JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
SHORT REPORT

Role of insulin receptor substrate-1 for diethylnitrosamine plus high-fat diet-induced hepatic tumorigenesis in mice

Khadbaatar Zolzaya, Akinobu Nakamura, Kazuki Tajima, Yasuo Terauchi*

Department of Endocrinology and Metabolism, Graduate School of Medicine, Yokohama City University, Yokohama, Japan

Keywords
Diethylnitrosamine, Hepatic tumorigenesis, Insulin receptor substrate-1

***Correspondence**
Yasuo Terauchi Tel.: +81-45-787-2639
Fax: +81-45-784-3012
E-mail address: terauchi-tky@umin.ac.jp

J Diabetes Invest 2014; 5: 27–30

doi: 10.1111/jdi.12128

ABSTRACT

We investigated the role of insulin receptor substrate (Irs)-1 for diethylnitrosamine (DEN) plus high-fat (HF) diet-induced hepatic tumorigenesis in mice. We gave DEN by intraperitoneal injection at the dose of 80 mg/kg to 18-week-old wild-type (WT) and Irs1-knockout (*Irs1*$^{-/-}$) mice, which were fed a HF diet from 8 weeks-of-age until they were killed (52 weeks). The *Irs1*$^{-/-}$ mice showed significantly lower plasma alanine aminotransferase levels, triglyceride contents in the liver and also lower expression levels of the genes encoding inflammatory cytokines than the WT mice. The incidence of DEN plus HF diet-induced hepatic tumors was 71.4% in the WT mice, whereas it was just 14.3% in the *Irs1*$^{-/-}$ mice. The present study showed that Irs1 played an important role in DEN plus HF diet-induced hepatic tumorigenesis.

INTRODUCTION

Recent epidemiological studies have showed obesity and diabetes mellitus as independent risk factors for hepatocellular carcinoma (HCC)[1]. Insulin receptor substrate (Irs)-1 is a key mediator of the signaling events that lie downstream of the insulin receptor. Irs1-deficient mice show normal glucose tolerance, peripheral insulin resistance and marked growth retardation[2–4]. In our previous study, we showed that Irs1 inhibition protected against high-fat (HF) diet-induced non-alcoholic steatohepatitis (NASH) and liver tumorigenesis[5]. Diethylnitrosamin (DEN) is an organic chemical that is highly toxic and widely used in cancer research to induce liver tumors in experimental animals. However, it is unclear how Irs1 plays a role in DEN-initiated hepatic tumorigenesis. In the present study, we investigated the effect of Irs1 on DEN plus HF diet-induced hepatic tumorigenesis in mice.

MATERIALS AND METHODS

Animals

Irs1-knockout (*Irs1*$^{-/-}$) mice were generated as described elsewhere[5]. We backcrossed these mice with C57Bl/6J mice more than 10 times. Male littermates derived from the intercrosses were fed standard chow (SC) until 8 weeks-of-age, and then shifted to a HF diet throughout the study period until 52 weeks-of-age. When they were 18 weeks-of-age, they were given an intraperitoneal injection of normal saline or DEN (80 mg/kg; Sigma-Aldrich, Tokyo, Japan). The mice were housed under a 12-h light–dark cycle. The animals were maintained according to standard animal care procedures laid down in the institutional guidelines.

Diet Protocol

Standard chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and a HF diet (High Fat Diet 32; Clea Japan, Inc., Tokyo, Japan) were used. The compositions of each of these diets are described elsewhere[5].

Measurement of Biochemical Parameters

Blood glucose was measured by a portable glucometer using Glutest Neo (Sanwa Chemical Co., Nagoya, Japan). Insulin levels were determined with an insulin enzyme-linked immunosorbent assay kit (Morinaga, Yokohama, Japan). Plasma alanine aminotransferase (ALT) was assayed by an enzymatic method (Wako Pure Chemical Industries Ltd., Osaka, Japan). The triglyceride content in the liver was determined as described elsewhere[5].

Received 23 April 2013; revised 8 June 2013; accepted 26 June 2013

Histopathological Evaluation

Liver samples were immersion-fixed overnight in 10% formalin at 4°C. The tissues were then routinely processed for paraffin embedding, and 5-μm sections mounted on glass slides were processed for hematoxylin–eosin (HE) staining.

