Table 5
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PCN

Gene	Accession no.		Fold change	*t* Test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	1.336*	0.000
		Fetal liver	0.963	0.622
		Placenta	1.093	0.753
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.102	0.567
		Fetal liver	1.050	0.787
		Placenta	1.109	0.785

* $P < 0.05$.

normalization was performed in each experimental datum, fold changes (average of signals of treated groups/average of signals of control groups) were calculated. Students *t* test or Welchs *t* test was done. In this study, we picked up the probes focusing on drug metabolizing CYPs isozymes (Table 1) and two orphan nuclear receptors for CYP inducers, Nr1i2 and Nr1i3. Among CYP isozymes examined, probes of which significance level was $P < 0.05$ and Absolute Call was present, were picked up.

Reverse transcriptase polymerase chain reaction (RT–PCR) for CYP3A1 mRNA

Total RNA was prepared as described above. For RT–PCR analysis, we selected CYP3A1 gene because it was the only gene which was commonly up-regulated in dam's liver, placenta, and fetal liver. PCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of the rat mRNA. PCR was carried out with 1 μl of cDNA sample in a 100-μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U of rTaq, 10 × PCR buffer, and dNTP mixture (Takara, Ohtsu, Japan). This was immediately followed by preheating at 95°C for 7 min, denaturation at 95°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min using Takara PCR Thermal Cycler SP (Takara). Annealing temperatures and cycle numbers are shown in Table 2. Optimal cycle numbers were determined in a preliminary experiment to ensure that the amplification was in the linear range and not during the plateau phase. PCR products were identified by electrophoresis on 2% agarose gel (Nippon Gene Co. Ltd.) followed by ethidium bromide (Invitrogen) staining. Fluorescent-gel imaging was carried out using an ultraviolet-CCD video system Fas-III (Toyobo, Tokyo, Japan). The relative band density against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was represented as the mean ± standard deviation (SD) for three dams, and statistical analysis was carried out using Students *t* test or Welchs *t* test.

Results

Histopathological findings

In the dam's liver, there were no histopathological changes in both CO and Sa groups (Figs. 1a, c). In PCN group, slight swelling of hepatocytes was observed (Fig. 1b). After the PB treatment, moderate vacuolization of hepatocytes was observed (Fig. 1d).

In the fetal liver, slight swelling of hepatocytes was observed in PCN group (Fig. 1f). There were no histopathological changes in CO (Fig. 1e), Sa, and PB groups. In the placenta, there were no histopathological changes in all groups.

Findings of microarray analysis

The selected results of microarray analysis on CYPs in the dam's liver, placenta and fetal liver are shown in Tables 3 and 4. Among 40 probes for drug metabolizing CYPs isozymes, probes showing significant changes were picked up. In Tables 5 and 6, the results of microarray analysis on two orphan nuclear receptors are shown.

In dam's liver, the gene expression of CYP3a18, Cyp3A1 (CYP3A subfamily), and cytochrome P-450e (CYP2B subfamily) prominently increased in PCN group (Table 3). The gene expression of Cyp2a1 (CYP2A subfamily) increased moderately, and that of Cyp2f1 (CYP2F subfamily), CYP4F4 (CYP4F subfamily), p450Md mRNA for cytochrome P-450 (CYP2C subfamily), CYP3A9 (CYP3A subfamily), and Cyp2b3 (CYP2B subfamily) also showed a

Table 6
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PB

Gene	Accession no.		Fold change	*t* test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	0.901	0.265
		Fetal liver	1.000	0.998
		Placenta	0.982	0.957
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.167	0.390
		Fetal liver	1.263	0.127
		Placenta	1.752	0.189

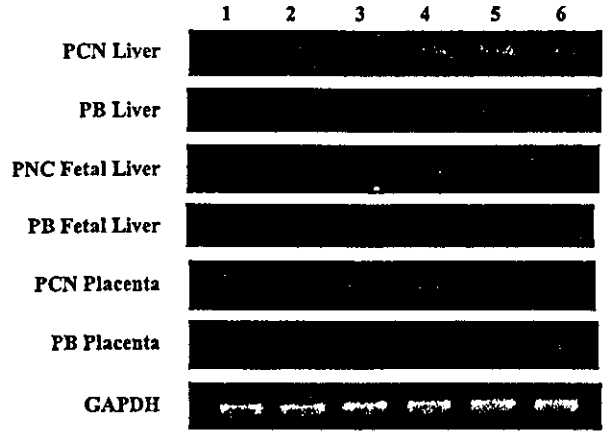

Fig. 2. The expression of CYP3A1 mRNA by RT–PCR in dam's liver, fetal liver, and placenta. Agarose gel electrophoresis. 1–3, rat number of control group; 4–6, rat number of treated group.

tendency of significant increase (Table 3). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G (CYP 1A subfamily) and Cyp4a1 (CYP4A subfamily) decreased in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2f1, Cyp2a1, Cyp2d2 (CYP2D subfamily), and cytochrome P450 PB1 (CYP2C subfamily) also showed a tendency of significant increase (Table 4). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G and Cyp2e1 (CYP2E subfamily) decreased in PB group (Table 4).

In fetal liver, the gene expression of Cyp3A1, pregnenolone 16-alpha-carbonitrile-inducible cytochrome P450 (CYP3A subfamily), and p450Md mRNA for cytochrome P-450 prominently increased in PCN group (Table 3), and that of Cyp2a1 also significantly increased (Table 3). On the other hand, significantly down-regulated genes coding CYPs were not detected in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2a1 showed a tendency of increase. The expression of CYP4A3 gene (CYP 4A subfamily) showed a tendency of decrease (Table 4).

In placenta, the expression of Cyp3A1 gene showed a non-significant increase in PCN group (Table 3), while it showed a significant increase in PB group (Table 4). There were no significantly down-regulated genes detected in either PCN or PB groups (Tables 3 and 4).

The expression of Nr1i2 gene was significantly elevated only in dam's liver of PCN group (Table 5), and the expression of Nr1i3 gene did not show significant changes in any groups (Table 6).

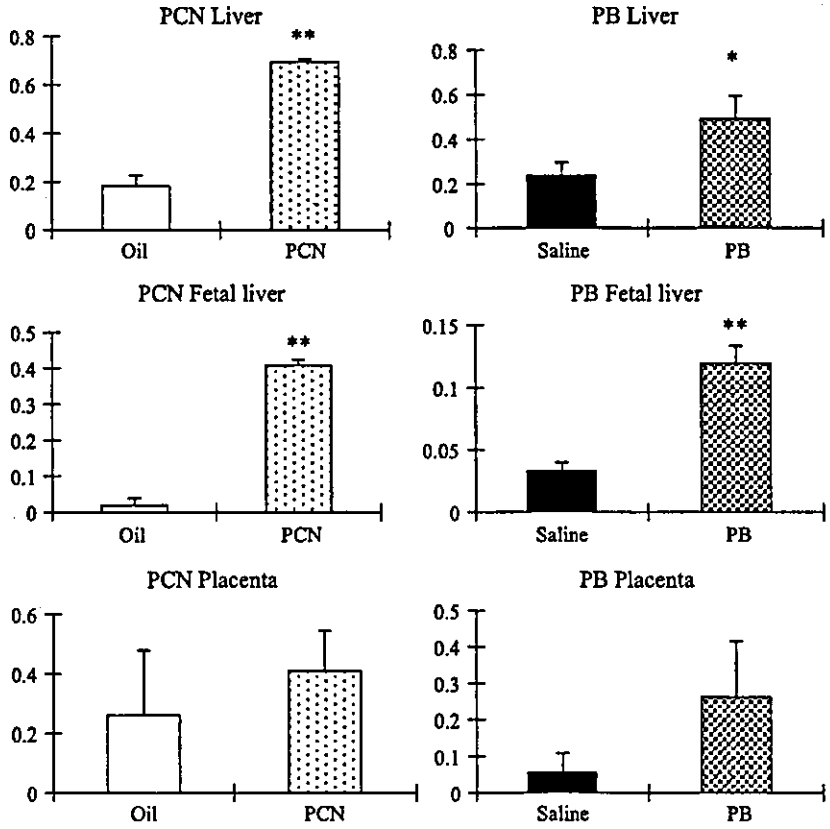

Fig. 3. The relative CYP3A1 band density to GAPDH by RT–PCR in dam's liver, fetal liver, and placenta. (□): CO group; (▨): PCN group; (■): Sa group; (▩): PB group. $*P < 0.05$ and $**P < 0.01$: significantly different from the control group.

Findings of RT–PCR for CYP3A1 mRNA

We performed RT–PCR on CYP3A1 mRNA, because it was the only gene which showed an up-regulation commonly in dam's and fetal livers and placenta. As shown in Figs. 2 and 3, a significant increase in CYP3A1 mRNA expression was observed in dam's and fetal livers. Although not significant, CYP3A1 mRNA expression also increased in placenta.

Discussion

Previously, we have reported the expression of nine CYP isozymes protein, against which antibodies were commercially available, in pregnant rat's liver, placenta, and fetal liver after treatment with dexamethasone, PCN or PB by Western blot analysis and immunohistochemistry (Ejiri et al., 2003, in press). In this study, the expression of CYPs genes was examined after PCN or PB treatment by microarray analysis, which can analyze 40 drug metabolizing CYP isozymes gene expression at one time.

