 1373261_at, 1369093_at, 1368798_at, 1368342_at) in 24 hr of exposure (complete linkage method, Euclidean distance). A cluster consisting of 3 fibrates (CFB,WY, GFZ), 3 NSAIDs (asprin [ASA], indomethacin [IM] and diclofenac sodium [DFNa]), valproic acid (VPA) and benzbromarone (BBr) was identified on the left side. Although the middle dose of ANIT belonged to the same cluster, its high dose did not.



		PC 1	PC2	PC3
in vivo	24 hr	59.92559	7.75393	5.10374
	29 day	61.68536	9.71071	6.16794
in vitro	24 hr	59.38938	12.54682	4.78532

Fig. 5. Two-dimensional visualization of principal component analysis of the 32 compounds *in vivo* and *in vitro* using the commonly up-regulated 41 genes between *in vivo* and *in vitro*. The upper two panels show the *in vivo* studies and the lower left panel shows the *in vitro* study. Within each plot, the highest contributing factor to the overall variability is shown on the x-axis as the first component (PC1). The y-axis shows the second highest component (PC2). These plots show the principal separation of samples due to putative PPARα activity toward the negative direction on the x-axis, PC1. The contributions (%) of principal components for each experiment are summarized in the table on the lower right.

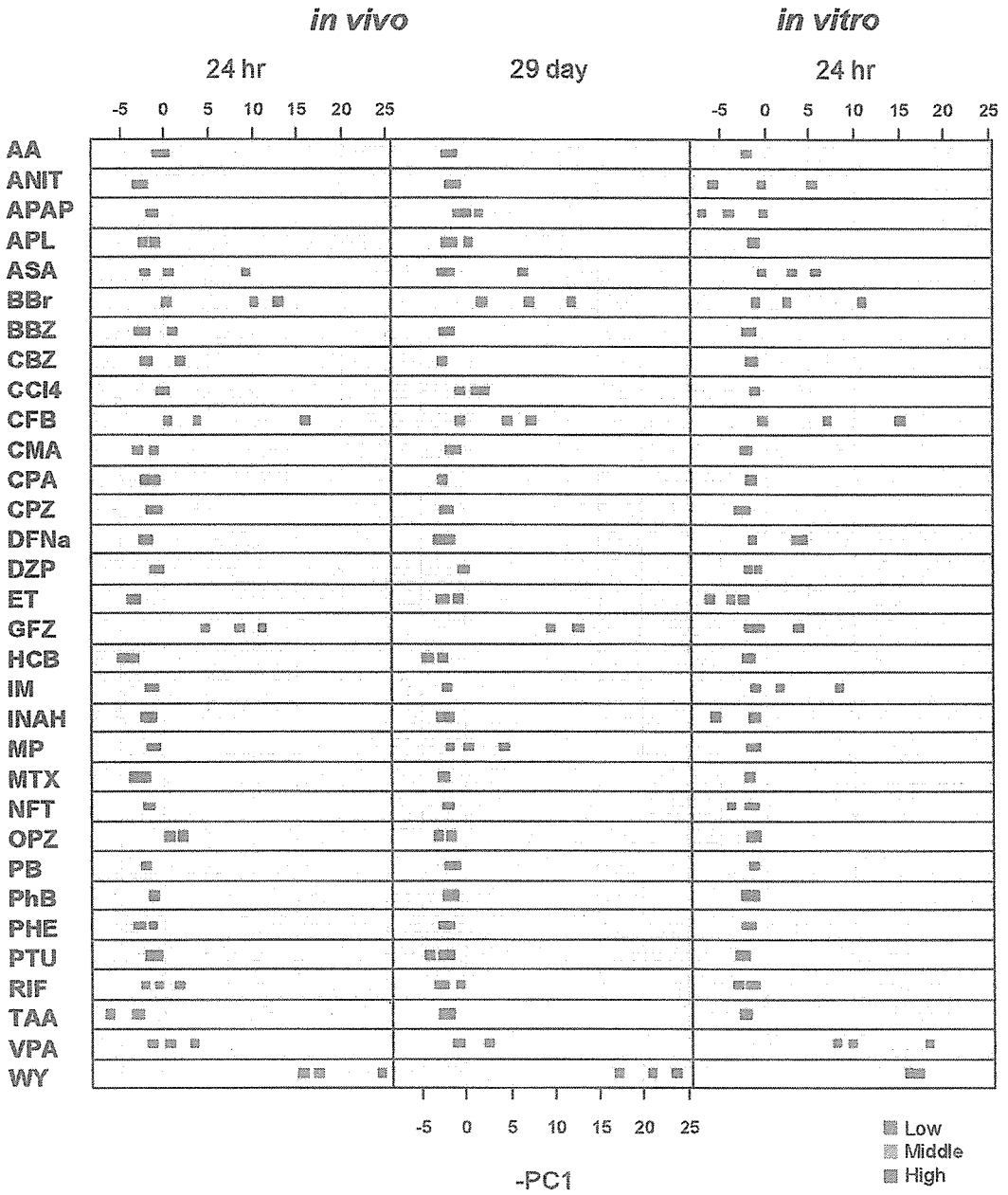


Fig. 6. Plotting of the first principal component (PC1) from the 32 compounds examined *in vivo* and *in vitro*. The left two panels show the *in vivo* studies, i.e., 24 hr after single dose and 24 hr after the last dose of the 28-day repeated dose, and the right panel shows the *in vitro* study, 24 hr exposure. The x-axis for each shows the negative value of PC1, as in Fig. 5, and the y-axis shows the compounds aligned alphabetically. The abbreviations of the compounds are found in Table 1. Note that the three fibrates (CFB, GFZ, WY), aspirin (ASA), and benzbromarone (BBr) show high values in this parameter for *in vivo* experiments and that diclofenac sodium (DFNa) and indomethacin (IM) show high values in addition to the drugs above.

et al., 1997). It appears that peroxisome proliferators also act on non-parenchymal cells other than Kupffer cells, and that they reduce the production of ECM in fibroblasts. Stellate cell, a hepatic non-parenchymal cell, possesses both of the characteristics of lipocyte and fibroblast, and its ability to produce ECM was found to be increased in liver fibrosis (Tanaka *et al.*, 1991). It is possible that production of ECM in stellate cells could be stimulated by peroxisome proliferators. Based on these ideas, it would be reasonable to conclude that the reduction of expression of genes related to ECM and cytoskeletons by peroxisome proliferators observed *in vivo* were not reproduced in the *in vitro* system, considering that these changes were a reflection of those occurring in non-parenchymal cells in liver. In the present study, relative liver weight was increased by a factor of two in peroxisome proliferators. The expression changes in genes classified to cell adhesion and cellular morphogenesis should have been associated with this obvious hypertrophy of the liver.

