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RAPID COMMUNICATION

Inhibition of hepatic interleukin-18 production by rosiglitazone in a rat model of nonalcoholic fatty liver disease

Hai-Ning Wang, Yan-Rong Wang, Guo-Qiang Liu, Zhe Liu, Pei-Xian Wu, Xiao-Ling Wei, Tian-Pei Hong

Hai-Ning Wang, Yan-Rong Wang, Guo-Qiang Liu, Zhe Liu, Pei-Xian Wu, Xiao-Ling Wei, Tian-Pei Hong, Department of Endocrinology, Peking University Third Hospital, Beijing 100191, China
Author contributions: Wang HN and Wang YR contributed equally to this work. Wang YR and Hong TP designed the research; Wang HN, Liu Z, Wu PX, and Wei XL performed the research; Hong TP, Wang HN and Liu GQ analyzed the data and wrote the paper.
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Correspondence to: Tian-Pei Hong, MD, PhD, Professor, Department of Endocrinology, Peking University Third Hospital, Beijing 100191, China. tpho66@bjmu.edu.cn
Telephone: +86-10-82265515 Fax: +86-10-62017700
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Abstract

AIM: To investigate the effects of rosiglitazone (RGZ) on expression of interleukin-18 (IL-18) and caspase-1 in liver of non-alcoholic fatty liver disease (NAFLD) rats.

METHODS: Twenty-eight Sprague-Dawley (SD) rats were randomly divided into control, NAFLD, and RGZ treated NAFLD groups. A NAFLD rat model of NAFLD was established by feeding the animals with a high-fat diet for 12 wk. The NAFLD animals were treated with RGZ or vehicle for the last 4 wk (week 9-12) and then sacrificed to obtain liver tissues. Histological changes were analyzed with HE, oil red O and Masson's trichrome staining. Expressions of IL-18 and caspase-1 were detected using immunohistochemical staining and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis.

RESULTS: The expression levels of both IL-18 and caspase-1 were higher in the liver of NAFLD group than in the control group. Steatosis, inflammation and fibrosis, found in the liver of NAFLD rats, were significantly improved 4 wk after RGZ treatment. The elevated hepatic IL-18 and caspase-1 expressions in NAFLD group were also significantly attenuated after RGZ treatment.

CONCLUSION: RGZ treatment can ameliorate increased hepatic IL-18 production and histological changes in liver of NAFLD rats. The beneficial effects of RGZ on NAFLD may be partly due to its inhibitory effect on hepatic IL-18 production.

Key words: Insulin resistance; Interleukin-18; Non-alcoholic fatty liver; Rosiglitazone

Peer reviewer: David Adams, Professor, Liver Research Laboratories, Institute for Biomedical Research, Queen Elizabeth Hospital, University of Birmingham, Birmingham B15 2TT, United Kingdom

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INTRODUCTION

Interleukin-18 (IL-18), previously called interferon-gamma (IFN-γ) inducing factor, is originally identified as a pro-inflammatory cytokine derived from Kupffer cells in animals with acute liver injury induced by endotoxin[1]. IL-18 is closely related to and acts synergistically with IL-12. However, its amino acid sequence and structure motifs resemble the IL-1 family. IL-18 expression has been demonstrated in a variety of cell types originated from both immune and non-immune systems, suggesting that IL-18 may have a wide range of cellular sources and functions apart from being a macrophage-derived inducer of IFN-γ production from type 1 T helper cells[2]. IL-18 is intracellularly synthesized as a non-functional precursor protein, pro-IL-18. Like pro-IL-1β, pro-IL-18 is then processed by caspase-1 into a bioactive mature form[3].

Non-alcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver diseases, represents a spectrum of liver disease extending from simple fatty liver through steatohepatitis to cirrhosis

in the absence of a history of significant alcohol use. NAFLD is considered one of the clinical features of metabolic syndrome in which insulin resistance plays a central role[4]. Several lines of evidence show that IL-18 may be important in the pathogenesis of inflammatory processes, which contribute to the development of insulin resistance. It has been shown that elevated serum IL-18 levels are associated with insulin resistance in obese subjects, women with polycystic ovary syndrome, and patients with type 2 diabetes mellitus[5-7]. Furthermore, hepatic IL-18 level is elevated in insulin resistance-related obese mice with NAFLD[8].

