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Abstract

The present study was performed to examine a role of adipose differentiation-related protein (ADRP) in the process of liver steatosis. Immunohistochemical findings indicated that ADRP protein expression is increased in the hepatocytes in patients with fatty liver when compared with normal liver.   ADRP protein expression is localized in the surface of lipid droplets in the hepatocytes.   Increased expression of ADRP mRNA and protein was similarly observed in fatty liver in ob/ob mice and the liver steatosis induced by high fat diet in mice.   The up-regulation of ADRP mRNA and protein in the liver by high fat diet was identified in the surface of lipid droplets in a time dependent manner.   Recent studies demonstrated that up-regulation of PPARγ in the hepatocytes is deeply involved in liver steatosis.   To clarify whether ADRP expression is increased by PPARγ activation in hepatocytes, we examined the effect of a PPARγ ligand, troglitazone, on ADRP mRNA expression in HepG2 cells. ADRP mRNA expression was increased by troglitazone in dose and time dependent manners.   All these results suggest that ADRP is up-regulated

in liver steatosis in human and mice and that high fat diet increases

expression of ADRP through PPARγ activation, followed by induction of

liver steatosis.

Key words

fatty liver; ADRP, PPARgamma; troglitazone, high fat diet

Introduction

Lipid droplets are cytoplasmic organelles which serve as storage sites for neutral lipids. Adipose differentiation-related protein (ADRP) is intrinsically associated with the surface of lipid droplets and is believed to function in the intracellular mobilization and storage of neutral lipids [1-3]. ADRP abundance is directly proportional to the levels of intracellular lipid found within cells and ADRP is increased in specific diseases involving fat accumulation [1, 4, 5]. There is however little evidence whether ADRP expression is indeed increased in human fatty liver that must store lipid.

The molecular mechanisms, which serve to modulate transcription from the human ADRP gene, have not been described. However, several reports have demonstrated that certain agonists of peroxisome proliferator-activated receptors (PPARs) are capable of both modulating human ADRP gene transcription and stimulating lipid droplet formation in some cell types [6-9]. PPARs are a family of transcription factors involved in lipid homeostasis and they play a pivotal role in the regulation

of intracellular fatty acid oxidation and storage [10-13]   The individual

PPARs (PPARα, PPARδ and PPARγ) are activated by many long-chain

saturated and unsaturated fatty acids, or by eicosanoids [14]. Activated

PPARs form a heterodimeric DNA binding complex with retinoid X

receptor and subsequently bind to a specific recognition site, termed a

PPAR response element (PPRE) located in the promoter of target genes.

The PPRE is composed of a direct repeat of a consensus six-base

(AGGTCA) half site separated by one base, typically A or T [15].   It has

been very recently demonstrated that a PPRE regulates transcription of the

gene for human ADRP [16].   The study has identified a PPRE within the

human ADRP promoter and has demonstrated experimentally that the

human ADRP PPRE is a functional PPAR binding motif in human

hepatocytes.   It is therefore considered that ADRP gene expression is

regulated by PPARs in the human hepatocytes.

        We have very recently demonstrated that lipid accumulation in the

liver was observed under microscopy as early as 2 weeks after high fat diet

containing approximately 80 % cholesterol and that high fat diet for 12 weeks developed a fatty liver phenotype, establishing a novel model of diet-induced liver steatosis [17]. Using the model, we have found that PPARγ mRNA and protein expression was specifically up-regulated in the liver by high fat diet. It has been furthermore demonstrated that down- or up-regulation of PPARγ expression by itself prevents or develop liver steatosis in mouse models [18, 19]. These results suggest that the PPARγ signaling pathway may be involved in the high fat diet-induced liver steatosis. Since ADRP expression is transcriptionally regulated by PPARs as described above, we made a hypothesis that ADRP is involved in the liver steatosis through PPARγ activation. The present study was performed to clarify 1) whether ADRP is increased in human fatty liver and liver steatosis models and 2) the mechanism of up-regulation of ADRP expression in the liver steatosis.

Materials and Methods

Human liver samples

Liver samples obtained from eleven patients who admitted to

Aasahikawa Medical College Hospital for diagnosis and treatment of

hepatic disease were analyzed in the present study.   All eleven cases

received liver biopsy.   Samples of the liver tissue were fixed in 4%

paraformaldehyde, embedded in paraffin, cut, and stained with hematoxylin

and eosin (H&E).   Histopathologic evaluation was performed using H &

E staning samples.   All cases were diagnosed histologically to have fatty

liver (n=8) and normal liver (n=3). Remained liver samples were used for

immunohistochemistry for ADRP detection.   The study was conducted

under informed consent and approved by the ethical committee on human

research in Asahikawa Medical College.