In the present study, down-regulation of genes classified to the immune response and coagulation was also *in vivo*-specific. PPARα is known to function as an inhibitory factor for inflammation, and PPARα agonists were reported to inhibit the expression of mRNA of fibrinogen, an acute phase protein (Kockx *et al.*, 1999; Corton *et al.*, 1998), and induction of fibrinogen gene by IL-6 (Gervois *et al.*, 2001). Moreover, it was also reported that WY-14643 inhibits induction of IL-6 and cyclooxygenase-2 by IL-1 in human aortic smooth muscle cells through inhibition of the translocation of NF-κB from the cytosol to the nucleus. It is thus expected that inhibition of the inflammatory response by PPARα agonists not only affects the hepatic parenchymal cell (producing acute phase proteins) but also affects the mechanism relating to non-parenchymal cells (including Kupffer cell) that releases inflammatory cytokines. It would be reasonable to conclude that the reason down-regulation of genes classified to the immune response and coagulation was observed *in vivo* but not *in vitro* was again due to the involvement of non-parenchymal cells. However, it might be due simply to the fact that the basal level of the mRNAs of these genes was down-regulated during our culture condition, since there was a report that PPARα agonist could inhibit the expression of fibrinogen mRNA by IL-6, using human hepatocyte culture (Gervois *et al.*, 2001).

As discussed above, the profiling of *in vivo* data represents gene expression in multiple cellular populations, whereas the profiling of *in vitro* data is focused on gene expression of hepatic parenchymal cells. The advantage of the *in vitro* system is that the direct effects of chemicals on hepatic parenchymal cells can be assessed, and in certain cases, the sensitivity and specificity of the test can be improved by eliminating noise due to gene expression of non-parenchymal cells. On the other hand, the *in vitro* system has an apparent disadvantage when indirect toxicity to parenchymal cells via non-parenchymal cells is involved or direct toxicity to non-parenchymal cells is involved.

In hierarchical clustering analysis of the *in vivo* data stored in our database, benzbromarone and aspirin were classified into the same cluster of the three peroxisome proliferators. It has been long known that benzbromarone is a PPARα ligand (Bichet *et al.*, 1990). As for aspirin, some NSAIDs including indomethacin, ibuprofen and fenoprofen, were reported to activate PPARα (Lehmann *et al.*, 1997), suggesting that aspirin belongs to PPARα agonists as well. In the hierarchical clustering of the *in vitro* data, two NSAIDs (indomethacin and diclofenac) and valproic acid were additionally located to the same cluster that included the three fibrates, and benzbromarone and aspirin. It has been reported that valproic acid induced the increase of liver weight and the activation of β-oxidation in rodents, suggesting that the drug has some PPARα agonist-like activity (Horie and Suga, 1985). Although the middle dose of ANIT belonged to the same cluster, its high dose showed a quite different profile. There is no report suggesting a relationship between ANIT and PPARα so far. One possibility is that ANIT is a potential PPARα agonist, and inconsistent results at high concentrations showed that cytotoxicity overwhelmed the inducing effects. At the middle dose of ANIT, various genes related to lipid metabolism (including β-oxidation), e.g., Acaa1, Acaa2, Cpt1a, Cpt1b, Pdk4, Ehhadh, Hmgcs2, Mte1, Cyp4a14, Cyp4b1, Cyp8b1, and Angptl4 were up-regulated more than twice of control *in vitro*. It would be interesting to examine the direct effect of ANIT on PPARα. It should be considered that any expression changes in β-oxidation-related genes do not necessarily indicate the direct involvement of PPARα.

The reason why the *in vitro* system was more sensitive than that of *in vivo* for detecting PPARα agonist-like activity is the high concentration of the drugs *in vitro*. In the standard protocol in our project, the maximal dose of the drugs *in vivo* is set to the level which the animals can tolerate for 28 days of repeated administration, while that for *in vitro* is independently determined according to the direct cytotoxicity of the cul-

tured hepatocytes. Therefore, in the case of chemicals causing severe toxicity to organs other than the liver, the practical concentration around the hepatocyte becomes much lower *in vivo* than *in vitro*. Since the main lethal cause in the case of NSAIDs is intestinal perforation, the doses employed were relatively low compared with that needed to elicit PPARα activation *in vivo*, and actually, a reduction of plasma lipid was barely observed. The PPARα activity of aspirin could possibly have been detected because its ulcerogenicity to intestine is much lower than that of the other NSAIDs.

One of the aims of the present project is the prediction of *in vivo* effects from *in vitro* experiments that have the advantages of saving chemicals, cost, and time. In the case of PPARα agonists, we could not find any common genes in down-regulated ones between *in vivo* and *in vitro*. On the other hand, 41 genes up-regulated *in vitro* were also up-regulated *in vivo*. We considered these as useful markers to predict PPARα activity *in vivo* from *in vitro*, and applied them to PCA. As shown in Fig. 5, PC1 appeared to have a PPARα agonist-like attribute, and 32 chemicals were aligned by this parameter in Fig. 6. This presentation conveniently identifies potential PPARα agonists both *in vivo* and *in vitro*. We are now incorporating this computing module into our toxicity prediction system and it will be useful in identifying other properties when appropriate marker genes are extracted.

