Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 215–222
BBRC
www.elsevier.com/locate/ybbrc

Increased expression of PPARγ in high fat diet-induced liver steatosis in mice

Mitsutaka Inoue [a], Takaaki Ohtake [a], Wataru Motomura [a], Nobuhiko Takahashi [b], Yayoi Hosoki [a], Shigeki Miyoshi [a], Yasuaki Suzuki [a], Hiroyuki Saito [a], Yutaka Kohgo [a], Toshikatsu Okumura [b,*]

[a] *Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan*
[b] *Department of General Medicine, Asahikawa Medical College, Asahikawa, Japan*

Received 26 July 2005
Available online 19 August 2005

Abstract

The present study was performed to examine a hypothesis that peroxisome proliferator-activated receptor γ (PPARγ) is implicated in high fat diet-induced liver steatosis. Mice were fed with control or high fat diet containing approximately 10% or 80% cholesterol, respectively. Macroscopic and microscopic findings demonstrated that lipid accumulation in the liver was observed as early as 2 weeks after high fat diet and that high fat diet for 12 weeks developed a fatty liver phenotype, establishing a novel model of diet-induced liver steatosis. Gene profiling with microarray and real-time PCR studies demonstrated that among genes involved in lipid metabolism, adipogenesis-related genes, PPARγ and its targeted gene, CD36 mRNA expression was specifically up-regulated in the liver by high fat diet for 2 weeks. Immunohistochemical study revealed that PPARγ protein expression is increased in the nuclei of hepatocytes by high fat diet. It was also shown that protein expression of cAMP response element-binding protein (CREB), an upstream molecule of PPARγ, in the liver was drastically suppressed by high fat diet. All these results suggest for the first time that the CREB-PPARγ signaling pathway may be involved in the high fat diet-induced liver steatosis.

Keywords: CD36; CREB; High fat diet; Fatty liver; PPARγ

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcriptional factor that belongs to the nuclear hormone receptor superfamily and forms a heterodimer with a retinoid X receptor [1–3]. PPARγ is predominantly expressed in adipose tissues and to a lesser extent in many tissues including liver [4–6]. In the adipose tissue, PPARγ plays a key role in lipid metabolism. For instance, immortalized fibroblasts lacking PPARγ lose the potential for differentiation to mature adipocytes [7], indicating that PPARγ is required for differentiation of preadipocytes to mature adipocytes. In addition to the major role as a key factor in adipogenesis, increasing evidence has demonstrated that PPARγ is involved in a number of biological systems. These include glucose metabolism, inflammatory response, angiogenesis, and cancer cell proliferation [8–17]. Thus, PPARγ has multifunctional roles in cell behavior.

Several murine models of obesity and diabetes, including ob/ob, A-ZIP, aP2/DTA, and KKAy, develop fatty livers that express enhanced levels of PPARγ in the liver [18–22]. These results suggest that PPARγ might be implicated in the pathophysiology of fatty liver observed in the animals. With regard to the functional role of PPARγ expression in hepatic steatosis, a couple of reports have demonstrated that liver-specific disruption of PPARγ in leptin-deficient mice improves fatty liver [23] and steatosis in the mouse liver was induced by PPARγ overexpression [24], indicating that PPARγ expression plays a key role in the development of lipid accumulation in the hepatocytes.

* Corresponding author. Fax: +81 166 68 2844.
E-mail address: okumurat@asahikawa-med.ac.jp (T. Okumura).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.070

Fatty liver is a common disease in patients accompanied with obesity and diabetes, and one of the major causes to develop obesity and diabetes in human is high fat diet [25]. However, little is known whether high fat diet induces PPARγ expression in the liver that is going to accumulate lipid. In the present study, we tried to prove the hypothesis that high fat diet by itself increases PPARγ expression in the liver, followed by induction of liver steatosis.

Materials and methods

Animals studies. Nine-week-old male C57Bl/6Ncrj mice (Charles River Japan, Tokyo, Japan) were housed in 12 h light/dark cycle (light on 7 a.m.), temperature 22 °C, and allowed ad libitum access to diet and water. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat (F2HFD2, Oriental Yeast, Tokyo, Japan). Control mice were fed with normal diet that contained 13.2% of calories as fat (MF, Oriental Yeast Company, Tokyo, Japan). Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. The animals were fasted for 10 h before blood and tissue collection. After a drop of blood was obtained from the tail vein for glucose measurements, each mouse was anesthetized with diethyl ether and weighed. Blood was collected by cardiac puncture and subsequently assayed for biochemical parameters. Livers and epididymal fat pads were dissected, weighed, and frozen in liquid nitrogen. Samples of the resected liver were used for later analysis for histology, Western blotting, and PCR study. All experiments were carried out in accordance with rules and guidelines of the Animal Experiment Committee in Asahikawa Medical College.

Biochemical analyses. Blood glucose was measured by Glucocard Data (Arkray, Tokyo, Japan). Serum lipid such as total cholesterol, HDL, LDL or triglyceride, and alanine aminotransferase (ALT) was measured by Automatic Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan).

Histopathologic evaluation. Samples of the remaining liver tissue were fixed in 4% paraformaldehyde, embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E).

To detect fat deposition in the liver, frozen sections were rinsed with distilled water, stained with 0.18% Oil-Red O (Sigma–Aldrich, St. Louis, MO, USA) with 60% 2-propanol (Sigma–Aldrich, St. Louis, MO, USA) for 20 min at 37 °C, and then rinsed with distilled water.