In PCN group, the expression of Cyp3A1 gene was mainly elevated in both dam's and fetal livers, and its degree was more prominent in the latter. These findings of Cyp3A1 gene expression corresponded to the results of CYP3A1 protein expression (Ejiri et al., 2003). In placenta, although CYP3A1 protein was hardly induced by PCN (Ejiri et al., 2003), Cyp3A1 gene expression showed a mild but not significant increase. On the other hand, the expression of cytochrome P-450e and Cyp2b3 genes increased in dam's liver but not in fetal liver. This may be due to the difference of drug metabolizing enzyme profiles between dams and fetuses.

In PB group, the expressions of Cyp3a1 and cytochrome P-450e genes were elevated mainly in dam's and fetal livers. In our previous study on the inductions of CYPs proteins by PB, CYP3A1 protein was clearly induced in fetal liver by Western blot analysis, while CYP2B1 protein was not detected by either Western blot analysis or immunohistochemical staining (Ejiri et al., in press). Cyp3A1 gene expression in placenta significantly increased in PB group in the present study, but significant changes were not observed in Western blot analysis and immunohistochemical staining (Ejiri et al., in press).

In our previous report, the expression of CYP2B1 protein in dam's liver was observed in PB group but not in control group, and it was not detected in fetal liver even in PB group (Ejiri et al., in press). Among four probes for CYP2B subfamily, only cytochrome P-450e (phenobarbital-inducible) gene showed an apparently significant increase in this study. The relation between the increased gene expression of cytochrome P-450e and the protein expression of CYP2B1 in our previous report is still obscure.

As mentioned above, the existence of the orphan nuclear receptors, which induce CYPs by some inducers such as PCN or PB, is attracting researchers' attention (Masuyama et al., 2000; Mikamo et al., 2003; Xiong et al., 2002; Zhang et al., 1999). For example, it is said that Nr1i2 is concerned with an induction of CYP3A subfamily by PCN, and Nr1i3 is concerned with an induction of CYP2B subfamily by PB. In this study, a significant elevation in Nr1i2 gene expression was observed only in dam's liver of PCN group, while Nr1i3 gene expression showed no significant changes in all groups. In dam's liver, as mentioned above, Nr1i2 was induced in PCN group, followed by significant increase of Cyp3A1 gene expression. However, in fetal liver, Nr1i2 was not induced in PCN group, but a prominent induction of Cyp3A1 was observed. In addition, in dam's liver, although the expressions of cytochrome P-450e and Cyp3A1 genes were induced in PB group, the expression of Nr1i3 gene did not show significant change. Further study should be done to clarify the relationship between orphan receptors and CYPs gene expression.

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Induction of cytochrome P450 isozymes by phenobarbital in pregnant rat and fetal livers and placenta

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Abstract

Cytochrome P450 (CYP) isozymes are important in metabolizing xenobiotics. They are found in extrahepatic tissues such as placenta as well as liver. Previously, we reported that CYP3A1 was detected in the cytoplasm of giant cells in the trophoblastic region of placenta of rats through pregnancy. In this study, we examined the changes in the expression of CYP proteins in the pregnant rat and fetal livers and placenta after treatment with phenobarbital (PB), one of the antiepileptic drugs which is well known to induce several phase I and phase II drug metabolizing enzymes in the liver. Namely, F344 pregnant rats were treated with PB (80 mg/kg, i.p.) from 13 days of gestation (DG) to 16 DG. All animals were sacrificed on 17 DG, and Western blot analysis and immunohistochemical staining on nine CYP proteins (CYP1A1, CYP2B1, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP4A1) and histological examination were done in the dam's liver, placenta, and the fetal liver. Western blot analysis revealed that CYP3A1 protein was significantly induced, CYP2B1 protein was detected, and CYP2D1 protein was significantly decreased in the dam's liver after PB-treatment. In placenta, only CYP3A1 was detected with no difference between control and PB-treated animals. The results of immunohistochemical staining corresponded closely to those of Western blot analysis in the dam's liver and placenta. In the fetal liver, CYP3A1 and CYP2C6 proteins were significantly induced after the PB-treatment, but their immunostainability was not prominent. The present results are considered useful as a basis for further investigation of drug metabolism in pregnant animals.

Keywords: CYP isozymes proteins; Dam's liver; Fetal liver; Placenta; Phenobarbital

Introduction

Cytochrome P450 isozymes (CYPs) are very important in metabolizing xenobiotics. CYPs are divided into several groups by amino acid sequence, and foreign chemicals are metabolized mainly by families CYP 1–4 (Nelson et al., 1993). The majority of CYPs are found in the liver, and some CYPs are found in extrahepatic tissues such as kidneys, adrenal glands, lungs, small intestines, brain, skin, and placenta (Black and Coon, 1987; Simmons and Kasper, 1989).

Although its enzyme files are restricted, placenta is thought to be able to metabolize many foreign chemical compounds. Throughout pregnancy, placenta plays a vital role in maintenance of pregnancy because it has many important endocrine and metabolic functions (Hakkola et al., 1996a; Juchau, 1980).

Previously, we examined the expression of CYP proteins in rat placenta, and reported that CYP3A1 was detected in the cytoplasm of trophoblastic giant cells throughout pregnancy (Ejiri et al., 2001). We examined the induction of CYP3A1 in pregnant rat and fetal livers and placenta, and reported that CYP3A1 was prominently induced by pregnenolone-16α-carbonitrile (PCN) and dexamethasone (DEX) in pregnant rat and fetal livers, but with no significant induction in placenta (Ejiri et al., 2003).

Phenobarbital (PB), an antiepileptic drug, is known to induce several phase I and phase II drug-metabolizing

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enzymes, especially CYP2B and CYP3A (Asoh et al., 1999). PB administered to pregnant women may exert a variety of effects on the fetus and could contribute to malformations, low birth weight, and so on (Asoh et al., 1999; Bittigau et al., 2003; Gupta and Yaffe, 1982; Holmes et al., 2001). It is said that many chemicals including PB pass through the placenta to the developing fetuses and are rapidly distributed to fetal tissues (Hansen et al., 1999; Papich and Davis, 1986; Welsh, 1982).

This study examines changes in the expression of CYP proteins in pregnant rat and fetal livers and placenta following PB treatment.

Materials and methods

Animals

Ten pregnant rats of the Fischer 344 (F344) strain were purchased from Saitama Experimental Animal Co. (Saitama, Japan). The animals were individually housed in plastic cages in an animal room under controlled conditions (temperature: 23 ± 2 °C; relative humidity 55 ± 5%; light/dark cycle: 14/10 h), and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. The day of a vaginal plug being recognized was designated as 0 day of gestation (0 DG).

Chemicals

Phenobarbital sodium (PB) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PB was dissolved in physiological saline immediately before use, and the concentration was adjusted to 80 mg/ml.

Treatments

From 13 to 16 DG, five dams were daily treated with 80 mg/kg of PB (PB-treated group) intraperitoneally (i.p.) and the remaining 5 dams with 0.1 ml/kg of saline i.p. (control group), respectively.

All animals were sacrificed by exsanguination under ether anesthesia at 17 DG. At necropsy, body weights of dams and fetuses, and liver weight of dams were recorded. Then, the livers of dams and fetuses and placentas were sampled, and half were stored at −80 °C until used for Western blot analysis. The remaining half were fixed in 10% neutral-buffered formalin for histological and immunohistochemical examination.

Western blot analysis

The samples were homogenized with 0.1 M phosphate buffer (PB)–0.15 M KCl–1 mM EDTA Na–1 mM DTT (pH 7.4). The homogenates were centrifuged at 9000 × *g* for 20 min, and then the supernatants were centrifuged at 105,000 × *g* for 60 min. After discarding the supernatant, the pellets were suspended with the same buffer and recentrifuged. The pellets were resuspended with 0.1 M PB–0.15 M KCl–20% glycerol–1 mM EDTA Na–1 mM DTT (pH 7.4), and stored at −80 °C until used. After proteins (10 or 20 μg of mother liver, 30 μg of fetal liver, and 40 μg of placenta) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was treated with anti-rat CYP antibodies. The anti-rat CYP antibodies used in this study were as follows: CYP1A1, CYP2C6, CYP2E1 and CYP4A1 (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan), and CYP2B1, CYP2C12, CYP3A1, CYP2D1 and CYP2D4 (Chemicon International INC. Temecula, CA). The optimal working dilution was determined based on the results of preliminary examinations. As to the second antibody, anti-rabbit IgG, peroxidase-linked species-specific whole antibody (Amersham Pharmacia Biotech UK Limited, England) (optimal dilution, 1:10,000) and anti-goat IgG, peroxidase-linked species-specific whole antibody (optimal dilution, 1:10,000) were used. ECL Plus system (Amersham Pharmacia Biotech Ltd.) was used to visualize the band, and Quantity One v3.0 (PDI, Inc, NY, USA) was used to quantitate the reactive proteins.

Histology and immunohistochemistry

Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE) for histological examinations. For immunohistochemical examinations, paraffin sections were stained by LSAB method with streptavidine. The above-mentioned antibodies were used as the primary antibody and its optimal working dilutions were determined based on the results of preliminary examinations. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction, and then counterstained with methyl green.

Statistical analysis

Body weight gain ((body weight at sacrifice − body weight at the beginning of treatment) / body weight at the beginning of treatment × 100) of dams, body weight of fetuses, and relative liver weight of dams (liver weight / body weight × 100) were expressed as mean ± standard deviation (SD) of five dams. Statistical analysis was by the unpaired t-test between the PB-treated and control groups.

Results

Body weights and relative liver weights

No deaths occurred to dams, and there was no difference in fetal mortality between the PB-treated and control groups. The body weight gain of dams and the body weight of fetuses tended to be depressed in the PB-treated group, although not

significantly (Fig. 1). The relative liver weight of dams significantly increased in the PB-treated group (Fig. 1).