In conclusion, our database efficiently works to classify a certain category of drugs (PPARα agonist in the present case) based on gene expression profiling. For these data, the gene expression profile *in vitro* is useful and sensitive to the direct toxicity of the chemicals in hepatic parenchymal cells, whereas indirect toxicities mediated by other cells or secondary toxicity due to pathophysiological changes such as blood pressure or inflammation in other organs might be overlooked. In order to predict *in vivo* effects from the *in vitro* system, it is important to identify genes commonly mobilized *in vivo* and *in vitro*. The scoring system (using the principal component that largely contributes the target effect) in the present study appeared to be quite useful and convenient to identify compounds with target activity among the ones stored in our database.

ACKNOWLEDGMENT

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GENE EXPRESSION PROFILE IN LIVER OF DIFFERING AGES OF RATS AFTER SINGLE ORAL ADMINISTRATION OF ACETAMINOPHEN

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ABSTRACT — In order to verify the influence of the rat age on hepatotoxicity, male Sprague-Dawley rats of 6 (young) and 12 (adult) weeks of age were orally administered acetaminophen (APAP), isoniazid (INH), or carbon tetrachloride (CCl4). Liver samples were obtained in a time-course manner, and changes in gene expression examined by an Affymetrix GeneChip. APAP caused more prominent hepatic injury with respect to pathology and blood biochemistry in adults than in young rats, whereas no obvious age-related differences were observed in INH- or CCl4-treated rats. Comparing gene expression in control rats, CYP3A13 was higher and GSTY2c was lower in adults, suggesting that production of the active metabolite of APAP is higher and its detoxification is lower in adults. The total amount of glutathione and total SH in rat liver was found to be higher in adult rats whereas the extent of its reduction by APAP was larger in adults. A detailed analysis of genes showing age-related differences revealed that some of them were different not in their extent but in their time course, i.e., the stress responses occurred earlier in the young than in the adult, resulting in a difference at 24 hr after dosing. These results suggest that the age-related difference in toxicity would be attributed to a higher expression of CYP3A13, producing the active metabolite of APAP as well as the lower expression of the detoxification enzyme, GSTY2c, in adult rats. Furthermore, these differences affect the time course of APAP toxicity. The present study clearly depicts the advantage of the multi-time, multi-dose protocol employed in our project for analyzing the mechanism of toxicity by gene expression profiling.

KEY WORDS: Toxicogenomics, Acetaminophen, Hepatotoxicity, Age-related difference, Rat

INTRODUCTION

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan which started in 2002 (Urushidani and Nagao, 2005). Its aim is to construct a large-scale toxicology database of transcriptome for the prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medical compounds, have been selected, using both *in vitro* studies with primary hepatocyte of rat and human, and *in vivo* studies in rat. Of these, the standard protocol of *in vivo* study in TGP consists of a single administration test and a repeated administration test with multi-time and multi-dose. The gene expression of the liver is comprehensively analyzed by using Affymetrix GeneChip® with the traditional toxicity parameters. Although the ages of the rats were varied in a time-course sacrifice between 6 to 10-weeks old, we did not previously know the age-difference effect in the susceptibility of hepatotoxicity. In starting of TGP, we

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conducted an exploratory study to clarify whether the age-related difference was present in hepatotoxicity by using the first 3 chemicals in the TGP database, i.e., APAP, isoniazid (INH), and carbon tetrachloride (CCl4). Of these 3 chemicals, APAP alone showed a significant age-related difference in hepatotoxicity. In this report, we focus on the age-difference in susceptibility of APAP hepatotoxicity and elucidate the cause of the difference.

MATERIALS AND METHODS

Chemicals

APAP, INH, and CCl4 were purchased from Sigma Co. Ltd., (St. Louis, MO). All other chemicals and reagents used in the present study were of HPLC or analytical grade and are commercially available.

Animal treatment

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks or 11-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 using a computerized stratified random grouping method based on body weights for each age. On the administration day, the animals were 6 and 12 weeks-old, respectively. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21 to 25°C with a relative humidity of 40 to 70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, OrientalYeast Co., Japan).

Five rats in each group were orally administered APAP or INH by suspending in 0.5% methylcellulose solution, and with CCl4 by dissolving in corn oil. The highest dose level for each administration was determined by a 1 week dose-finding study (data not shown) and subsequently the middle and low doses were determined, i.e., 50, 300, 1000 mg/kg for APAP, 10, 50, 200 mg/kg for INH, and 30, 100, 300 mg/kg for CCl4. Blood samples were obtained at 3, 6, 9, and 24 hr post-dose with a needle and a heparinized syringe from the abdominal artery under ether anesthesia. Plasma was obtained after centrifugation and stored below −20°C until use. Aspartate aminotansferase (AST), Alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined by COBAS MIRA plus autoanalyzer (Roche Diagnostics, Basel, SZ). After blood collection, the animals were euthanized by exsanguination from the abdominal veins and arteries under ether anesthesia. The liver samples were collected in 10% neutral buffered formalin at necropsy and embedded in paraffin, and then sectioned and stained with hematoxylin-eosin. Histopathological evaluation of the liver specimens was conducted by using light microscopy and graded as - (no change), +/− (minimal), + (slight), ++ (moderate), and +++ (severe).

Measurement of hepatic glutathione and SH contents

The pre-weighed (ca. 0.1 g each) liver samples were homogenized with 5% 5-sulfosalicylic acid (Sigma-Aldrich), and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was used for measurement of total glutathione (GSH) content in liver using the Total Glutathione Quantification Kit (Dojindo Laboratories) according to the manufacturer's instructions. In this kit, GSH oxidized by DTNB is reduced by glutathione reductase; namely, an enzyme recycling method is employed. We modified this method in order to measure the total free SH contents in the sample. In brief, the manufacturer's instructions were followed except that the glutathione reductase solution was replaced with a buffer solution and the dilution factor for the sample and GSH standard solutions was reduced ca. 1/5 − 1/10 fold. The reaction time was 10 min at room temperature and the measurement was done by a pseudo-end point method. The results were expressed as SH equivalent to the standard GSH per wet weight (g) in both measurements.

