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ラットを用いた鉄欠乏状態から惹起される肝臓中の異所性脂肪蓄積の影響

Effect of hepatic lipotoxicity induced by iron deficiency in rats.

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要旨：鉄は生体内において必須微量元素であり、重要な栄養素の一つである。鉄欠乏状態はヘモグロビン合成機能障害による酸素運搬能力の低下から、各種代謝異常を誘引することが知られており、特に脂質代謝異常による生体への影響は様々な疾病につながることが予想される。そこで本研究では、鉄欠乏時の代謝異常が生体内に及ぼす影響について検討した。Wistar系雄ラットを用いAIN-93Gを基本組成とし、ミネラル混合中に鉄を含む鉄充足食を与える群（control群）、鉄を除いた鉄欠乏食を与える群（iron-deficiency群）、鉄欠乏食の飼料摂取量に合わせて鉄充足食を与える群（pair-fed群）の3群を設け、5週間の飼育観察を行った。飼育後、全血ならびに肝臓を摘出し各種実験に用いた。血中ヘモグロビン（Hb）、ヘマトクリット（Hct）、中性脂肪（TG）および肝臓TG含量を測定した結果、iron-deficiency群でHb、Hctの低下、血中および肝臓TG含量は高値を示した。また肝臓中の炎症関連因子のmRNA発現量および脂質過酸化生成物であるマロンジアルデヒドを測定した結果、TNF-αにおいてはiron-deficiency群で高値傾向を示したものの大差はなかった。以上の結果から、鉄欠乏状態は肝臓への異所性脂肪蓄積を誘引するものの、その他の影響については確認できなかった。

Abstract: Iron deficiency remains a public health concern affecting the worldwide population. The iron is an important element in a wide variety of biochemical reactions in human and animals. Iron deficiency has a potent influence on various energy production pathways particularly those of oxidative functions and capacity and lipotoxicity causing the development of metabolic disorders, but the pathological mechanism is not defined well. To further characterize the lipotoxicity response to iron-deficiency, male Wister rats were fed either a control or iron-deficient diet or were pair-fed the control diet to the level of intake of the iron-deficiency group for five weeks. In addition to reductions in hemoglobin (Hb) and hematocrit (Hct), the iron-deficient group also exhibited higher serum and hepatic triacylglycerol (TG) compared to the
Iron deficiency and iron-deficiency anemia are global health problems, and although the prevalence of iron-deficiency anemia has recently declined somewhat, iron deficiency continues to be the top-ranking problem worldwide. Iron is crucial in biological functions, including respiration, energy production, DNA synthesis, and cell proliferation. Particularly, intracellular stable energy production, a chemical process in which oxygen is used to make energy from carbohydrates, uses the redox properties of iron. The most of iron utilization in eukaryotes is focused on oxygen metabolism, either as an oxygen carrier or as an electron carrier that can facilitate oxygen-based chemical reactions. In addition, iron is a cofactor for several key enzymes and its loss may negatively affect electron transport systems that are responsible for energy metabolism in mitochondria. However, the biological relevance of the effect of iron deficiency on energy metabolism is still not clear. Perhaps the various metabolic pathway links between glucose, lipid, protein and anemia are complex and not yet fully elucidated.

The laboratory animal models of iron-deficiency have shown increased risk for developing metabolic disease, such as accumulated ectopic fat, hyperglycemia, and hyperlipidemia. In particular, hypertriglyceridemia is commonly reported in iron-deficient animals, there are changes in hepatic TG content in response to iron deficiency and an increased abundance of lipid droplets has been observed in the skeletal muscles of iron-deficient rats. This suggests iron-deficiency increases abundance of lipids in organs what is an important common risk-factor for lipid peroxidation. Moreover, peroxidation of membrane lipids may cause cell necrosis and megamitochondria and the aldehyde products of lipid peroxidation, 4-hydroxynonenal and MDA, may contribute to inflammation by activating NF-κB that is a transcription factor regulating the expression of several proinflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin 8.

The purpose of this study was to clarify the effects of iron deficiency on lipotoxicity and lipid peroxidation, with special reference to rat liver. We investigated MDA levels and inflammation-related TNF-α mRNA expression levels in rats fed an iron-deficient diet.

