in mice revealed obesity phenotype but amelioration of insulin resistance [12]. These results suggest that expression levels of lipin-1 in adipocytes closely and positively correlate with whole glucose metabolism. To elucidate the mechanism by which obesity-related lipin-1 suppression in adipose tissue may become a clue to a new target for treating obesity and insulin resistance.

Since TNF-α plays pivotal and exacerbating roles in adipocytes functions in obesity [14], we made to elucidate whether TNF-α may be implicated in decreased lipin-1 expression in adipocytes. We used fully differentiated 3T3-L1 adipocytes as a model of adipocytes in the present study. Since there are functional differences between lipin-1A and -1B [3,8], we analyzed both lipin-1A and -1B isoforms throughout in this study. As clearly demonstrated in this study, TNF-α decreased lipin-1A and -1B expression in 3T3-L1 adipocytes in dose- and time-dependent manners. Very recently, Lu et al. [22] have reported in harmony with our result that TNF-α decreased lipin-1 expression in 3T3-L1 adipocytes. However, they have not mentioned the mechanism by which TNF-α decreased lipin-1 expression. Therefore we next tried to clarify the molecular mechanism by which TNF-α decreased lipin-1 expression in 3T3-L1 adipocytes.

Nuclear factor-kappa B (NF-κB) acts as a key transcription factor for many biological actions induced by TNF-α [14]. TNF-α suppresses various genes in 3T3-L1 adipocytes including insulin signal molecules. Ruan et al. [23] demonstrated that inactivation of NF-κB abolished the suppression of >98% of the genes normally suppressed by TNF-α through the expression of a non-degradable mutant of NF-κB inhibitor, IκBα-DN. We therefore tried to find the possibility that NF-κB could be involved in the suppressive effect of TNF-α on lipin-1 expression by using SN50, a cell-permeable inhibitory peptide for NF-κB. SN50 failed to block the inhibition of lipin-1 mRNA expression by TNF-α, indicating that NF-κB dose not play a role in the mechanism.

MAPKs are activated by TNF-α in phosphorylation cascades and will eventually phosphorylate and activate distinct sets of kinases and transcription factors [14]. Therefore, we tested the possibility that MAPKs influenced decreased lipin-1 expression by TNF-α. The possibility is unlikely because each MAPK inhibitor did not block the suppressive effect of TNF-α on lipin-1 mRNA expression.

Ceramide is an intracellular lipid and acts as an intermediate molecule linking TNF-α to cellular insulin resistance by inhibiting insulin signalings [24]. Because TNF-α is known to increase intracellular ceramide, we hypothesized that ceramide may be involved in the decreased lipin-1 expression by TNF-α. We also used desipramine to evaluate an intrinsic sphingomyelin–ceramide signaling. The present results provided that $C_2$-Ceramide itself did not decrease both the lipin-1 expression and desipramine did not reverse the suppressive effect of TNF-α on the lipin-1 expression, suggesting ceramide pathway is not involved in the TNF-α action on lipin-1 expression.

Recently, canonical Wnt/β-catenin/TCF4 signaling has been involved in the inhibition of adipogenesis by TNF-α [25]. We applied the concept that TNF-α affects β-catenin signaling to make a hypothesis that this signaling might play a role in TNF-α-induced lipin-1 mRNA suppression. Because GSK-3β phosphorylates β-catenin and targets it for ubiquitin-mediated degradation, inhibition of GSK-3β enhances β-catenin/TCF4 pathway through accumulation of β-catenin [26]. If this pathway is involved in the inhibitory action of TNF-α on lipin-1 expression, inhibition of GSK-3β reduces lipin-1 expression similarly to the action of TNF-α, or may enhance the action of TNF-α. As demonstrated in this study, inhibition of GSK-3β alone or in combination with TNF-α did not affect the lipin-1 expression, suggesting that β-catenin pathway is not involved in the suppressive effect of TNF-α on lipin-1 expression.

Janus tyrosine kinase (Jak) family including Jak1, Jak2, Jak3 and Tyr2 is a receptor associated kinase and has been activated by various cytokines (e.g., erythropoietin, interleukin (IL)-3, and interferon (IFN)-γ). In 3T3-L1 adipocytes, Guo et al. [17] reported that murine TNF-α induces tyrosine phosphorylation and activation of the intracellular Jak1, Jak2 and Tyr2. Because TNF-α affects mostly on Jak2 in the report, we investigated whether Jak2 could be involved in the lipin-1 suppression by TNF-α. The present study showed that AG490, a Jak2 inhibitor, clearly reversed the TNF-α-induced suppression of both the lipin-1A and -1B expression (Fig. 4A, B). Lu et al. [22] have reported that IL-1β and IFN-γ in addition to TNF-α suppressed lipin-1 expression in adipocytes. Because IFN-γ activates Jak2 signaling [27], involvement of Jak2 as showed in this study should be a pivotal role in the mechanism. The recovery level of lipin-1B was weaker than that of lipin-1A. There might be some additional signals involving in the suppression of lipin-1B. Because lipin-1A and -1B are produced from same lipin-1 gene by alternative splicing, such lipin-1B specific signals might affect lipin-1 gene expression including mRNA splicing mechanism.

The precise roles of Jak2 in glucose and lipid metabolism have not been elucidated, although Jak is known as the intracellular signaling molecule under stimulation of leptin [28] and growth hormone [29]. Thirone et al. [30] reported that siRNA-mediated gene knock-down for Jak2 in L6 skeletal muscle cells relieves insulin resistance induced by ceramide or knock-down for insulin receptor substrate-1. The report concluded that inhibition of Jak2 might be useful strategy to relieve insulin resistance of metabolic outcomes. Because amelioration of insulin resistance is thought to be obtained by the recovery of TNF-α-induced lipin-1 suppression by inhibiting Jak2 revealed in this study, there is a same metabolic direction between our and their results in the meaning of Jak2 to insulin resistance.

Activation of Jak2 by TNF-α is accompanied by the tyrosine phosphorylation of members of the STAT (signal transducers and activators of transcription) family of transcription factors and the induction of STAT DNA-binding activity [27]. This raises the possibility that STAT may be involved in the reduced lipin-1 expression by TNF-α at transcriptional level. Further investigation is needed to be elucidated the precise involvement of Jak/STAT pathway in this mechanism.

Great majority of the suppressed genes by TNF-α have been mediated by NF-κB [23]. In this regard, mediation of Jak2 not NF-κB pathway for the suppression of lipin-1 gene expression by TNF-α is considered as a rare pathway among the overall gene suppressing effects of TNF-α. This rarity may take advantage of quest for specific means to recover lipin-1 expression in terms of not interfering with other common signalings of TNF-α.

In summary, our data showed that an obesity-related cytokine, TNF-α, reduces both the lipin-1A and lipin-1B mRNA expression. Our novel findings are Jak2 signaling could be involved in decreased lipin-1A and -1B mRNA expression induced by TNF-α. These findings suggest that Jak2 signaling could be a target for preventing adipose lipin-1 depletion, connecting to the new treatment for obesity.

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## References

[1] M. Péterfy, J. Phan, P. Xu, K. Reue, Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin, Nat. Genet. 27 (2001) 121–124.
[2] J. Donkor, M. Sariahmetoglu, J. Dewald, D.N. Brindley, K. Reue, Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns, J. Biol. Chem. 282 (2007) 3450–3457.