Microarray experiment

An aliquot of the sample (about 30 mg) was obtained from the left lateral lobe of the liver of each animal immediately after sacrifice, and put into RNA later® (Ambion, Austin, TX, USA) overnight for 4°C and then frozen. Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. The purity of the RNA was checked by gel electrophoresis, and the 260/280 nm ratio was between 2.0-2.2. Total RNA was isolated using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA).

Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA) containing 15923 probe sets. The procedure was conducted basically according to the manufacturer's instructions. Briefly, 5 μg of total RNA was used for the synthesis of cDNA with the Superscript Choice

System (Invitrogen, Carlsbad, CA, USA) and T7-$(dT)_{24}$-oligonucleotide primer (Affymetrix). After the cDNA was purified by cDNA Cleanup Module (Affymetrix), biotin-labeled cRNA was synthesized by using the BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). The cRNA was purified by an IVT cRNA Cleanup Spin Column (Affymetrix), and then fragmented. Twenty μg of the fragmented cRNA was hybridized to a RAE230A probe array for 18 hr at 45°C at 60 rpm. After hybridization, the array was washed and stained by streptavidin-phycoerythrin using the Fluidics Station 400 (Affymetrix). Finally, the array was scanned by Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0.

Statistical analysis

The data from biochemistry was expressed as the mean value of the 5 animals in each group. In the present experiments, the data of blood biochemistry showed large variations and we thus employed Mann-Whitney's U-test (Snedecor and Cochran, 1989) for analyzing the drug effects.

Gene expression data were analyzed by using GeneSpring® version 6.1 (Silicon Genetics, Santa Clara, CA, USA). Expression data was normalized by the mean value (global normalization; adjusted to an arbitrary value of 500). After filtering the genes by flags (present or marginal call at least 12 of the 24 samples in each experiment), the fold change determination to the concurrent control samples was performed in order to extract drug-related changes. In some cases, Student's *t*-test (Snedecor and Cochran, 1989) with Benjamin's adjustment ($p<0.05$) was also used for the age-difference comparison using the control samples.

RESULTS

Phenotype change by APAP, INH and CCl4

Time-course changes in plasma AST, ALT and LDH after administrations of APAP, INH, and CCl4 are shown in Table 1. In APAP-treated rats, significant increases of ALT and AST were noted in adult rats at 24 hr after treatment of 1000 mg/kg. Young rats treated with the same dosage showed a tendency of increment of these enzyme activities, but it was not statistically significant. In the cases of INH and CCl4, no obvious increase of these enzymes was detected. Although a significant statistical difference in young rats was detected 9 hr after INH-treatment, it was a decrease in the measure and thus considered not to be toxic.

Histopathological findings of the liver are shown in Table 2. At 24 hr post-dose of APAP, necrosis, increased eosinophility and vacuolation of hepatocyte were observed with inflammatory cells infiltration at 1000 mg/kg in both ages. Of these, hepatocyte necrosis in adult rats was more evident than that in young rats. In the young rats, minimal necrosis (grade +/–) was only noted in 2 out of 5 animals (Photo 1A), whereas all of the adult rats had necrosis: 2 of them were grade + (Photo 1B) and the remaining 3 were grade +/–.

No obvious changes were found in the liver with this dose range of INH. Although CCl4 caused some pathological changes in the liver, no obvious age-related differences were noted. Both biochemistry and histopathology indicated that hepatotoxicity of APAP was evident, i.e., increases in the leak of the enzymes and centrilobular necrosis of hepatocytes, and adult rats appeared to be more susceptible to hepatotoxicity than the young ones.

Age-dependent gene expression

Based on the biochemical and histopathological data, the age-related difference appeared to be specific for APAP among the three chemicals. This suggested that there should be some difference in the factors specifically involved in APAP-induced hepatotoxicity. In order to elucidate the cause of the difference, we tried to extract genes with different expression by age from control animals.

Nine control animals in each time point were employed for both young and adult rats from the TGP database, and the genes that changed more than 2-fold between the ages with statistical significance ($p < 0.05$) in all time points (3, 6, 9, and 24 hr) were extracted (Table 3). Of the total 11 genes extracted, 7 genes were lower and 4 genes were higher in adult rats than in young ones. They contained "hemoglobin beta chain complex" and "aminolevulinic acid synthase 2" (the rate-limiting enzyme in hepatic porphyrin-heme biosynthesis), both of which appeared to reflect the biosynthesis of red blood cells in the liver, and this appears to be reasonable, because extramedullary hematopoiesis decreases with age. As for changes related to APAP toxicity, it was noteworthy that the lower value of glutathione *S*-transferase Yc2 (GSTYc2) and the higher value of Cyp3A13 in adult rats were clearly indicated. Fig.1 shows the time- and dose-dependent effects of APAP on the expression of these two genes. GSTYc2 (Fig. 1A) was found to be always higher in young than in adult rats. Nine hr after

Table 1. Changes in Blood biochemistry after treatment with 1000 mg/kg of Acetaminophen (APAP), 200 mg/kg of Isoniazid (INH) and 300 mg/kg carbon tetrachloride (CCl4).

