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# GH-independent IGF-I action is essential to prevent the development of nonalcoholic steatohepatitis in a GH-deficient rat model

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ABSTRACT

The progression to nonalcoholic steatohepatitis (NASH) from simple steatosis is associated with the mitochondrial dysfunction, enhanced oxidative stress, and inflammation. Recently, it has been reported that the prevalence of NAFLD (nonalcoholic fatty liver disease)/NASH is increased in patients with adult growth hormone deficiency (AGHD), suggesting that the deficiencies in GH and insulin-like growth factor (IGF-I) are involved in the development of NAFLD/NASH; however, the precise underlying mechanism remains to be elucidated. To clarify the mechanisms and the specific contribution of GH and IGF-I in these conditions, we examined the liver of a GH-deficient rat model, spontaneous dwarf rat (SDR) and the effect of GH and IGF-I administration. SDR showed steatosis and fibrosis in the liver in line with the phenotype observed in AGHD. Serum AST and ALT levels and triglyceride content in the liver were significantly increased in the SDR compared with the control. Intriguingly, the mitochondrial morphology in the SDR hepatocyte was impaired and the area was significantly decreased. Furthermore, oxidative stress in the SDR liver was enhanced. These changes were improved not only by GH but also by IGF-I administration, suggesting that GH-independent IGF-I action plays an essential role in the liver. In conclusion, we demonstrated that GH-deficient rat exhibits NASH and IGF-I plays an essential role to prevent the development of NASH. The improved mitochondrial function and reduced oxidative stress may contribute the effect of IGF-I in the liver.



## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. NAFLD refers to the accumulation of hepatic steatosis not due to excess alcohol consumption [1]. NAFLD includes both nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH). Histologically, NAFLD occurs as a spectrum from mild hepatic steatosis only, to NASH characterized by hepatocellular injury, inflammation, and fibrosis to cirrhosis. NASH is a serious disease because it is intractable and progressive. The progression to NASH is associated with multiple factors, but it generally consists of two steps; first, the insulin resistance in conjunction with an accumulation of fat in visceral adipose tissue and within hepatocytes; and second, the increased oxidative stress, mitochondrial dysfunction, and inflammation [2]. Recently, it has been reported that various hormones including growth hormone (GH) are involved in the development of NASH [3].

In patients with hypothalamic and pituitary dysfunction display a development of NAFLD [4]. Several reports have suggested that such hepatic impairment may be particularly related to growth hormone deficiency (GHD). Adult GHD (AGHD) is characterized by a decreased serum IGF-I level, increased visceral adiposity, and abnormal lipid profile [5]. Ichikawa et al. showed that hepatic steatosis was more frequently observed in hypopituitary patients with GHD than in patients without GHD [6]. In another case of AGHD accompanied by NASH and hyperlipidemia, GH replacement therapy drastically reversed NASH [7]. Moreover, it has been reported that the prevalence of NAFLD is 6.4-fold higher in patients with AGHD than in age-, sex-, and BMI-matched control subjects. In addition, GH replacement therapy improved these hepatic abnormalities in the patients [8]. These data strongly suggest that the deficiencies in GH and IGF-I are involved in the development of NAFLD/NASH [3]; however, the precise underlying mechanism remains to be elucidated.

With regard to the mechanistic action of GH, it has been shown that specific deletion of GH receptor (GHR) in hepatocytes leads to steatosis with an impaired lipid metabolism [9]. Furthermore, liver-specific deletion of JAK2 [10] or STAT5 [11,12] in mice results in steatosis, suggesting the importance of downstream signaling of

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GHR in the lipid metabolism of the hepatocytes. On the other hand, in an animal model of liver cirrhosis, exogenous IGF-I expression in the liver reduced fibrosis and ultimately lead to amendment of cirrhosis, suggesting an anti-fibrotic action of IGF-I [13]. GH has a pronounced lipolytic effect, particularly in the visceral fat [14]. Because visceral adiposity is closely related with development of NAFLD, it is plausible that GH exerts its effect by reducing the visceral fat as an indirect mechanism *in vivo*. These findings suggest that direct, indirect, and plural actions of GH and IGF-I are closely inter-reacted and contribute to occurrence of NAFLD. However, the relative contributions of GH and IGF-I in the liver remain controversial. To elucidate the specific role of GH and IGF-I in the liver, we analyzed a GH-deficient animal model, spontaneous dwarf rat (SDR) and investigated the effect of GH and IGF-I administration on the liver.