Ribonucleic Acid Preparation and Real-Time Quantitative Polymerase Chain Reaction

Total ribonucleic acid was prepared from portions of the liver using Isogen reagent (NipponGene, Tokyo, Japan), in accordance with the manufacturer's instructions, and used as the starting material for the complementary DNA preparation. Synthesis of complementary DNA and TaqMan quantitative polymerase chain reaction were carried out according to previously described methods[5]. The relative expression levels were compared after normalization to the expression level of beta-actin.

Statistical Analysis

Results were expressed as means ± SE (n). Differences between two groups were analyzed for statistical significance by the Student's t-test. $P < 0.05$ was considered to show statistical significance.

RESULTS

Metabolic Changes in *Irs1*$^{-/-}$ Mice

The bodyweight of the DEN-treated *Irs1*$^{-/-}$ mice on HF diet was approximately 60% of that of the DEN-treated wild-type (WT) mice on a HF diet (Figure 1a). However, fed-state blood glucose levels were increased in the *Irs1*$^{-/-}$ mice as compared with those in the WT mice after 28 weeks on the HF diet (Figure 1b). Also, the fasting insulin level was significantly higher in the *Irs1*$^{-/-}$ mice (Figure 1c). In contrast, the plasma ALT level, liver weight and triglyceride content of the liver were significantly lower in the *Irs1*$^{-/-}$ mice than in the WT mice (Figure 1d–f). These results showed that DEN-treated *Irs1*$^{-/-}$ mice on HF diet were dramatically protected against HF diet-induced hepatic steatosis despite showing hyperglycemia and hyperinsulinemia.

DEN Plus HF Diet-Induced Tumorigenesis in *Irs1*$^{-/-}$ Mice

Because *Irs1*$^{-/-}$ mice on a long-term HF diet were protected against hepatic steatosis and tumorigenesis in our previous study[5], we were prompted to evaluate the potential effect of Irs1 on hepatic tumorigenesis in DEN-treated *Irs1*$^{-/-}$ mice on a HF diet. The incidence of DEN plus HF diet-induced hepatic tumors in the WT mice was 71.4% (5/7: multiple tumors in four mice and a single tumor in one mouse; Figure 2a). In contrast, the incidence in *Irs1*$^{-/-}$ mice was just 14.3% (1/7: single tumor in one mouse; Figure 2b). Furthermore, HE staining of liver sections from the WT mice showed diffuse fat droplets (Figure 2c), whereas stained liver sections from the *Irs1*$^{-/-}$ mice showed almost normal liver histology (Figure 2d). The expression levels of genes encoding inflammatory cytokines, such as tumor necrosis factor-α and monocyte chemoattractant protein-1, were significantly decreased in the *Irs1*$^{-/-}$ mice as compared with those in the WT mice (Figure 2e).

DISCUSSION

In the present study, we found that DEN plus HF diet-treated *Irs1*$^{-/-}$ mice were protected from hepatic tumorigenesis as compared with DEN plus HF diet-treated WT mice. These results