Immunohistochemistry. Immunohistochemistry for PPARγ and cAMP response element-binding protein (CREB) was performed. Immunohistochemical staining was carried out by using the avidin–biotin complex method. Slides were deparaffinized. Sections were permeabilized, blocked in Block Ace (Dainippon Seiyaku, Osaka, Japan) for 1 h at room temperature, and then incubated in primary antibody overnight at 4 °C. The rabbit polyclonal antibody against PPARγ or CREB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:100 dilution in PBS-T. Each section was incubated with biotinylated guinea pig anti-rabbit IgG for 1 h and with avidin–biotin-alkaline phosphatase complex for 1 h. Finally, the sections were reacted with Alkaline Phosphatase Substrate Kit II (Vector Laboratories, Burlingame, CA, USA). Endogenous alkaline phosphatase activity was blocked by Levamisol solution (Vector Laboratories, Burlingame, CA, USA).

Microarray. Total RNA was isolated from the livers of mice fed with high fat diet or normal diet for 4 weeks. Reverse transcription, second-strand synthesis, and probe labeling were all performed using 10 μg of total liver RNA as a template for cDNA synthesis. Biotin-labeled cRNA was produced using the above cDNA as a template, purified, fragmented, and hybridized to Panorama Mouse Micro Array (Sigma–Aldrich Japan K.K. Genosys Division, Tokyo, Japan). After hybridization, bound cRNA was fluorescently labeled using R-phycoerythrin-streptavidin, and the fluorescence was intensified by the antibody amplification method. Signal intensity of the microarray was scanned by ScanArray Lite (PE Lifescience, Tokyo, Japan). The data were analyzed using DNASIS Array (HITACHI Software Engineering, Tokyo, Japan).

RNA isolation and first strand cDNA synthesis. Total hepatic RNA was isolated from small pieces of liver (80–100 μg) using QIAGEN RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse-transcribed using the RETROscript (Ambion, Austin, Texas, USA). From each mouse, 1 μg of total RNA was mixed with 2 μl of Random decamers and nuclease-free water in a total volume of 12 μl and heated at 80 °C for 3 min. The mixture was then chilled on ice and incubated with 2 μl of 10× RT buffer, 4 μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase, at 44 °C for 60 min. The reaction mixtures were further incubated for 10 min at 92 °C. The cDNA was stored at −30 °C until used for real-time PCR.

Primer of PPARγ and CD36. Mouse 36B4 was used as endogenous amplification control. The use of this universally expressed housekeeping gene allows for correction of variations in the efficiencies of RNA extraction and reverse transcription. The specific primer pairs (Table 1) were used according to the previous reports for PPARγ1, CD36 or 36B4 [26–28].

Real-time PCR. Real-time PCRs were performed in a LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green fluorescence. In this system, all reactions were run in glass capillaries with a total volume of 20 μl. The reaction mixture consisted of 2 μl of FastStart DNA Master SYBR Green I (FastStart *Taq* DNA polymerase, reaction buffer, deoxynucleoside triphosphate mixture [with dUTP instead of dTTP], SYBR Green I dye, and 10 mM $MgCl_2$) (Roche Diagnostics GmbH, Mannheim, Germany). Each primer was added to a final concentration of 0.5 μM, and $MgCl_2$ was then added to obtain a final concentration of 4 mM. In each experiment, 1 μg of extracted RNAs from liver tissues was reverse-transcribed. The PCR consisted of the following steps: (1) 50 °C for 2 min to prevent carry over of DNA; (2) 95 °C for 10 min to activate ampliTaq Gold polymerase and (3) 50 cycles each consisting of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s.

Western blotting analysis. The protein expression of CREB was studied in mice liver (50 μg) by Western blot analysis. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) following the manufacturer's suggested procedure. Fifty micrograms of protein was separated by 10% SDS–PAGE (PAG Mini Daiichi, Daiichi Pure Chemicals, Tokyo, Japan). After electrophoresis, the proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA), blocked overnight in Block Ace (Dainipponn Seiyaku, Osaka, Japan) at 4 °C, reacted with primary polyclonal antibody against human CREB (Cell Signaling Technology, Beverly, MA, USA) or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a control for 1 h, washed with TBS-T, reacted with secondary polyclonal antibody against rabbit IgG (CHEMICON International, Temecula, CA, USA) for 1 h, and washed with TBS-T. After reaction with horseradish peroxidase-conjugated anti-rabbit IgG, immune complexes were visualized by using the ECL plus detection reagents (Amersham International, NJ, USA) following the manufacturer's suggested procedure. Normal rabbit IgG was used simultaneously as a control.

Table 1
Specific primer pairs used in the present study

mRNA	Primers	
	Sense	Antisense
PPARγ1	CCACCAACTTCGGAATCAGCT	TTTGTGGATCCGGCAGTTAAGA
CD36	TCCTCTGACATTTGCAGGTCTATC	AAAGGCATTGGCTGGAAGAA
36B4	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

Statistical analysis. The results are expressed as means ± SEM. Statistical analysis was performed by repeated-measures ANOVA and subsequent Fisher's LSD test. A value of $p < 0.05$ was considered statistically significant.

Results

High fat diet-induced obesity and liver steatosis

Body weight, epididymal fat weight, and liver weight in both high fat and normal diet groups were measured for 2, 4, 6 or 12 weeks. As shown in Fig. 1A, high fat diet increased body weight, fat weight, and liver weight in a time-dependent manner. A significant increase in body weight by high fat diet was observed at 4 weeks. Epididymal fat and liver weight were significantly increased at 6 weeks. Macroscopic findings demonstrated that the liver in mice fed with high fat for 12 weeks was larger and seemed to be a fatty liver phenotype (Fig. 1B). These findings may indicate that high fat diet used in the present study develops obesity and fatty liver.