Findings of Western blot analysis

In the dam's liver, although CYP2B1 protein was not detected in the control group, it was easily detected in the PB-treated group (Figs. 2 and 3). CYP3A1 protein was significantly induced, while CYP2D1 protein was significantly decreased after PB-treatment (Figs. 2 and 3). CYP1A1, 2C6, 2C12, 2E1, and 4A1 proteins were not induced following the PB-treatment, and CYP2D4 protein was not detected in either the control or PB-treated groups.

In the placenta, only CYP3A1 protein was detected, and the value of band intensity was not significantly changed after the PB treatment (Figs. 2 and 3).

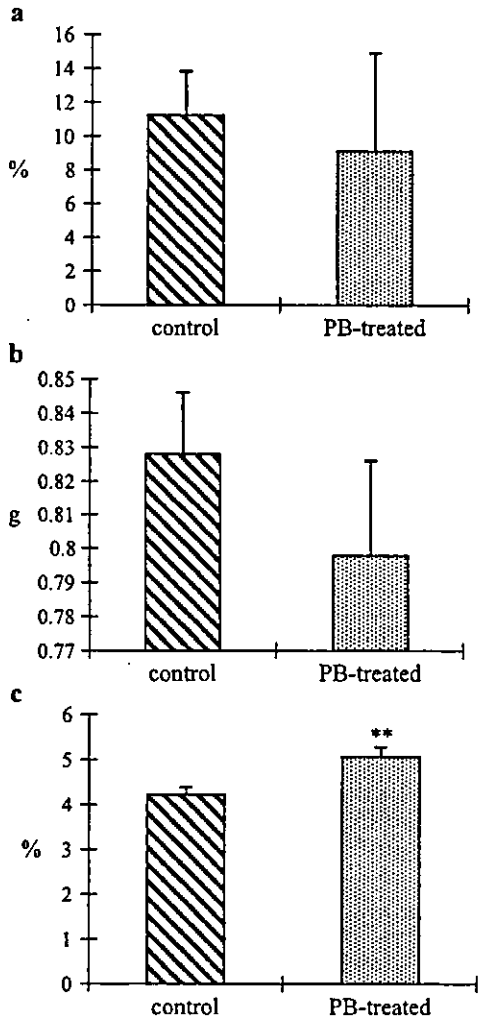

Fig. 1. Changes in body weight gain (a), fetal body weight (b) and relative liver weight in dams (c) after PB-treatment. (▨) control group, (▩): PB-treated group. Data are expressed as mean + SD of 5 dams. $^{*}P < 0.05$ and $^{**}P < 0.01$: significantly different from the control group.

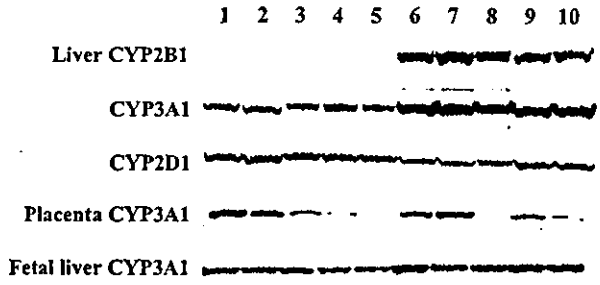

Fig. 2. The results of Western blot analysis. 1–5: rat number of the control group; 6–10: rat number of the PB-treated group.

In the fetal liver, CYP3A1 protein was significantly induced in the PB-treated group (Figs. 2 and 3). CYP2C6 protein was also significantly but slightly induced in the PB-treated group (Fig. 3). CYP2D1, 2E1, and 4A1 proteins showed no significant changes after the PB treatment, and CYP 1A1, 2B1, 2C12, and 2D4 proteins were not detected in both the control and PB-treated groups.

Histological findings

In the dam's liver, there were no histopathological changes in the control group (Fig. 4a). After the PB treatment, moderate vacuolization of hepatocytes was observed (Fig. 4b). There were no histopathological changes in the placenta and fetal liver in both the control and PB-treated groups.

Immunohistochemical findings

Among nine CYPs used in this study, CYP1A1, 2C6, 2D1, 2E1 and 3A1 proteins were clearly observed in the dam's liver in both the control and PB-treated groups. Positive signals for CYP3A1 were observed only in one layer of hepatocytes surrounding the central vein in the control group (Fig. 5a), while CYP3A1-positive hepatocytes were observed in the central and midzonal areas of the hepatic lobule in the PB-treated group (Fig. 5b). Positive signals for CYP1A1, 2C6, 2D1, and 2E1 were observed mainly in the central and midzonal areas of the hepatic lobule in the control group. The stainability for CYP2D1 protein was weakened in the PB-treated group compared with that in the control group (Figs. 5c and d), while the stainabilities for CYP1A1, 2C6, and 2E1 were not changed after the PB treatment.

Hepatocytes positive for CYP2B1 appeared after the PB treatment, although there were no CYP2B1-positive hepatocytes in the control group (Figs. 5e and f). A few hepatocytes weakly positive for CYP4A1 was observed in both the control and PB-treated groups, and there were no positive signals for CYP2C12 and 2D4 proteins in either the control or PB-treated groups.

In the placenta, CYP3A1 protein was detected immunohistochemically in the cytoplasm of trophoblastic giant cells

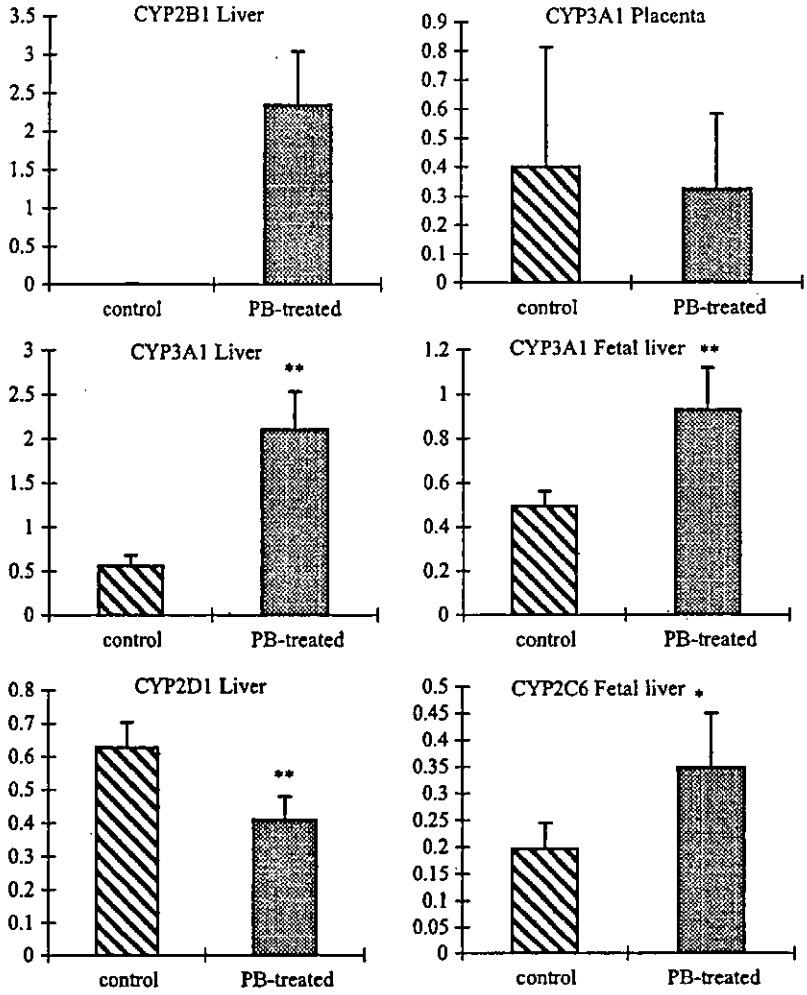

Fig. 3. Changes in CYP isozymes expression in dam's liver, placenta and fetal liver after PB treatment. (▨): control group; (▩): PB-treated group. $*P < 0.05$ and $**P < 0.01$: significantly different from the control group.

in both the control and PB-treated groups (Figs. 5g and h). Other CYPs used in this study were not detected immunohistochemically.

In the fetal liver, only a few signals positive for CYP3A1 and CYP2C6 proteins were detectable in both the control and PB-treated groups. The remaining CYPs were not detected, although Western blot analysis showed positive results on CYP2D1, 2E1, and 4A1.

Discussion

PB is commonly used as an antiepileptic drug. Because epileptic treatment is often long-term and pregnant women are sometimes treated with antiepileptics, there are many studies on adverse effects of antiepileptic drugs on mothers, fetuses, and newborn infants (Asoh et al., 1999; Bittigau et al., 2002, 2003;

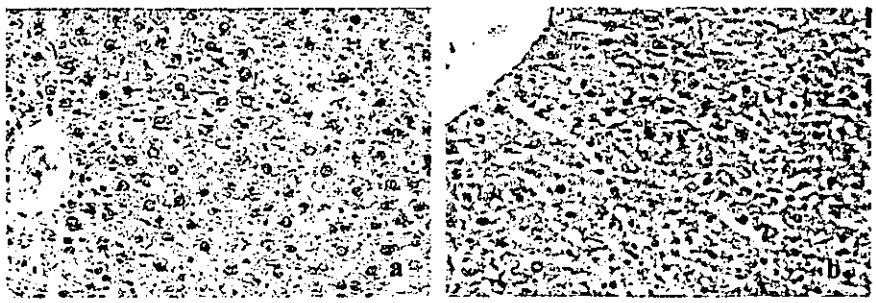

Fig. 4. Histology of dam's liver of the control (a) and PB-treated groups (b). Moderate vacuolization of hepatocytes is seen in b. HE, ×200.