Compound	Age (week)	Dose (mg/kg)	Time (hr)	Number of Amimals	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
APAP	6	0	3	5	66.2 ± 9.6	35.0 ± 8.7	111.2 ± 47.3
			6	5	63.4 ± 7.8	32.2 ± 4.1	105.2 ± 32.0
			9	5	53.4 ± 4.2	28.6 ± 3.4	81.6 ± 24.0
			24	5	59.0 ± 6.3	37.0 ± 3.1	105.2 ± 33.8
		1000	3	5	66.2 ± 7.3	36.8 ± 5.2	88.2 ± 14.2
			6	5	61.8 ± 4.2	31.2 ± 1.8	119.2 ± 21.4
			9	5	60.2 ± 4.1	30.8 ± 5.9	107.2 ± 30.1
			24	5	76.4 ± 14.1	51.0 ± 17.2	134.4 ± 31.9
	12	0	3	5	68.4 ± 17.1	29.2 ± 5.4	115.6 ± 29.8
			6	5	56.6 ± 7.3	25.0 ± 2.5	122.6 ± 44.9
			9	5	52.6 ± 2.2	25.2 ± 2.2	100.8 ± 54.6
			24	5	65.6 ± 12.5	29.8 ± 5.3	122.8 ± 62.1
		1000	3	5	63.4 ± 11.1	30.8 ± 7.7	109.2 ± 24.7
			6	5	58.6 ± 3.0	26.6 ± 3.8	93.0 ± 15.0
			9	5	52.0 ± 3.7	26.4 ± 4.7	121.8 ± 62.2
			24	5	287.8 ± 221.0 *	78.4 ± 62.3 *	315.4 ± 208.1
INAH	6	0	3	5	59.8 ± 7.2	34.0 ± 4.5	92.0 ± 19.8
			6	5	55.2 ± 7.2	26.8 ± 4.9	114.2 ± 36.1
			9	5	55.4 ± 5.0 *	27.4 ± 2.3	82.6 ± 29.5
			24	5	59.6 ± 8.2	33.2 ± 4.7	109.4 ± 46.5
		200	3	5	58.2 ± 5.8	31.4 ± 3.6	86.6 ± 22.9
			6	5	50.0 ± 2.2	24.0 ± 2.0	92.0 ± 16.2
			9	5	39.2 ± 6.7	24.6 ± 1.3	94.6 ± 26.1
			24	5	47.6 ± 6.7	21.2 ± 6.6	96.0 ± 25.6
	12	0	3	5	54.8 ± 10.5	25.0 ± 7.6	93.4 ± 44.9
			6	5	49.8 ± 11.7	24.2 ± 3.6	87.0 ± 38.6
			9	5	46.0 ± 16.2	22.6 ± 4.4	112.0 ± 85.9
			24	5	50.8 ± 9.1	26.6 ± 9.7	99.0 ± 30.6
		200	3	5	51.2 ± 10.8	27.2 ± 4.0	85.2 ± 34.6
			6	5	42.4 ± 3.6	22.4 ± 3.9	77.0 ± 29.2
			9	5	29.0 ± 7.1	19.2 ± 4.9	83.0 ± 30.4
			24	5	41.4 ± 11.0	14.2 ± 4.1	89.8 ± 41.2
CCl4	6	0	3	5	66.6 ± 2.7	37.2 ± 7.2	209.0 ± 109.8
			6	5	62.8 ± 5.1	31.4 ± 2.1	189.2 ± 78.5
			9	5	62.6 ± 9.9	30.0 ± 4.2	163.0 ± 55.4
			24	5	66.6 ± 4.0	37.2 ± 2.7	186.2 ± 67.2
		300	3	5	67.8 ± 4.3	39.4 ± 4.5	201.4 ± 74.9
			6	5	65.2 ± 5.7	29.8 ± 4.6	205.8 ± 48.3
			9	5	63.2 ± 5.8	28.4 ± 1.5	200.2 ± 59.1
			24	5	75.0 ± 13.3	42.2 ± 4.6	192.6 ± 38.2
	12	0	3	5	73.0 ± 18.8	37.8 ± 7.6	156.6 ± 30.4
			6	5	90.6 ± 38.4	47.6 ± 25.2	181.2 ± 32.5
			9	5	131.8 ± 162.9	123.2 ± 206.7	239.6 ± 100.7
			24	5	63.4 ± 10.6	34.6 ± 4.0	171.6 ± 51.0
		300	3	5	65.6 ± 9.9	35.6 ± 5.7	213.2 ± 58.6
			6	5	75.4 ± 21.1	37.0 ± 16.5	179.2 ± 71.4
			9	5	72.6 ± 17.8	31.4 ± 10.7	235.8 ± 110.0
			24	5	68.8 ± 1.5	36.6 ± 4.1	201.8 ± 60.6

Data are expressed as mean ± SD of 5 measurements. * $p<0.05$; significantly different from control by Mann-Whitney's U-test.

dosing, APAP clearly up-regulated the expression of the enzyme and the values after the induction were still higher in young than adult rats. In the case of CYP3A13, its expression was always lower in young than in adult rats and was not affected by APAP at all (Fig. 1B).

In order not to miss other molecular species, the expressions of CYP and GST were checked. There are

Table 2. Histopathological changes of liver by 1000 mg/kg of acetaminophen (APAP), 200 mg/kg of isoniazid (INH) and 300 mg/kg of carbon tetrachloride (CCl4).

Compound	Age (Week)	Time (hr)	Findings	Number of animals							
				Control				High dose			
			Grade	−	±	+	++	−	±	+	++
APAP	6	3	No abnormal findings								
		6	No abnormal findings								
		9	Increased eosinophility of hepatocyte	5				2	3		
		24	Necrosis of hepatocyte	5				3	2		
			Increased eosinophility of hepatocyte	5					1	2	2
			Vacuolar change of hepatocyte	5				4		1	
			Inflammatory infiltration	5					1	4	
	12	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	Necrosis of hepatocyte	5					3	2	
			Increased eosinophility of hepatocyte	5						2	3
			Vacuolar change of hepatocyte	5				4	1		
			Inflammatory infiltration	5					2	3	
INAH	6	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	No abnormal findings								
	12	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	No abnormal findings								
CCl4	6	3	Degeneration, hydropic	5				2	2	1	
		6	Degeneration, hydropic	5				1	2	2	
			Cellular infiltration	5				4	1		
		9	Degeneration, hydropic	5				1	4		
			Cellular infiltration	5				4	1		
		24	Degeneration, hydropic	5				1	4		
			Cellular infiltration	5				2	3		
			Degeneration, fatty	5					1	4	
	12	3	Degeneration, hydropic	5				2	2	1	
		6	Degeneration, hydropic	5				2	3		
			Cellular infiltration	5					5		
		9	Degeneration, hydropic	5				2	3		
			Cellular infiltration	5				1	4		
		24	Degeneration, hydropic	5				3	2		
			Cellular infiltration	5					5		
			Degeneration, fatty	5					2	3	

Grade indication: no change (−), minimal (±), slight (+), moderate (++).

Table 3. Differentially expressed genes in control animals between 6 and 12 week-old rats.