Fig. 2A shows H&E staining of the liver tissues. No obvious change was observed in the control liver throughout the period tested until 12 weeks. On the other hand,

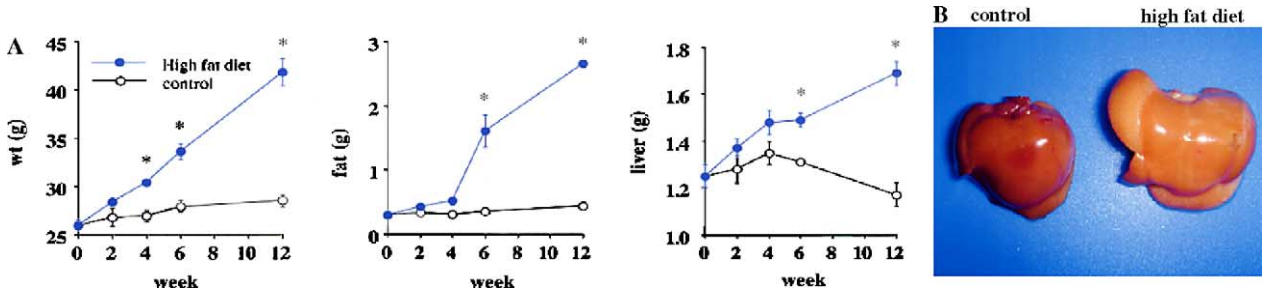

Fig. 1. (A) Body weight, fat weight, and liver weight in mice fed with control or high fat diet. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories. Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. Each data represents mean ± SEM of five animals. *$p < 0.01$, when compared with control. (B) Representative macroscopic observation of livers in mice fed with control or high fat diet for 12 weeks.

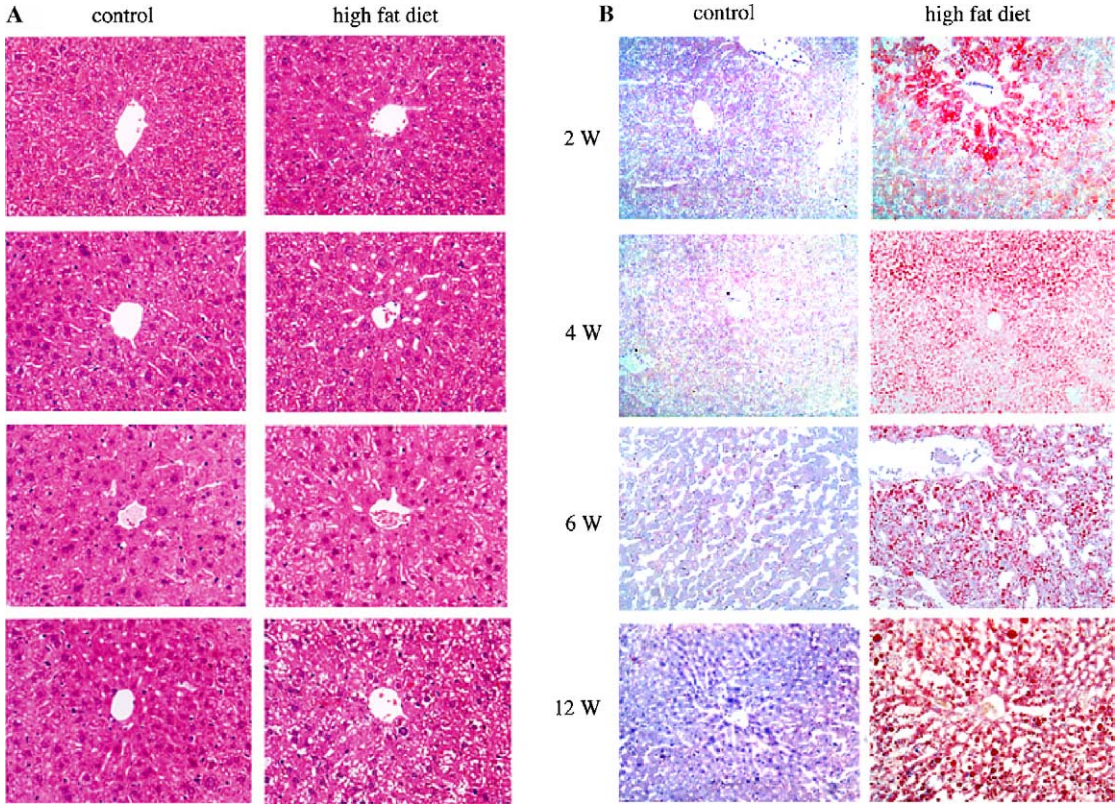

Fig. 2. Representative H&E (A) and Oil-Red O (B) staining of liver tissue in mice fed with control diet and high fat diet. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories. Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. Liver tissues were stained with H&E or Oil-Red O.

high fat diet-induced lipid deposition in hepatocytes especially around the central vein. A drastic liver steatosis was observed in the liver of a mouse fed with high fat diet for 12 weeks. To further clarify whether lipid accumulation was indeed induced in the liver, the liver tissues were stained with Oil-Red O (Fig. 2B). The Oil-Red O stain clearly revealed that triglyceride accumulation was evident in hepatocytes as early as 2 weeks after high fat diet. The proportion of lipid droplet in the liver increased in a time-dependent manner. These results illustrate that the high fat diet tested in the present study could make a model representing liver steatosis to examine sequential mechanisms of diet-induced lipid accumulation in the hepatocytes.