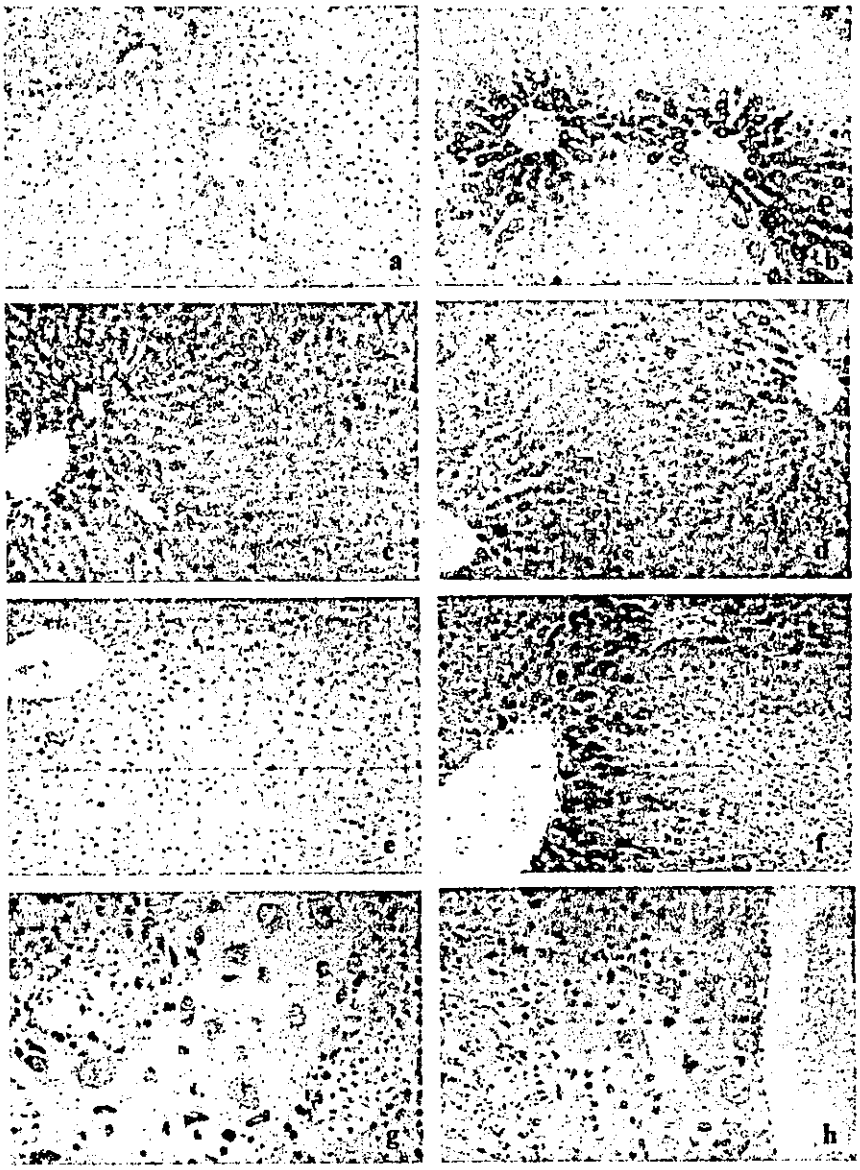

Fig. 5. Immunohistochemistry for CYP isozymes. CYP3A1 in dam's liver (a and b), CYP2D1 in dam's liver (c and d), CYP2B1 in dam's liver (e and f) and CYP3A1 in placenta (g and h). a, c, e, and g: control group; b, d, f, and h: PB-treated group. Immunostaining, ×100 (a, b, c, d, e, and f) or ×200 (g and h).

Bruno and Harden, 2002; Holmes et al., 2001; Nulman et al., 1999). As mentioned, antiepileptic drugs taken by pregnant women to prevent seizures are among the most common causes of malformations (neural tube defects, orofacial clefts, digital anomalies, and microcephaly), growth retardation, developmental delay, low birth weight, smaller head circumference, and so on (Asoh et al., 1999; Bittigau et al., 2003; Gupta and Yaffe, 1982; Holmes et al., 2001). In this study, although not significant, fetal body weight tended to be depressed. We did not examine the offspring for malformations. The relative liver weight was significantly increased after the PB-treatment, corresponding to the well-known fact that PB administration causes an increase in the liver weight.

In Western blot analysis, changes in the expression of CYP proteins were observed in the dam's liver, placenta, and fetal liver. In the dam's liver, seven (CYP1A1, 2C6, 2C12, 2D1, 2E1, 3A1 and 4A1) CYP proteins out of nine used in this study were detected spontaneously. Among these seven CYP proteins (CYP1A1, 2C6, 2C12, 2D1, 2E1, 3A1 and 4A1), only CYP3A1 was significantly increased after the PB treatment. On the other hand, CYP2B1 was not detected in the control group in the present study, although it is said that CYP2B1 protein is detected in the rat liver (Lupp et al., 2001). The reason for this difference is not clear, but it may be due to the difference in specificity and sensitivity of antibodies used, or the difference in the amount of CYP2B1 protein in the

liver. CYP2B1 protein was easily detected in the PB-treated group.

In the fetal liver of the control group, CYP proteins which can be detected (CYP2C6, CYP2D1, CYP2E1, CYP3A1 and CYP4A1) were more restricted than those in the dam's liver of the control group by Western blot analysis. In addition, they were barely detectable by immunohistochemical staining. Although CYP3A1 protein was prominently detected by Western blot analysis, it was not detected by immunohistochemical staining in either the control or PB-treated groups. It seems to depend on the quantity of protein in the fetal liver. In our previous study, CYP3A1 protein was easily detected in the fetal liver by Western blot analysis and immunohistochemical staining after exposure of pregnant rats to PCN and DEX (Ejiri et al., 2003). PCN and DEX may be more potent in the induction of CYP3A1 in the fetal liver than PB.

Moreover, in the fetal liver, CYP2B1 was not detected in either group, although it has been reported that the induction of CYP2B1 is observed in rat fetuses and neonates after the PB administration to dams (Asoh et al., 1999).

In the placenta, as reported in our previous paper (Ejiri et al., 2001, 2003), only CYP3A1 was detected in the control group, and there was no difference in the intensity of expression between the control and PB-treated groups; no other CYP proteins were induced after the PB administration by Western blot analysis and immunohistochemical staining.

The relative lack of enzyme induction in the placenta implies transplacental passage without chemical reaction therein. Accordingly, the placenta serves little or no role in fetal protection against phenobarbital as a prototype of maternal medication.

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Gene expression profiling in streptozotocin treated mouse liver using DNA microarray

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Abstract

Streptozotocin (SZ) is known to exert toxic effects not only on pancreatic islet beta cells but also on other organs including liver. For analyzing changes in genes expression associated with SZ toxicity, we performed DNA microarray analyses on the liver obtained from SZ-treated mice. Eight-week-old male ICR mice were treated i.p. with 200 mg/kg of SZ, and the blood and liver were taken at 6, 24 and 48 h after the treatment. Labeled cRNA prepared from total RNA of the liver was hybridized to the GeneChip Murine Genome U74A V.2 (Affymetrix). The number of the probe sets, which were clearly up-regulated or down-regulated, were over 100 at 6 and 24 h after the SZ-treatment, and it decreased at 48 h after the treatment. Many of the up-regulated genes were categorized into cell cycle/apoptosis related genes, immune/allergy related genes and stress response/xenobiotic metabolism related genes. On the other hand, genes related to glucose, lipid and protein metabolisms were down-regulated. These changes started prior to the elevation of the serum glucose levels, indicating the direct action of SZ on the liver rather than the secondary effect of diabetes. This may be related with the previously reported hepatic changes such as lipid peroxidation, mitochondrial swelling and inhibition of hepatocyte proliferation observed before the development of hyperglycemia (Exp. Toxic Pathol. 55 (2004) 467)

Keywords: Streptozotocin; Diabetes; Hepatic alteration; Gene expression; DNA microarray; Mouse

Introduction

Although streptozotocin (SZ), an extract from *Streptomyces achromogenes*, was originally developed as an antibiotic and/or antitumor agent (Vavra et al., 1960; White, 1963), it has been attracting a great attention as a useful tool for the induction of diabetes mellitus and its complications in laboratory rodents (Sibay et al., 1971; Steffes and Mauer, 1984; Kume et al., 1992) because of its toxic action on islet ß cells. We have previously reported the details of SZ-induced hepatic lesions in the acute (6–48 h after the treatment) and the subacute (4–12 weeks after the treatment) phase (Kume et al., 1994a,b; Doi et al., 1997, Kume et al., 2004). Those studies characterized the pathological changes such as the appearance of oncocytic hepatocytes, cytomegalic

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hepatocytes and bile duct hyperplasia in the subacute phase. In addition, it was also clarified that several hepatic changes including lipid peroxidation, mitochondrial swelling, peroxisome proliferation and inhibition of hepatocyte proliferation occurred before the elevation of the serum glucose levels, which indicated that those changes were attributable to the direct effects of SZ on hepatocytes rather than the secondary effects of diabetes or hyperglycemia (Kume et al., 2004).

Several reports have focused on the molecular genetic effects associated with SZ-induced diabetes (Wada et al., 2001; Dhahbi et al., 2003; Susztak et al., 2004); however, no researchers reported the changes in genes expression in SZ-treated animals before they showed hyperglycemia. This study, using the Affymetrix GeneChip, revealed molecular genetic changes in the liver before (6 and 24 h after the treatment) and after (48 h after the treatment) the induction of hyperglycemia.