Immunohistochemistry

Immunohistochemistry for ADRP was performed.   Slides were

deparaffinized. Sections were permeabilized, blocked in BlockAce

(Dainippon Seiyaku, Osaka, Japan) for 1hr at room temperature and then

incubated in primary antibody over night at 4 degree. The guinea-pig

polyclonal antibody against ADRP (Progen Biotechnique, Heidelbelg,

Germany) was used at a 1:100 diluted in PBS-T. Each section was

incubated with biotinylated goat anti guinea-pig IgG for 1hr and with

streptavidin Alexa 488 and TO-PRO-3 (Molecular Probes, Eugene, Oregon,

USA) for 1 hr. Immunodetection was carried out using confocal

microscopy.

Animal studies

Liver steatosis was made in mice according to our recent

publication [17]. Briefly, 9 weeks old male C57Bl/6Ncrj mice (Charles

River Japan, Tokyo, Japan) were housed in 12h light/dark cycle (light on 7

a.m.), temperature 22 degree, 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 company Ltd, Tokyo,

Japan). Control mice were fed with normal diet that contained 13.2 % of

calories as fat (MF, Oriental Yeast Company Ltd, Tokyo, Japan). Mice in

both high fat and normal fat diet were sacrificed after 2, 4, 6, 12 or 24

weeks. 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.

Cell culture

A human hepatocyte cell line, HepG2, was obtained from the

American Type Culture Collection (Rockville, MD, USA) and was cultured

in Dulbecco's modified Eagles medium (GIBCO, Grand Island, NY, USA)

supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 2.5 g/ml

amphotericine and 10% fetal bovine serum. Cells were incubated at 37

degree in a humidified atmosphere of 5% CO_2 in air.

Reagents and Treatments

Troglitazone, a PPARγ ligand, was kindly provided from Sankyo

Pharmaceutical Co. (Tokyo, Japan). Troglitazone was dissolved in dimethyl

sulfoxide (DMSO) with a final concentration of DMSO 0.05 % in the

culture medium.

RNA isolation and first strand cDNA synthesis.

Total hepatic RNA was isolated from cultured cells or 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 degree for 3 min. The

mixture was then chilled on ice and incubated with 2 μl of 10×   RT buffer,

4ul dNTP mix, 1 μl RNase inhibitor, and 1ul reverse transcriptase, at 44

degree for 60min. The reaction mixtures were further incubated for 10min at 92 degree. The cDNA was stored at –30 degree until used for real-time PCR.

Primer of ADRP

Human GAPDH and Mouse 36B4 were 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 are shown in Table 1,

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.

Western blotting analysis

        The protein expression of ADRP 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 degree, reacted with primary

polyclonal antibody against ADRP (Progen Biotechnique, Heidelbelg, Germany) or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a control for 1h, washed with TBS-T, reacted with secondary polyclonal antibody against guinea-pig IgG or rabbit IgG (CHEMICON International, Temecula, CA, USA) for 1 hr, and washed with TBS-T. After reaction with horseradish peroxidase-conjugated anti guinea-pig or rabbit IgG immune complexes were visualized by using the ECL plus detection reagents (Amersham International, NJ, USA) following the manufacturer's suggested procedure.

Statistical analysis

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

Results

Human

Immunohistochemical examination for detecting ADRP protein in human liver tissues was performed. Liver samples were obtained from paraffin-embedded tissues from patients with 3 normal and 8 fatty liver patients, respectively. Figure 1 shows the representative H & E staining of livers including fatty liver (Figure 1 d-f). As shown in Figure 2, ADRP protein was strongly expressed in liver samples obtained from three patients with fatty liver when compared with normal liver samples. ADRP protein was detected in the surface of lipid droplets in the hepatocytes.

Ob/Ob mice

Since strong steatosis is seen in the liver of ob/ob mice, we examined the expression of ADRP in this model. Figure 3A clearly demonstrates that ADRP protein is higher expressed around the central vein

when compared with control mice.   As shown in Figure 3B, ADRP protein

expression was localized in the surface of lipid droplet in the hepatocytes.

Real-time PCR revealed an increased expression of ADRP mRNA in the

liver of ob/ob mice (Figure 3C).

Mice fed with high fat diet

We next examined the expression of ADRP in the liver in a high

fat diet-induced liver steatosis model.   As demonstrated in Figure 4,

ADRP protein expression was increased in the liver by high fat diet.

Higher expression of ADRP in hepatocytes of mice fed with high fat diet is

seen as early as 2 weeks on the diet.   The increased expression o f ADRP

was time-dependent.   ADRP protein was detected in the surface of lipid

droplets in the hepatocytes as similarly as in human fatty liver sample

(Figure 5).   An increased expression of ADRP protein was also detected

by Western blotting for ADRP (Figure 6A).   ADRP mRNA in the liver

was up-regulated by high fat diet in a time-dependent manner (Figure 6B).

HepG2 cells

To clarify whether activation of PPARγ is involved in up-regulation of ADRP expression, the effect of PPARγ activation by troglitazone on ADRP mRNA expression was examined.   Troglitazone significantly up-regulated ADRP mRNA expression in a human hepatic cell line, HepG2.   The up-regulation of ADRP mRNA expression was dose- and time-dependent (Figure 7).