## 2. Methods

### 2.1. Animal study

Rat experiments were performed according to the guidelines of the animal ethics committee of Kobe University Graduate School of Medicine. GH-deficient rat, spontaneous dwarf rat (SDR) was purchased from Japan SLC (Hamamatsu, Japan), and the age-matched originate strain Sprague–Dawley (SD) rats were used as control. To examine the phenotype in liver of SDR and SD rat, five males for each group (16-week-old) were used. Rats were housed under controlled environmental conditions (12-h light, 12-h dark), with normal chow and water *ad libitum*. For the continuous 4 weeks administration of GH and IGF-I, control rat and SDR were anesthetized and osmotic minipumps (model 2004, Alzet Co., Palo Alto, CA) were implanted subcutaneously, containing rat GH (20 μg/μl; NIDDK National Hormone and Pituitary Program) or recombinant human IGF-I (10 μg/μl; Astellas Pharma Inc. Tokyo, Japan) or vehicle (saline) as previously described [15]. The dose of GH and IGF-I was determined to restore GH and IGF-I levels in the physiological range [15]. The effect of GH and IGF-I administration was verified by the increase in the body weight and serum rat and human IGF-I concentration.

### 2.2. Histological analysis

After 4 weeks of administration, at the age of 20 weeks, liver specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-μm-thick sections, and evaluated by H&E and Masson-trichrome staining. For the evaluation of fibrotic area, at least three whole sections form each animal were scanned and the images were quantified using NIH image J software as previously described [16].

### 2.3. Biochemical analysis

Serum human IGF-I and rat IGF-I were determined using IGF-I ELISA kit (R&D systems, Inc., Minneapolis, MN, and Diagnostic Systems Laboratories, Inc., Webster, TX). The serum ALT and AST levels were measured by ALT and AST kits (Stanbio, Boerne, TX). Tissue triglyceride content was measured by triglyceride E-test after extraction from the liver (Wako, Osaka, Japan). Tissue TBARS content was determined by the TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI).

### 2.4. Quantitative real-time PCR

Quantitative real-time PCR (QRPCR) was performed as previously described [17], on a LightCycler system using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Switzerland). Each value was normalized to β-actin expression. The primer sequences are as follows; glycerol-3-phosphate acyltransferase sense, AACCTGGCGGAGCACATTC, antisense, GAGCAGGCAGGCCAC AAT; glyceraldehyde-3-phosphate dehydrogenase sense, AAGTATAAGTAAGGGCTGGCCTAAATG, antisense, AGGCTGGCACTGCACA AGA; carnitine palmitoyltransferase I sense, TGGGCATGATCGCAA AGA, antisense, GAGGACGCCGCTCACAAT.

### 2.5. Immunohistochemistry and electron microscopy

Immunohistochemistry was performed as previously described [7]. After antigen retrieval with citric acid buffer, the sections were incubated with monoclonal anti-8-hydroxy-2-deoxyguanosine/8-hydroxyguanosine (8OHdG/8OHG; 1:100; JaICA, Japan) and anti-nitrotyrosine monoclonal antibody (MAB5404; 1:10; Chemicon), visualized by using standard immunohistochemical methods, and counterstained by hematoxylin. Electron microscopy was performed as previously described [18]. The relative mitochondrial area was quantified using image J software. At least five cells for each animal were analyzed for the quantification.

### 2.6. Statistical analysis

Results were expressed as mean ± SE unless otherwise specified. Statistical analysis was performed using JMP Statistical Database Software (SAS Institute, Inc., Cary, NC). Two-way ANOVA followed by Tukey's post hoc test was used. Statistical significance was determined as $P < 0.05$.