Materials and methods

The study was approved by the Ethical Committee at Tanabe Seiyaku Co., Ltd. and all efforts were made to minimize animal suffering.

Animals and treatments

Eighteen 8-week-old male Crj:CD-1(ICR) mice (Charles River Japan Inc., Kanagawa, Japan) were used. The animals were housed in polycarbonate cages in a barrier system animal room under controlled conditions (temperature: 23±2 °C, humidity: 55±5%, 12 h light/dark cycle) and fed CRF-1 pellets (Oriental Yeast Co. Ltd. Tokyo, Japan) and tap water ad libitum throughout the experimental period. Half of the animals (three groups of three mice) were injected intraperitoneally with 200 mg/kg b.w. of SZ (Sigma, St. Louis, MO, USA) at around 9:00 a.m. SZ was dissolved in 0.05 M citrate buffer solution (pH 4.5) just before used. At 6, 24 and 48 h after SZ-injections, whole blood was taken from the *aorta abdominalis* of the three mice in each group under ether anesthesia. After that, the livers were removed and the small pieces of the left lateral lobe of the liver were quickly frozen. The other nine mice, which were given vehicle alone and killed in the same way, served as vehicle controls.

RNA extraction

Total liver RNA was isolated from the frozen tissue fragments using ISOGEN reagent (Nippon Gene, Tokyo, Japan). The RNA fractions from the three mice in each group were pooled for GeneChip analysis.

Affymetrix GeneChip analysis

Pooled total RNA from each group was labeled as described in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). mRNA was reverse-transcribed into cDNA using SuperScript Choice system (Invitrogen Life Technologies) and T7-(dT) 24 primer (Amersham Biosciences, Piscataway, NJ). The cDNA was converted to labeled cRNA using Bioarray HighYield RNA Transcript Labeling Kit (Affymetrix), which was purified using RNeasy Mini Kit (QIAGEN, Valencia, CA). The labeled cRNA was hydrolyzed in fragmentation buffer (40 mM Tris-acetate pH8.1, 100 mM KOAc, 30 mM MgOAc) to a size of approximately 35-200 nucleotides.

Ten microgram of the fragmented cRNA was hybridized with the Murine Genome U74AV2 array (Affymetrix) in hybridization cocktail (0.05 ug/uL cRNA, 50 pM control oligonucleotide B2, 1.5 pM bioB, 5 pM bioC, 25 pM bioD, 100 pM cre, 0.1 mg/mL herring sperm DNA, 0.5 mg/mL acetylated BSA, 100 mM MES, 1 M Na+, 20 mM EDTA, 0.01% Tween20). Hybridization was carried out overnight (16 h) at 45 °C, followed by washing, and staining with streptavidin-phycoerythrin (SAPE, Molecular Proves, Eugene, OR). Hybridization assay procedures including preparation of solutions were carried out as described in the Affymetrix GeneChip Expression Analysis Technical Manual. The distribution of fluorescent material on the array was determined using a confocal laser scanner (GeneArray Scanner, Affymetrix).

Array data processing

Signal quantification, background adjustment, judgment of detection call and other analysis were performed using the Microarray Suite (MAS) ver. 5.0 (Affymetrix). All arrays were globally scaled to a target value of 200. Genes were only considered for further analysis, if their corresponding probe sets had a signal intensity over 300 and their detection call was P (present). Pair-wise comparison analysis was performed between SZ-treated mice and control mice. The signal log ratio (SLR) was calculated for each probe set using the following formula: $\log_2$ (signal intensity in SZ-treated mice/that in control mice). Probe sets with SLR greater or equal to 1.0 was judged as 'up-regulated'. On the other hand, probe sets with SLR less or equal to −1.0 was judged as 'down-regulated'.

Annotation information on the probe sets on the U74A V.2 array was downloaded from the NetAffyx provided by Affymetrix. The probe sets judged as 'up-regulated' or 'down-regulated' were categorized according to the annotation information and protein information from Protein Knowledgebase provided by Swiss Institute of Bioinformatics (Swiss-Prot).

Signal transduction relationship between the up- or down-regulated genes was looked over and graphically displayed, using a database TransPath (BioBase, Wolfenbüttel Germany) accessorily. That analysis was performed exploratory without regard for the SLR.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of 10 genes were examined by RT-PCR to confirm the results of the GeneChip analysis. For GADD 45, Mdm2, Bcl2l, Wig1, Bax, Cdkn1a, Ppara, Srebf1, Hmgcs2 and Trp53, samples from 6 h after the treatment were used, and samples from 24 h after the treatment was used for Scd1, according to the results of the GeneChip analysis.

Frozen livers were thawed on ice, and total RNA was isolated using ISOGEN reagent. The quality of the isolated RNA was assessed by electrophoresis on RNA 6000 Nano kit (Agilent Tech.) based on the integrity of 28S and 18S bands.

The forward and reverse primers were designed using Primer Express software version 1.5 (Applied Biosystems) from the mouse mRNA sequence. The mRNA sequences were obtained from nr (All GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences). No longer "non-redundant"). Each primer was homology searched by NCBI BLAST search to ensure that it was specific for the target mRNA transcript. The primers were synthesized by QIAGEN K.K. (Tokyo, Japan).

RT-PCR were conducted in one step using the SYBR® Green RT-PCR Kit in a ABI PRISM 7700 Sequence Detector system (Applied Biosystems) according to the manufacturer's protocol.

Samples were deemed positive at any given cycle when the value of the emitted fluorescence was greater than the threshold value calculated by the instrument's software (Sequence Detector Ver.1.6.3). The threshold cycle, which is defined as the cycle at which PCR amplification reaches a significant value (i.e., usually 15 times the standard deviation of the baseline), is given as a mean value.

The data are expressed as the ratio of target mRNA to GAPDH mRNA, and the data are then shown as a fold change relative to control at each time point.

Results

Comparison analysis of the expression profiles was performed between SZ-treated mice and control mice from the GeneChip data. Fig. 1 shows the number of the up-regulated (SLR≥1.0) or the down-regulated (SLR≤−1.0) probe sets in the liver of SZ-treated mice. The number of probe sets, which were clearly up-regulated or down-regulated, was over 100 at 6 and 24 h after the SZ-treatment, and it decreased at 48 h after the treatment. There were only a small number of genes which showed similar changes among the time points examined.

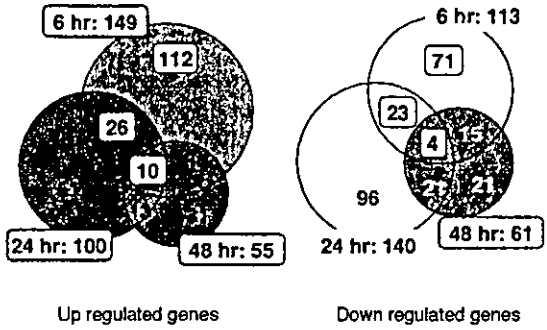

Fig. 1. Up- or down-regulated probe sets in the liver of the SZ-treated mice. Condition: Up regulation: signal $\log_2$ ratio to control group≥1, signal intensity >300. Down regulation: signal $\log_2$ ratio to control group≤−1, signal intensity of control group>300.

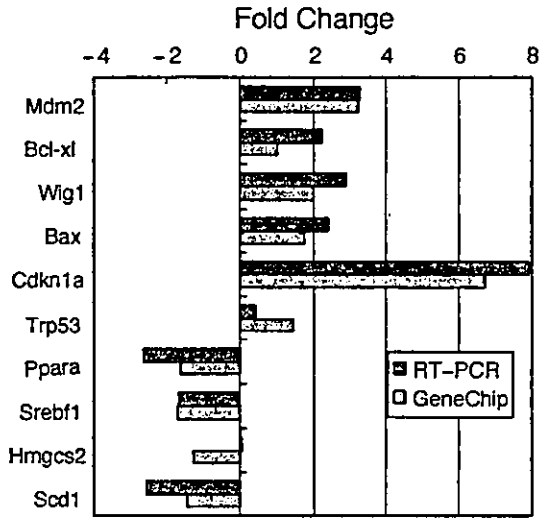

Fig. 2. Validation of the GeneChip data with RT-PCR. The expression of 11 genes was analyzed using GeneChip and RT-PCR. The changes in the expression of these genes were similar in the direction and the magnitude between the two techniques.