Discussion

Immunohistochamical studies indicated that increased levels of ADRP protein was identified in the liver tissue of fatty liver patients when compared with normal control.   So far, a couple of papers showed that ADRP is increased in specific diseases involving fat accumulation [1]. There is however no evidence that ADRP is indeed increased in the hepatocytes in fatty liver patients, indicating that the present study provides

the first evidence of increased expression of ADRP in hepatocytes in human liver steatosis. With regard to species other than human, it has been very recently demonstrated that ADRP mRNA expression was elevated in ob/ob mouse livers compared with wild-type mouse livers [20]. The present study clearly showed an increased expression of ADRP mRNA and protein in the steatotic liver of ob/ob mice that are obesity and diabetes. We have furthermore examined the effect of high fat diet that is capable of inducing liver steatosis on ADRP expression in the wild type mice liver to assess whether high fat diet by itself is involved in ADRP gene transcription. High fat diet increased ADRP mRNA and protein expression in the hepatocytes that is going to accumulate lipid in a time-dependent manner. A significant increase in ADRP expression was seen in the liver as early as 2 weeks on high fat diet. As shown in our previous study [17], lipid accumulation is significant within 2 weeks.

A number of factors are capable of contributing to liver steatosis [21]. These include overeating, obesity, hyperlipidemia, hyperglycemia

and insulin resistance. Multiple factors should be involved in fatty liver in human patients. To access whether high fat diet by itself is implicated in the upregulation of ADRP of hepatocytes, we examined the time-course change of ADRP expression in mice fed with high fat diet. Increased expression of ADRP protein was seen as early as 2 weeks after high fat diet. According to our recent publication [17], microscopic findings revealed that fat accumulation had started in mouse liver by the high fat diet within 2 weeks. Within 2 weeks on high fat diet, neither obesity nor hyperglycemia was observed, suggesting that these factors should be excluded as factors that contribute to liver steatosis and up-regulation of ADRP expression observed in mice fed with high fat diet for 2 weeks.

Targett-Adams et al. [16] have demonstrated that a PPAR response element (PPRE) with the sequence 5'-AGGTGA A AGGGCG-3' within promotor region of ADRP gene. Mutational analysis revealed that ADRP PPRE specifically mediated the up-regulation of transcription in response to activation by agonists of PPAR subtypes in both rat and human

hepatocye-derived cell lines. From these results, they suggested that PPARs control transcription of ADRP by means of a functional PPRE located within its promotor.

PPARγ is a member of PPARs and is predominantly expressed in adipose tissues and a lesser extent in many tissues including liver [22-27]. 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 [28], 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 have demonstrated that PPARγ is involved in a number of biological systems. These include glucose metabolism, inflammatory response, angiogenesis and cancer cell proliferation [29-37]. 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 [38-42].   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 demonstrated that liver-specific

disruption of PPARγ in leptin-deficient mice improves fatty liver [18] and

steatosis in the mouse liver was induced by PPARγ overexpression [19],

indicating that PPARγ expression plays a key role in the development of

lipid accumulation in the hepatocytes.   We have very recently established

that high fat diet induces liver steatosis in mice [17].   Briefly, 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.   With regard to the mechanism by which high fat

diet induces fatty liver, 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.

These results suggest that the PPARγ signaling pathway may be involved in

the high fat diet-induced liver steatosis.   Since PPARγ expression is

increased in the liver steatosis by high fat diet [17] and PPARγ control

transcription of ADRP by means of a functional PPRE located within its

promotor as described above, we would speculate that increased expression

of ADRP in liver steatosis observed in the present study is mediated by

up-regulation of PPARγ.   To clarify whether PPARγ is implicated in

increased expression of ADRP in hepatocytes, we next examined the effect

of PPARγ activation by its selective ligand on the expression of ADRP in a

human hepatocyte cell line.   We have previously demonstrated that a

human hepatocyte cell line, HepG2, expresses functional PPARγ [36].

Using the cell line, the present study clearly demonstrated that troglitazone

increased expression of ADRP mRNA in time- and dose-dependent manners.   It is therefore suggested that PPARγ activation in human hepatocytes up-regulates ADRP gene expression.

In conclusion, all these results suggest that high fat diet induces ADRP expression in hepatocytes probably through PPARγ, followed by induction of liver steatosis.   These evidence may help us develop a novel therapeutic strategy for human fatty liver disease.

Acknowledgements

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Figure legends

Figure 1

Representative H & E staining of liver tissue in patients with normal liver

(a-c) and fatty liver (d-f).

Figure 2

Representative ADRP immunostaining of liver tissue in normal liver (a-c)

and fatty liver (d-f).   Each g-i shows the high power view of d-f,

respectively.

Figure 3

Representative ADRP immunostaining of liver tissue in control and ob/ob

mice (A) and high power view of ADRP immunostatining in ob/ob mice

(B) were shown.   ADRP mRNA expression in the liver was evaluated by

real-time PCR, and ADRP mRNA relative expression was illustrated (C).