[3] M. Péterfy, J. Phan, K. Reue, Alternatively spliced lipin isoforms exhibit distinct expression pattern, subcellular localization, and role in adipogenesis, J. Biol. Chem. 280 (2005) 32883–32889.
[4] J. Phan, M. Péterfy, K. Reue, Lipin expression preceding peroxisome proliferator-activated receptor-gamma is critical for adipogenesis in vivo and in vitro, J. Biol. Chem. 279 (2004) 29558–29564.
[5] B.N. Finck, M.C. Gropler, Z. Chen, T.C. Leone, M.A. Croce, T.E. Harris, J.C. Lawrence Jr., D.P. Kelly, Lipin 1 is an inducible amplifier of the hepatic PGC-1alpha/PPARalpha regulatory pathway, Cell Metab. 4 (2006) 199–210.
[6] Z. Chen, M.C. Gropler, J. Norris, J.C. Lawrence Jr., T.E. Harris, B.N. Finck, Alterations in hepatic metabolism in fld mice reveal a role for lipin 1 in regulating VLDL-triacylglyceride secretion, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1738–1744.
[7] M.L. Reitman, The fat and thin of lipin, Cell Metab. 1 (2005) 5–6.
[8] K. Reue, P. Zhang, The lipin protein family: dual roles in lipid biosynthesis and gene expression, FEBS Lett. 582 (2008) 90–96.
[9] A. Yao-Borengasser, N. Rasouli, V. Varma, L.M. Miles, B. Phanavanh, T.N. Starks, J. Phan, H.J. Spencer 3rd., R.E. McGehee Jr., K. Reue, P.A. Kern, Lipin expression is attenuated in adipose tissue of insulin-resistant human subjects and increases with peroxisome proliferator-activated receptor gamma activation, Diabetes 55 (2006) 2811–2818.
[10] M.A. Croce, J.C. Eagon, L.L. LaRiviere, K.M. Korenblat, S. Klein, B.N. Finck, Hepatic lipin 1beta expression is diminished in insulin-resistant obese subjects and is reactivated by marked weight loss, Diabetes 56 (2007) 2395–2399.
[11] V. van Harmelen, M. Rydén, E. Sjölin, J. Hoffstedt, A role of lipin in human obesity and insulin resistance. relation to adipocyte glucose transport and GLUT4 expression, J. Lipid Res. 48 (2007) 201–206.
[12] J. Phan, K. Reue, Lipin, a lipodystrophy and obesity gene, Cell Metab. 1 (2005) 73–78.
[13] G.S. Hotamisligil, N.S. Shargill, B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance, Science 259 (1993) 87–91.
[14] W.P. Cawthorn, J.K. Sethi, TNF-α and adipocyte biology, FEBS Lett. 582 (2008) 117–131.
[15] N. Takahashi, Y. Qi, H.R. Patel, R.S. Ahima, A novel aminosterol reverses diabetes and fatty liver disease in obese mice, J. Hepatol. 41 (2004) 391–398.
[16] Y.Z. Lin, S.Y. Yao, R.A. Veach, T.R. Torgerson, J. Hawiger, Inhibition of nuclear translocation of transcription factor NF-kB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence, J. Biol. Chem. 270 (1995) 14255–14258.
[17] D. Guo, J.D. Dunbar, C.H. Yang, L.M. Pfeffer, D.B. Donner, Induction of Jak/STAT signaling by activation of the type 1 TNF receptor, J. Immunol. 160 (1998) 2742–2750.
[18] N. Meydan, T. Grunberger, H. Dadi, M. Shahar, E. Arpaia, Z. Lapidot, J.S. Leeder, M. Freedman, A. Cohen, A. Gazit, A. Levitzki, C.M. Roifman, Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor, Nature 379 (1996) 645–648.
[19] H.C. Chen, S.J. Smith, Z. Ladha, D.R. Jensen, L.D. Ferreira, L.K. Pulawa, J.G. McGuire, R.E. Pitas, R.H. Eckel, R.V. Farese Jr., Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1, J. Clin. Invest. 109 (2002) 1049–1055.
[20] S. Neschen, K. Morino, L.E. Hammond, D. Zhang, Z. Liu, A.J. Romanelli, G.W. Cline, R.L. Pongratz, X. Zhang, C.S. Choi, R.A. Coleman, G.I. Shulman, Prevention of hepatic steatosis and hepatic insulin resistance in mitochondrial acyl-CoA:glycerol-*sn*-3-phosphate acyltransferase 1 knockout mice, Cell Metab. 2 (2005) 55–65.
[21] N. Takahashi, M. Nagamine, S. Tanno, W. Motomura, Y. Kohgo, T. Okumura, A diacylglycerol kinase inhibitor, R59022, stimulates glucose transport through a MKK3/6-p38 signaling pathway in skeletal muscle cells, Biochem. Biophys. Res. Commun. 360 (2007) 244–250.
[22] B. Lu, Y. Lu, A.H. Moser, J. Shigenaga, C. Grunfeld, K.R. Feingold, LPS and proinflammatory cytokines decrease lipin-1 in mouse adipose tissue and 3T3-L1 adipocytes, Am. J. Physiol. Endocrinol. Metab. 295 (2008) E1502–1509.
[23] H. Ruan, N. Hacohen, T.R. Golub, L. Van Parijs, H.F. Lodish, Tumor necrosis factor-α suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kB activation by TNF-α is obligatory, Diabetes 51 (2002) 1319–1336.
[24] W.L. Holland, S.A. Summers, Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism, Endocr. Rev. 29 (2008) 381–402.
[25] W.P. Cawthorn, F. Heyd, K. Hegyi, J.K. Sethi, Tumour necrosis factor-α inhibits adipogenesis via a β-catenin/TCF4(TCF7L2)-dependent pathway, Cell Death Differ. 14 (2007) 1361–1373.
[26] C.N. Bennett, S.E. Ross, K.A. Longo, L. Bajnok, N. Hemati, K.W. Johnson, S.D. Harrison, O.A. MacDougald, Regulation of Wnt signaling during adipogenesis, J. Biol. Chem. 277 (2002) 30998–31004.
[27] C. Schindler, D.E. Levy, T. Decker, JAK-STAT signaling: from interferons to cytokines, J. Biol. Chem. 282 (2007) 20059–20063.
[28] R.S. Ahima, S.Y. Osei, Leptin signaling, Physiol. Behav. 81 (2004) 223–241.
[29] H. Eriksson, M. Ridderstråle, H. Tornqvist, Tyrosine phosphorylation of the growth hormone (GH) receptor and Janus tyrosine kinase-2 is involved in the insulin-like actions of GH in primary rat adipocytes, Endocrinology 136 (1995) 5093–5101.
[30] A.C.P. Thirone, L. JeBailey, P.J. Bilan, A. Klip, Opposite effect of JAK2 on insulin-dependent activation of mitogen-activated protein kinases and Akt in muscle cells: possible target to ameliorate insulin resistance, Diabetes 55 (2006) 942–951.

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# Body iron metabolism and pathophysiology of iron overload

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**Abstract** Iron is an essential metal for the body, while excess iron accumulation causes organ dysfunction through the production of reactive oxygen species. There is a sophisticated balance of body iron metabolism of storage and transport, which is regulated by several factors including the newly identified peptide hepcidin. As there is no passive excretory mechanism of iron, iron is easily accumulated when exogenous iron is loaded by hereditary factors, repeated transfusions, and other diseased conditions. The free irons, non-transferrin-bound iron, and labile plasma iron in the circulation, and the labile iron pool within the cells, are responsible for iron toxicity. The characteristic features of advanced iron overload are failure of vital organs such as liver and heart in addition to endocrine dysfunctions. For the estimation of body iron, there are direct and indirect methods available. Serum ferritin is the most convenient and widely available modality, even though its specificity is sometimes problematic. Recently, new physical detection methods using magnetic resonance imaging and superconducting quantum interference devices have become available to estimate iron concentration in liver and myocardium. The widely used application of iron chelators with high compliance will resolve the problems of organ dysfunction by excess iron and improve patient outcomes.

**Keywords** Hemochromatosis · Hepcidin · Iron metabolism · Iron overload · Non-transferrin-bound iron (NTBI)

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## 1 Introduction

Iron is an essential metal for hemoglobin synthesis of erythrocytes, oxidation–reduction reactions, and cellular proliferation, whereas excess iron accumulation causes organ dysfunction through the production of reactive oxygen species (ROS). The total amount of body iron is approximately 3–4 g, two-thirds of which is composed of red blood cell (RBC) iron and recycled iron by RBC destruction; the remainder is stored in ferritin/hemosiderin, while only 1–2 mg of iron are absorbed in the intestinal tract and circulated in the blood [1]. Body iron metabolism is a semi-closed system, and is critically regulated by several factors including the newly identified peptide hepcidin. In the circulation, iron is usually bound to transferrin (Tf), and most of the Tf-bound iron is utilized for bone marrow erythropoiesis [1]. As there is no active mechanism to excrete iron from the body, a progressive accumulation of body iron easily occurs as a result of long-term transfusions in patients with anemia of genetic disorders such as thalassemia, sickle cell disease (SCD), and Diamond Blackfan syndrome, and of bone-marrow failures such as aplastic anemia (AA) and myelodysplastic syndromes (MDS). In order to consider pathophysiological mechanisms of organ injury by iron overload, an understanding of molecular mechanisms of body iron metabolism is essential.

**Table 1** Molecules involved in body iron metabolism

| |
|---|
| **Molecules for intestinal iron absorption** |
| Divalent metal transporter 1 (DMT1) |
| Duodenal cytochrome *b* (Dcytb) |
| Heme carrier protein (HCP) |
| Hemeoxygenase-1 |
| Ferroportin |
| Hephaestin |
| Transferrin |
| **Molecules for bone marrow iron uptake** |
| Transferrin receptor 1 |
| Transferrin |
| **Molecules for reutilization of senescent red blood cells** |
| Hemeoxygenase-1 |
| Ferroportin |
| Transferrin |
| **Molecules for hepatic iron storage** |
| Ferritin |
| Hemosiderin |
| Transferrin |
| Transferrin receptor 1 |
| Transferrin receptor 2 |
| Non-transferrin-bound iron |
| HFE |
| $\beta$2-microglobin |
| Divalent metal transporter 1 |
| ZIP14 |
| Hemojuvelin |
| **Molecules for systemic iron regulation** |
| Hepcidin |
| (Unknown erythroid regulator?) |

## 2 Molecular mechanisms of body iron metabolism

Table 1 shows a list of molecules involved in body iron metabolism, categorized as functions including intestinal absorption, erythroid iron uptake, reutilization of senescent RBCs, hepatic iron storage, and systemic regulation.

### 2.1 Intestinal iron absorption

Ingested iron is classified as non-heme iron and heme iron. Non-heme iron derived from plants is mainly composed of inorganic ferric Fe(III) iron, and is absorbed into enterocytes through the divalent metal transporter 1 (DMT1) after reduction of Fe(III) to Fe(II) by duodenal cytochrome *b* [2, 3]. In contrast, heme-iron derived from meat is absorbed through a heme carrier protein into enterocytes, where it is degraded by hemeoxygenase-1 (HO-1). Iron within enterocytes is then transferred from the luminal to the vascular site of the cell, and released into the circulation via the metal transporter, ferroportin in the form of Fe(II). Excreted Fe(II) is thereafter oxidized to Fe(III) by hephaestin, a homolog of ceruloplasmin, and the resulting ferric iron is bound to serum Tf [4].

### 2.2 Red blood cell iron reutilization in the reticuloendothelial system (RES) and iron load by blood transfusion

The average life span of circulating RBCs is approximately 120 days, indicating that 20 mg of iron derived from 20 ml of RBCs are processed by RES/macrophages on a daily basis. Within macrophages, heme derived from phagocytized RBCs is catabolized by HO-1, and free iron is released.

Intra-cellular iron is released into the circulation via ferroportin, and the iron is donated to Tf and reutilized for bone marrow erythropoiesis.