Probe sets, of which SLR was over 1.5 or was under −1.5, were picked up and tabulated in Table 1. Genes corresponding to those probe sets were categorized according to the information mainly from the

Table 1. Hepatic gene expression in the streptozotocin treated mice

Title	GeneName	6hr	24hr	48hr	Affy ID
Carbohydrate and lipid metabolism					
amylase 2, pancreatic	Amy2	-2	0.5	-2.9	97524_f_at
amylase 2, pancreatic	Amy2	-2.2	-0.5	-3.3	97523_f_at
apolipoprotein A-IV	Apoa4	0.5	0.7		100078_at
carboxyl ester lipase	Cel	-1.9	-0.1	-3.5	99939_at
carboxylesterase 1	Ces1	-1.7	1.2	0.1	103519_at
colipase, pancreatic	Clps	-1.1	-2.1	-3.3	160132_at
cytosolic acyl-CoA thioesterase 1	Cte1			0.8	103581_at
cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	-0.7	-0.9		99404_at
cytochrome P450, family 8, subfamily b, polypeptide 1	Cyp8b1	-1.4	-3.8	-0.8	103284_at
fatty acid synthase	Fasn	0.9	-1.5	-0.2	98575_at
farnesyl diphosphate synthetase	Fdps	0.4	-2	0.3	160424_f_at
farnesyl diphosphate synthetase	Fdps	0.4	-2.8	-0.2	99098_at
glucokinase	Gck	-0.8	-2.4	-1.8	102851_at
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Hmgcs1	-0.9	-1.5	0	94325_at
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	-0.9	-2.1	-1.2	92590_at
isopentenyl-diphosphate delta isomerase	Idi1	-0.3	-2.5	-0.1	98269_at
lipase, hepatic	Lipc	-0.6	-1.9	-1.2	98982_at
low density lipoprotein receptor-related protein 1	Lrp1	-1.6	-0.4	-0.1	101073_at
nuclear receptor subfamily 0, group B, member 2	Nr0b2	-1.5	-0.8	-0.5	97123_at
NAD(P) dependent steroid dehydrogenase-like	Nsdhl	-0.1	-1.7	0	98631_g_at
phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	Pik3r1		0.2	0.2	96592_at
phospholipid scramblase 1	Plscr1		0.1	0	102839_at
pancreatic lipase related protein 1	Pnliprp1	-2.1	1.4	-3.2	92601_at
peroxisome proliferator activated receptor alpha	Ppara	-1.8	-0.2	0.3	102668_at
sterol-C4-methyl oxidase-like	Sc4mol	-0.9	-2	0.2	160388_at
stearoyl-Coenzyme A desaturase 1	Scd1	-0.4	-1.4	-2.3	94058_at
stearoyl-Coenzyme A desaturase 1	Scd1	-0.4	-1.7	-2.6	94057_g_at
sterol regulatory element binding factor 1	Srebf1	-1.7	-1.1	-0.6	93284_at
thyroid hormone responsive SPOT14 homolog (Rattus)	Thrsp	-0.2	-1.7	0.7	160306_at
Protein/amino acid metabolism					
betaine-homocysteine methyltransferase	Bhmt	0.1	-1.9	-0.3	94049_at
carboxylesterase 3	Ces3	-1.5	-1.2	-1.3	101536_f_at
carnitine palmitoyltransferase 1, liver	Cpt1a	-1.5	0.1	1.2	93320_at
cysteine sulfinic acid decarboxylase	Csad	-1	-2.4	-1.3	99184_at
elastase 1, pancreatic	Ela1	-0.6	-1.7	-2.9	93783_at
elastase 2	Ela2	-1.2	0.1	-2.6	94037_at
F-box only protein 21	Fbxo21	-2.9	-1.7	0.5	104109_at
guanidinoacetate methyltransferase	Gamt	-0.5	-2	-0.6	101408_at
histidine ammonia lyase	Hal	-1.8	-1.1	-0.5	92833_at
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Herpud1	-1.7	-0.7	-0.8	95057_at
murinoglobulin 1	Mug1	-0.9	-1.8	-0.7	92837_f_at
protein tyrosine phosphatase, receptor type, D	Ptprd	-2.4	-1	0.2	93465_at
tyrosine aminotransferase	Tat	-0.1	-1.9	-0.7	98326_at
trypsin 2	Try2	-1.8	0.8	-3.3	92873_f_at
trypsin 4	Try4	-2.2		-3.8	101043_f_at
Cell cycle/apoptosis					
B-cell translocation gene 2, anti-proliferative	Btg2			0.8	101583_at
cyclin D1	Ccnd1	-0.3	1.2	0.5	94232_at
cyclin G1	Ccng1				160127_at
cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a				94891_at
cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a				98067_at
dual specificity phosphatase 6 (MKP3)	Dusp6			1.4	93265_at
early growth response 1	Egr1		0	1	98579_at
etoposide induced 2.4 mRNA	Ei24	0.6		0.6	99629_at
G0/G1 switch gene 2	G0s2	-1.2	-1.5	-0.1	97531_at
Jun-B oncogene	Junb		1.3	0.9	102362_i_at
transformed mouse 3T3 cell double minute 2	Mdm2				98110_at
nucleolar protein 1	Nol1		0	-0.1	96804_at
p53 apoptosis effector related to Pmp22	Perp-pending	0.1		0.5	97825_at
serum-inducible kinase	Snk			1.4	92310_at
transducer of ErbB-2.1	Tob1	0.8			99532_at
Bcl2-associated X protein	Bax			1.1	93536_at
Tnf receptor associated factor 4	Traf4		1.1	0.1	162482_at
wild-type p53-induced gene 1	Wig1			1	92282_at
Immune and inflammation					
ras homolog gene family, member AB	Arhb			0.8	101030_at
chemokine (C-X-C motif) ligand 1	Cxcl1			-0.1	95349_g_at
chemokine (C-X-C motif) ligand 1	Cxcl1	1.4		-0.1	95348_at
chemokine (C-X-C motif) ligand 12	Cxcl12	-1.7	-1.5	-0.7	100112_at
intercellular adhesion molecule	Icam1			0.9	96752_at
interferon-stimulated protein	Isg15	0.5	1.1		98822_at
lymphocyte antigen 6 complex, locus D	Ly6d	0.9		0.8	160553_at

Continued

Table. 1 (*continued*)

Title	GeneName	6hr	24hr	48hr	Affy ID
mannose binding lectin, serum (C)	Mbl2	1.1			97427_at
nuclear, factor, erythroid derived 2, like 2	Nfe2l2				92562_at
nuclear factor I/B	Nfib	-2.4	-0.7	0	99440_at
orosomucoid 2	Orm2	0.3	1.2	-1.8	94734_at
polymeric immunoglobulin receptor	Pigr	-0.8	-1.7	-1.2	99928_at
S100 calcium binding protein A11 (calizzarin)	S100a11	1.1		1.1	98600_at
serine (or cysteine) proteinase inhibitor, clade A, member 6	Serpina6	-0.7	-2.4	-1.1	96227_at
serine (or cysteine) proteinase inhibitor, clade F), member 1	Serpinf1	-0.2	-0.8	-1.5	93574_at
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Tap1		1.1	0.9	103035_at
Stress response and xenobiotic metabolism					
cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	-0.1	-0.8	-1.7	102998_at
cytochrome P450, family 2, subfamily b, polypeptide 10	Cyp2b10		-0.7	-0.8	102701_at
cytochrome P450, family 2, subfamily b, polypeptide 9	Cyp2b9	0.3		-2.2	101862_at
flavin containing monooxygenase 3	Fmo3	1.1	-1.7		104421_at
heat shock protein 1B	Hspa1b	-1.1	-1.5	0.3	100948_at
nicotinamide N-methyltransferase	Nnmt		1.2	0.4	101473_at
serum amyloid A 2	Saa2	-1.4	-0.4	-1.8	103465_f_at
serum amyloid A 3	Saa3			0.8	102712_at
thioether S-methyltransferase	Temt	-1.3	-3.3	-2.1	97402_at
thioredoxin-like 2	Txnl2	0.4		1	95696_at
Cytoskelton etc.					
actin, gamma, cytoplasmic	Actg		0.5	0.4	96573_at
cadherin 2	Cdh2	-2.1	-0.2	0.3	102852_at
gephyrin	Gphn	-3	-0.6	0.1	99441_at
p300/CBP-associated factor	Pcaf	-1.8	-0.4	-0.2	104070_at
tubulin, beta 2	Tubb2		0.2	-0.8	94835_f_at
tubulin, beta 3	Tubb3		1.4	0.1	160462_f_at
Miscellaneous					
S-adenosylhomocysteine hydrolase	Ahcy	-0.1	-1.5	-0.2	98024_at
aminolevulinic acid synthase 2, erythroid	Alas2	-2.4	-0.2	-0.8	92768_s_at
angiogenin	Ang	0.6		1	94392_f_at
aquaporin 8	Aqp8	0	-1.2	-1.9	102200_at
ATPase, H+ transporting, V1 subunit D	Atp6v1d	0.5			96951_at
bystin-like	Bysl		-0.1	0.1	160227_s_at
carbonic anhydrase 14	Car14	-0.1	-1.6	-0.7	98079_at
carbonic anhydrase 3	Car3	-0.6	-2.3	-0.8	160375_at
carbonic anhydrase 5a, mitochondrial	Car5a	-0.8	-1.7	-0.4	98137_at
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	Ccrn4l	0			99535_at
calcitonin gene-related peptide-receptor component protein	Crcp		0.3	0.6	103334_at
D site albumin promoter binding protein	Dbp	-2.6			160841_at
deiodinase, iodothyronine, type I	Dio1	-2.6	-1.9	-3.1	95552_at
Down syndrome critical region homolog 1 (human)	Dscr1		0.8	0.7	100555_at
ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	-1.6	-0.8	-0.3	97317_at
UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminyl)-galactosylglucosylceramide-beta-1, 4-N-acetylgalactosaminyltransferase	Galgt1		-0.4	-0.2	103367_at
glial cell line derived neurotrophic factor family receptor alpha 1	Gfra1	-1.6	-0.6	0.1	93872_at
growth hormone receptor	Ghr	-1.8	-1	-0.5	99107_at
hemoglobin alpha, adult chain 1	Hba-a1	0.9	-0.8	-2.2	94781_at
hemoglobin, beta adult major chain	Hbb-b1		-0.6	-2.5	103534_at
hemoglobin, beta adult major chain	Hbb-b1	0.8	-0.4	-2.1	101869_s_at
insulin-like growth factor binding protein 2	Igfbp2	-0.5	-2.2	0	98627_at
inositol 1,4,5-triphosphate receptor 5	Itpr5	-2.5	-0.3	-0.2	101441_i_at
kallikrein 6	Klk6	-3.1		-2.5	100061_f_at
lipin 1	Lpin1	-0.1			98892_at
nucleolar protein 5A	Nol5a		0.3	0.4	95108_at
nuclear receptor subfamily 1, group D, member 2	Nr1d2	-0.4	1.2		99076_at
regenerating islet-derived 1	Reg1	-2.1	0.4	-2.5	160213_at
v-rel reticuloendotheliosis viral oncogene homolog A (avian)	Rela		0.2	-0.5	97813_at
renin 1 structural	Ren1		1.2		98480_s_at
sodium channel, voltage-gated, type I, beta polypeptide	Scn1b	0.6			102806_at
syncollin	Sycn	-1.1	-0.1	-6.5	95509_at
thyrotroph embryonic factor	Tef	-0.1	1.3		160117_at

Data is shown as signal $\log_2$ ratio to each control group.