Probe Set ID	Gene ID	Gene Symbol	Fold change v.s. 6W				P-value				Gene Title
			3H	6H	9H	24H	3H	6H	9H	24H	
1387123_at	NM_012753	Cyp17a1	0.23	0.34	0.26	0.24	3.9E-04	4.1E-02	1.6E-03	1.9E-03	cytochrome P450, family 17, subfamily a, polypeptide 1
1371089_at	AA945082	Yc2	0.21	0.28	0.15	0.30	2.4E-04	7.0E-03	3.5E-03	8.6E-03	glutathione *S*-transferase Yc2 subunit
1368160_at	NM_013144	Igfbp1	0.23	0.14	0.23	0.33	1.4E-03	2.0E-03	4.7E-02	2.8E-02	insulin-like growth factor binding protein 1
1371102_x_at	X05080	Hbb	0.42	0.43	0.27	0.35	9.4E-03	2.0E-03	7.9E-05	3.1E-03	hemoglobin beta chain complex
1371245_a_at	BI287300	---	0.45	0.39	0.24	0.38	1.4E-02	9.4E-04	3.4E-05	2.5E-03	---
1367985_at	NM_013197	Alas2	0.42	0.41	0.32	0.42	7.0E-04	4.6E-03	2.5E-05	1.4E-03	aminolevulinic acid synthase 2
1387022_at	NM_022407	Aldh1a1	0.42	0.41	0.35	0.44	3.5E-03	1.2E-02	3.5E-03	1.1E-02	aldehyde dehydrogenase family 1, member A1
1369465_at	NM_012584	Hsd3b	2.6	2.4	2.7	2.0	1.9E-03	2.9E-02	1.7E-03	3.1E-02	steroid delta-isomerase, 3 beta
1390146_at	BF414998	RGD1306105_predicted	2.5	3.4	3.1	2.9	2.0E-03	1.6E-03	1.7E-03	1.1E-03	similar to RIKEN cDNA 2610318G18 (predicted)
1370387_at	U46118	Cyp3a13	6.4	5.1	4.7	3.4	1.4E-03	1.3E-02	3.0E-04	1.4E-03	cytochrome P450, family 3, subfamily a, polypeptide 13
1368171_at	NM_017061	Lox	2.7	5.6	7.4	3.6	1.9E-02	2.5E-02	7.9E-05	2.7E-02	lysyl oxidase

Nine control animals for each time point were employed for both young and adult rats from the TGP database, and the genes that changed more than 2-fold between the ages with statistical significance ($p < 0.05$, Student's *t*-test with Benjamin's adjustment) in all time points (3, 6, 9, and 24 hr) were extracted. Fold changes of 12-week vs. 6-week and their p values at each time point are also shown.

19 probe sets annotated with GST, while GSTYc2 was the only one with a difference between young and adult. A comparison between young and adult rats was made at 24 hr after the treatment with 1000 mg/kg of APAP and significant differences were detected in three GSTs, i.e., GST mu 2, microsomal GST 3 (predicted), and GST omega, all of which showed lower values in adult than in young rats (data not shown). There are 63 probe sets annotated with CYP, and only CYP17A1 and CYP2C40 (in addition to CYP3A13) were found to be differentially expressed between young and adult rats, and the former two had much lower values in adult than in young rats. No probe sets with different sensitivity to APAP treatment between ages were detected in CYPs. As CYP17A1 and CYP2C40 are involved in steroid biosynthesis and arachidonic acid metabolism, respectively, CYP3A13 was found to be the only candidate for CYP with age-related difference that produces active metabolites of APAP.

The above results could explain the observation that adult rats showed high sensitivity to APAP. In order to explain the specificity of the chemicals, we checked the expressions of CYP2E1 and *N*-acetyltransferase, which are considered to be involved in production of active metabolites of INH and/or CCl4. As described above, CYP2E1 had no age-related difference, and this was the same for the INH- or CCl4-treated group. There are 6 probe sets annotated with *N*-acetyltransferase, and none of them showed significant age-related differences regardless of treatment with INH (data not shown).

Measurement of GSH and SH

It is a consensus that APAP is converted into an active metabolite that is detoxified by GSH conjugation and hepatotoxicity emerges when intracellular GSH is depleted. From the present results, it was suggested that adult rats had a high ability to produce the active metabolite but low capacity of detoxification and that subsequently this caused GSH-depletion more easily as compared with young rats. This was confirmed by direct measurements of hepatic GSH and SH contents by the DTNB method. The results are shown in Fig. 2. Both GSH and free SH contents per wet weight of liver in adult rats showed a value twice as high as that in young rats. Twenty-four hr after treatment with 1000 mg/kg of APAP, these measures did not change in young rats, whereas they showed a remarkable decrease in adults with a statistical significance in free SH contents. This supported our assumption that the high sensitivity to APAP in adult rats is attributed to the high degree of GSH-depletion.