## 3. Results

### 3.1. A GH-deficient rat model exhibited steatosis, heptocyte injury, and fibrosis in the liver and GH and IGF-I administration restored these changes

To elucidate the effect of GH-deficient status in the liver, we evaluated GH-deficient rat model. We used spontaneous dwarf rat (SDR) as a GH-deficient animal model, whose serum GH is completely absent because of the splice site mutation in the GH-1 gene [19]. Interestingly, SDR at the age of 20-week-old exhibited steatosis, hepatocyte injury, and fibrosis in the liver (Fig. 1A). The serum ALT and AST levels were elevated compared with those in the control (Fig. 1B). The triglyceride content in the liver was also significantly increased (Fig. 1C) as comparable with the results of oil red O staining (Fig. 1A). The quantitative analysis of fibrotic area showed that fibrosis in the liver of SDR was significantly increased compared with that in the control (Fig. 1D). To clarify the contribution of GH and IGF-I in these conditions, we examined the effect of GH or IGF-I administration for 4 weeks. The administration of GH restored serum IGF-I to the comparable levels in the control (Fig. 1E). The administration of human IGF-I also restored to the comparable levels of serum human IGF-I with that of rat IGF-I levels in the control (data not shown). Surprisingly, not only GH but also IGF-I reversed these abnormalities in the liver observed in SDR, particularly, the effect on the fibrosis was predominant when IGF-I was administered than that in GH administration (Fig. 1D).

### 3.2. Lipid metabolism and mitochondrial function in GH-deficient rat

To dissect the molecular mechanisms of the effect of GH and IGF-I, we analyzed the expression of key genes for triglyceride synthesis and mitochondrial function. The expressions of glycerol-3-phosphate acyltransferase and glyceraldehyde-3-phosphate dehydrogenase were significantly increased in SDR and GH or IGF-I administration decreased these changes. Intriguingly, the expression of