Swiss-Prot, although the genes, of which annotation was unclear (EST etc.), were excluded from the table.

The regulated genes showed a broad range, but many of the up-regulated genes were categorized into cell cycle/apoptosis related genes, immune/allergy related genes and stress response/xenobiotic metabolism related genes. On the other hand, many of the down-regulated genes belonged to glucose, lipid and protein metabolism related genes.

Ten genes were picked up among the up- or the down-regulated genes judged from GeneChip data, and were also analyzed by RT-PCR. Fig. 2 shows the fold changes

of each gene expression in SZ-treated mice from that in control mice analyzed by the GeneChip and the RT-PCR. The changes in these genes expression were similar in the direction and the magnitude between the two techniques. Only the expression changes of Hmgcs2 were different by using the RT-PCR and by using the GeneChip.

Discussion

Gene expression analysis was performed on the SZ-treated mouse liver in the acute phase of the treatment.

Affymetrix oligonucleotide microarrays generally yield data with a high degree of reliability as judged by Northern blot or quantitative PCR (Cao et al., 2001; Dhahbi et al., 2003). The changes in the expression of the 9 of 10 genes were similar in direction and magnitude between the two techniques as shown in Fig 2. Thus, we judged the GeneChip data were enough reliable, and we will discuss the expression profiles using the GeneChip data hereinafter. However, even only one gene (Hmgcs2) showed different results between the two techniques, when we pick up particular gene, quantitative PCR or other techniques should be used.

The number of up-regulated or down-regulated genes was over 100 at 6 and 24 h after the treatment. However, unexpectedly, there were only a small number of genes which showed similar changes among the time points examined. Judging from the clinicopathological examinations reported before, the 6 h after the administration was the stage when the temporal hypoglycemia was induced by an abrupt and transient insulin release from the injured pancreatic islets (Kume et al., 2004). In histopathological analysis, hepatocytes showed degenerative changes at 6 h, and they became almost normal at 24 or 48 h after the administration (Kume et al., 2004). Thus, the pathological condition seemed to be dynamically changing from 6 to 48 h after the administration, and this may explain why there were only a few genes showing similar changes among the time points examined.

Many of the up-regulated genes were categorized into cell cycle/apoptosis related genes, immune/allergy related genes and stress response/xenobiotic metabolism related genes. On the other hand, many of the down-regulated genes belonged to glucose, lipid and protein metabolism related genes. From the data, we found out the relation and the transduction pathway using a signal transduction pathway analyzing software TransPath, and the hypothetical transduction pathways were figured.

Fig. 3 indicates the relationship between the changes in apoptosis related genes expression. Numeral represents the SLR (the signal of the probe sets of the SZ treated mice/the control mice) at the corresponding time. Bcl2-associated X protein (bax), BCL2-like 11 (Bcl2l11) and other related genes were up-regulated from 6 to 48 h after the administration. Those genes belonged to Bcl2 family and were known to work on apoptosis acceleration (LeBlanc et al., 2002, O'Connor et al., 1998). Apoptotic protease activating factor 1 (Apaf1), which is known to mutually act with those genes (Robles et al., 2001), was also activated. Apaf1 interacts with caspase-9 and induces apoptosis (Cecconi et al., 1998). Moreover, p53, one of the main transcriptional factors of apoptosis (Kaelin, 1999), was up-regulated about two times at 6 h after the administration, and some of the up-regulators of p53 including 'p53 apoptosis effector related to Pmp22' (perp), wild-type p53-induced gene 1 (wig1) (Hellborg et al., 2001), and transformed mouse 3T3 cell double minute 2 (mdm2) (Gottlieb et al., 2002) were also up-regulated. Thus, the activation of apoptosis is expected. On the other hand, the up-regulation of bcl-xl and bag3, which were known to act as an inhibitor of apoptosis (Schott et al., 1995; Lee et al., 1999), was also observed. This may be related with the pathological findings of no increase in apoptotic figures at any time points (Kume et al., 2004), although nuclear chromatin margination, one of the ultrastructural characteristics of apoptosis, was detected in hepatocyte primary cultures after SZ-treatment (to be published elsewhere).

Fig. 4 shows the relationship between the changes in the cell cycle related genes expression. P53 activation was induced and the cascade for some related genes such as growth arrest and DNA-damage-inducible 45 (GADD45), and cyclin-dependent kinase inhibitor 1A (Cdkn1a, p21) was observed. GADD45 induces G2/M cell cycle checkpoint and make G2/M arrest (Wang et al., 1999), while Cdkn1a induces G1/S arrest (Cazzalini et al., 2003). Thus the present results indicated an existence of cell cycle arrest. This may be supported by the immunohistochemical analysis, in which the ratio of the proliferating cell nuclear antigen (PCNA) positive hepatocytes was low at 24 and 48 h after the SZ-treatment (Kume et al., 2004).

Most of genes related with the lipid and glucose metabolism were down-regulated. In Fig. 5, a cascade which leads to nuclear receptors PPARs or RXR was shown. Almost all factors were down-regulated, and serum lipids levels seemed to increase via a decrease in the expression of LDL-R or other factors. On the other hand, some fatty acid synthesis-related factors such as stearoyl-CoA desaturase (Ntambi, 1995) were also down-regulated, although these factors act to decrease serum lipids. More over, sterol regulatory element binding factor 1 (Srebf1), which acts in cholesterol synthesis (Wang et al., 1994), was down-regulated, and the downstream factors such as HmgCoA Synthases (Sakakura et al., 2001) were also down-regulated, although the cascade has not figured. These findings

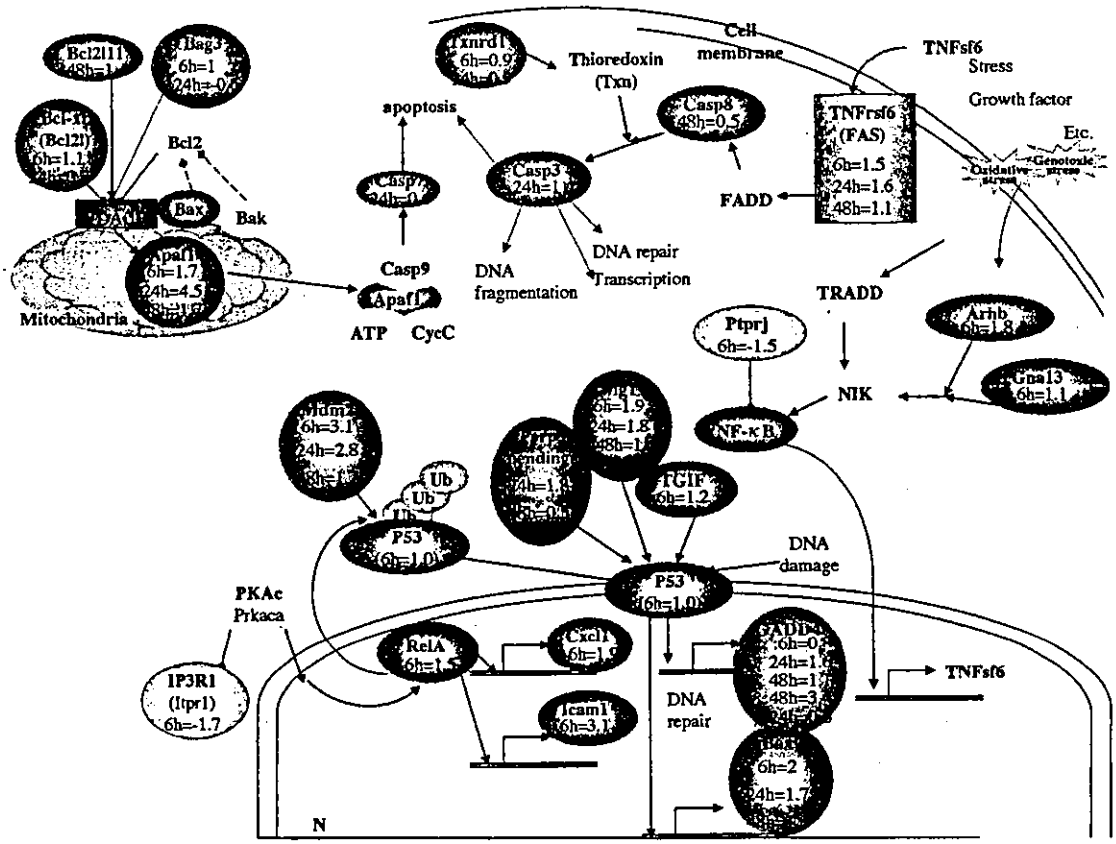

Fig. 3. Hypothetical signal transduction in the liver of the SZ- treated mice (figured by using TransPath program). Numeral represents the SLR of the signal of the probe sets of the SZ-treated mice/the control mice (1): Apoptosis related genes.