Rosiglitazone (RGZ), a selective ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ), is an insulin sensitizer that has been used in a number of insulin-resistant conditions, including NAFLD. Several clinical studies showed that RGZ could improve liver enzyme levels and histological changes in NAFLD patients by increasing insulin sensitivity[9-11]. However, whether the beneficial effect of RGZ on NAFLD is associated with reduced IL-18 expression in the liver remains unclear. This study analyzed the expression of IL-18 and caspase-1 in the liver of NAFLD rats, and investigated the effects of RGZ on hepatic IL-18 production and liver histology.

MATERIALS AND METHODS

Animal and experimental protocol

Twenty-eight male Sprague-Dawley (SD) rats, weighing 140-160 g, were housed in individual cages at 22℃ with free access to food (standard chow diet) and water for 1 wk before initiation of the experiment. The study protocol was approved by the Animal Care and Use Committee of Peking University Health Science Center.

The rats were randomly divided into control group (n = 6), NAFLD group (n = 11), and RGZ-treated NAFLD group (n = 11). Rats in the control group were maintained on the standard chow diet for 12 wk. A rat model of NAFLD was induced by a high-fat diet (standard chow diet + 10% lard + 2% cholesterol) for 8 wk as previously described[12]. Subsequently, rats in the RGZ-treated NAFLD group were treated with RGZ maleate (Avandia®, 4 mg/kg per day) *via* gavage, whereas rats in the NAFLD group were given normal saline for another 4 wk. At the end of study, all rats were sacrificed after 12 h of fasting. Blood samples were collected for biochemical assays. The liver was removed and weighted after rinsed with ice-cold saline, and sampled for histological study and RNA extraction.

Biochemical analyses

Serum insulin concentrations were determined with a radioimmunoassay kit (Beijing Atom HighTech Co., Ltd., Beijing, China). Serum leptin and adiponectin levels were measured with an ELISA kit (Invitrogen, Carlsbad, CA, USA). Free fatty acid (FFA) concentrations were analyzed using a commercially available kit (Randox, Antrim, UK). Additional blood biochemical parameters, including glucose, triglycerides, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), were assayed using an automatic biochemical analyzer. Homeostasis model assessment (HOMA) was employed to estimate the insulin resistance index (HOMA-IR), which reflects both peripheral and hepatic insulin resistance[13]. Liver weight index (%) was calculated as liver weight/body weight × 100.

Histological studies

The sections of liver tissues from the center of the largest hepatic lobes were fixed in 10% buffered formaldehyde, and then embedded in paraffin. A 5 μm-thick section cut from a paraffin-embedded block was stained with HE and Masson's trichrome. To visualize the fat droplet accumulation, frozen liver tissue sections were stained with oil red O. Steatosis, necro-inflammatory grade and stage of fibrosis were assessed as previously described[14]. Liver histology was evaluated blindly.

Immunohistochemical staining

Goat IL-18 polyclonal antibody was obtained from Santa Cruz (Santa Cruz, CA, USA). Rabbit caspase-1 polyclonal antibody was purchased from Lab Vision (Fremont, CA, USA). Immunohistochemical staining of 5 μm-thick paraffin-embedded liver tissue sections was performed according to its manufacturer's protocol (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA). Briefly, liver sections were deparaffinized in xylene and rehydrated in graded ethanol. After endogenous peroxidase and biotin were blocked, the tissues were pre-incubated with 3% horse serum for 30 min to prevent non-specific reactions. The sections were then incubated with primary antibodies diluted at 1:150 for 60 min. On negative control sections, the step with primary antibodies was omitted. Polyclonal antibodies were detected using a biotinylated anti-goat or rabbit IgG diluted at 1:200 in 5% bovine serum albumin for 30 min. The sections were incubated with R.T.U. Vectastain Elite ABC reagent for 30 min, stained with diaminobenzidine for 5 min and counterstained with hematoxylin before they were mounted.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the liver tissues using a TRIzol reagent (Gibco, Carlsbad, CA, USA) according to its manufacturer's instructions. cDNA was prepared with the SuperScription First-Strand Synthesis System (Invitrogen) as previously described[15] and amplified by polymerase chain reaction (PCR). The sequences of primers used for PCR are 5'-GGCTCTTGTGTCAACT TCAAA-3' and 5'-TTATCAGTCTGGTCTGGGATT-3' (232 bp) for *IL-18*, 5'-TCCTGAGGGCAAAGAGGAA GC-3' and 5'-GGCAAGACGTGTACGAGTGGGT-3' (479 bp) for *caspase-1*, 5'-GCTCGTCGTCGACAACG GCTC-3' and 5'-CAAACATGATCTGGGTCATCT TCTC-3' (353 bp) for *β-acti*, respectively. Each semi-quantitative RT-PCR analysis was performed with a set