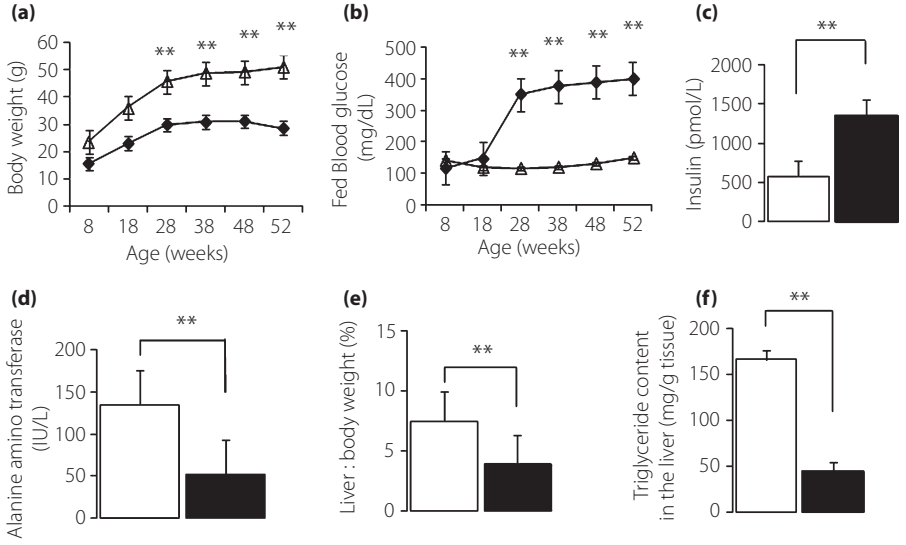

Figure 1 | Metabolic changes in the diethylnitrosamine (DEN)-treated Irs1-knockout (*Irs1*$^{-/-}$) mice on a high-fat (HF) diet. Changes in (a) bodyweight and (b) fed-state blood glucose levels in the DEN-treated wild-type (WT) and *Irs1*$^{-/-}$mice on a HF diet (WT: open triangles, *Irs1*$^{-/-}$: filled diamonds; $n = 7$). (c) Plasma fasting insulin, (d) plasma alanine aminotransferase, (e) ratio of the liver weight to bodyweight and (f) hepatic triglyceride content in the DEN-treated WT and *Irs1*$^{-/-}$ mice on a HF diet (WT: open bar, *Irs1*$^{-/-}$: filled bar; $n = 7$). Values are means ± SE. $^{**}P < 0.01$.

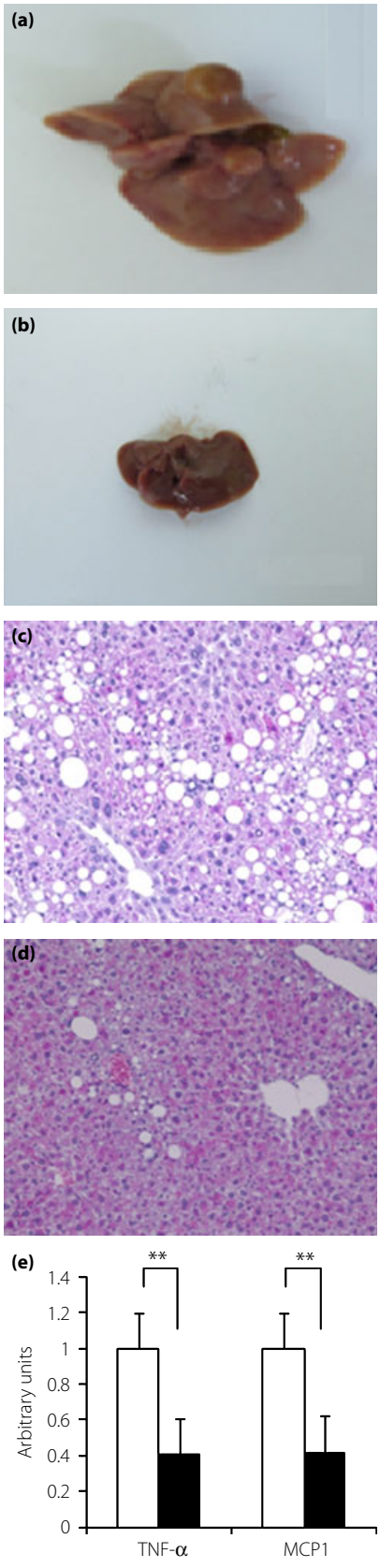

Figure 2 | Diethylnitrosamine (DEN) plus high-fat (HF) diet-induced tumorigenesis in Irs1-knockout (*Irs1*$^{-/-}$) mice. Macroscopic observation in the (a) DEN-treated wild-type (WT) and (b) *Irs1*$^{-/-}$ mice on a HF diet. (c,d) Histopathological findings of the livers from the (c) wild-type (WT) and (d) *Irs1*$^{-/-}$ mice on hematoxylin–eosin stained sections. (e) The messenger ribonucleic acid expression levels of inflammatory cytokine-related genes in the two groups of mice (WT: open bar, *Irs1*$^{-/-}$: filled bar, $n = 4$). Values are means ± SE. $^{**}P < 0.01$. MCP1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α.

suggest that Irs1 might play an important role in DEN plus HF diet-induced hepatic tumorigenesis.