In this model, we analyzed biochemical and molecular changes in the process of steatosis in the liver. Biochemical analysis revealed that serum total cholesterol was significantly higher in mice fed with high fat diet even after 2 weeks. On the other hand, serum triglyceride was not increased in mice with high fat diet before 4 weeks. Fasting blood glucose was not different between the both groups at 4 weeks. Increased fasting blood glucose was observed after 12 weeks on high fat diet, indicating that diabetes developed by high fat diet for 12 weeks. Serum ALT in high fat diet was not different from that in control throughout the period (Table 2).

Genes profiling in the liver

To investigate what kind of genes in the liver were influenced by high fat diet, we performed global transcriptional profiling using RNA isolated from liver in mice fed with high fat diet for 4 weeks. Biotin-labeled RNA probes from either high fat diet-induced liver or normal diet-induced liver were hybridized to Affymetrix microarray chips containing approximately 22,000 genes. When a 2-fold change is used as the cut-off for either up- or down-regulation, we found that 639 genes were up-regulated and 1407 genes were down-regulated in the high fat diet group. Among all, we focused on genes involved in lipid metabolism such as fatty acid oxidation, fatty acid synthesis or adipogenesis. Table 3 shows the data in the liver sample obtained from mice fed with high fat diet for 4 weeks when compared with normal diet. No significant change of expression was observed in genes involved in fatty acid oxidation, PPARα, acyl-CoA synthase, CPT-1 or UCP-2, and fatty acid synthesis, SREBP-1, fatty acid synthase or GPAT. In contrast, adipogenesis-related genes such as PPARγ, CD36, and fatty acid binding protein were increased in the liver by high fat diet when a 2-fold change is used as cut-off for up-regulation.

Table 2
Biochemical characteristics

	2 weeks	4 weeks	6 weeks	12 weeks
T.Cho (mg/dL)				
Control	85 ± 3	87 ± 9	71 ± 10	71 ± 15
HFD	138 ± 6*	133 ± 11*	165 ± 20*	125 ± 17*
HDL-C (mg/dL)				
Control	65 ± 2	68 ± 8	52 ± 8	55 ± 12
HFD	105 ± 4*	98 ± 9*	126 ± 15*	98 ± 13*
LDL-C (mg/dL)				
Control	7 ± 1	6 ± 2	4 ± 1	4 ± 1
HFD	21 ± 2*	18 ± 4*	21 ± 4*	17 ± 6*
ALT (IU/L)				
Control	26 ± 6	43 ± 9	24 ± 3	21 ± 3
HFD	30 ± 5	33 ± 5	24 ± 2	24 ± 5
Glucose (mg/dL)				
Control	90 ± 5	93 ± 5	107 ± 15	133 ± 6
HFD	91 ± 6	96 ± 11	135 ± 31	262 ± 26*

Mice of high fat diet group were fed with high fat diet that contained 82% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories. Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. Each data represents mean ± SEM of five animals.

* $p < 0.01$, when compared with control.

Table 3
Lipid metabolism-related genes in the liver in mice fed with high fat diet

Gene	Fold induction
Fatty acid oxidation	
PPARα	1.46
Acyl-CoA synthase	1.56
CPT-1	1.68
UCP-2	0.89
Fatty acid synthesis	
SREBP-1	0.99
Fatty acid synthase	0.79
GPAT	0.77
Adipogenesis	
PPARγ	2.49
CD36	4.38
Fatty acid binding protein	2.12

Expression of PPARγ mRNA and protein in the liver

Based on the array data, we next examined the time-course changes of PPARγ and CD36 mRNA expression in the liver by real-time PCR. As shown in Fig. 3, both PPARγ1 and CD36 mRNA expression in the liver was up-regulated by high fat diet on as early as 2 weeks. It was also shown that the up-regulation of both the genes took place in a time-dependent manner.

Fig. 4A shows the representative liver sample in mice fed with high fat diet on 12 weeks stained with PPARγ antibody. In mice fed with normal diet, PPARγ-positive cells were not identified while immunoreactive PPARγ was clearly identified in the nuclei of hepatocytes in mice fed with high fat diet. PPARγ-positive cell number in a field was counted and the mean number of positive cells is shown in Fig. 4B. The PPARγ immunoreactive cell number was time-dependently increased in mice fed with high fat diet, while PPARγ-positive cell was not identified in the liver in mice fed with control diet, suggesting that PPARγ