In patients with genetic anemias and bone marrow failures, regular transfusion is required in order to overcome the intractable symptoms. Transfused RBCs are taken up and degraded by RES/macrophages, in which the recycled iron is overloaded and the excess iron saturates the binding capacity of Tf. This excess iron appears in the circulation as a form of non-Tf-bound iron (NTBI) [1, 5], and causes organ dysfunction by the production of ROS. One milliliter of blood contains approximately 0.5 mg of iron, and there is no active mechanism for excretion of this excess iron. In Japan, one unit of blood corresponds to 200 ml of whole blood or 140 ml of concentrated RBCs, both of which contain approximately 100 mg of iron. As the critical level of iron overload at which organ dysfunction occurs in the liver is approximately 7 mg/g dry liver weight [6], according to the formula derived by Angelucci [body iron accumulation (mg/kg) = liver iron concentration (LIC; mg/g dry weight $\times$ 10.6)] [7], only 40 Japanese units of transfusion are required to reach this level.

### 2.3 Iron uptake and utilization in liver

The liver is a major storage organ of iron, in which excess iron is stored as ferritin and hemosiderin. In addition to these proteins, an additional fraction of free iron is present in the form of the labile iron pool (LIP) within cells. The LIP is biologically active in intracellular metabolism via oxidation–reduction reactions, cell proliferation, and cell signaling, but is toxic if present in excess. As shown in Fig. 1, hepatocytes have essentially two pathways for uptake of iron from the circulation: Tf-bound iron ($Fe_2$-Tf) at physiological iron concentrations, and NTBI in iron overload conditions [3].

Concerning the uptake of $Fe_2$-Tf, there are three pathways involved: two are dependent on and one is independent of transferrin receptor (TfR) recycling.

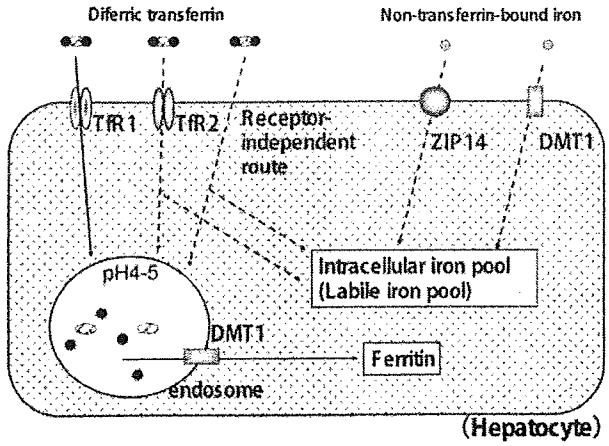


**Fig. 1** Routes for iron uptake by hepatocytes. Hepatocytes have several pathways for iron uptake from the circulation. Concerning uptake of Tf-bound iron ($Fe_2$-Tf) at physiological concentrations, there are three pathways involving TfR1, TfR2, and TfR-independent mechanisms. The pathway via TfR1 is a classical one and is well elucidated. When serum $Fe_2$-Tf binds to TfR1, the $Fe_2$Tf-TfR1 complex is internalized by endocytosis, and iron is released within the endosome when endosomal pH is acidic. The resulting apotransferrin-TfR1 complex is then recycled back to the cell surface for re-utilization. Released iron into the endosome is transferred to the cytoplasm by DMT1; the resulting cytoplasmic free iron is used for iron-related biological functions, and the rest of the iron is stored as ferritin. In addition to TfR1, TfR2 and the mechanism that is independent of TfR1 and TfR2, are also considered to be important routes for iron uptake in hepatocytes, but the details of these routes remain to be elucidated. Concerning the hepatic uptake of NTBI, which is present in the serum during conditions of iron overload, DMT1 and ZIP14 are considered to be involved

Transferrin receptor 1 (TfR1) is a classical functional receptor, expressed highly in erythroblasts, but less so in hepatocytes. When serum $Fe_2$-Tf binds to TfR1, $Fe_2$-Tf is internalized by endocytosis. Internalized $Fe_2$Tf-TfR1 complexes within the endosome release iron when endosomal pH is acidified. The resulting apotransferrin-TfR1 complex is then recycled back to the cell surface for re-utilization. Transferrin receptor 2 (TfR2), a new homolog of TfR1, is ubiquitously expressed on hepatocyte surfaces and possesses a similar mechanism of recycling, but the binding affinity is rather weak: the functional role of TfR2 for cellular iron uptake is still obscured. In hepatocytes, there is another $Fe_2$-Tf uptake mechanism that is independent of TfR recycling, which is also considered to be important [8].

In iron-overloaded conditions, NTBI appears in the circulation and is taken up through two molecules such as DMT1 and ZIP14 on hepatocytes [9].

### 2.4 Bone marrow iron metabolism and erythropoiesis

Bone marrow erythroblasts require large amounts of iron for hemoglobin synthesis. TfR1 is strongly expressed in erythroblasts and functions as the uptake system of extracellular $Fe_2$-Tf. Within erythroblasts, iron is transferred to mitochondria and is incorporated into the center of the heme ring, which is synthesized by condensation of $\delta$-aminolevulinic acid, a product made by erythroid $\delta$-aminolevulinic acid synthase (eALAS). It is noteworthy that the synthesis of eALAS is also regulated by an iron-responsive-element binding protein (IRP) as well as TfR1 [10]. It is well known that genetic abnormalities of this pathway cause the phenotype of ringed sideroblastic anemias [11].

### 2.5 Systemic regulation of body iron metabolism

It has been postulated for a long time that a soluble factor acts to synchronize body iron metabolism between different organs. Recently, a basic peptide called hepcidin, an antimicrobial purified from urine, was found to have this role [12]. Hepcidin is considered to be a negative regulator that inhibits both intestinal iron absorption and reticulo-endothelial iron release. It is mainly synthesized in the liver, in which production is enhanced during iron overload and inflammation [13]. In some patients with genetic hemochromatosis, an abnormality of *hepcidin* gene has been reported. In these patients, hepcidin production was suppressed and iron absorption increased [14]. Furthermore, hepcidin expression is also down-regulated even in patients without a genetic abnormality of hepcidin. These reports strongly suggest that hepcidin plays an important role in tissue iron deposition in many iron-overloaded conditions including HFE hemochromatosis [15]. Currently, several additional molecules such as TfR2 and hemojuvelin (HJV) are also known to be involved in its regulation [16]. Furthermore, it is becoming clear that there is a role for hepcidin even in secondary iron overload. In a mouse model of $\beta$-thalassemia, representing ineffective erythropoiesis, there is an upregulation of hepcidin and a down-regulation of ferroportin, explaining how hepcidin also contributes to the formation of secondary hemochromatosis associated with ineffective erythropoiesis [17].

## 3 Forms of iron in serum and tissue

As free iron is extremely toxic to cells, the body has a number of protective mechanisms with which to bind iron in various tissue compartments. In serum, iron is usually bound to Tf, but some is present as NTBI when iron concentration exceeds the iron binding capacity of plasma Tf. It is also noted that ferritin is present in serum, although its biological role in iron transport is unclear.

### 3.1 Iron in plasma: Tf-bound iron and non-Tf-bound iron (NTBI)

It is well known that plasma Tf is capable of binding and transporting ferric iron to cells via TfRs. The binding capacity of Tf to inorganic iron is very strong, and this characteristic behavior prevents iron from existing in its free form under normal physiological conditions. As the Tf saturation in normal physiological conditions is up to 35%, this suggests that there is sufficient capacity to prevent the release of free toxic iron into the circulation [18]. However, when the iron binding capacity of Tf is saturated in the iron-overloaded state, an additional iron compartment NTBI, appears in the circulation. This compartment is biologically more toxic than Tf-bound iron. Among the NTBI fractions, labile plasma iron (LPI) is the most toxic. Unlike Tf-bound iron, the cellular uptake of NTBI is not dependent on the TfR, and therefore the resulting iron is diffusely distributed throughout the organs, independent of the presence of the TfR [5, 19]. Unlike serum iron, TIBC and percent-Tf-saturation measurements, the inter-institutional difference of NTBI and LPI measurements are too great and these parameters have not been standardized.

### 3.2 Iron in tissue: tissue ferritin and labile iron pool (LIP)

Within cells, iron is stored in the proteins ferritin or hemosiderin. Ferritin is a cytoplasmic protein consisting of 25 heterodimeric subunits of H and L that stores iron as ferric hydroxide phosphate in a controlled manner. Each molecule can store up to 4,500 Fe(III) within the protein shell [20], and release greater quantities of iron when the body is iron deficient. Most ferritin is present in liver, spleen, and bone marrow, and a trace amount is found in the blood as serum ferritin. It is noteworthy that the synthesis of ferritin is post-transcriptionally regulated by the cytoplasmic transacting factor IRP. IRP activates ferritin synthesis when iron is excess in the cell [21]. This adaptive response is important for preventing cells from free iron toxicity.

In addition to ferritin iron, LIP is present within cells in order to facilitate biological actions involving iron atoms, and can become cytotoxic or carcinogenic when the concentration exceeds the protective capacity of ferritin. Most of the LIP is free ferric iron bound to citrate or adenosine diphosphate, and a small amount of LIP is reduced to ferrous iron, which is responsible for oxidation–reduction reactions and the Fenton reaction. Iron toxicity is developed thorough the production of ROS.