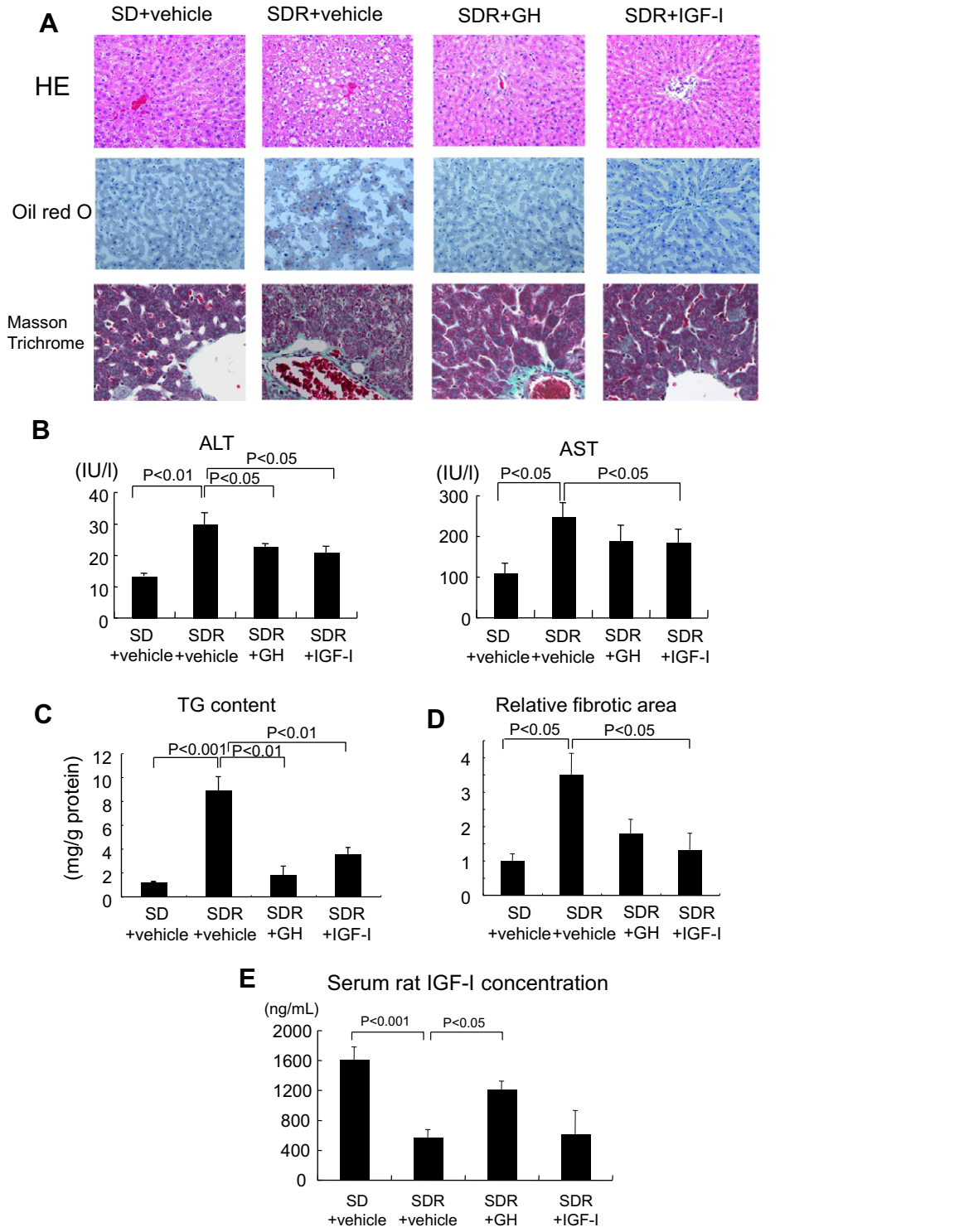


**Fig. 1.** (A) Histological analysis of the liver in control (SD) and GH-deficient rat (SDR). HE staining revealed steatosis, hepatic cell injury (ballooning necrosis), and disrupted lobular structure in the liver in SDR. Oil red O staining showed lipid deposit in hepatocytes. Masson-trichrome staining showed perivascular and pericellular fibrosis in the SDR liver. Four-week administration of GH or IGF-I restored these changes in SDR. (B) Serum AST and ALT levels were significantly elevated in SDR compared with the control and GH or IGF-I administration decreased these levels. (C) In SDR, tissue triglyceride content in the liver was significantly increased and GH or IGF-I administration reversed these changes. (D) In SDR, the fibrotic area assessed by Masson-trichrome staining in the liver was significantly increased compared with the control and IGF-I administration improved the fibrotic area. (E) Serum rat IGF-I concentrations. GH administration partially restored serum rat IGF-I levels in SDR to that in the control rat. Results were expressed as mean ± SE. Statistical significance was determined as $P < 0.05$.

the mitochondrial rate-limiting enzyme for β-oxidation, carnitine palmitoyltransferase I (CPT-1) was significantly decreased in SDR and both GH and IGF-I administration partially restored these changes (Fig. 2).

### 3.3. Mitochondrial morphology in GH-deficient rat

The decrease in the expression of CPT-1 suggested an impaired β-oxidation concomitant with the mitochondrial dysfunction,

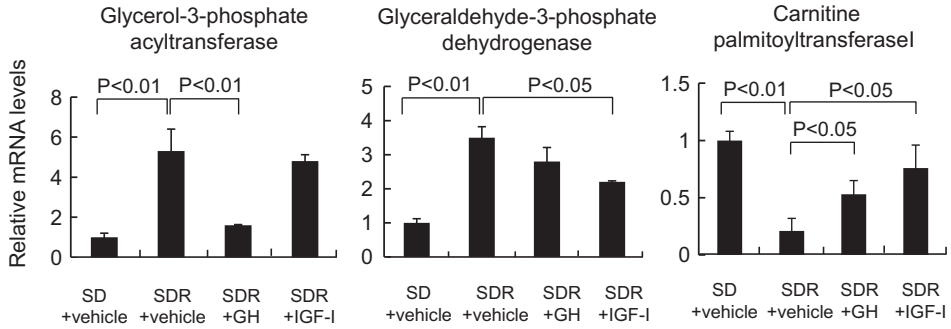


**Fig. 2.** Quantitative analysis of expression of glycerol-3-phosphate acyltransferase, glyceraldehyde-3-phosphate dehydrogenase, and carnitine palmitoyltralsferase-I by quantitative real-time PCR. Glycerol-3-phosphate acyltransferase and glyceraldehyde-3-phosphate dehydrogenase are important enzymes for triglyceride synthesis and carnitine palmitoyltransferase-I is a rate-limiting enzyme of mitochondrial β-oxidation. Results were expressed as mean ± SE. Statistical significance was determined as $P < 0.05$.