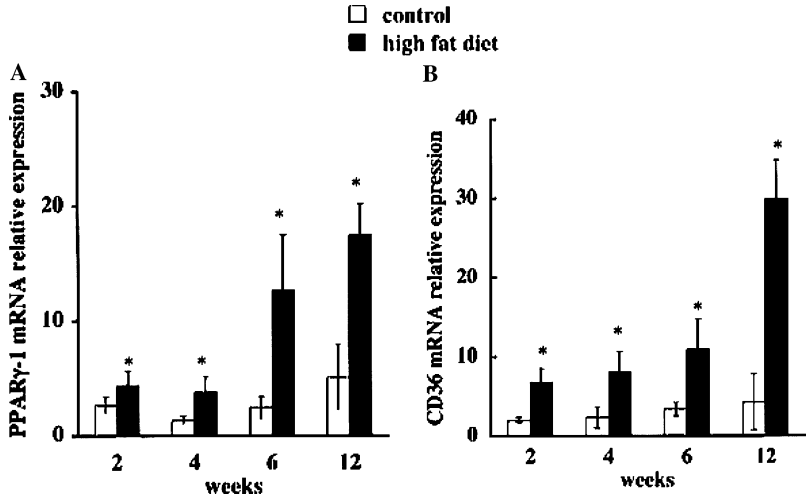

Fig. 3. PPARγ1 (A) and CD36 (B) mRNA expression in the liver by high fat diet. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories as fat. Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. Total hepatic RNA was isolated and real-time PCRs were performed. Each data represents mean ± SEM of five animals. $*p < 0.01$, when compared with control.

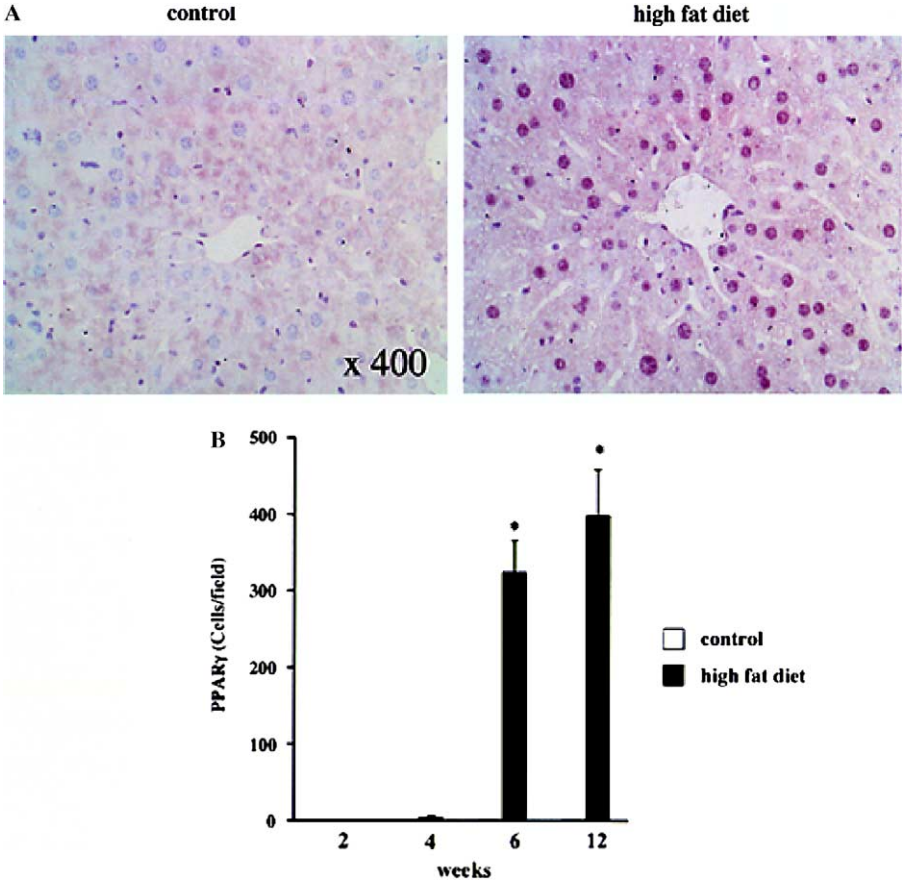

Fig. 4. (A) Representative liver tissue in mice fed with high fat diet on 6 weeks stained with PPARγ antibody. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories as fat. Mice in both high fat and normal fat diet were sacrificed after 12 weeks. Liver tissues in control or high fat diet were stained with PPARγ antibody. (B) Time course change of PPARγ immunoreactive cell number in the liver by high fat diet. Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, or 12 weeks. PPARγ-positive cell number was counted. Each data represents mean ± SEM of five animals. $*p < 0.01$, when compared with control.

protein expression in the nuclei of hepatocytes is indeed up-regulated by high fat diet, being in good agreement with up-regulation of PPARγ mRNA expression as described above.

Protein expression of CREB in the liver

We next tried to clarify the mechanism by which high fat diet induces PPARγ expression. Since CREB is known as an upstream molecule that regulates PPARγ expression [29], we made a hypothesis that CREB might mediate the high fat diet-induced up-regulation of PPARγ in the liver. To address the problem, the protein expression of CREB in the liver was examined by Western blotting. As illustrated in Fig. 5A, protein expression of CREB in the liver was drastically inhibited by high fat diet on 4 weeks. Immunohistochemical study also demonstrated that CREB immunoreactivity was weaker in the liver fed with high fat diet on 2 and 4 weeks in a time-dependent fashion (Fig. 5B), strongly supporting the results shown by Western blotting that CREB protein expression in the liver is downregulated by high fat diet.