Gene expression changes by APAP

Numbers of altered genes with 2-fold or more of

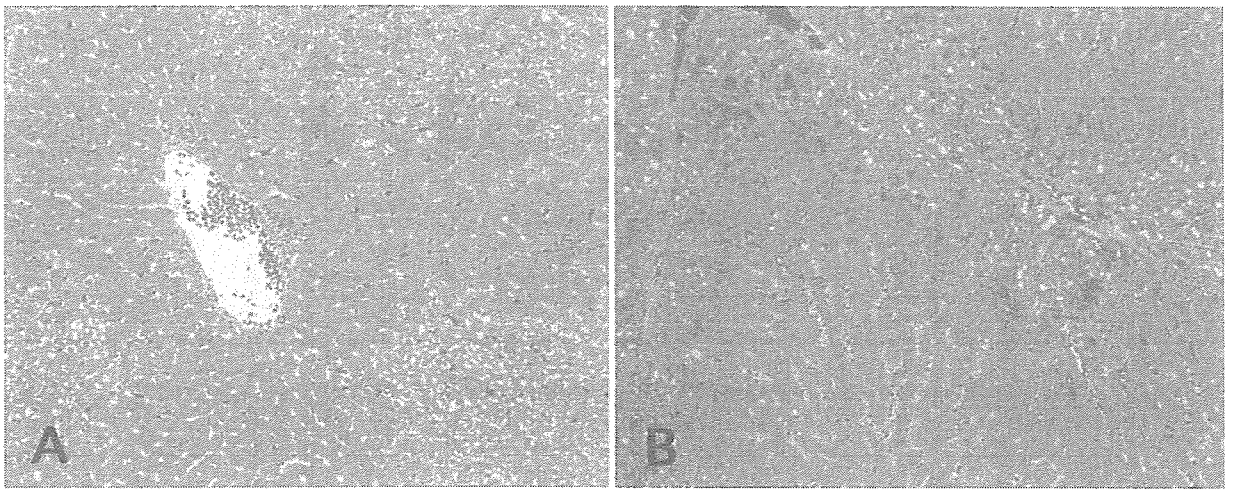

Photo 1. H&E-stained sections of rat liver 24 hr after oral administration of 1000 mg/kgAPAP. A: 6-week-old rat; centrilobular necrosis +/−, eosinophility ++, inflammatory infiltration +. B: 12-week-old rat; centrilobular necrosis +, eosinophility ++, inflammatory infiltration +.

the corresponding control in the highest doses of APAP, INH, and CCl4 are shown in Table 4. In this analysis, we did not use statistical filtering because we considered that it was favorable to overview the drug-related changes in order to compare the age difference between young and adult rats. The numbers of genes affected by APAP were greater in adult rats than those in young rats for all time points. In contrast, INH and CCl4 did not show any clear tendency in terms of the age-related difference in the numbers of mobilized genes.

The altered genes with more than 3-fold change vs. control at 24 hr post-dose of APAP in adult rats are listed in Table 5 (up-regulated genes), and those with less than 1/3 vs. control at 24 hr in adult rats are listed in Table 6 (down-regulated genes). The fold change of

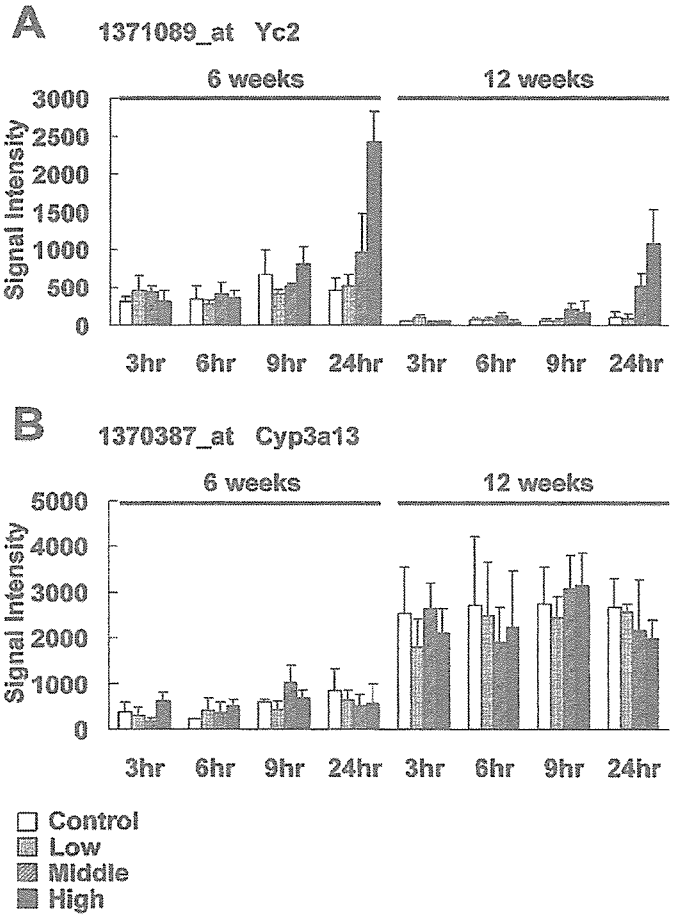

Fig. 1. Time- and dose-dependent effects of APAP on the expression of glutathione *S*-transferase Yc2 (1371089_at) and Cyp3a13 (1370387_at). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and expressed as mean ± SD (N=3).

each gene in young rats is also included in these tables. Response to oxidative stress (Hspla, Hsplb, Hmoxl, Txnrdl, Hspb8, Hspca, Hspb8), glutathione metabolism (Gss, Gstp, Gsr, Gclc), and response to DNA damage (Ddit4, Prpl9, Apexl, Gadd45a) were up-regulated in adult rats. Cell proliferation (pcna, Naplll, Gnl3), part of glutathione metabolism (Gsr, Gclc), UDP-glucuronocyltransferase (RGD:708541, RGD:708417) were up-regulated in both young and adult rats. However, microsomal enzymes (Cyp7Al, Cyp3All, Cyp17Al, Comt, Gulo, G6pc, Secll, RGD:2467, G6pc, Fmol, Gulo) and mitochondrial enzymes (Glul, Oat, Ehhadh, Abcc8, Otc, Abat, Ivd, Abcc9, Aadat, Ca5a, Acsll) were dominantly down-regulated in adult rats. Of these, some mitochondrial enzymes (Oat, Ehhadh and Ivd) were also down-regulated in young rats. These results indicated that the gene expression changes in adult rats were also greater than those in young ones, reflecting the degree of liver damage.

Changes of some representative genes are depicted as Fig. 3 and Fig. 4, which show time- and dose-dependency. In genes such as Egrl, Dnajbl (predicted), Ddit3, and Hspca (Fig. 3A-D), their expression was highly up-regulated 24 hr after treatment of the highest dose and the extent of induction was remarkably different between ages. On the other hand, in the genes such as Hsp ala, Hmox-1, Gadd45, and Txnrd (Fig. 4A-D), their induction was larger in adult than young 24 hr after treatment, whereas induction in the young ones occurred at an earlier time point than the adults, i.e., the extent of induction of these genes were much higher in the young than in the adult at early time points. Namely, part of the protective response against APAP started earlier in young rats.