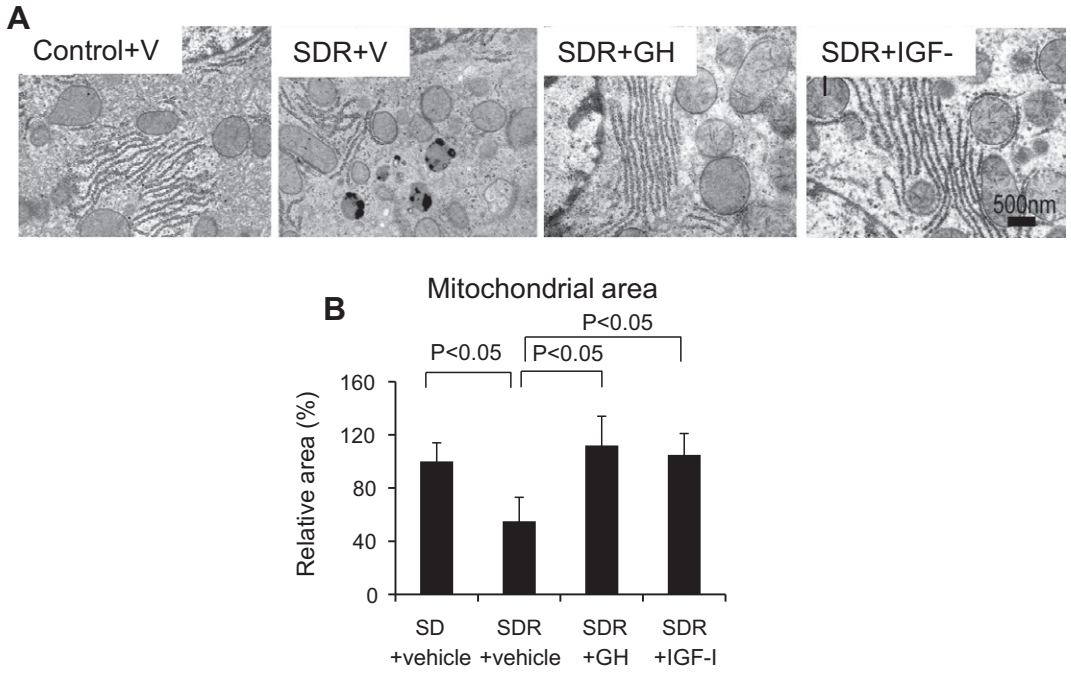


**Fig. 3.** (A) Ultrastructure assessed by electron microscopy. In the SDR, the size of the mitochondria was smaller, the shape was irregular, and the ultrastructure of clistae was disorganized as compared with the control. There were abnormal electron depots in a part of mitochondria in the hepatocyte of SDR. (B) The quantitative analysis of mitochondrial area revealed that the area was significantly decreased in SDR as compared with control and GH and IGF-I administration restored. Results were expressed as mean ± SE. Statistical significance was determined as $P < 0.05$.

which plays an important role in the development of NASH. We then investigated the mitochondrial morphology in the SDR hepatocyte. As expected, the mitochondrial morphology was severely impaired in the SDR liver (Fig. 3A). In particular, the size of the mitochondria was smaller, the shape was irregular, and the ultrastructure of clistae was disorganized as compared with the control. Furthermore, there were abnormal electron depots in a part of mitochondria in the hepatocyte (Fig. 3A). The quantitative analysis of mitochondrial number and area revealed that although the number was not changed, the area was significantly decreased in the SDR as compared with control and GH or IGF-I administration significantly increased it (Fig. 3B) accompanied with the restoration of the morphological changes.

### 3.4. Oxidative stress in GH-deficient rat

Accumulation of lipids in the hepatocyte impairs the oxidative capacity of mitochondria, increases the reducing state of the electron transport chain complexes and generation of reactive oxygen species (ROS), and further induces mitochondrial damage and impaired function. These process are closely involved in the development of NASH [20]. We then examined the oxidative stress in the SDR liver. As shown in Fig. 4A, immunohistochemcal analysis using anti-nitrotyrosine antibody showed an enhanced oxidative stress in the SDR liver. The other oxidative stress marker, 8OHdG staining revealed similar results (data not shown). Interestingly, GH or IGF-I administration improved these changes in the liver (Fig. 4A). In addition, an oxidative stress marker, tissue TBARS content was significantly increased in the SDR liver compared with control. The elevated TBARS level was significantly decreased by GH and IGF-I administration (Fig. 4B).