Discussion

Recent studies have established a role for hepatic PPARγ in the development and maintenance of steatosis in the liver [18–22]. A liver specific knockout of PPARγ in ob/ob mice results in decreased lipid stores in the livers of these animals and reduced expression of several genes important to adipocyte differentiation and lipid metabolism [23]. In contrast, liver steatosis was induced by overexpression of PPARγ1 gene in mice [24]. In other words, up-regulation of PPARγ gene in the liver would result in steatosis while lipid accumulation would be prevented by down-regulation of PPARγ gene in the hepatocytes. Matsui et al. [30] have very recently demonstrated that triglyceride content of the liver of PPARγ (+/−) mice on high fat

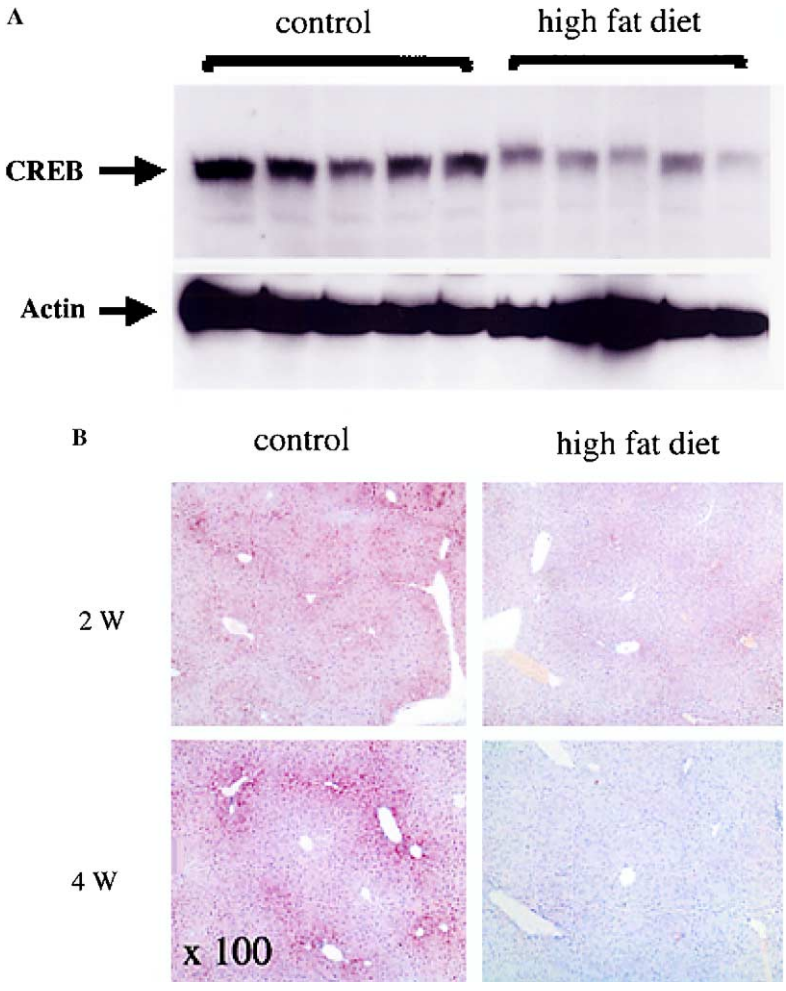

Fig. 5. (A) Western blot for CREB in the liver of mice fed with high fat diet for 4 weeks. Mice of high fat diet group were fed with high fat diet that contained 82.0% of calories as fat. Control mice were fed with normal diet that contained 13.2% of calories as fat. Mice in both high fat and normal fat diet were sacrificed after 4 weeks and the liver was resected. CREB protein expression was detected by Western blot. (B) Immunohistochemistry for CREB in the liver of mice fed with high fat diet. Mice in both high fat and normal fat diet were sacrificed after 2 or 4 weeks and the liver was resected. Each liver tissue was stained with CREB antibody.

diet was reduced by approximately 30% when compared with wild-type mice on the same high fat diet, supporting the concept that increased expression of PPARγ is required for accumulation of fat in the liver. Thus, PPARγ should be considered as a key molecule in the pathophysiology of liver steatosis.

High fat diet is known as a cause to develop fatty liver. There is however no evidence that PPARγ is indeed implicated in the process of liver steatosis induced by high fat diet. From a different point of view, we do not know if high fat diet influences PPARγ expression in the liver. To clarify the above speculation, in the present study, we first established an animal model that high fat diet induces lipid accumulation in the liver. Macrosopic findings indicated that high fat diet for 12 weeks developed obesity and a fatty liver phenotype. This evidence made us possible to analyze a serial process of formation of liver steatosis by high fat diet.

High fat diet on as early as 2 weeks, Oil-Red O stain clearly revealed that lipid droplets are identified in the hepatocytes especially around the central vein, strongly indicating that initial process leading to steatosis had started before 2 weeks. Within 2 weeks, high fat diet had not increased body weight and plasma glucose, suggesting that neither obesity nor diabetes is implicated in the high fat diet-induced steatosis at 2 weeks. So far, murine models of fatty liver have both marked obesity and diabetes [18–22]. Obesity and diabetes accompanied with fatty liver made it difficult to rule out the effects of obesity and diabetes on liver steatosis through adipocytokines and insulin-resistance-related biochemical signaling. The present evidence that liver steatosis was induced before development of obesity and diabetes raised a hypothesis that an initial step for the high fat diet-induced liver steatosis is independent of obesity and diabetes.

With regard to mechanisms by which high fat diet induces liver steatosis in mice, we analyzed gene profiling in the liver using DNA chips. Among genes known as lipid metabolism, adipogenesis related genes such as PPARγ and CD36 [31–33] were specifically up-regulated while genes that are involved in fatty acid oxidation and fatty acid synthesis failed to be regulated by high fat diet. These results suggest that adipogenesis rather than fatty acid oxidation and fatty acid synthesis may play a role in the high fat-induced liver steatosis. It was furthermore suggested that adipogenesis-related genes, PPARγ and its targeted gene, CD36, in the liver might be implicated in the high fat diet-induced liver steatosis.