### 3.3 Serum ferritin

In 1972, Jacobs et al. [22, 23] in the UK reported that ferritin was also present in serum, although its amount was very low. By quantitative phlebotomy, it was found that serum ferritin (SF) correlated with total body iron stores. Although it is still not clear how SF is produced, it is the most convenient laboratory test available to estimate body iron stores at the present time. However, the level of SF is also affected by acute and chronic inflammation and infections. Therefore, data should be interpreted carefully when using SF as a biological marker for evaluation of body iron stores, as shown in Table 2. There is a difference between the standard values of SF concentration in males and females (normal range 10–220 μg/L in males; 10–85 μg/L in females). It is clear that low SF values less than

**Table 2** Considerations needed to use serum ferritin as a biological marker for the evaluation of body iron store

| | |
|---|---|
| ● | **There is a sex difference of standard values of serum ferritin concentration** |
| ✓ | Male: 10–220 μg/L, Female: 10–85 μg/L |
| ● | **Serum ferritin will be increased in various clinical conditions other than iron overload** |
| ✓ | Chronic inflammation (effect of inflammatory cytokines) |
| ✓ | Chronic liver damage (release from destroyed hepatocytes) |
| ✓ | Malignancies (release from destroyed tumors) |
| ✓ | The conditions needed to be considered for differential diagnosis dependent on the value of serum ferritin |
| | **Slight elevation (250–500 μg/L)** |
| | Malignancies, chronic liver damage, chronic inflammation, mild iron overload |
| | **Mild elevation (500–1000 μg/L)** |
| | Early stage of iron overload, ineffective erythropoiesis (thalassemia, etc) |
| | The frequency of the conditions except iron overload decreases |
| | **Moderate elevation (1000–5000 μg/L)** |
| | Iron overload, Adult Still's disease, hemophagocytic syndrome |
| | **Severe elevation (more than 5000 μg/L)** |
| | Iron overload (hemochromatosis) |

12 μg/L are usually representative of body iron deficiency. On other hand, patients with SF levels that are higher than the normal range may be indicative of conditions such as iron overload, inflammation, collagen disease, malignancy, and hepatic diseases [24]. This characteristic feature of the SF assay is considered to be a disadvantage for monitoring iron overload. Especially in Japan, the significance of SF as an inflammation marker has been over-stressed because there are few patients with hereditary hemochromatosis showing significantly high values of more than a couple of thousand or ten thousand microgram per liter.

Systemic measurements of SF in various diseases were conducted mainly in the late 1970s, just after the development of this assay, and it was found that AA and sideroblastic anemia patients who had received blood transfusions had SF levels of more than 1,000 μg/L, whereas patients without transfusions had lower levels. These old data have suggested previously that anemic patients who had ineffective erythropoiesis without transfusion support could maintain their SF levels at values less than 1,000 μg/L, even though adaptive increases in intestinal iron absorption were noted [25]. Therefore, the interpretation of the value of SF for the assessment of body iron status is simplified if other clinical conditions such as inflammation and malignancy are excluded by other modalities. The clinical studies concerning the relationship between blood transfusion and SF have been conducted mainly in the Europe and US, showing that there is a clear-cut positive correlation between the amount of chronic blood transfusion and the elevation of SF in patients with β-thalassemia [26, 27]. Furthermore, the concentration of heart iron is increased when SF levels become greater than 1,800 μg/L, and the prevalence of cardiac events is significantly increased when SF levels are more than 2,500 μg/L [6, 28]. Similar results concerning the relationship between SF and organ dysfunction of liver and heart were shown in a Japanese retrospective study in transfusion-dependent patients with bone-marrow-failure syndromes [29]. In this study, 90% of patients with either cardiac or hepatic complications had high SF levels of more than 1,000 μg/L. Coincidentally, this level of SF also represents the threshold of the target value at which iron chelation therapy should be initiated in patients with transfusion iron overload, according to the guidelines of the International MDS Symposium [30].

## 4 Measurement of body iron stores: comparison with serum ferritin

Direct and indirect methods are available for the estimation of body iron. As previously mentioned, the measurement of SF is the most convenient and cost-effective technique, although other factors can also influence its value. There is no argument that the gold standard for iron determination is direct tissue iron determination. Notably, other methods that are becoming increasingly important include physical methods such as the superconducting quantum-interference device (SQUID) and magnetic resonance imaging (MRI).

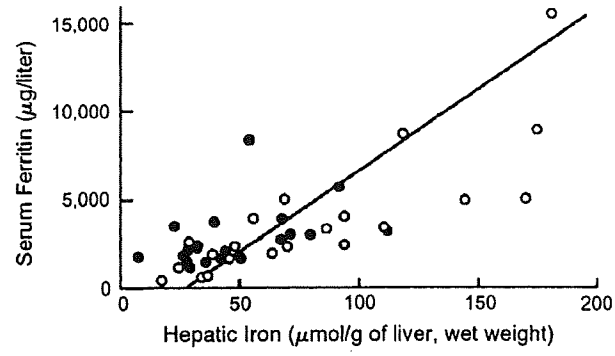


**Fig. 2** Comparison of hepatic iron and serum ferritin concentrations. Indirect estimation is compared with the reference method, based on the direct measurement of hepatic iron levels by chemical analysis or magnetic-susceptibility studies. *Open circles* denote the values at the start of the trial (before deferiprone therapy), and *solid circles* the values at the time of the final analysis. The *diagonal line* denotes the simple linear least-squares regression between the two variables. (From [31]. Reproduced with permission. Olivieri NF et al. N Engl J Med. 1995;332:918–22. Copyright ©1995 Massachusetts Medical Society. All rights reserved)

### 4.1 Direct measurement

Liver is the major organ for iron storage and has the largest capacity to store excess iron. The measurement of hepatic iron concentration by liver biopsy is the most reliable means to assess body iron storage; however, this procedure is invasive and cannot be used in all cases [7]. Figure 2 compares the indirect estimation of body iron based on serum ferritin and LIC. Open circles denote the values at the start of the trial (before treatment with deferiprone), and solid circles denote the values at the time of the final analysis. The correlation between these measurements was significant ($R = 0.73$; $P < 0.005$) [31]. Concerning the determination of cardiac iron deposition, myocardial biopsy can be used; however, this procedure is not often conducted without special experimental reasons due to its high technical risk.

In patients with β-thalassemia, there is a correlation between LIC and cumulative amounts of RBC transfusions [26] and the risk of organ dysfunction is enhanced when LIC values are greater than 7 mg/kg wet tissue, and LIC levels of over 15 mg/kg wet tissue increase the risk of early cardiac death due to iron deposition in the myocardium [6]. Studies in the deferasirox clinical development program in β-thalassemia also demonstrated a correlation between the reduction in LIC and SF values ($R = 0.63$).

### 4.2 Physical measurement of body iron

As iron is one of the heavy metals, an increased concentration of biological iron consisting of ferritin and hemosiderin can be detected by body imaging procedures. Until recently, abdominal echograms and computed tomography (CT) produced images at high iron concentrations, although these two modalities are not quantitative and are only capable of detecting iron overload under conditions of extremely high iron deposition [32]. Recently, quantitative procedures such as SQUID [33] and MRI have been introduced, which use the physical characteristics of iron. However, SQUID apparatus is only available in a couple of institutions in the Europe and US because of its cost. On other hand, LIC determinations by MRI are widely available. This method utilizes the specific characteristic of iron that shortens T1, T2, and T2* relaxation times. The measurable range of iron concentration by R2 (in a 1.5-T MRI magnet) is 0.3–42.7 mg Fe/g dry tissue, which covers the concentrations observed in iron-overloaded livers.

In addition to LIC measurement, the determination of cardiac iron concentration is clinically important because one of the major causes of death in iron overload is sudden cardiac arrest. The most reliable non-invasive method of cardiac iron is MRI R2*, which was developed by Anderson et al. [34]. The advantage of MRI R2* is the shorter time period required to acquire an image as only one breath period is necessary by this procedure.

Of the patients with LIC values below 350 μmol/g, all but one had myocardial iron within normal (≤8 μmol/g) or nearly normal ranges. When liver iron levels reached a threshold of 350 μmol/g, iron deposition became evident in the myocardium. At the same time, there was a proportional increase in urinary iron excretion, indicating raised levels of labile iron. SF levels of >1,800 μg/L were also associated with myocardial deposition.

## 5 Toxic effect of iron overload on organ function

Iron overload induces organ damage in liver, heart, pancreas, thyroid, and the central nervous system. The main cause of this organ damage is due to the overproduction of ROS in the presence of excess iron.

### 5.1 Mechanism of iron toxicity

The production of ROS by iron is mainly through the Fenton reaction, which eventually forms hydroxyl radicals from superoxide or hydrogen peroxide [35]. Among ROS, the hydroxyl radical is the most toxic fraction and it targets carbohydrate, protein, and nucleic acids. It is known that the reaction of hydroxyl radicals with the nucleic acid base 8-hydroxyguanine (8-OHG) is highly correlated with teratogenicity and carcinogenicity by oxidative stresses. Another powerful ROS showing similar reactivity as the hydroxyl radical is lipid hydroxyl-peroxide: ROOH. In iron overload, lipid peroxidative products such as malondialdehyde and 4-hydroxy-2-nonenal are increased, which form the radicals ROO-(alkyl oxyradical) and RO-(alkoxy radical). These lipid-based radicals possess longer half lives than hydroxyl radicals, and also have a stronger capacity for chronic cell toxicity and DNA damage.

### 5.2 Iron overload syndrome

Pathological conditions representing body iron overload are designated as iron overload syndromes, and iron deposition causes organ dysfunction including cell death, fibrosis, and carcinogenesis. Iron overload syndromes are classified as genetic or secondary as shown in Table 3.