## 4. Discussion

In this study we demonstrated that the GH-deficient animal model, SDR exhibited steatosis, hepatocyte injury, and fibrosis in

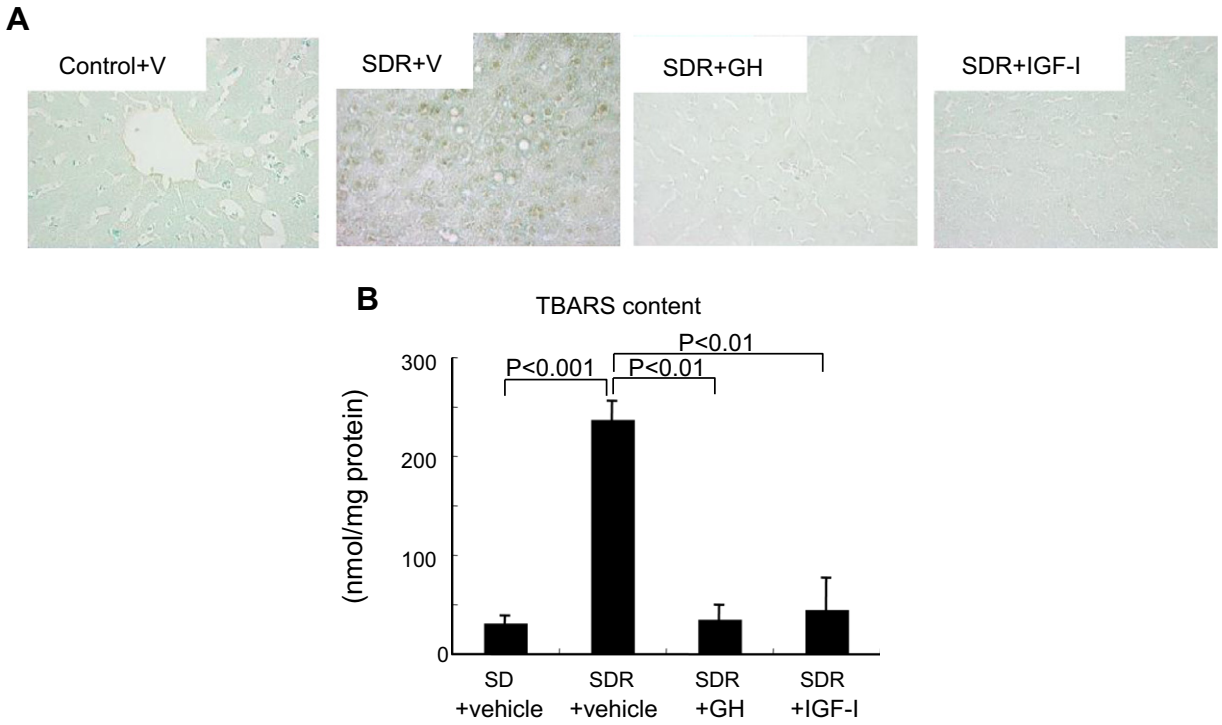


**Fig. 4.** (A) Immunochemical analysis using anti-nitrotyrosine antibody. Oxidative stress in the SDR liver was enhanced compared with the control and GH and IGF-I administration improved it. (B) Oxidative stress marker, TBARS concentration in the SDR liver was significantly increased compared with the control and GH and IGF-I administration reversed these changes. Results were expressed as mean ± SE. Statistical significance was determined as $P < 0.05$.

the liver in conjunction with an elevation of liver enzyme levels, indicating that an absence of GH lead to NASH. These results were compatible with the previous reports in mice [10–12] and humans [6,8], in which deficiency in GH or GH signaling is associated with hepatic steatosis. Furthermore, we demonstrated that administration of GH and IGF-I restored these biochemical and histological changes, clearly demonstrating a presence of GH-independent IGF-I action to prevent the development of NASH.