1. Introduction

2. Materials and Methods

2.1. Animal care and diet

All experimental procedures on rats were approved by the Committee on the Ethics of Animal
Experiments of Kanto-Gakuin University Institutional Animal Care and Use Committee. Three-week-old male Wistar rats (Japan Charles River Inc., Kanagawa, Japan) were categorized into three groups. First, we obtained 16 rats for ad libitum feeding groups. All rats were allowed access to the control diet (30 mg Fe/kg diet), based on the AIN-93G diet (35), for three days before starting dietary treatments. After the acclimation period, rats were randomly assigned to one of two diet groups (n=8, respectively) for five weeks. The dietary manipulations consisted of ad libitum feeding with control diet (control) or iron-deficiency diet (iron-deficiency, 8 mg Fe/kg diet). After one week, eight rats were obtained and assigned to pair-feeding with the control diet group (pair-fed). For pair feeding, each pair-fed rat was paired with an iron-deficiency rat in order of weight-match. Accurate pair feeding was achieved by maintaining equal food intake between the iron-deficiency rat and the pair-fed rat. All of the rats had free access to ion-exchanged water. The rats were fasted for 18 h before evaluation. Body weights were measured at the completion of the feeding period, the rats then were killed, and liver and blood samples were taken. Blood was collected from the tail vein for Hb and Hct measurement. Hb was measured using the Hemoglobin B-test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Centrifugation in a capillary tube system determined Hct. Serum samples were obtained by centrifugation at 3000 rpm for 30 min at 4°C, and used for biochemical measurements.

2.2. Serum and hepatic TG measurements

Serum TG level was determined by the Nagahama Life-science Laboratory of Oriental Yeast Co., Ltd. (Nagahama, Shiga, Japan).

Liver lipids were extracted using Bligh and Dyer method (37). Frozen liver tissue (50 mg) was added to a homogenization tube containing 0.25 mL saline. The pellets were homogenized to form a suspension. Suspension of 0.1 ml was mixed with 0.25 ml saline and was extracted with 3.6 ml of a chloroform/methanol (1:2) mixture and centrifuged at 3000 rpm for 10 min at room temperature. After centrifugation, the lower liquid phase was isolated and transferred to a separate vial. Hepatic TG concentration was then measured using the Triglyceride E-test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.3. RT-PCR analyses of TNF-α

To evaluate the mRNA transcript level of TNF-α in the livers of each group, the total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s instructions. Then, total RNA was random primed with hexamers and reverse-transcribed to cDNA using the SuperScrip III reverse transcription kit (Thermo Fisher Scientific, Waltham, MA). The cDNA product was PCR-amplified by TaKaRa EX Taq (Takara Bio Inc., Shiga, Japan). Primer sequences and PCR conditions are described below: The primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan). The primers were TNF-α, (sense) 5’-CGTCGTAGCAAACCACCAAG-3’ and (antisense) 5’-CAACAGACCTGATGCAGTCCAG-3’ and β-actin, (sense) 5’-GAAGAGCTATGAGCTGCCTG-3’ and (antisense) 5’-AGCAATGCCTGGGTACATGG-3’. PCR amplification was performed with a program of 2 min incubation at 95 °C followed by 30 reaction cycles (denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min). PCR products were analyzed by agarose gel electrophoresis in 3% agarose gels and visualized by GR Red staining. The gels were scanned and band density was quantified using the Image J software (the U.S. National Institutes of Health).

2.4. Measurement of hepatic MDA adducts.

The degree of lipid peroxidation was assessed by measuring MDA adducts in the homogenized liver tissue using the OxiSelect™ MDA Adduct ELISA Kit (Cell Biolabs, San Diego, CA). The MDA values were expressed as pmol/mg of protein.
2.5. Statistical Analysis

All analyses were showed as mean ± standard deviation (SD). Comparisons between groups were made by means of Tukey–Kramer test. When unequal variance occurred between groups, we used the Steel–Dwass test. Software used in this study was JMP 12 (JMP® 12 SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at p < 0.05.