Hereditary hemochromatosis is the most common genetic disorder in Western countries [36], and its clinical

**Table 3** Classification of iron overload

| | |
|---|---|
| **Hereditary hemochromatosis and related disorders** | |
| Hereditary hemochromatosis | Type 1 |
| | *HFE* gene (6p21.3) mutation |
| | Type 2 |
| | Subtype A: *hemojuvelin* gene (1q21) mutation |
| | Subtype B: *hepcidin* gene (19q13) mutation |
| | Type 3 |
| | *Transferrin receptor 2* gene (7q22) mutation |
| | Type 4 |
| | *Ferroportin* gene (*2q32*) mutation |
| *Ferritin* gene mutation | *H-ferritin* gene mutation (mRNA iron-responsive-element mutation) |
| *DMT1* gene mutation | |
| *Ceruloplasmin* gene mutation | |
| Atransferrinemia | *Transferrin* gene mutation |
| **Secondary iron overload** | |
| Ineffective erythropoiesis | Thalassemia, sideroblastic anemia, myelodysplastic syndromes |
| Administration of iron for long periods | Take orally or intravenous injection |
| Transfusion for long periods | |
| Dietary iron overload | |
| Liver dysfunction | Alcoholic liver injury, chronic hepatitis (type C), non-alcoholic steatohepatitis |
| Others | Porphyria |

manifestation is systemic iron deposition mainly in liver, heart, brain, and endocrine organs. This organ damage is considered to be a result of tissue injuries by iron-induced oxidative stresses [37]. In 1996, the causative gene was identified as *HFE* in the human chromosome 6 [38], and approximately 85% of patients with hereditary hemochromatosis in Western counties have a homologous mutation of C282Y in their *HFE* gene. Thereafter, other genes such as *hemojuvelin* (*HJV*), *TfR2*, *ferroportin*, and *hepcidin* (*HAMP*) gene were identified [39]. In spite of the lack of genetic background, iron overload is commonly observed as a secondary condition. The most common condition occurs in patients who require long-term blood transfusions due to severe anemias. This condition includes genetic disorders such as thalassemia and SCD, and anemia refractory to conventional treatments. In these patients, ineffective erythropoiesis and continuous accumulation of exogenous iron by transfusion are considered to be responsible for the iron overload. The resulting organ failures such as liver failure, cardiac failure, and severe diabetes mellitus affect patients' outcome [1]. In addition to these classical conditions, there are many diseases that show mild iron deposition or dysregulation of body iron distribution. Such conditions include chronic hepatitis C, alcoholic liver disease, non-alcoholic steatohepatitis, and insulin resistance, and iron is an important cofactor that modifies these disease conditions. Furthermore, it is becoming clear that excess iron is also hazardous as it promotes atherosclerosis, carcinogenesis, diabetes, and other lifestyle-related disorders [40].

### 5.3 Organ dysfunction by excess iron

The liver is the most important organ for iron storage with the largest capacity to sequester excess iron. The periodical change of organ dysfunction by long-term transfusions has been studied in patients with homozygous β-thalassemia. Usually, within 2 years of transfusion, abnormalities of liver function tests (LFTs) such as transaminase are not prominent; LFTs are within the normal range or slightly elevated. During these periods, the liver biopsy examination shows a slight fibrosis with mild inflammation and iron deposition. Clinically, the liver is hardened and palpable, and serum transaminase levels are moderately elevated, while other LFTs are within the normal range or slightly elevated. Therefore, it is important for transfusion-dependent patients that clinicians make a correct staging in order to confirm whether any liver lesions are fibrotic or cirrhotic by examining CT, MRI, and biochemical analyses including serum transaminase determinations.

The most important adverse event of long-term transfusion is a sudden death due to cardiac failure. It was reported that approximately 70% of deaths in patients with β-thalassemia are cardiogenic [41]. Signs of cardiac dysfunction include cardiac hypertrophy, arrhythmia, and endocarditis, which eventually cause cardiac failure. Left ventricular disturbance is prominent and is represented as the decrease of ventricular ejection fraction (VEF) by cardiac echogram. As this decrease of VEF appears prior to the clinical signs of cardiac failure and the enlargement of cardiac shadow in chest X-rays, the cardiac echogram is the most useful modality for the follow-up of myocardial damage by iron overload [42]. MRI is also useful to assess the ventricular function, and the deposition of iron in cardiac muscles is detectable by an increase in signal intensity. Furthermore, MRI calculation of T2* or R2* allows the possibility of semi-quantitation of iron concentrations, even at relatively low concentrations [43].

According to a follow-up study in patients with β-thalassemia, organ dysfunction by iron overload appears firstly in the liver when serum ferritin exceeds 1,000 μg/L, and other organ involvements including heart follow in accordance with the further development of iron deposition. Significant cardiac iron deposition is usually observed when LICs are more than 15 mg/g dry weight or serum ferritin levels are more than 1,800–2,500 μg/L [6].

Clinically, in order to detect organ dysfunctions, serum ferritin determinations should be conducted once every 1–3 months. When serum ferritin levels exceed 1,500 μg/L, patients should be examined for the symptoms of cardiac failure or arrhythmias [44], and periodical cardiac echograms may also be useful in diagnosis.

In addition to iron deposition in the liver and heart, pancreatic beta cells are another important target of iron toxicity, which cause glucose intolerance and diabetes mellitus. An additional factor leading to the development of glucose intolerance is hepatic disturbance of insulin utilization, which accelerates beta cell depletion due to hyperinsulinemia [45]. From a clinical perspective, serial determinations of blood glucose, urine sugar, and glycoalbumin are useful, whereas glycohemoglobin is not as useful owing to the effect of transfusions. Endocrinopathies by long-term transfusion include developmental disturbances, incomplete puberty, and thyroid dysfunctions [46]. In patients with thalassemia and SCD, special attention should be paid to early onset symptoms such as disturbances of development and sexual immaturity.

## 6 Conclusion

Iron is essential for the body, but extremely toxic when excess amounts are present. As the body has no active excretion pathways for iron, a continuous load of iron exceeding 1–2 mg/day will result in iron overload, and organ failures including liver and heart. The recent

understanding of body iron metabolism at a molecular level enables us to elucidate the mechanism of iron toxicity more precisely. Improvement of patients' outcomes is becoming promising if a correct early diagnosis is made, and suitable management of these intractable conditions using iron chelation with high compliance is conducted.

## References


1. Andrews NC. Disorders of iron metabolism. N Engl J Med. 1999;341:1986–95.
2. McKie AT, Latunde-Dada GO, Miret S, et al. Molecular evidence for the role of a ferric reductase in iron transport. Biochem Soc Trans. 2002;30:722–4.
3. Trinder D, Fox C, Vautier G, Olynyk JK. Molecular pathogenesis of iron overload. Gut. 2002;51:290–5.
4. Sargent PJ, Farnaud S, Evans RW. Structure/function overview of proteins involved in iron storage and transport. Curr Med Chem. 2005;12:2683–93.
5. Cabantchik ZI, Breuer W, Zanninelli G, Cianciulli P. LPI-labile plasma iron in iron overload. Best Pract Res Clin Haematol. 2005;18:277–87.
6. Olivieri NF, Brittenham GM. Iron-chelating therapy and the treatment of thalassemia. Blood. 1997;89:739–61.
7. Angelucci E, Brittenham GM, McLaren CE, et al. Hepatic iron concentration and total body iron stores in thalassemia major. N Engl J Med. 2000;343:327–31.
8. Ikuta K, Zak O, Aisen P. Recycling, degradation and sensitivity to the synergistic anion of transferrin in the receptor-independent route of iron uptake by human hepatoma (HuH–7) cells. Int J Biochem Cell Biol. 2004;36:340–52.
9. Liuzzi JP, Aydemir F, Nam H, Knutson MD, Cousins RJ. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. Proc Natl Acad Sci USA. 2006;103:13612–7.
10. Schranzhofer M, Schifrer M, Cabrera JA, et al. Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. Blood. 2006;107:4159–67.
11. Fleming MD. The genetics of inherited sideroblastic anemias. Semin Hematol. 2002;39:270–81.
12. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem. 2001;276:7806–10.
13. Inamura J, Ikuta K, Jimbo J, et al. Upregulation of hepcidin by interleukin-1beta in human hepatoma cell lines. Hepatol Res. 2005;33:198–205.
14. Ganz T. Hepcidin in iron metabolism. Curr Opin Hematol. 2004;11:251–4.
15. Bridle KR, Frazer DM, Wilkins SJ, et al. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet. 2003;361:669–73.
16. Pietrangelo A. Hemochromatosis: an endocrine liver disease. Hepatology. 2007;46:1291–301.
17. Gardenghi S, Marongiu MF, Ramos P, et al. Ineffective erythropoiesis in β-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood. 2007;109:5027–35.
18. Cazzola M, Huebers HA, Sayers MH, MacPhail AP, Eng M, Finch CA. Transferrin saturation, plasma iron turnover, and transferrin uptake in normal humans. Blood. 1985;66:935–9.
19. Breuer W, Hershko C, Cabantchik ZI. The importance of non-transferrin bound iron in disorders of iron metabolism. Transfus Sci. 2000;23:185–92.
20. Koorts AM, Viljoen M. Ferritin and ferritin isoforms I: structure-function relationships, synthesis, degradation and secretion. Arch Physiol Biochem. 2007;113:30–54.
21. Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta. 1996;1275:161–203.
22. Jacobs A, Beamish MR, Allison M. The measurement of circulating ferritin. J Clin Pathol. 1972;25:1003.
23. Jacobs A, Miller F, Worwood M, Beamish MR, Wardrop CA. Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. Br Med J. 1972;4:206–8.
24. Piperno A. Classification and diagnosis of iron overload. Haematologica. 1998;83:447–55.
25. Saito H, Hayashi D, Ohya T, Ohya F, Yamada H. Clinical evaluation on serum ferritin (author's transl). Rinsho Ketsueki. 1979;20:1317–25.
26. Galanello R, Piga A, Forni GL, et al. Phase II clinical evaluation of deferasirox, a once-daily oral chelating agent, in paediatric patients with β-thalassaemia major. Haematologica. 2006;91:1343–51.
27. Cappellini MD, Cohen A, Piga A, et al. A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with beta-thalassemia. Blood. 2006;107:3455–62.
28. Jensen PD, Jensen FT, Christensen T, Eiskjaer H, Baandrup U, Nielsen JL. Evaluation of myocardial iron by magnetic resonance imaging during iron chelation therapy with deferrioxamine: indication of close relation between myocardial iron content and chelatable iron pool. Blood. 2003;101:4632–9.
29. Takatoku M, Uchiyama T, Okamoto S, et al. Retrospective nationwide survey of Japanese patients with transfusion-dependent MDS and aplastic anemia highlights the negative impact of iron overload on morbidity/mortality. Eur J Haematol. 2007;78:487–94.
30. Gattermann N. Guidelines on iron chelation therapy in patients with myelodysplastic syndromes and transfusional iron overload. Leuk Res. 2007;31(Suppl 3):S10–5.
31. Olivieri NF, Brittenham GM, Matsui D, et al. Iron-chelation therapy with oral deferiprone in patients with thalassemia major. N Engl J Med. 1995;332:918–22.
32. Long JA Jr, Doppman JL, Nienhus AW, Mills SR. Computed tomographic analysis of beta-thalassemic syndromes with hemochromatosis: pathologic findings with clinical and laboratory correlations. J Comput Assist Tomogr. 1980;4:159–65.
33. Brittenham GM, Farrell DE, Harris JW, et al. Magnetic-susceptibility measurement of human iron stores. N Engl J Med. 1982;307:1671–5.
34. Anderson LJ, Westwood MA, Holden S, et al. Myocardial iron clearance during reversal of siderotic cardiomyopathy with intravenous desferrioxamine: a prospective study using T2* cardiovascular magnetic resonance. Br J Haematol. 2004;127:348–55.
35. Crichton RR, Wilmet S, Legssyer R, Ward RJ. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem. 2002;91:9–18.
36. Yen AW, Fancher TL, Bowlus CL. Revisiting hereditary hemochromatosis: current concepts and progress. Am J Med. 2006;119:391–9.
37. Pietrangelo A. Hereditary hemochromatosis—a new look at an old disease. N Engl J Med. 2004;350:2383–97.
38. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet. 1996;13:399–408.
39. Franchini M. Hereditary iron overload: update on pathophysiology, diagnosis, and treatment. Am J Hematol. 2006;81:202–9.