of *IL-18* or caspase-1 primers in combination with a set of primers for house-keeping gene *β-actin* as an internal standard. The conditions of PCR were as follows: 1 cycle at 94℃ for 5 min; 30 cycles at 94℃ for 30 s, at 60℃ for 30 s, at 72℃ for 45 s; 1 cycle at 72℃ for 5 min. The PCR products were separated on a 2% agarose gel which was dried and then scanned using an ultraviolet gel imaging system (BioRad, Hercules, CA, USA). Gene expression levels were represented as ratios of target gene to co-amplified internal standard.

Statistical analysis

Data are expressed as mean ± SE. Analysis of variance was used to compare the means of three groups, followed by Newman-Keuls test to determine the statistical significance between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of RGZ on hepatic histology

The liver tissues from all groups were stained with HE and analyzed (Figure 1A-C). In contrast to the control group, typical steatosis and portal and lobular inflammation were observed in the NAFLD group after 12 wk of feeding with a high-fat diet (Figure 1A and B). In the NAFLD group, steatosis was observed in about 65% of hepatocytes (mean score: 3 *vs* 0 in the control group). Moderate infiltration of mononuclear and polymorphonuclear cells was also found (mean score: 2 *vs* 0). Fat droplet accumulation and fibrosis (mean score: 1 *vs* 0) were observed in sections stained with oil red O (Figure 1D-F) and Masson's trichrome (Figure 1G-I) in the NAFLD group (Figure 1E and H), but not in the control group (Figure 1D and G). These findings suggest that the animal model of NAFLD was successfully established. Furthermore, markedly attenuated steatosis (mean score: 0.6), inflammation (mean score: 0.5) and fibrosis (mean score: 0.2) were observed 4 wk after treatment with RGZ (Figure 1C and I).

Effects of RGZ on hepatic metrology and biochemistry

Increased liver weight and liver weight index observed in the NAFLD group were significantly improved after RGZ treatment (Table 1). The hepatic surface was smooth, red-brown, and soft in the control group, whereas the liver in the NAFLD group was enlarged in dimmer color with a moderate texture. The liver feature of the RGZ-treated NAFLD group was in between the above two groups (data not shown). Moreover, serum ALT, AST and ALP levels were significantly elevated in the NAFLD group and significantly reduced in the RGZ-treated NAFLD group compared with the untreated NAFLD group (Table 1).

Effects of RGZ on insulin resistance and metabolic parameters

Increased serum insulin, leptin, FFA, and HOMA-IR levels were observed in the NAFLD group, which were attenuated in the RGZ-treated NAFLD group. In contrast, decreased serum adiponectin levels were found in the NAFLD group, which were ameliorated in the RGZ-treated NAFLD group. Fasting blood glucose concentration was significantly elevated in the NAFLD group and decreased in the RGZ-treated NAFLD group. The levels of serum triglycerides were significantly higher in the NAFLD group, and reduced after RGZ treatment. Serum cholesterol levels were significantly higher in the NAFLD group, and tended to become lower in the RGZ-treated NAFLD group although the difference was not statistically significant (Table 1).

Table 1 Effects of RGZ on blood biochemistry and hepatic parameters (mean ± SE)