3. Results and Discussion

The body weight gain in the iron-deficiency group was significantly lower (p < 0.05). There was no significant difference of body weight gain between the iron-deficient and pair-fed groups (Table 1). Hb and Hct in the iron-deficient group were significantly lower (p < 0.05) than in the control group (Table 1). An iron-deficient diet decreased Hb concentration and Hct value in this study, suggesting that iron-deficient anemia occurred in rats fed the iron-deficient diet. Additionally, although the pair-fed rats had restricted food intake, their iron levels were almost equal to that of control rats. The serum TG level was significantly higher (p < 0.05) in the iron-deficient group than in the control and pair-fed groups (Table 1). Furthermore hepatic TG level was increased (p < 0.05) in the iron-deficient group than in the pair-fed group (Table 1). Perhaps, iron deficiency induces low energy levels and protects against diet-induced metabolic disorders by enhancing TG levels. These results suggest the hypothesis that iron deficiency induces impairment of carbohydrate metabolism, which can cause adiposopathy and multiple metabolic disorders (to be submitted).

Anemia results in an increase oxidative stress in red blood cells. Additionally, triacylglycerol over storage in hepatocytes is the basis for hepatic lipotoxicity and further, oxidative stress from steatotic hepatocytes leads to lipid peroxidation, impaired mitochondrial and peroxisomal oxidation of fatty acids, and cytokine release. Endotoxin-inducible cytokines, particularly TNF-α, are associated with the pathogenesis of Nonalcoholic Fatty Liver Disease (NAFLD) in experimental animals. Therefore we examined whether hepatic lipotoxicity supported on the iron-deficiency treatment, was resulting in inflammatory responses and MDA accumulation in rat liver. TNF-α mRNA expression indicated a slight increase in the iron-deficient group than in the control and pair-fed groups, hepatic MDA adducts were hardly any different in each group. (Fig. 1a,b). Other studies have found that the increase of lipid peroxidation and hepatic inflammation may be associated with in the degree of fat infiltration in the liver. Our study suggests that MDA accumulation and TNF-α induced inflammatory effect

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body weight gain, Hb, Hct levels, serum TG and hepatic TG of control, pair-fed and iron-deficiency rats.1</th>
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<tr>
<td></td>
<td>control</td>
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<tr>
<td>Body weight gain (g)</td>
<td>280±30</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.8±2.7</td>
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<tr>
<td>Hct (%)</td>
<td>47.1±4.7</td>
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<td>Serum TG (mg/dL)</td>
<td>87±24</td>
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<tr>
<td>Hepatic TG (mg/g liver)</td>
<td>70±36</td>
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1 Values are mean ±SD. a p<0.05 versus control, b p<0.05 versus pair-fed by the Tukey–Kramer test. c p<0.05 versus pair-fed by the Steel–Dwass test.
have not occurred in hepatic cells after five weeks, but long-term continued monitoring is required to determine this more precisely.

In our study, serum TG and hepatic TG levels were significantly increased in rats treated with iron deficiency. The presumed mechanism for this effect is the reduced oxygen transport associated with anemia, and energetic efficiency is affected at all levels of iron deficiency in human and animals. Our data suggest that several fatty hepatic diseases may be due, in part, to inadequate dietary iron. Further, tissue iron deficiency may also induce lipotoxicity through reduced cellular oxidative capacity. We analyzed mRNA expression of enzymes associated with energy-metabolism isolated from each group. The results showed hardly any difference in each enzyme mRNA expression (data not shown). For all of the diseases that contribute to iron-deficiency anemia has yet to be elucidated, it appears the possibility that iron deficiency anemia may extend to unexpected diseases.

4. Conclusion

Increased serum and hepatic TG levels in iron-deficient rats suggest the possibility of fatty liver diseases as lipotoxicity. However, no alteration was found, at least in hepatic MDA levels and the expression levels of hepatic TNF-α mRNA.

Fig. 1  The TNF-α mRNA expression and MDA adducts in rat liver.
Values are mean±SD. a. RT-PCR analysis. TNF-α mRNA levels normalized for β-actin mRNA levels were expressed as relative ratio compared with the control group. b. Level of MDA adducts.
5. Acknowledgments

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6. References

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ラットを用いた鉄欠乏状態から惹起される肝臓中の異所性脂肪蓄積の影響