Each data represents mean $\pm$ SEM of 5 animals.   *, $p < 0.01$, when

compared with contro4

Figure 4

Mice were fed with high-fat diet that contained 82.0 % of calories as fat or normal diet that contained 13.2 % of calories.   Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6, 12 or 24 weeks.   Liver tissues were stained with ADRP antibody and representative samples were shown.

Figure 5

Mice in high fat and diet were sacrificed after 24 weeks.   Representative ADRP immuostaining was shown.

Figure 6

(A) Western blot for ADRP in the liver of mice fed with high fat diet for xx 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 xx weeks and the liver was resected.
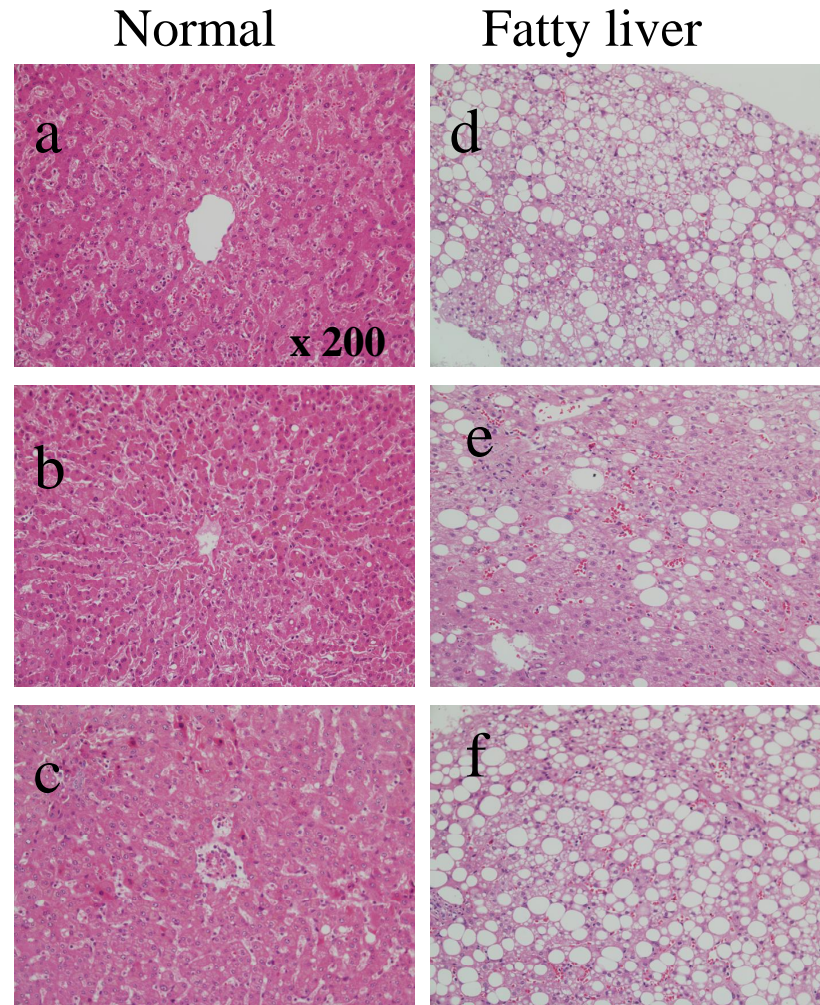
ADRP protein expression was detected by Western blot. Lower panel

shows the relative intensity of ADRP protein expression evaluated by

densitometry.

(B) ADRP mRNA expression in the liver of mice fed with high fat diet.

Mice in both high fat and normal fat diet were sacrificed after 2, 4, 6 or 12

weeks and the liver was resected.   ADRP mRNA expression was detected

by real-time PCR.

Figure 7

Dose-response (A) and time-course (B) effect of troglitazone on ADRP

mRNA expression in HepG2 cells.   HepG2 cells were treated with several

doses of troglitazone and ADRP mRNA expression was detected by

real-time PCR.

Table1   Primers used

----

human ADRP   CTCATGGGTAGAGTGGAAAAGGAGCATTGG   (sense)

                  TTGGATGTTGGACAGGAGGGTGTGGCACGT   (antisense)

human GAPDH   TCATCTCTGCCCCCTCTGCT   (sense)

                  CGACGCCTGCTTCACCACCT   (antisense)

mouse ADRP   AAGAGGCCAAACAAAAGAGCCAGGAGACCA   (sense)

                  ACCCTGAATTTTCTGGTTGGCACTGTGCAT   (antisense)

mouse   36B4   CGACCTGGAAGTCCAACTAC   (sense)

                  ATCTGCTGCATCTGCTTG (antisence)