DEN is well-known for its effects of inducing hepatocyte DNA damage and mutation[6], and a HF diet could promote DEN-initiated hepatic tumorigenesis, just like phenobarbital[7,8]. What was the mechanism by which *Irs1*$^{-/-}$ mice were protected from DEN plus HF diet-induced hepatic tumorigenesis? We propose that HF-induced hepatic steatosis and subsequent inflammation in the liver could be related to the pathophysiology of this condition. It has been reported that the steatotic host microenvironment likely sets the stage for tumor development[9], and that the tumor-promoting effect of HF-induced obesity in HCC depends, to a large extent, on the low-grade inflammatory response it induces, which involves elevated production of tumor-promoting cytokines, such as tumor necrosis factor[8]. Indeed, the present results showed that the triglyceride content of the liver was significantly lower in the *Irs1*$^{-/-}$ mice treated with DEN plus HF diet than in the WT mice treated with DEN plus HF diet, and that the expression levels of genes encoding inflammatory cytokines were significantly decreased in the *Irs1*$^{-/-}$ mice treated with DEN plus HF diet as compared with the findings in the WT mice treated with DEN plus HF diet.

Then, why is Irs1 the key factor in DEN plus HF diet-induced hepatic tumorigenesis? Very recently, we showed that *Irs1*$^{-/-}$ mice fed a HF diet were dramatically protected against hepatic steatosis despite exhibiting severe hyperinsulinemia, as compared with the WT mice fed the same diet[5]. From these results, we assumed that the protection against hepatic steatosis in the *Irs1*$^{-/-}$ mice on a HF diet was caused by downregulation of Irs1-mediated insulin actions in the liver[5], as persistent Irs1 signaling promotes lipogenesis and hepatic steatosis[10]. Thus, we suggest that the protective effect on DEN plus HF diet-induced tumorigenesis in *Irs1*$^{-/-}$ mice is probably secondary to the antisteatotic and thereby anti-inflammatory effect.

A limitation of the present study was that it still remained unclear as to what promotes DEN plus HF diet-induced liver tumorigenesis, HF-induced hepatic steatosis or Irs1 signaling. To eliminate this problem, it might be useful to evaluate the effect of the addition of a hepatopromoter, such as phenobarbital. Further study will be required.

In conclusion, we showed that *Irs1*$^{-/-}$ mice were protected against DEN plus HF diet-induced liver tumorigenesis as compared with WT mice. The present study showed that Irs1 played an important role in DEN plus HF diet-induced hepatic tumorigenesis. Thus, blockade of Irs1 signaling might be an effective strategy to prevent hepatic tumorigenesis.

ACKNOWLEDGEMENTS

We thank Mitsuyo Kaji and Eri Sakamoto (Department of Endocrinology and Metabolism, Graduate School of Medicine, Yokohama City University, Japan) for their excellent technical assistance and animal care. This work was supported in part by a Grant-in-Aid for Scientific Research (B) 19390251 and (B) 21390282 from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; a Medical Award from the Japan Medical Association; a Grant-in-Aid from the Uehara Memorial Foundation; a Grant-in-Aid from the Daiichi-Sankyo Foundation of Life Science; a Grant-in-Aid from the Naito Foundation (to YT); and a Grant-in-aid from Yamaguchi Endocrine Research Foundation (to AN). The authors declare that they have no conflicts of interest.

REFERENCES

1. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; 51: 1820–1832.
2. Tamemoto H, Kadowaki T, Tobe K, *et al.* Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 1994; 372: 182–186.
3. Araki E, Lipes MA, Patti ME, *et al.* Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 1994; 372: 186–190.
4. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 2001; 414: 799–806.
5. Nakamura A, Tajima K, Zolzaya K, *et al.* Protection from non-alcoholic steatohepatitis and liver tumourigenesis in high fat-fed insulin receptor substrate-1-knockout mice despite insulin resistance. *Diabetologia* 2012; 55: 3382–3391.
6. Sarma DS, Rao PM, Rajalakshmi S. Liver tumour promotion by chemicals: models and mechanisms. *Cancer Surv* 1986; 5: 781–798.
7. Wang Y, Ausman LM, Greenberg AS, *et al.* Nonalcoholic steatohepatitis induced by a high-fat diet promotes diethylnitrosamine-initiated early hepatocarcinogenesis in rats. *Int J Cancer* 2009; 124: 540–546.
8. Park EJ, Lee JH, Yu GY, *et al.* Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* 2010; 140: 197–208.
9. VanSaun MN, Lee IK, Washington MK, *et al.* High fat diet induced hepatic steatosis establishes a permissive microenvironment for colorectal metastases and promotes primary dysplasia in a murine model. *Am J Pathol* 2009; 175: 355–364.
10. Guo S, Copps KD, Dong X, *et al.* The Irs1 branch of the insulin signaling cascade plays a dominant role in hepatic nutrient homeostasis. *Mol Cell Biol* 2009; 29: 5070–5083.