40. Bonkovsky HL, Lambrecht RW, Shan Y. Iron as a co-morbid factor in nonhemochromatotic liver disease. Alcohol. 2003;30:137–44.
41. Zurlo MG, De Stefano P, Borgna-Pignatti C, et al. Survival and causes of death in thalassaemia major. Lancet. 1989;2:27–30.
42. McGowan JH, Cleland JG. Reliability of reporting left ventricular systolic function by echocardiography: a systematic review of 3 methods. Am Heart J. 2003;146:388–97.
43. Anderson LJ, Holden S, Davis B, et al. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. Eur Heart J. 2001;22:2171–9.
44. Telfer PT, Prestcott E, Holden S, Walker M, Hoffbrand AV, Wonke B. Hepatic iron concentration combined with long-term monitoring of serum ferritin to predict complications of iron overload in thalassaemia major. Br J Haematol. 2000;110:971–7.
45. Olivieri NF. The β-thalassemias. N Engl J Med. 1999;341:99–109.
46. Fung EB, Harmatz PR, Lee PD, et al. Increased prevalence of iron-overload associated endocrinopathy in thalassaemia versus sickle-cell disease. Br J Haematol. 2006;135:574–82.

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METABOLISM, CANCER AND GENETICS

# Dysregulation of systemic iron metabolism in alcoholic liver diseases

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**Key words**
alcohol, hepcidin, iron, steatohepatitis, transferrin receptor 1.

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## Abstract

Alcoholic liver diseases (ALD) are frequently associated with iron overload. Until recently, the effects of ethanol in hepatic iron uptake and intestinal iron absorption have not been clarified in detail. Two possible mechanisms for iron overload are the uptake of iron into hepatocytes in a specific manner through the increased expression of transferrin receptor (TfR) 1; and increased intestinal iron absorption by the lowering of hepcidin. It is worthwhile to examine whether a similar mechanism is present in the development of steatosis and non-alcoholic steatohepatitis (NASH). Hepatocytes have several iron uptake pathways. Ethanol increases transferrin (Tf)-mediated uptake via a receptor-dependent manner, but downregulates the non-Tf-bound iron uptake. According to immunohistochemical study, TfR1 was increased in hepatocytes in 80% of hepatic tissues of patients with ALD, but was not detected in normal hepatic tissues. In an experimental model, ethanol exposure to the primary cultured-hepatocytes in the presence of iron increased TfR1 expression and $^{59}$Fe-labeled Tf uptake. In patients with ALD, intestinal iron absorption is increased by oral iron uptake assay. The regulatory hormone for iron homeostasis, hepcidin is downregulated in ethanol-loaded mice liver. As well as ALD, a similar mechanism was present in the mouse model fed with a high-fat diet, a model of the initial phenomenon of steatosis. The common mechanism for hepatic iron deposition and the triggering role of iron may be present in the development of ALD and non-alcoholic fatty liver disease/NASH.

## Introduction

Body iron metabolism is strictly regulated in physiological conditions, but it is becoming clear that several factors including alcohol, hepatitis C virus (HCV) infection, steatohepatitis etc. affect iron metabolism and the outcomes of their own diseases.[1] Alcoholic liver diseases (ALD), which are characterized by fatty liver, fibrosis, hepatitis and cirrhosis, are frequently associated with mild to severe iron overload. In advanced cases, such as cirrhosis, the reticuloendothelial iron deposition is dominant, in which endotoxemia and hypercytokinemias are deeply involved. However, in ALD of earlier stages, such as fatty liver and fibrosis, iron deposition is very mild and iron is preferentially present in hepatocytes. These findings indicate that alcohol itself or its metabolites primarily affect and dysregulate overall body iron metabolism, including hepatocyte iron uptake and intestinal iron absorption in a specific manner such as via the newly discovered hormone, hepcidin. Concerning the fundamental pathogenesis of ALD, the production of reactive oxygen species (ROS) is considered to be responsible. During the oxidation process of ethanol, superoxide ($O_2^-$) is produced and is transformed to hydroxyl radical ($OH^-$), which is the most potent oxidant via the Fenton reaction in the presence of free iron.[2] Actually, in the intragastric infusion model of ALD, supplementation of carbonyl iron advances fibrosis and cirrhosis.[3] Two possible mechanisms of the role of alcohol in the early stage of disease can be seen in Fig. 1; one is increased uptake of iron into hepatocytes and the other is increased intestinal iron absorption.[4]

## Iron accumulation in hepatocytes by ethanol

It is well known that Japanese patients with ALD have a phenotype that is rather mild compared with that of severe alcoholic siderosis seen in the USA.[5] In our study dealing with Japanese ALD,[6] as well as in the rat model,[7] there is a positive correlation between iron deposition and histological intensity of a lipid-peroxidation product, 4-hydroxy-2-nonenal (HNE)-protein adduct, suggesting that free iron responsible for the Fenton reaction may be present predominantly in hepatocytes, and that ROS-induced cell damage is increased.

Hepatocytes have several pathways for iron uptake: transferrin (Tf)-mediated and non-mediated pathways.[8] Plasma iron is usually bound to Tf and iron-bound Tf is taken up via its specific receptor. In addition, non-Tf-bound iron (NTBI) is thought to contribute iron uptake to hepatocytes through either a divalent metal transporter (DMT1)[9] or ZIP14.[10] As shown in Fig. 2, we have found that ethanol augmented $^{59}$Fe-bound Tf, but inhibited $^{59}$Fe-citrate



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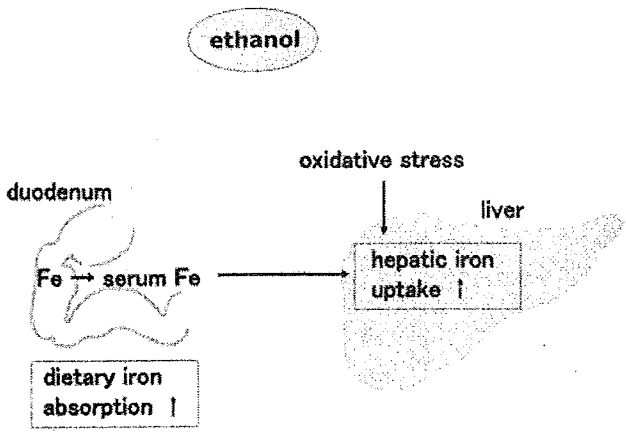


**Figure 1** Two possible mechanisms of dysregulated hepatic iron accumulation in alcoholic liver disease (ALD) in the early stage of the disease. One is increased uptake of iron into hepatocytes and the other is increased intestinal iron absorption.