Figure 1

Normal x200   Fatty liver x200   x 800

Figure 2

Figure 3

A

control   *ob/ob*

x200

B   *ob/ob*

x 1200

C

Figure 4

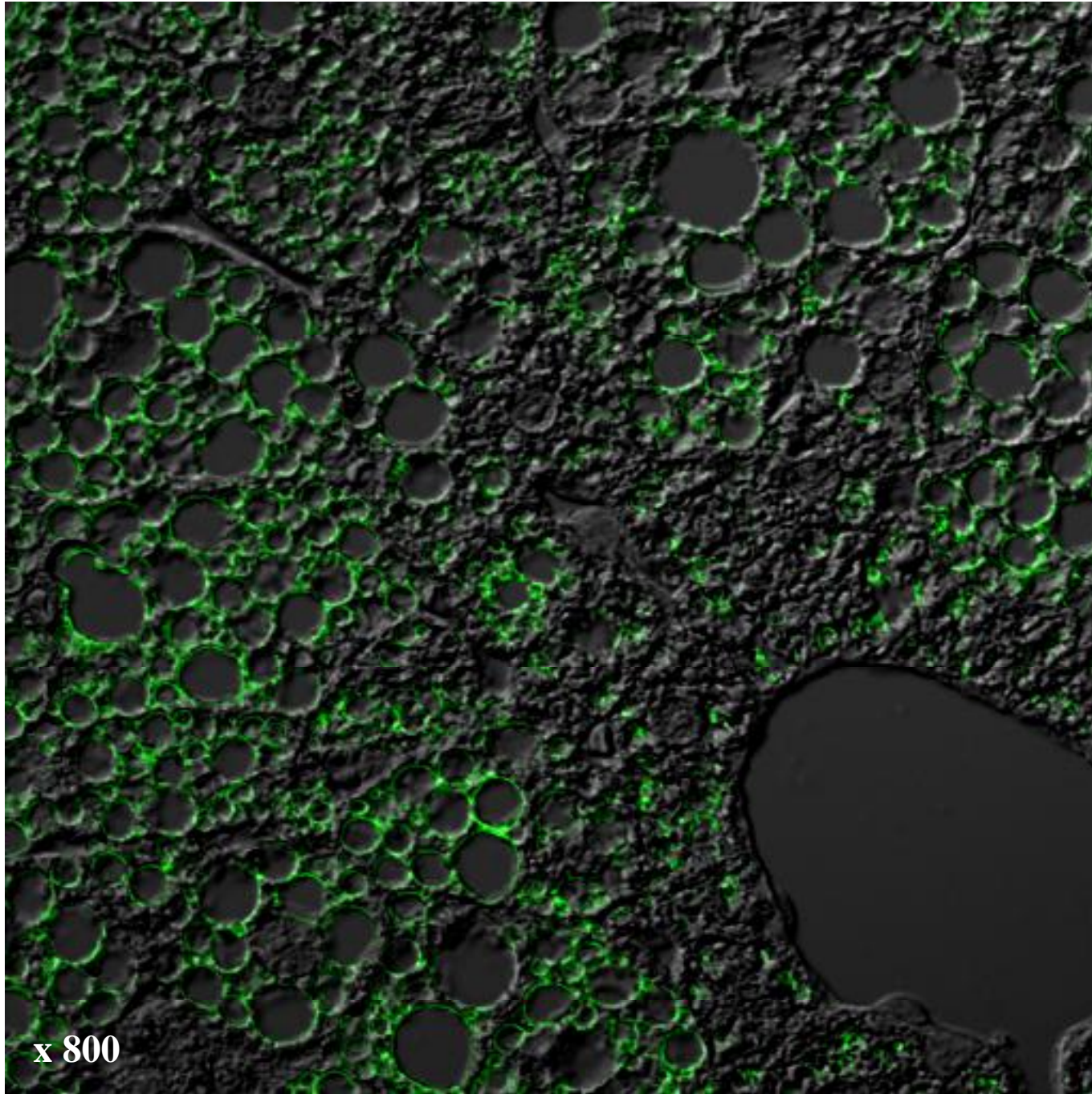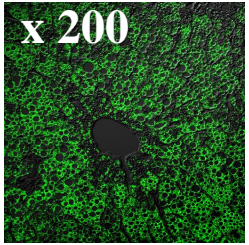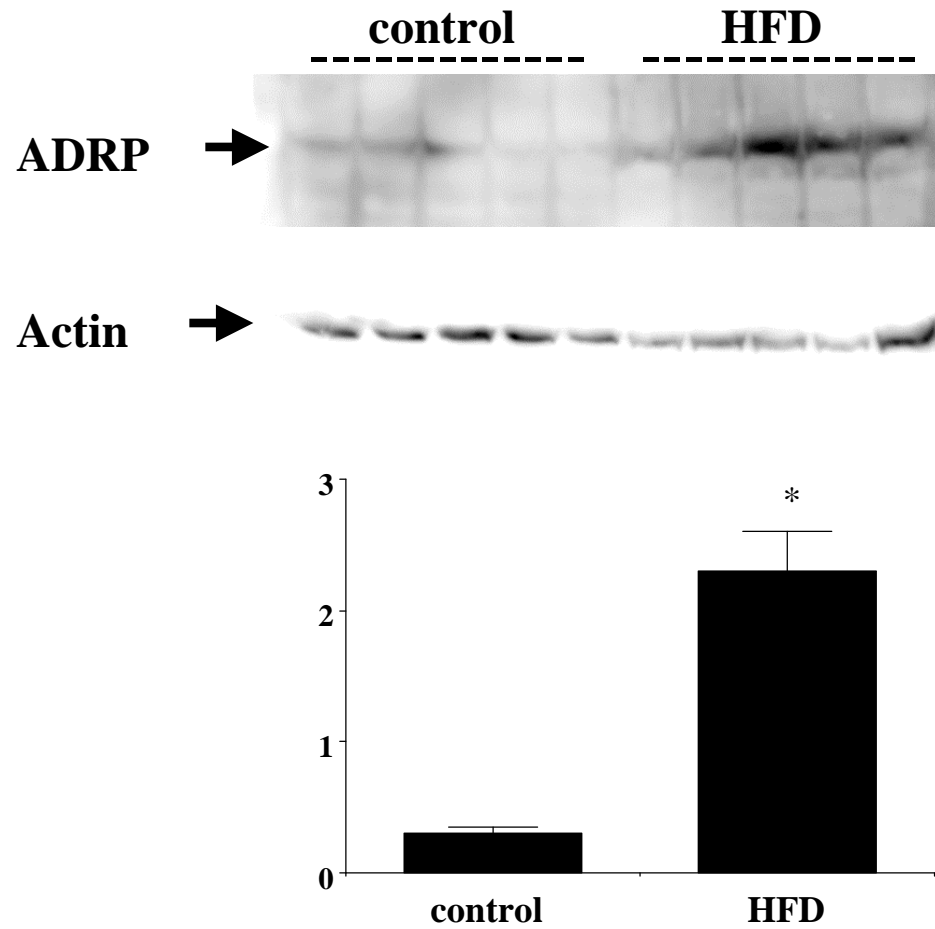|	2 w	4 w	6 w	12 w	24 w
control					
HFD					