Parameters	Control (n = 6)	NAFLD (n = 11)	RGZ-treated NAFLD (n = 11)
Glucose (mmol/L)	6.48 ± 0.32	7.31 ± 0.20[a]	6.26 ± 0.16[c]
Insulin (mU/L)	20.41 ± 1.85	27.03 ± 1.48[a]	21.07 ± 1.19[c]
HOMA-IR	5.90 ± 0.58	8.93 ± 0.48[a]	5.95 ± 0.41[c]
Leptin (μg/L)	2.63 ± 0.13	4.21 ± 0.09[a]	3.75 ± 0.05[a,c]
Adiponectin (mg/L)	2.22 ± 0.05	1.64 ± 0.07[a]	1.91 ± 0.10[a,c]
Free fatty acid (μmol/L)	361.0 ± 42.6	539.1 ± 32.1[a]	401.0 ± 32.9[c]
Triglycerides (mmol/L)	0.51 ± 0.04	1.21 ± 0.04[a]	0.82 ± 0.06[a,c]
Cholesterol (mmol/L)	1.45 ± 0.14	2.69 ± 0.11[a]	2.38 ± 0.11[a]
Body weight (g)	563.2 ± 9.6	581.6 ± 9.7	571.4 ± 9.1
Liver weight (g)	13.90 ± 0.53	26.51 ± 1.96[a]	15.86 ± 1.06[a,c]
Liver weight index (%)	2.48 ± 0.05	4.51 ± 0.17[a]	3.00 ± 0.16[c]
Alanine aminotransferase (U/L)	57.2 ± 5.8	100.9 ± 7.2[a]	53.6 ± 5.8[c]
Aspartate aminotransferase (U/L)	164.4 ± 11.8	197.5 ± 9.1[a]	151.2 ± 9.5[c]
Alkaline phosphatase (U/L)	84.8 ± 6.1	174.6 ± 11.6[a]	87.3 ± 4.9[c]

[a]$P < 0.05$ *vs* control group, [c]$P < 0.05$ *vs* NAFLD group. HOMA-IR: Homeostasis model assessment-insulin resistance index.

Effects of RGZ on IL-18 and caspase-1 expression in liver tissues

Immunohistochemical staining was used to analyze the expression of IL-18 (Figure 1J-L) and caspase-1 (Figure 1M-O) proteins in the liver tissues. Positive expression of either IL-18 or caspase-1 was rarely detected in liver tissues from the control group with very week staining in some Kupffer cells (Figure 1J and M). However, the NAFLD group exhibited a strong expression of both IL-18 and caspase-1 in the liver lobules. Hepatocytes and/or infiltrating inflammatory cells within the lobules were the major cell types expressing IL-18 and caspase-1 (Figure 1K and N). Compared to the untreated NAFLD group, the expression of hepatic IL-18 and caspase-1 was significantly inhibited 4 wk after treatment with RGZ (Figure 1L and O).

RT-PCR analysis further showed that constitutive *IL-18* or *caspase-1* mRNA expression was found in liver tissues from the control group. The mRNA levels of *IL-18* and *caspase-1* were significantly higher in liver tissues from the NAFLD group than in those from the control group. The mRNA expression of *IL-18* and *caspase-1* was significantly reduced in the RGZ-treated