(NTBI), suggesting that Tf-bound iron may have an important role for hepatic iron uptake by ethanol. Although there are two molecules of Tf receptor, TfR1 and TfR2, TfR1 has a high affinity to serum Tf and is considered to be functional. However, in normal hepatocytes, TfR2 is constitutively expressed, but TfR1 is downregulated, suggesting that TfR1 does not contribute to the steady-state iron uptake. By immunohistochemical study of TfR1, the expression was increased in hepatocytes in 80% of hepatic tissues in Japanese patients with ALD, but was not detected in normal hepatic tissues.[11] It is noteworthy that the mean duration of abstinence of patients who demonstrated positive TfR1 expression in hepatocytes was significantly shorter than that of patients who demonstrated negative TfR1 expression. Taken together, it is possible that ethanol may augment TfR1. In the rat primary hepatocyte culture, the expression of TfR1 is upregulated in the presence of ethanol and iron by western blotting and $^{35}S$-methionine metabolic labeling, suggesting that ethanol or its metabolite may affect the regulation of TfR1 and iron uptake. This increased TfR1 expression was regulated by increasing the activity of iron regulatory protein (IRP).[12]

## Role of hepcidin in alcoholic iron overload

Body iron homeostasis is regulated strictly among processes such as dietary iron absorption, transport in circulation, and utilization or storage in bone marrow and liver. Increase in intestinal iron absorption is one of the mechanisms of the increase of body iron in alcoholics.[13] In patients with hereditary hemochromatosis, serum pro-hepcidin was lower than that in normal controls, suggesting that iron absorption is increased even with high iron storage.[14] It was also speculated that downregulation of hepcidin might be one of the important factors for the pathogenesis of iron overload in ALD.[15] In the ethanol-loaded mouse model which has a mild steatotic change, the hepcidin 1, 2 mRNA and protein expressions were significantly lower than in those of control.[16] In addition, alcohol-loading might disrupt the sensing signal of inflammatory cytokines and then downregulate hepcidin expression, following the increased iron absorption from the small intestine. Concerning the mechanism of hepcidin downregulation by alcohol, a decreased hepcidin expression in mouse liver is accompanied by increases of DMT1 and ferroportin 1, and a decrease of hepcidin promoter activity and DNA-binding activity of CCAAT/enhancer-binding protein (C/EBP).[17] In hemochromatotic hfe(-/-) mice treated with ethanol, a further decrease in hepcidin mRNA expression was observed, in association with the decrease of C/EBP alpha, which may have implications for the liver injury observed in alcoholic liver disease and genetic hemochromatosis in combination with alcohol.[18]

## Steatosis as an inducer of dysregulation of iron metabolism

Non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD) is a clinical entity characterized by the histopathological changes nearly identical to those induced by alcohol intake. In US population, approximately 25% are obese, and at least 20% of the obese individuals have hepatic steatosis, and it is suggested that obesity and steatosis affect liver disease progression.[19] A mild or moderate excess iron is frequently accumulated in liver tissue with NASH. Actually, the prevalence of the *HFE* gene mutation associated with hereditary hemochromatosis is increasing in patients with NASH, with the evidence strongly suggesting that iron is one of the important factors for the development of NASH.[20] It was also reported that phlebotomy is effective against NASH and the rise of oxidative stress markers related to the grade of iron overload in the liver.[21] However, the mechanism of iron overload in NASH is still unknown. Recently, our study using the high-fat diet mouse model suggested a strong link between the activation of peroxisome proliferator-activated receptor gamma (PPARγ) and the downregulation of cAMP response element-binding protein (CREB)[22] in addition to an increase of adipose differentiation-related protein (ADPR).[23] Among these molecules, the downregulation of CREB may be crucial, because CREB activation contributes to survival signals such as anti-apoptotic protein Bcl-2[24] and iron chelator desferrioxamine-increased CREB binding to the D-loop DNA of the mitochondrial genome in neurons.[25] The downregulation of CREB, which is associated with an activation of PPARγ by high-fat diet stimulation, may potentiate further dysregulation of iron metabolism. Actually, we found a significant increase in mRNAs of TfR1 and DMT1, and a decrease of hepcidin mRNA in association with bodyweight gain, mild steatosis and increased HNE immunostaining in this model (Miyoshi *et al.*, unpubl. data, 2007). As iron accumulation in the liver tissue after 16 weeks on a high-fat diet was not yet significant, our data strongly suggests that initial high-fat diet introduction upregulates TfR1 expression and downregulates hepcidin expression in the liver tissue, and upregulates DMT1 expression in the duodenum. Taken together, a high-fat diet itself has a capacity to accelerate intestinal iron absorption and hepatic iron uptake, as does ethanol. Therefore, it seems likely that iron is one of the important factors triggering NASH/NAFLD to develop, rather than a secondary factor.

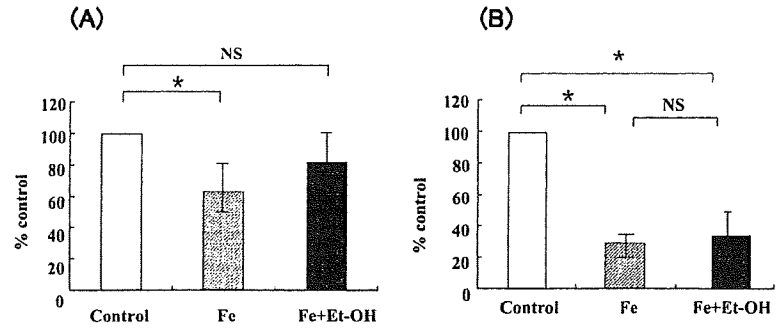


**Figure 2** (a) Transferrin-bound iron uptake examined by incubation with $^{59}$Fe-transferrin for 1 h after 24 h in the iron-deficient condition (control), with 20 μM iron (Fe), and with 20 μM iron and 25 mM ethanol (Fe + Et-OH). $^{59}$Fe-transferrin uptake of iron-loaded hepatocytes was decreased to 63% compared with control hepatocytes. Additional ethanol exposure had a higher uptake at 82% of the control hepatocytes. There was no significant difference between control and iron- and ethanol-loaded hepatocytes. The experiment was repeated four times. (b) Non-transferrin-bound iron uptake examined by incubation with $^{59}$Fe ferric chloride for 24 h in the iron-deficient condition (control), with 20 μM iron (Fe), and with 20 μM iron and 25 mM ethanol (Fe + Et-OH). Non-transferrin-bound $^{59}$Fe uptake of iron-loaded hepatocytes was decreased to 29% compared with control hepatocytes. The additional ethanol exposure produced 34% of the iron uptake compared with the control hepatocytes. There was a significant difference between control and iron- and ethanol-loaded hepatocytes. The experiment was repeated four times. NS, not significant. $^*P < 0.05$. (From [12] with modifications with authors' permission.)

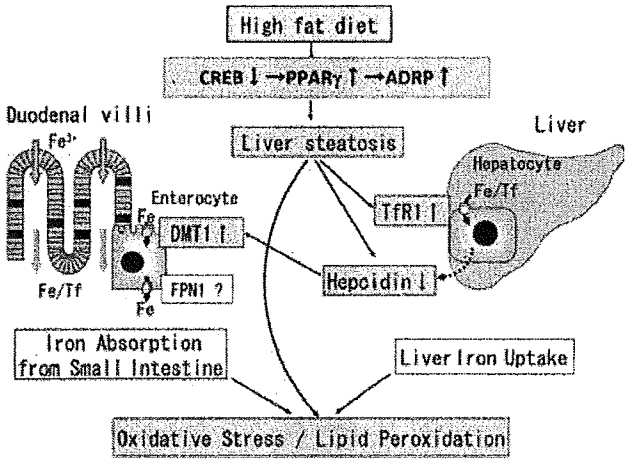


**Figure 3** A high-fat diet itself has the capacity to accelerate intestinal iron absorption and hepatic iron uptake as well as ethanol. It seems likely that iron is one of the important factors triggering the development of non-alcoholic steatohepatitis/non-alcoholic fatty liver disease, rather than a secondary factor.

## Conclusion

It is important to rationalize the finding of mild deposition of iron in earlier stages of ALD and to clarify the molecules involving the hepatic iron uptake in the presence of ethanol. In addition to the upregulation of TfR1 expression in hepatocytes, which is implicated in hepatic iron overload in alcoholic liver diseases, the decrease of hepcidin is also responsible for the increase of iron uptake. As shown in Fig. 3, a similar mechanism may be present in NASH or NAFLD through the production of ROS by a high-fat diet. A common pathway via steatosis/iron/oxidative stress should be considered for the development of liver fibrosis and carcinogenesis by iron as the initial progression stage.

## Conflict of interest

No conflict of interest has been declared by the authors.

## References

1 Kohgo Y, Ikuta K, Ohtake T, Torimoto Y, Kato J. Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance. *World J. Gastroenterol.* 2007; **13**: 4699–706.
2 Bacon BR, Britton RS. The pathology of hepatic iron overload: a free radical- mediated process? *Hepatology* 1990; **11**: 127–37.
3 Tsukamoto H, Horne W, Kamimura S *et al.* Experimental liver cirrhosis induced by alcohol and iron. *J. Clin. Invest.* 1995; **96**: 620–30.
4 Kohgo Y, Ohtake T, Ikuta K *et al.* Iron accumulation in alcoholic liver diseases. *Alcohol. Clin. Exp. Res.* 2005; **29**: 189S–193S.
5 Takada A, Takase S, Tsutsumi M. Characteristic features of alcoholic liver disease in Japan: a review. *Gastroenterol. Jpn.* 1993; **28**: 137–48.
6 Ohhira M, Ohtake T, Matsumoto A *et al.* Immunohistochemical detection of 4-hydroxy-2-nonenal-modified-protein adducts in human alcoholic liver diseases. *Alcohol. Clin. Exp. Res.* 1998; **22**: 145S–149S.
7 Li CJ, Nanji AA, Siakotos AN, Lin RC. Acetaldehyde-modified and 4-hydroxynonenal-modified proteins in the livers of rats with alcoholic liver disease. *Hepatology* 1997; **26**: 650–7.
8 Breuer W, Hershko C, Cabantchik ZI. The importance of non-transferrin bound iron in disorders of iron metabolism. *Transfus. Sci.* 2000; **23**: 185–92.
9 Shindo M, Torimoto Y, Saito H *et al.* Functional role of DMT1 in transferrin-independent iron uptake by human hepatocyte and hepatocellular carcinoma cell, HLF. *Hepatol. Res.* 2006; **35**: 152–62.
10 Liuzzi JP, Aydemir F, Nam H, Knutson MD, Cousins RJ. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. *Proc. Natl Acad. Sci. USA* 2006; **103**: 13612–7.
11 Suzuki Y, Saito H, Suzuki M *et al.* Up-regulation of transferrin receptor expression in hepatocytes by habitual alcohol drinking is