x 200

x 200

x 800
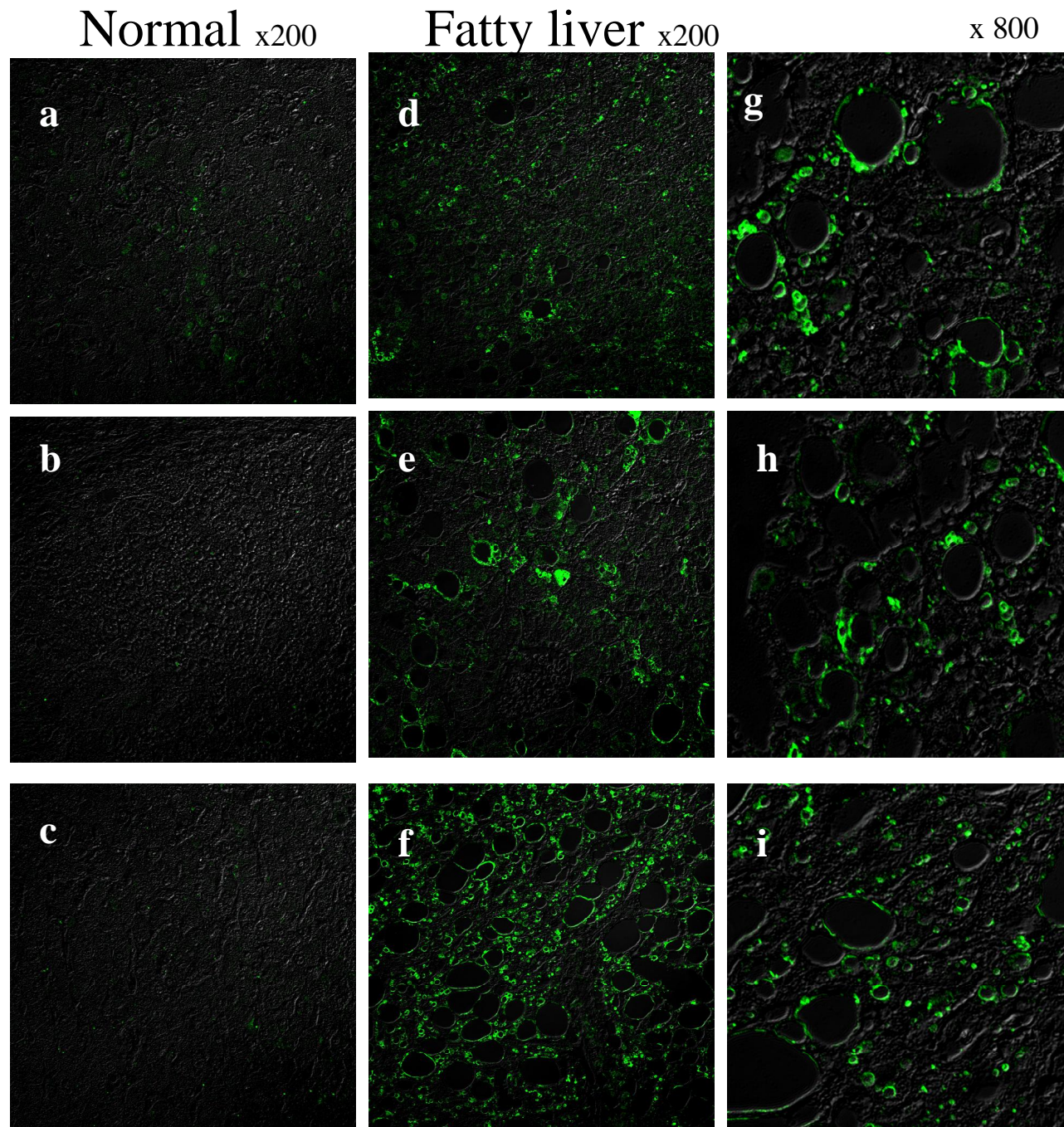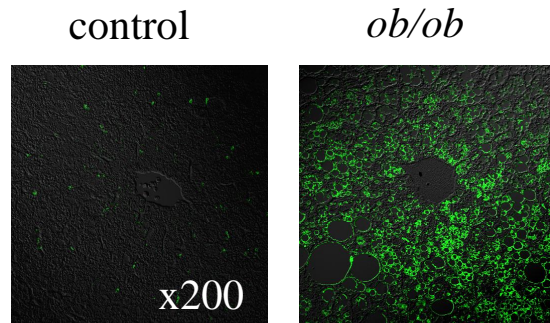