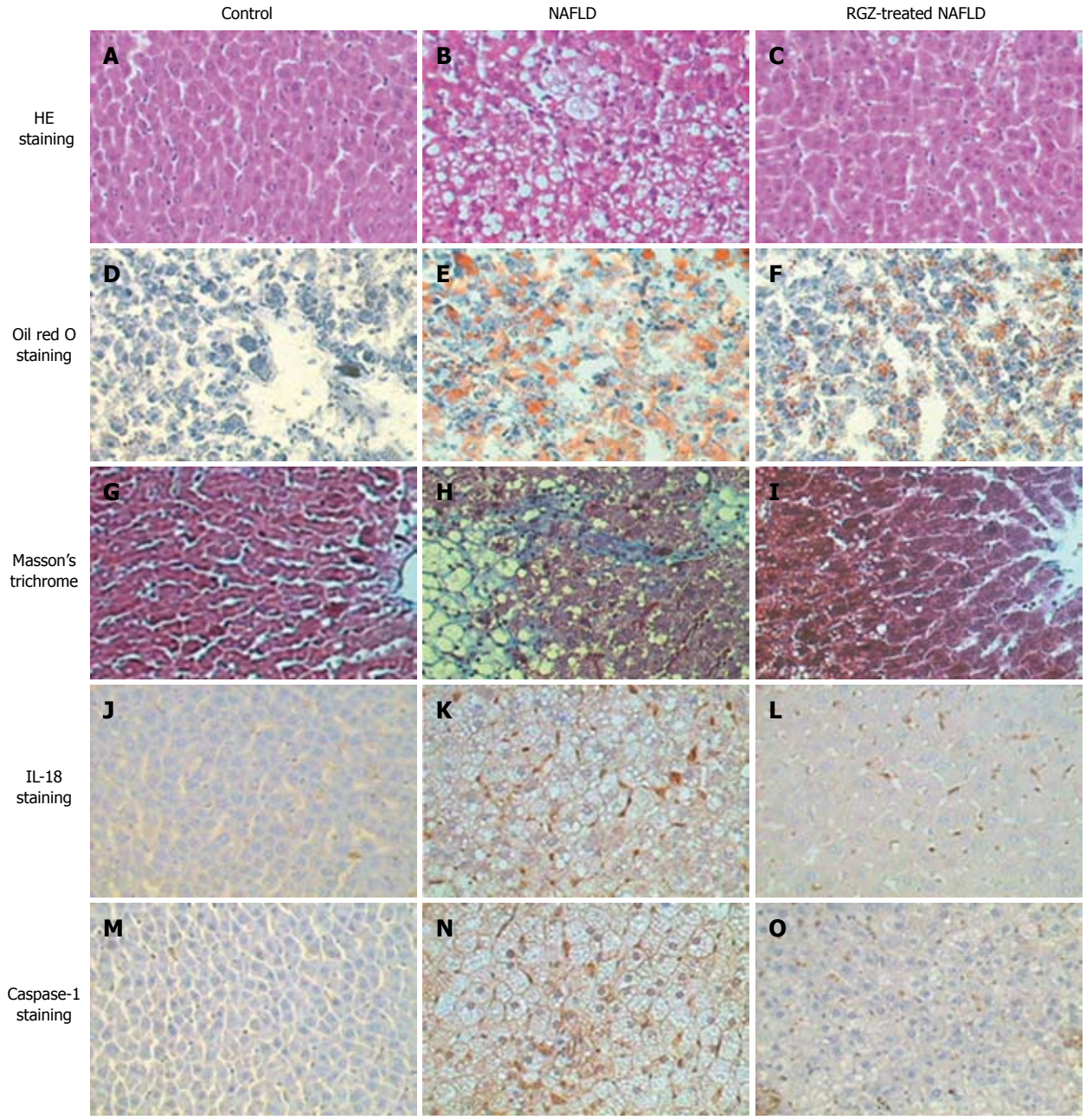

Figure 1 Histological studies of rat livers in normal control, NAFLD and RGZ-treated NAFLD groups (x 400). Liver tissue sections were stained with HE, A: Normal control group; B: NAFLD group; C: RGZ-treated NAFLD group. Liver tissue sections were stained with oil red O, D: normal control group; E: NAFLD group; F: RGZ-treated NAFLD group. Liver tissue sections were stained with Masson's trichrome, G: Normal control group; H: NAFLD group; I: RGZ-treated NAFLD group. Liver tissue sections were stained with immunohistochemistry for IL-18, J: Normal control group; K: NAFLD group; L: RGZ-treated NAFLD group. Liver tissue sections were stained with immunohistochemistry for caspase-1, M: Normal control group; N: NAFLD group; O: RGZ-treated NAFLD group. Histological changes in fatty liver disease and IL-18- or caspase-1-positive staining cells were rarely detectable in the control group. NAFLD rat liver showed steatosis and moderate inflammatory changes, fat droplet accumulation, mild fibrosis, strong IL-18- and caspase-1-positive staining. A significant improvement of steatosis, inflammation, fibrosis, and IL-18 and caspase-1 staining was observed in liver of the RGZ-treated NAFLD group.

NAFLD group compared to the NAFLD group, but remained higher in the RGZ-treated NAFLD group than in the control group (Figure 2).

DISCUSSION

Insulin resistance is closely associated with NAFLD, typically known as a part of the metabolic syndrome, and has been implicated as a contributing factor for the pathogenesis of NAFLD[4,16-18]. Treatment with RGZ, an oral anti-diabetic agent of the thiazolidinediones, leads to the improvement in insulin resistance with ameliorated histological and biochemical changes of liver injury in diabetic and non-diabetic patients with NAFLD[9-11]. Pioglitazone[19], another thiazolidinedione insulin-sensitizer, and metformin[20] exert similar effects in non-diabetic patients with NAFLD. These findings suggest that insulin resistance contributes to the development of NAFLD and that insulin sensitizers may represent important agents for the treatment of NAFLD. Furthermore, the beneficial effects of insulin-sensitizing agents, RGZ[21,22], pioglitazone[12] and