It is surprising that IGF-I restored hepatic abnormalities in the SDR because GHR is mainly expressed in the hepatocyte and liver-specific deletion of the GHR resulted in marked steatosis and particularly, 2 weeks of IGF-I infusion did not correct the steatosis in this model mouse, suggesting an essential and direct role of GH in the liver [9]. However, in this model, the contribution of IGF-I cannot completely be excluded. In the aspect of specific contribution of GH or IGF-I, our experimental model is suitable for evaluating the effect of IGF-I. In addition, several reports have suggested a pivotal role of IGF-I in the liver. IGF-I administration improved liver dysfunction and fibrosis in rat cirrhosis model [21] and mitochondrial function in aging rats [22]. In humans, NAFLD is associated with low circulating levels of IGF-I [23,24]. IL-6 and IGF-1 are independent prognostic factors of liver steatosis and NASH in morbidly obese patients [25]. In addition, a fibrotic marker, hyaluronic acid levels showed a negative relationship with IGF-1 and the IGF-1/IGFBP-3 ratio in patients with NAFLD [26]. Although it is obvious that there is an IGF-I-independent action of GH in the liver in the aspects of anti-steatotic action [9,12] and gene expression profiles [27], the results in this study strongly suggest a presence of GH-independent action of IGF-I in the liver.

In the development of NASH, insulin resistance, oxidative stress, mitochondrial dysfunction, and inflammatory cascade are believed to play integral roles [1]. Insulin resistance and excess adiposity are associated with increased lipid influx into the liver and increase de novo hepatic lipogenesis, promoting hepatic triglyceride accumulation. Defects in lipid utilization via mitochondrial oxidation and lipid export contribute to hepatic lipid build-up. Impaired mitochondrial function and excess of lipid accumulation promote an enhanced oxidative stress. Intriguingly, mitochondrial morphology was severely impaired in the SDR liver and GH and IGF-I restored these changes. Furthermore, the oxidative stress was enhanced in the SDR liver and GH and IGF-I improved the oxidative stress, suggesting that GH exerts via IGF-I in the regulation of mitochondrial function and oxidative stress. It has been reported that low dose of IGF-I improved rat cirrhosis model via protecting the mitochondrial function [21]. IGF-I improved mitochondrial function *in vitro* [28] and *in vivo* [22]. IGF-IR activation prevented oxidative stress and mitochondrial dysfunction, and apoptosis in HUCEC cells [28]. IGF-I administration reduced oxidative mitochondrial damage, corrected impaired mitochondrial function such as complex V ATPase activity, and decreased caspase activities [22]. In agreement with these previous results, our data demonstrated that IGF-I improved mitochondrial morphology and oxidative stress in the liver. These effects of IGF-I might contribute to improve hepatic abnormalities via these mechanisms independent of GH.

There are several other potential underlying mechanisms, in which IGF-I plays a role in the liver. It is well known that IGF-I improves insulin sensitivity *in vivo*. The specific elimination of liver-derived IGF-I results in insulin insensitivity in muscle, liver, and fat tissues [29]. Abdominal adiposity and insulin resistance play an essential role in the development of NASH. It is possible that IGF-I may ameliorate NASH at least in part via improving insulin sensitivity. In addition, IGF-I exerts a strong anabolic action particularly in protein metabolism in muscle, which is commonly disturbed in chronic liver disease. The improvement of the nutritional condition may also contribute as shown by a pilot study that IGF-I administration in cirrhotic patients demonstrated that serum albumin and energy metabolism improved after 120 days of administration [30]. Finally, although the mechanism has yet

not been clarified, IGF-I may have an anti-fibrotic effect in the liver. In an animal model of liver cirrhosis, exogenous IGF-I expression in the liver reduced fibrosis and ultimately lead to amendment of cirrhosis, suggesting an anti-fibrotic action of IGF-I [13].

Decreased levels of IGF-I are seen in patients with chronic liver disease including NASH [23,25]. The most of circulating IGF-I is produced in the liver [31] and reduced expression of GHR and decreased synthesis of IGF-I in the hepatocyte is related with the reduction of circulating IGF-I levels in chronic liver disease. Volzke et al. reported that hepatic steatosis was associated with low serum IGF-I and high IGFBP-3 levels, independent of alcohol consumption in 3863 subjects [32]. Although it has been established that serum IGF-I levels are important marker for hepatocellular functional capacity and for prognosis in NASH and cirrhosis [33], taken together with the previous reports of the effect of IGF-I on the liver, the present study suggests that liver may be an important target tissue for IGF-I, not only as an IGF-I-producing organ.

In conclusion, we demonstrated that GH-deficient rat exhibits NASH and IGF-I plays an essential role to prevent the development of NASH in the liver. The improved mitochondrial function and reduced oxidative stress may contribute the effect of IGF-I.

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