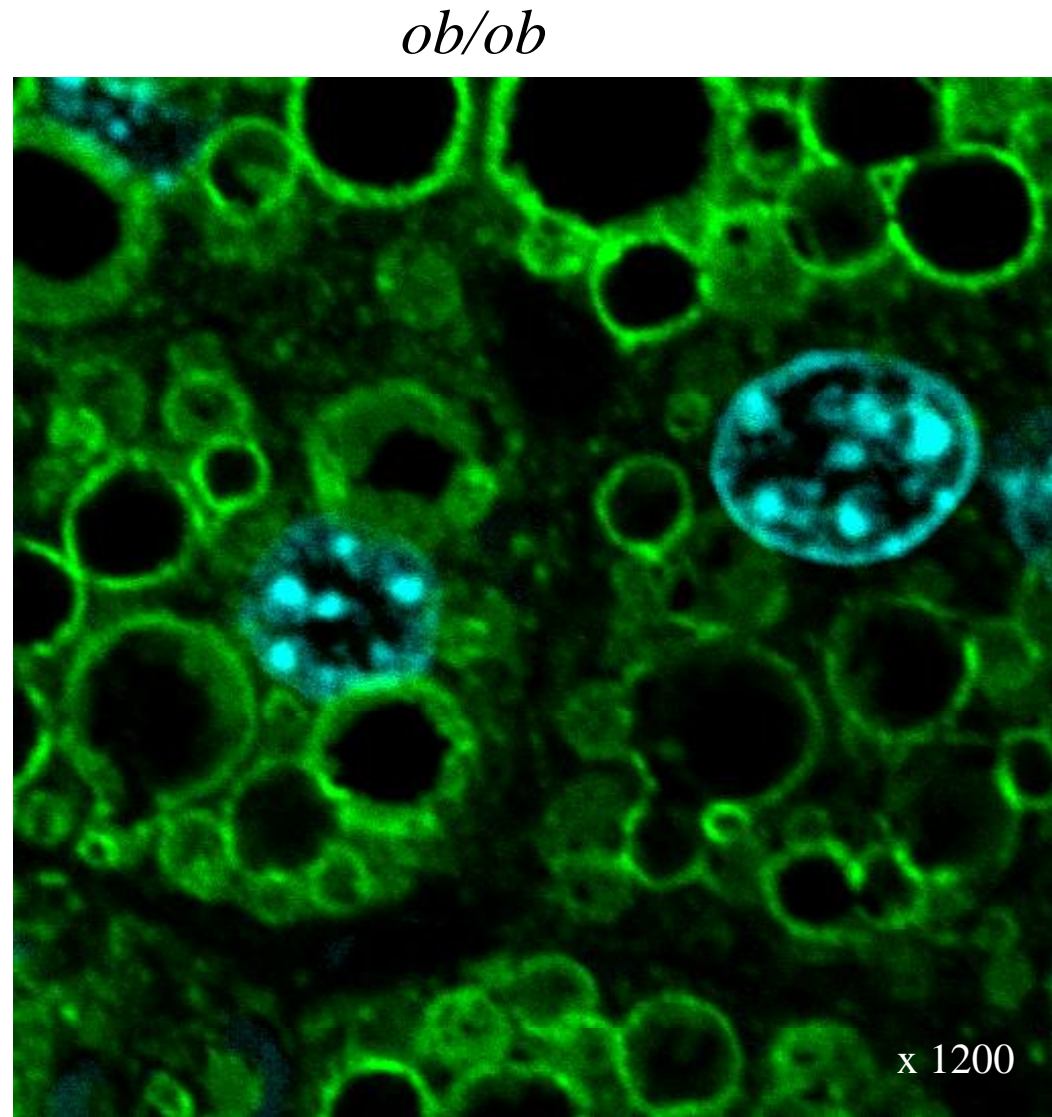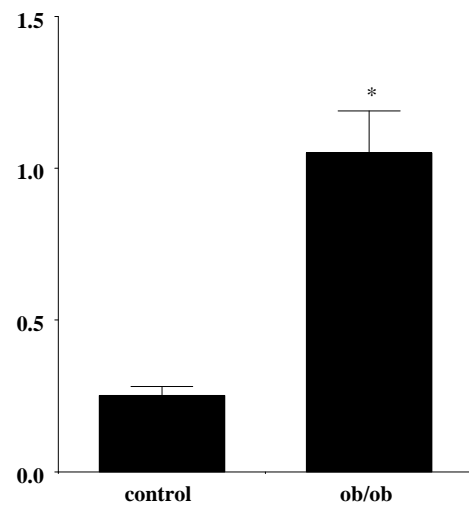