According to the gene profiling data described above, we examined the sequential analysis of PPARγ and CD36 mRNA expression in the liver. High fat diet significantly increased the expression of PPARγ and CD36 within 2 weeks. The up-regulation of PPARγ in the liver was confirmed by immunohistochemical study as demonstrated in the present study. It was also confirmed that PPARγ protein expression is identified in the nuclei of hepatocytes. The increased expression of PPARγ in the hepatocytes may be functionally active as a transcriptional factor because gene expression of CD36, a target gene of PPARγ, was also up-regulated in the liver of mice fed with high fat diet as demonstrated in the present study. Yu et al. [24] have demonstrated that PPARγ1 overexpression in the mice liver by adenoviral gene delivery induced liver steatosis. In the study, they showed that PPARγ protein expression was detected in the nuclei of hepatocytes. These results indicate that increased expression of PPARγ in the nuclei of hepatocytes results in lipid accumulation in the liver. It is therefore suggested that up-regulation of PPARγ in the nuclei of hepatocytes may be a vital molecular mechanism to induce lipid accumulation in the liver by high fat diet.

We next tried to clarify a mechanism by which high fat diet increases PPARγ expression in the liver. CREB is a transcriptional factor that plays a role in gluconeonegesis [34]. Recently, Herzig et al. [29] have demonstrated that mice infected with a dominant-negative CREB-expressing adenovirus showed a fatty liver phenotype and a pronounced increase in hepatic triglyceride content. It was also demonstrated that PPARγ and CD36 expression in the liver was elevated in the dominant-negative CREB mice. Based on these results, they raised a hypothesis that CREB control hepatic lipid metabolism through PPARγ. We therefore examined a possibility that CREB may mediate the high fat diet-induced expression of PPARγ in the liver. As clearly demonstrated by Western blot and immunohistochemistry, CREB protein expression was down-regulated in the liver in mice fed with high fat diet. Since down-regulation of CREB in the liver had been already observed as early as 2 weeks after high fat diet, we would speculate that inhibition of CREB activity in the liver by high fat diet may play a role in the up-regulation of PPARγ in the hepatocytes which is capable to developing liver steatosis. All these results suggest that the CREB–PPARγ pathway should be listed as a target signaling pathway to induce liver steatosis by high fat diet.

In summary, the present study provides a novel mouse liver steatosis model by high fat diet and raised a possibility that high fat diet results in liver steatosis through up-regulation of PPARγ. It is also suggested that inhibition of CREB in the liver may be involved in this process.

Acknowledgments

This work was supported in part by grants provided by Ministry of Education, Science, Sports and Culture, Japan.

References

[1] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.