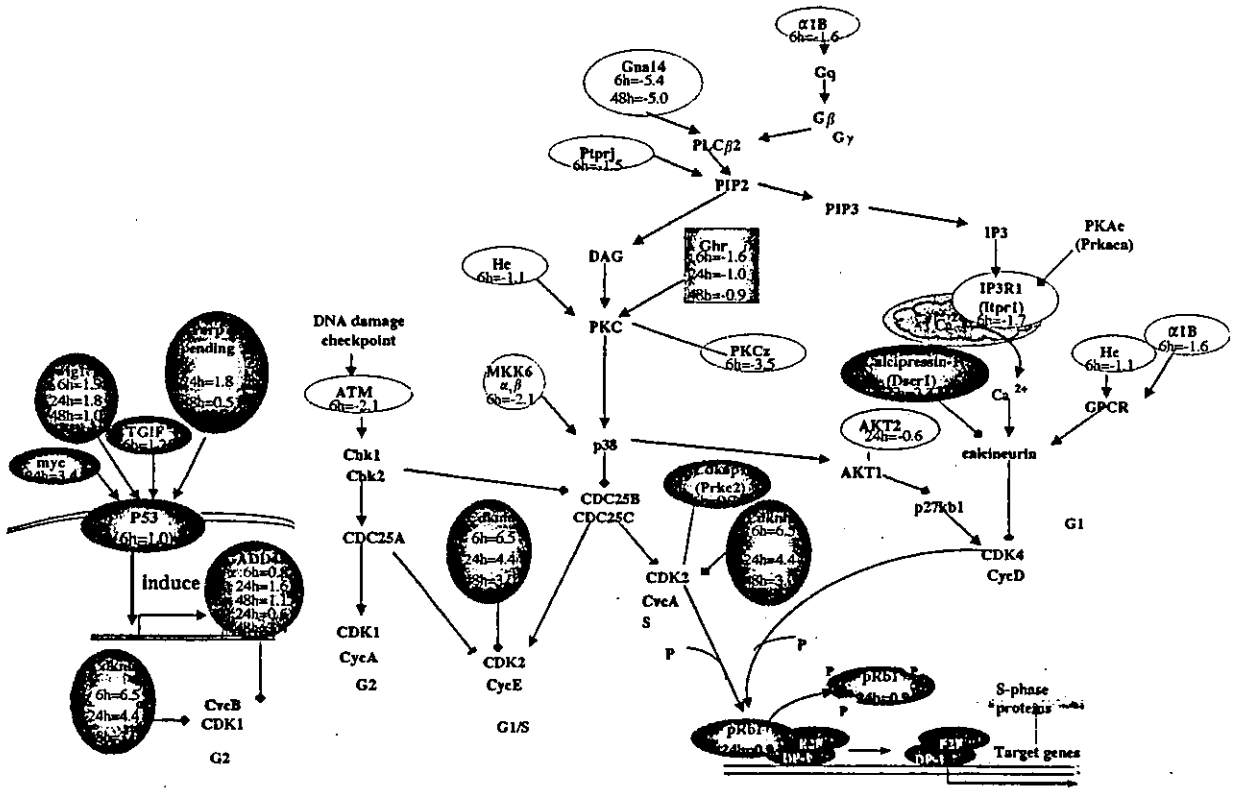

Fig. 4. Hypothetical signal transduction in the liver of the SZ-treated mice (figured by using TransPath program). Numeral represents the SLR of the signal of the probe sets of the SZ-treated mice/the control mice (2): Cell cycle related genes.

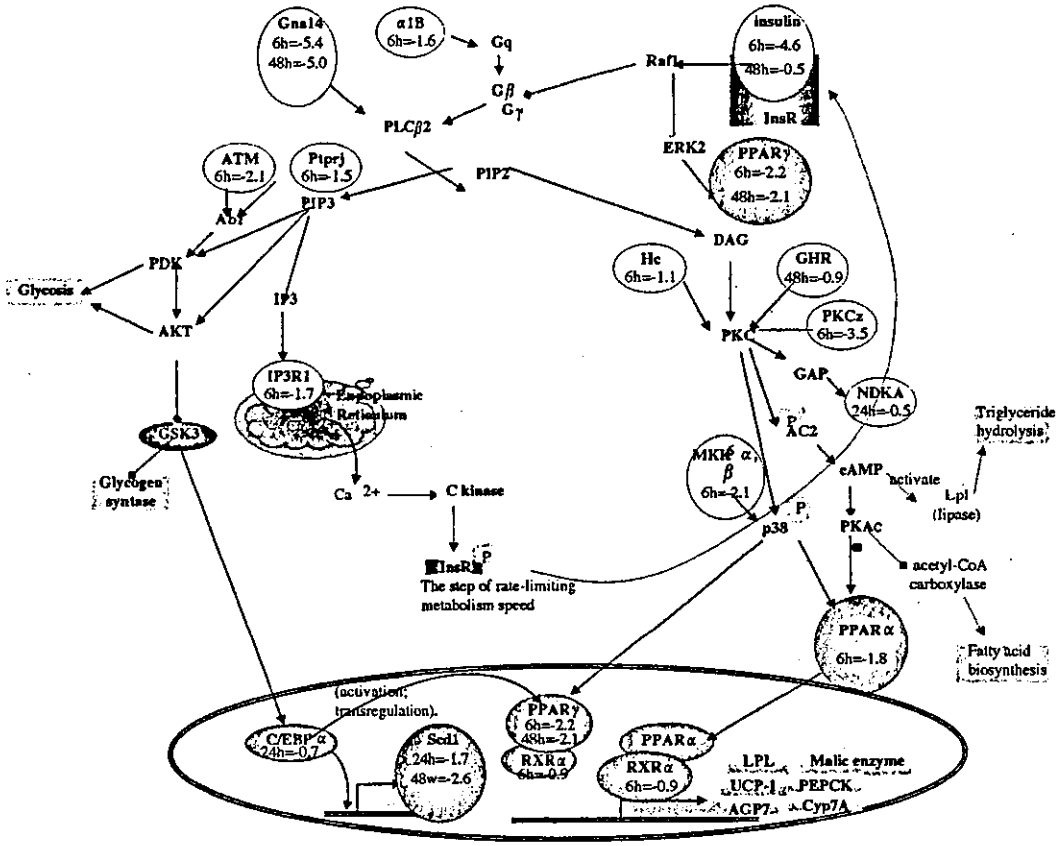

Fig. 5. Hypothetical signal transduction in the liver of the SZ-treated mice (figured by using TransPath program). Numeral represents the SLR of the signal of the probe sets of the SZ-treated mice/the control mice (3): PPAR related genes.

may not indicate that SZ controls these parameters directly, but may indicate that the energy for the lipid and glucose metabolism was not supplied due to the hepatocyte injury.

In conclusion, from the gene expression analysis of the liver of the SZ-treated mice, several changes suggesting the induction of apoptosis, inhibition of cell cycle and decreases in lipid metabolisms were observed. These changes started prior to the elevation of the serum glucose levels, indicating the direct action of SZ on the liver, rather than the secondary effect of diabetes. This may be related with the previously reported hepatic changes such as lipid peroxidation, mitochondrial swelling, and inhibition of hepatocyte proliferation observed before the development of hyperglycemia (Kume et al., 2004).

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Morphological and gene expression analysis in mouse primary cultured hepatocytes exposed to streptozotocin

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Abstract

Streptozotocin (SZ) is known to exert toxic effects not only on pancreatic islet beta cells but also on other organs including the liver. For analyzing direct effects of SZ on hepatocytes, we performed morphological analysis and DNA microarray analysis on mouse primary cultured hepatocytes. Hepatocytes were taken from non-treated Crj:CD-1(ICR) mice. The primary cultured hepatocytes were treated with SZ at concentrations of 0, 1, 3, 10, 30 and 100 mM. After the treatment for about 6 or 24 h, cell survival assay using tetrazolium salt (WST-1), light microscopic/electron microscopic analysis and gene expression analysis were performed. For the gene expression analysis, target (labeled cRNA) prepared from total RNA of the hepatocytes was hybridized to the GeneChip Murine Genome U74A V.2 (Affymetrix). The signal intensity calculation and scaling were performed using Microarray Suite Software Ver 5.0. IC50 of the cell survival assay was around 62 mM at 6 h exposure and 7 mM at 24 h exposure. Marked chromatin margination was observed in nuclei of the hepatocytes treated with SZ at concentrations of 3 or 10 mM. Gene expression analysis revealed similar expression changes to those of in vivo, i.e. up-regulation in cell proliferation/apoptosis related genes, and down-regulation of lipid metabolism related genes. These results potently supported the hypothesis that many of the hepatic alteration including histopathological and gene expression changes are induced by direct effect of SZ rather than by the secondary effect of the hyperglycemia or hypoinsulinemia.

Keywords: Streptozotocin; In vitro; Hepatocyte; Apoptosis; Gene expression; DNA microarray; Mouse

Introduction

Streptozotocin (SZ) has been attracting a great attention as a useful tool for the induction of diabetes mellitus and its complications in laboratory rodents (Sibay et al., 1971; Steffes and Mauer, 1984; Kume et al., 1992) because of its toxic action on islet β cells. However, SZ is known to exert toxic effects not only on pancreatic islet β cells but also on other organs including liver.

We have previously reported the details of SZ-induced hepatic lesions in the acute (6–48 h after the treatment) and the subacute (4–12 weeks after the treatment) phase (Kume et al., 1994a, b; Doi et al., 1997; Kume et al.,

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