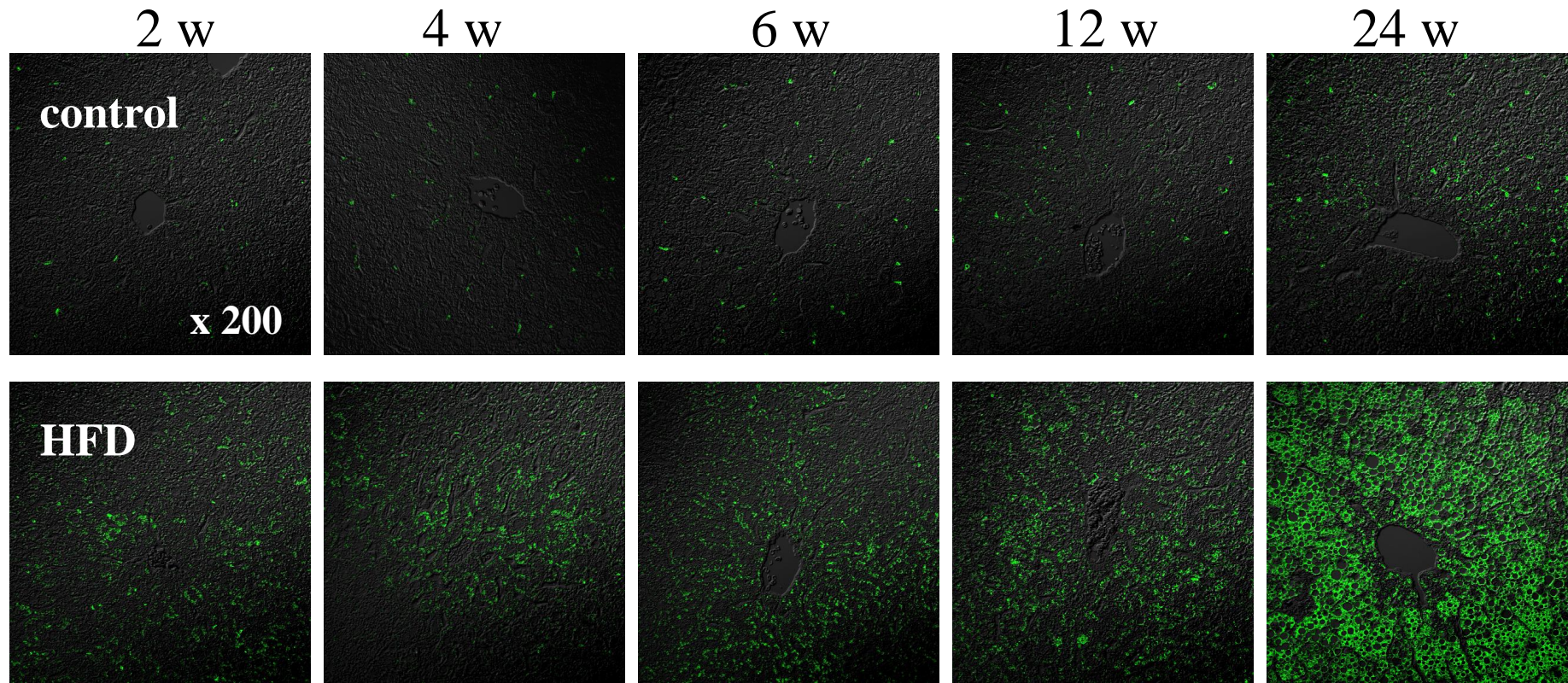
Figure 5

Figure 6

A

B

Figure 7