[2] T. Lemberger, B. Desvergne, W. Wahli, Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology, Annu. Rev. Cell. Dev. Biol. 12 (1996) 335–363.
[3] B.M. Spiegelman, J.S. Flier, Adipogenesis and obesity: rounding out the big picture, Cell 84 (1996) 377–389.
[4] P. Tontonoz, E. Hu, R.A. Graves, A.I. Budavari, B.M. Spiegelman, mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer, Genes Dev. 8 (1994) 1224–1234.
[5] Y. Zhu, C. Qi, J.R. Korenberg, X.N. Chen, D. Noya, M.S. Rao, J.K. Reddy, Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms, Proc. Natl. Acad. Sci. USA 92 (1995) 7921–7925.
[6] O. Braissant, F. Foufelle, C. Scotto, M. Dauca, W. Wahli, Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat, Endocrinology 137 (1996) 54–366.
[7] E.D. Rosen, C.H. Hsu, X. Wang, S. Sakai, M.W. Freeman, F.J. Gonzalez, B.M. Spiegelman, C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway, Genes Dev. 16 (2002) 22–26.
[8] J.J. Nolan, B. Ludvik, P. Beerdsen, M. Joyce, J. Olefsky, Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone, N. Engl. J. Med. 331 (1994) 1188–1193.
[9] M. Ricote, A.C. Li, T.M. Willson, C.J. Kelly, C.K. Glass, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation, Nature 391 (1998) 79–82.
[10] X. Xin, S. Yang, J. Kowalski, M.E. Gerritsen, Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo, J. Biol. Chem. 274 (1999) 9116–9121.
[11] A.C. Li, K.K. Brown, M.J. Silvestre, T.M. Willson, W. Palinski, C.K. Glass, Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice, J. Clin. Invest. 106 (2000) 523–531.
[12] P. Sarraf, E. Mueller, D. Jones, F. King, D.J. DeAngelo, J.B. Partridge, S.A. Holden, L.B. Chen, S. Singer, C. Fletcher, B.M. Spiegelman, Differentiation and reversal of malignant changes in colon cancer through PPARgamma, Nat. Med. 4 (1998) 1046–1052.
[13] J.A. Brockman, R.A. Gupta, R.N. DuBois, Activation of PPARγ leads to inhibition of anchorage-independent growth of human colorectal cancer cells, Gastroenterology 115 (1998) 1049–1055.
[14] P. Tontonoz, S. Singer, B.M. Forman, P. Sarraf, J.A. Fletcher, C.D. Fletcher, R.P. Brun, E. Mueller, S. Altiok, H. Oppenheim, R.M. Evans, B.M. Spiegelman, Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptorγ and the retinoid X receptor, Proc. Natl. Acad. Sci. USA 94 (1997) 237–241.
[15] N. Takahashi, T. Okumura, W. Motomura, Y. Fujimoto, I. Kawabata, Y. Kohgo, Activation of PPARγ inhibits cell growth and induces apoptosis in human gastric cancer cells, FEBS Lett. 455 (1999) 135–139.
[16] W. Motomura, T. Okumura, N. Takahashi, T. Obara, Y. Kohgo, Activation of peroxisome proliferator-activated receptor γ by troglitazone inhibits cell growth through the increase of $p27^{Kip1}$ in human pancreatic carcinoma cells, Cancer Res. 60 (2000) 5558–5564.
[17] M. Motomura, N. Takahashi, M. Nagamine, M. Sawamukai, S. Tanno, Y. Kohgo, T. Okumura, Growth arrest by troglitazone is mediated by $p27^{Kip1}$ accumulation which is resulted from dual inhibition of proteasome activity and Skp2 expression in human hepatocellular carcinoma cells, Int. J. Cancer 108 (2004) 41–46.
[18] R.A. Memon, L.H. Tecott, K. Nonogaki, A. Beigneux, A.H. Moser, C. Grunfeld, K.R. Feingold, Up-regulation of peroxisome proliferator-activated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice, Endocrinology 141 (2000) 4021–4031.
[19] R. Rahimian, E. Masih-Khan, M. Lo, C. van Breemen, B.M. McManus, G.P. Dube, Hepatic over-expression of peroxisome proliferator activated receptor gamma2 in the ob/ob mouse model of non-insulin dependent diabetes mellitus, Mol. Cell. Biochem. 224 (2001) 29–37.
[20] L. Chao, B. Marcus-Samuels, M.M. Mason, J. Moitra, C. Vinson, E. Arioglu, O. Gavrilova, M.L. Reitman, Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones, J. Clin. Invest. 106 (2000) 1221–1228.
[21] C.F. Burant, S. Sreenan, K. Hirano, T.A. Tai, J. Lohmiller, J. Lukens, N.O. Davidson, S. Ross, R.A. Graves, Troglitazone action is independent of adipose tissue, J. Clin. Invest. 100 (1997) 2900–2908.
[22] M. Bedoucha, E. Atzpodien, U.A. Boelsterli, Diabetic KKAy mice exhibit increased hepatic PPARgamma1 gene expression and develop hepatic steatosis upon chronic treatment with antidiabetic thiazolidinediones, J. Hepatol. 35 (2001) 17–23.
[23] K. Matsusue, M. Haluzik, G. Lambert, S.H. Yim, O. Gavrilova, J.M. Ward, B. Brewer, M.L. Reitman, F.J. Gonzalez, Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes, J. Clin. Invest. 111 (2003) 737–747.
[24] S. Yu, K. Matsusue, P. Kashireddy, W.Q. Cao, V. Yeldandi, A.V. Yeldandi, M.S. Rao, F.J. Gonzalez, J.K. Reddy, Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression, J. Biol. Chem. 278 (2003) 498–505.
[25] P. Angulo, Nonalcoholic fatty liver disease, N. Engl. J. Med. 346 (2002) 1221–1232.
[26] M. Bedoucha, E. Atzpodien, U.A. Boelsterli, Diabetic KKAy mice exhibit increased hepatic PPARgamma1 gene expression and develop hepatic steatosis upon chronic treatment with antidiabetic thiazolidinediones, J. Hepatol. 38 (2001) 17–23.
[27] N. Takahashi, Y. Qi, H.R. Patel, R.S. Ahima, A novel aminosterol reverses diabetes and fatty liver disease in obese mice, J. Hepatol. 41 (2004) 391–398.
[28] F.M. Gregoire, Q. Zhang, S.J. Smith, C. Tong, D. Ross, H. Lopez, D.B. West, Diet-induced obesity and hepatic gene expression alterations in C57BL/6J and ICAM-1-deficient mice, Am. J. Physiol. 282 (2002) E703–E713.
[29] S. Herzig, S. Hedrick, I. Morantte, S.H. Koo, F. Galimi, M. Montminy, CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma, Nature 426 (2003) 190–193.
[30] J. Matsui, Y. Terauchi, N. Kubota, I. Takamoto, K. Eto, T. Yamashita, K. Komeda, T. Yamauchi, J. Kamon, S. Kita, M. Noda, T. Kadowaki, Pioglitazone reduces islet triglyceride content and restores impaired glucose-stimulated insulin secretion in heterozygous peroxisome proliferator-activated receptor-gamma-deficient mice on a high-fat diet, Diabetes 53 (2004) 2844–2854.
[31] A.C. Nicholson, J. Han, M. Febbraio, R.L. Silversterin, D.P. Hajjar, Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis, Ann. N. Y. Acad. Sci. 947 (2001) 224–228.
[32] K. Motojima, P. Passilly, J.M. Peters, F.J. Gonzalez, N. Latruffe, Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner, J. Biol. Chem. 273 (1998) 16710–16714.
[33] R. Walczak, P. Tontonoz, PPARadigms and PPARadoxes: expanding roles for PPARgamma in the control of lipid metabolism, J. Lipid Res. 43 (2002) 177–186.
[34] S. Herzig, F. Long, U.S. Jhala, S. Hedrick, R. Quinn, A. Bauer, D. Rudolph, G. Schutz, C. Yoon, P. Puigserver, B. Spiegelman, M. Montminy, CREB regulates hepatic gluconeogenesis through the coactivator PGC-1, Nature 413 (2001) 179–183.