By analyzing the time- and dose-dependent manner, it was revealed that some of stress-related genes responded faster in young than adult rats by APAP. Since expression of these genes was not affected by INH or CCl4, it was not a feature of young rats but rather specific for APAP among these chemicals. Furthermore, reviewing the histopathology in Table 2, cell infiltration with minimal extent occurred at 9 hr only in the young rats, supporting the result of the above gene expression analysis.

DISCUSSION

The mechanism of hepatotoxicity by APAP has been proposed in some papers (Cohen and Khairallah, 1997; Parkinson, 2001; Bessems and Vermeulen, 2001; Irwin *et al.*, 2004). The most widely accepted mecha-

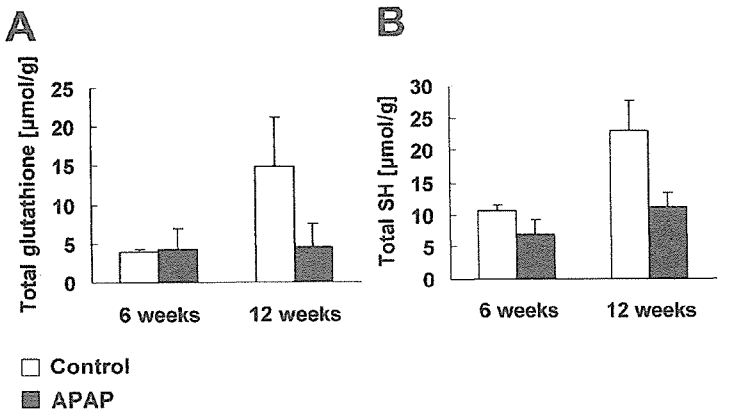

Fig. 2. Effects of APAP on hepatic content of total glutathione and SH of 6- and 12 week-old rats.
Both 6- or 12-week-old rats received 1000 mg/kg APAP orally and liver samples obtained 24 hr later. A: total glutathione contents expressed as μmol glutathione per g wet weight, B: total SH contents equivalent to μmol glutathione per g wet weight. Values are mean ± SD (N=3). A statistically significant reduction of total SH was observed in 12-week rats ($p<0.05$).

nism is that APAP is biotransformed to a reactive intermediate (*N*-acetyl-*p*-benzoquinone imine) by cytochrome P450s (Cyp2E1 and Cyp3A) in liver. The main detoxification pathway is considered to be GSH-conjugation. Finally, overflow of intermediate and/or depletion of GSH cause protein arylation and/or oxidation, and subsequently leads to necrosis of the liver. Expression changes by APAP in the present study were generally in agreement with the above mechanism of action, i.e., response to oxidative stress, glutathione metabolism, response to DNA damage, cell proliferation, and conjugation enzymes were up-regulated and several microsomal enzymes and mitochondrial enzymes were down-regulated. Of these, down-regulation of several microsomal enzymes and mitochondrial enzymes could be explained by the organelle function, because the microsome was the place where the reactive metabolite was produced and cell death was closely related to mitochondrial damage (Jaeschke and Bajt, 2006). These changes by APAP were also greater in adult rats than in young rats at 24 hr after dosing.

Table 4. Number of genes whose expression was changed more than 2-fold by 1000 mg/kg of APAP, 200 mg/kg of INH or 300 mg/kg of CCl4.

Compound	Age (week)	Time (hr)	Number of Genes	
			Increased	Decreased
APAP	6	3	27	67
		6	14	32
		9	19	39
		24	137	149
	12	3	32	88
		6	33	198
		9	34	67
		24	220	193
INAH	6	3	15	17
		6	6	13
		9	5	25
		24	3	34
	12	3	4	15
		6	1	20
		9	18	19
		24	6	62
CCl4	6	3	15	25
		6	10	73
		9	5	31
		24	21	39
	12	3	34	24
		6	4	24
		9	14	12
		24	9	64

Expression data was normalized by mean value (global normalization; adjusted to an arbitrary value of 500) and filtering of the genes by flags (present or marginal call at least 12 of the 24 samples in each experiment) was performed. The fold change of each of the probe sets by the drug was calculated and the numbers of the genes showing more than 2-fold or less than half of control value were counted for each age.

Although there have been several studies where hepatotoxicity of APAP in rodent was analyzed by gene expression (Reilly *et al.*, 2001; Ruepp *et al.*, 2002; Irwin *et al.*, 2004; Heinloth *et al.*, 2004), there is a limited number of papers regarding the age difference in the susceptibility of hepatotoxicity. A few papers described age-related differences such as weanling vs. mature (Allameh *et al.*, 1997), 11-day vs. 33-day-old (Green *et al.*, 1984), or 4-month vs. 25-month-old rats (Rikans and Moore, 1988), but the cause of the differences was not well clarified (Rikans, 1989; Tarloff *et al.*, 1996). There was no information about the difference in susceptibility concerning young to mature age, either. As 6 week-old rats are generally used for toxicological tests (and thus we employ this age for creating our toxicogenomics database), we are interested in the sensitivity of rats of this age against hepatotoxicants compared with matured rats, such as 12-week-olds.

In the present study, a single oral dose of APAP at 1000 mg/kg caused marginal hepatotoxicity in young rats, such as a tendency for an increase in plasma enzymes and minimal hepatocyte necrosis. Under the same condition, adult rats showed more prominent toxicity. This age-related difference should be based on APAP-specific mechanism(s), since no age-related differences were noted in INH or CCl4-induced hepatotoxicity. From the present results and literatures, the main mechanism was considered to be as follows: a) the expression of CYP3A13 that produces the active metabolite of APAP is higher in adult than in young rats, b) among the GST species that detoxify the active metabolite of APAP, the expression of GSTYc2 is lower in adult than in young, and c) the expression of GST, mu 2, microsomal GST 3 (predicted), GST omega 1 are inhibited by APAP.

The modification of APAP toxicity by modifying Cyp3A has been reported in rodents. Inhibition of Cyp3A prevented APAP hepatotoxicity (Kostrubsky *et al.*, 1997), whereas caffeine, dexamethazone, troglitazone or pregnenolone 16 alpha-carbonitrile, all of