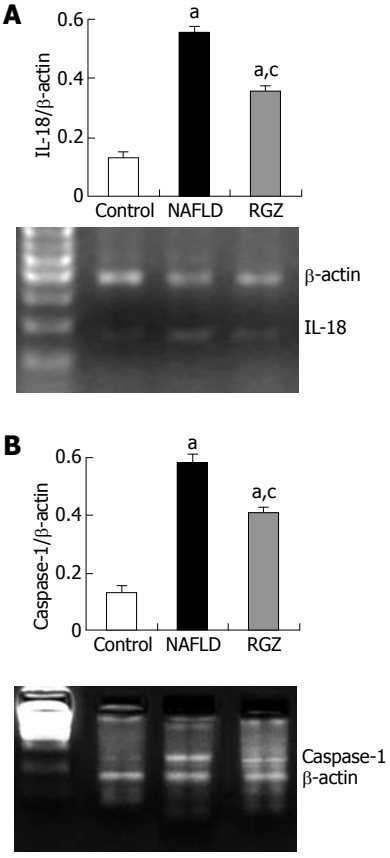

Figure 2 RT-PCR analysis of mRNA expression of *IL-18* (A) and caspase-1 (B) in liver tissues of the three groups. The histograms show the ratio of target gene expression to *β-actin*. Data are presented as mean ± SE from six independent experiments in the control group and eleven in the NAFLD and RGZ-treated NAFLD groups. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* NAFLD group.

metformin[21], on hepatic steatosis and inflammation have been confirmed in various animal models of NAFLD. Compared to metformin, RGZ appears to be a better drug for improving hepatic steatosis[21]. In line with the above reports[12,21,22], our study showed that increased liver weight and liver weight index, elevated serum liver enzyme levels and altered liver histological conditions including steatosis, inflammation and fibrosis were observed in the NAFLD rats, which were significantly improved 4 wk after RGZ treatment. In agreement with previous reports[12,21], the present study also showed that the levels of serum insulin, leptin, FFA and triglycerides as well as HOMA-IR were significantly increased, whereas the levels of serum adiponectin remarkably decreased in the NAFLD group. Four weeks after RGZ treatment, these abnormalities were significantly improved, suggesting that insulin resistance may play an important role in the pathogenesis of NAFLD.

Overnutrition-induced chronic inflammation is a key component in the pathogenesis of insulin resistance and metabolic syndrome. Pro-inflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver by inhibiting the insulin signal transduction[23]. A role of IL-18 has been recently postulated in the development of insulin resistance based on the observation that elevated serum IL-18 levels are associated with insulin resistance and hypoadiponectinemia[5-7,24]. In obese women, circulating levels of IL-18 are increased and positively associated with body weight and visceral fat, which can be ameliorated by caloric restriction-induced weight loss over 1 year[25]. A recent report showed that plasma IL-18 is associated with changes in insulin resistance and reduced after weight loss with a 15-wk life-style intervention. In addition, the expression of IL-18 in adipose tissue is increased in obese subjects but not affected by weight loss[26], indicating that changes in plasma IL-18 are related to insulin resistance rather than to obesity. Notably, the fact that increased serum IL-18 levels are associated with increased serum liver enzyme concentrations suggests that IL-18 might contribute to the development of liver disease associated with insulin resistance[27,28]. The role of IL-18 in the development of insulin resistance-related NAFLD is further supported by a report showing that both serum IL-18 concentration and hepatic *IL-18* mRNA expression are elevated in lipopolysaccharide-treated *ob/ob* mice, an animal model of fatty liver disease[8]. Moreover, IL-18 may play an important role in liver injury caused by hepatitis B virus infection[29], hepatic ischemia/reperfusion[30], and endotoxin exposure[31] since the liver injury can be reversed by blockage of IL-18 *via* either gene knockout[31] or neutralizing antibody[30]. Importantly, exogenous administration of IL-18 with IL-12 to BALB/c mice induces fatty liver in an IFN-γ and nitric oxide dependent manner[32], suggesting that IL-18 plays a pivotal role in the inflammatory cascade leading to NAFLD associated with insulin resistance.

In the present study, the expression of IL-18 and caspase-1 was extremely low in liver tissues from the control animals. However, the expression of IL-18 and caspase-1 was significantly increased in liver lobules from NAFLD rats. Hepatocytes and inflammatory cells within the lobules are the major cell types expressing IL-18 and caspase-1. IL-18 is synthesized as a precursor molecule without biological activity and requires caspase-1 for cleavage into a mature peptide and subsequent release[3]. Increased expression of caspase-1 in hepatocytes and infiltrating inflammatory cells indicates that both cell types may produce and secrete mature IL-18. This finding is generally consistent with a previous report showing that IL-18 production can be originated from Kupffer cells as well as injured hepatocytes[29]. As noted above, RGZ improves the histological and biochemical changes in NAFLD along with the improvement in insulin resistance[9-11]. In addition, increased circulating levels of IL-18 are significantly decreased after RGZ therapy for patients with metabolic syndrome[33] or type 2 diabetes mellitus[34]. The present study showed that increased expression of IL-18 and caspase-1 in livers of NAFLD rats was reduced 4 wk after treatment with RGZ. Therefore, it is conceivable that hepatic IL-18 production may have a critical role in the development of NAFLD and that the beneficial effects of RGZ on NAFLD may be mediated by inhibiting IL-18 expression possibly *via* PPAR-γ activation[35].

The mechanism by which hepatic IL-18 production

contributes to the development of NAFLD remains to be elucidated. IL-18 is a pro-inflammatory cytokine with multiple functions including stimulation of IFN-γ production[1-3], enhancement of IL-1β, and IL-8 production *via* direct stimulation of tumor necrosis factor-α (TNF-α) production[36]. A previous study showed that intraperitoneal administration of IL-18 with IL-12 induces mouse fatty liver in an IFN-γ dependent manner[32]. Macrophage-derived TNF-α contributes to insulin resistance and development of hepatic steatosis in diet-induced obesity[37]. It was also reported that either IFN-γ or TNF-α up-regulates gene expression or posttranslational processing of *IL-18* in several tissue cell types[15,38,39], indicating that IL-18 may contribute to the development of insulin resistance-related NAFLD *via* a complex cytokine network of IL-18 and many other cytokines such as TNF-α, IFN-γ and IL-1β.

In summary, increased hepatic IL-18 production along with liver histological and biochemical changes can be ameliorated after RGZ treatment. The beneficial effects of RGZ on NAFLD may be due to its direct anti-inflammatory properties, possibly *via* PPAR-γ activation, or secondary to improved insulin resistance.

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COMMENTS

Background

Interleukin-18 (IL-18), originally identified as a pro-inflammatory cytokine in endotoxin-induced liver injury, is an important mediator of innate and adaptive immunity. Recently, IL-18 has been reported to be associated with insulin resistance-related non-alcoholic fatty liver disease (NAFLD).

Research frontiers

Treatment with thiazolidinedione (TZD) leads to improvement in insulin resistance and ameliorates histological and biochemical changes of liver injury in patients with NAFLD. However, whether the beneficial effect of TZD on NAFLD is associated with reduced IL-18 expression in the liver remains unknown.

Innovations and breakthroughs

The data show that increased hepatic IL-18 and caspase-1 expression along with liver histological and biochemical changes can be ameliorated after rosiglitazone (RGZ) treatment in an animal model of NAFLD. Therefore, the beneficial effects of RGZ on NAFLD may be due to its direct anti-inflammatory properties or secondary to improved insulin resistance, *via* peroxisome proliferator-activated receptor gamma (PPAR-γ) activation.

Applications

IL-18 plays a pivotal role in the inflammatory cascade leading to NAFLD associated with insulin resistance. Therefore, blockage of hepatic IL-18 action may be a novel target for treatment of NAFLD.

Terminology

NAFLD represents a spectrum of liver disease extending from simple fatty liver through steatohepatitis to cirrhosis in the absence of a history of significant alcohol use. Low-grade inflammation, characterized by abnormal cytokine production, increased synthesis of acute-phase reactants and activation of inflammatory signaling pathways, is a key component in the pathogenesis of insulin resistance, obesity and steatohepatitis.

Peer review

This is an interesting paper. The authors showed that RGZ treatment can improve the increased hepatic IL-18 expression along with liver histological changes in a rat model of NAFLD.

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