implicated in hepatic iron overload in alcoholic liver disease. *Alcohol. Clin. Exp. Res.* 2002; **26**: 26S–31S.
12 Suzuki M, Fujimoto Y, Suzuki Y *et al.* Induction of transferrin receptor by ethanol in rat primary hepatocyte culture. *Alcohol. Clin. Exp. Res.* 2004; **28**: 98S–105S.
13 Duane P, Raja KB, Simpson RJ, Peters TJ. Intestinal iron absorption in chronic alcoholics. *Alcohol Alcohol.* 1992; **27**: 539–44.
14 Kulaksiz H, Gehrke SG, Janetzko A *et al.* Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. *Gut* 2004; **53**: 735–43.
15 Bridle K, Cheung TK, Murphy T *et al.* Hepcidin is down-regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. *Alcohol. Clin. Exp. Res.* 2006; **30**: 106–12.
16 Ohtake T, Saito H, Hosoki Y *et al.* Hepcidin is down-regulated in alcohol loading. *Alcohol. Clin. Exp. Res.* 2007; **31**: S2–8.
17 Harrison-Findik DD, Schafer D, Klein E *et al.* Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcripition and leads to increased duodenal iron transporter expression. *J. Biol. Chem.* 2006; **281**: 22 974–82.
18 Harrison-Findik DD, Klein E, Crist C, Evans J, Timchenko N, Gollan J. Iron-mediated regulation of liver hepcidin expression in rats and mice is abolished by alcohol. *Hepatology* 2007; **46**: 1979–85.
19 Harrison SA, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: what we know in the new millennium. *Am. J. Gastroenterol.* 2002; **97**: 2714–24.
20 Bonkovsky HL, Jawaid Q, Tortorelli K *et al.* Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. *J. Hepatol.* 1999; **31**: 421–9.
21 Nakashima T, Sumida Y, Furutani M *et al.* Elevation of serum thioredoxin levels in patients with nonalcoholic steatohepatitis, *Hepatol. Res.* 2005; **33**: 135–7.
22 Inoue M, Ohtake T, Motomura W *et al.* Increased expression of PPARgamma in high fat diet-induced liver steatosis in mice. *Biochem. Biophys. Res. Commun.* 2005; **336**: 215–22.
23 Motomura W, Inoue M, Ohtake T *et al.* Up-regulation of ADRP in fatty liver in human and liver steatosis in mice fed with high fat diet. *Biochem. Biophys. Res. Commun.* 2006; **340**: 1111–18.
24 Kitagawa K. CREB and cAMP response element-mediated gene expression in the ischemic brain. *FEBS J.* 2007; **274**: 3210–7.
25 Ryu H, Lee J, Impey S, Ratan RR, Ferrante RJ. Antioxidants modulate mitochondrial PKA and increase CREB binding to D-loop DNA of the mitochondrial genome in neurons. *Proc. Natl Acad. Sci. USA* 2005; **102**: 13 915–20.

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# Lipopolysaccharide induces adipose differentiation-related protein expression and lipid accumulation in the liver through inhibition of fatty acid oxidation in mice

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***Background.*** In the present study, we examined the effect of lipopolysaccharide (LPS) on liver histopathology with special reference to lipid metabolism in mice. ***Methods.*** Mice were injected with LPS intraperitoneally, and its effect on the liver was investigated pathologically and biochemically. ***Results.*** Oil-red O staining and adipose differentiation-related protein (ADRP) immunohistochemistry demonstrated that injection of LPS transiently induced lipid accumulation and ADRP expression in hepatocytes, especially around the portal vein. Microscopic observation revealed that lipid accumulation started 12 h after LPS injection. Time-course studies showed that LPS rapidly, within 2 h, decreased hepatic expression of nuclear hormone receptors, including peroxisome proliferator-activated receptor (PPAR) α. LPS inhibited the expression of PPARα-target genes involved in fatty acid oxidation in the liver such as those coding for enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, whereas LPS also suppressed the expression of genes related to fatty acid synthesis such as those for fatty acid synthase, stearoyl-CoA desaturase, and acetyl-CoA carboxylase α. ***Conclusions.*** LPS induces transient lipid accumulation and expression of ADRP in the liver through inhibition of fatty acid oxidation by down-regulation of the PPARα-related transcriptional mechanism.

**Key words:** LPS, adipose differentiation-related protein, liver steatosis, PPARα, fatty acid oxidation

## Introduction

Hyperlipidemia frequently accompanies infectious and inflammatory diseases.[1] The administration of endotoxin or lipopolysaccharide (LPS) has been used to mimic infection, and studies have demonstrated that a single administration of LPS is sufficient to produce hypertriglyceridemia,[2–4] suggesting that LPS is involved in the hyperlipidemia induced by infection and inflammation. The doses of LPS that produce hypertriglyceridemia in rodents are similar to those that produce fever, anorexia, and changes in acute-phase protein synthesis, suggesting that hypertriglyceridemia is a very sensitive, physiological part of the host response to infection rather than a manifestation of toxicity.[5] Although a number of lines of evidence obtained from biochemical and molecular biological studies have shown that LPS is capable to changing lipid and lipoprotein metabolism in the liver,[1] little is known about LPS-induced histopathological changes in the liver with particular reference to lipid metabolism. In the present study, we examined the effect of LPS on liver pathology associated with changes in lipid metabolism caused by LPS and the possible mechanism of that effect.

## Materials and methods

### *Animal studies*

Ten-week-old male C57Bl/6Ncrj mice (Charles River Japan, Tokyo, Japan) were housed under a 12 h light/dark cycle (light on at 7 a.m.) at 22°C and given food and water ad libitum. Mice were fed a normal diet in which 13.2% of calories were from fat (MF, Oriental Yeast, Tokyo, Japan). Mice were injected with LPS (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally, and each mouse was anesthetized with diethyl ether and weighed at several time points. To test the possibility

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that reduced food intake by itself could induce lipid accumulation in the liver, we also examined the effect of fasting for 24 h on the liver histology. Mice deprived of food but with free access to water were killed after fasting for 24 h and their livers were dissected out for later histological analysis. Blood was collected by cardiac puncture and subsequently assayed for biochemical parameters. Livers and epididymal fat pads were dissected out, weighed, and frozen in liquid nitrogen. Samples of the resected liver were used for later histological and polymerase chain reaction (PCR) analysis. All experiments were carried out in accordance with rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

*Biochemical analyses*

The serum biochemical markers alanine aminotransferase (ALT) and triglycerides were measured with an Automatic Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan).

*Histopathologic evaluation*

Samples of the liver tissue were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and the sections stained with hematoxylin and eosin (HE). To detect fat deposition in the liver, frozen sections were rinsed with distilled water, stained with 0.18% oil red O (Sigma-Aldrich) with 60% 2-propanol (Sigma-Aldrich) for 20 min at 37°C, and then rinsed with distilled water as in our previous study.[6] Immunohistochemistry for adipose differentiation-related protein (ADRP) was performed as described in our previous study.[7]

*RNA isolation and first-strand cDNA synthesis*

Total hepatic RNA was isolated from small pieces of mouse tissue (80–100 μg) by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed with 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°C for 3 min. The mixture was then chilled on ice and incubated with 2 μl 10× reverse transcriptase buffer, 4 μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase at 44°C for 1 h. The reaction mixtures were further incubated for 10 min at 92°C. The cDNA was stored at –30°C until used for real-time PCR.

*Real-time PCR*

Real-time PCR were performed in a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with a Taqman probe. Primers sets for tumor necrosis factor (TNF) α (Mm00443258_m1), peroxisome proliferator-activated receptor γ (PPARγ) (Mm00440945_m1), PPARα (Mm00440939_m1), retinoid X receptor (RXR) α (Mm00441182_m1), sterol regulatory element binding protein (SREBP) 1 (Mm00550338_m1), fatty acid synthetase (Mm00662319_m1), stearoyl-CoA desaturase (Mm00772290_m1), acetyl-CoA carboxylase (Mm01304283_g1), enoyl-CoA hydratase (Mm00470091_s1), very long acyl CoA dehydrogenase (Mm00444296_m1), carnitine palmitoyl transferase (Mm00487202_m1), and ADRP (Mm00475794_m1) were purchased from Applied Biosystems.

*Statistical analysis*

The results are expressed as means ± SEM. Statistical analysis was performed by repeated measures analysis of variance and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

## Results

LPS caused changes in body, liver, and fat weight in mice (Fig. 1). LPS at a dose of 30 mg/kg gradually caused a reduction in body weight, which reached a minimum 24 h after LPS administration (Fig. 1, left). LPS also caused reductions in the weights of liver and fat, at least during the first 12 h. The inhibitory effects of LPS on each parameter were dose dependent (Fig. 1, right).

Biochemical markers in serum of mice administered 30 mg/kg LPS also changed over time (Fig. 2). Serum ALT and triglycerides did not change during the first 6 h but significantly increased by 12 h after LPS administration. Thus, high doses of LPS, which induced liver steatosis in the present study (see below), also increased serum triglyceride levels, supporting previous evidence that a single dose of LPS is sufficient to produce hypertriglyceridemia.[5]

Histologically, a number of small lipid droplets were clearly seen in the liver 24 h after LPS administration (Fig. 3A, B). The lipid droplets were observed predominantly in hepatocytes around the portal vein rather than around the central veins. Inflammatory cells infiltrating the liver were rarely observed. Although accumulation of small lipid droplets was clearly seen after 24 h, droplets were not observed 48 or 72 h after LPS administration. We also evaluated the dose–response effect of LPS on the induction of lipid droplets in the liver 24 h after LPS injection and observed the accumulation of lipid droplets in mice administered LPS at doses of 0.3 mg/kg or larger (data not shown). To test the possibility that reduced food intake by itself could induce lipid accumu-

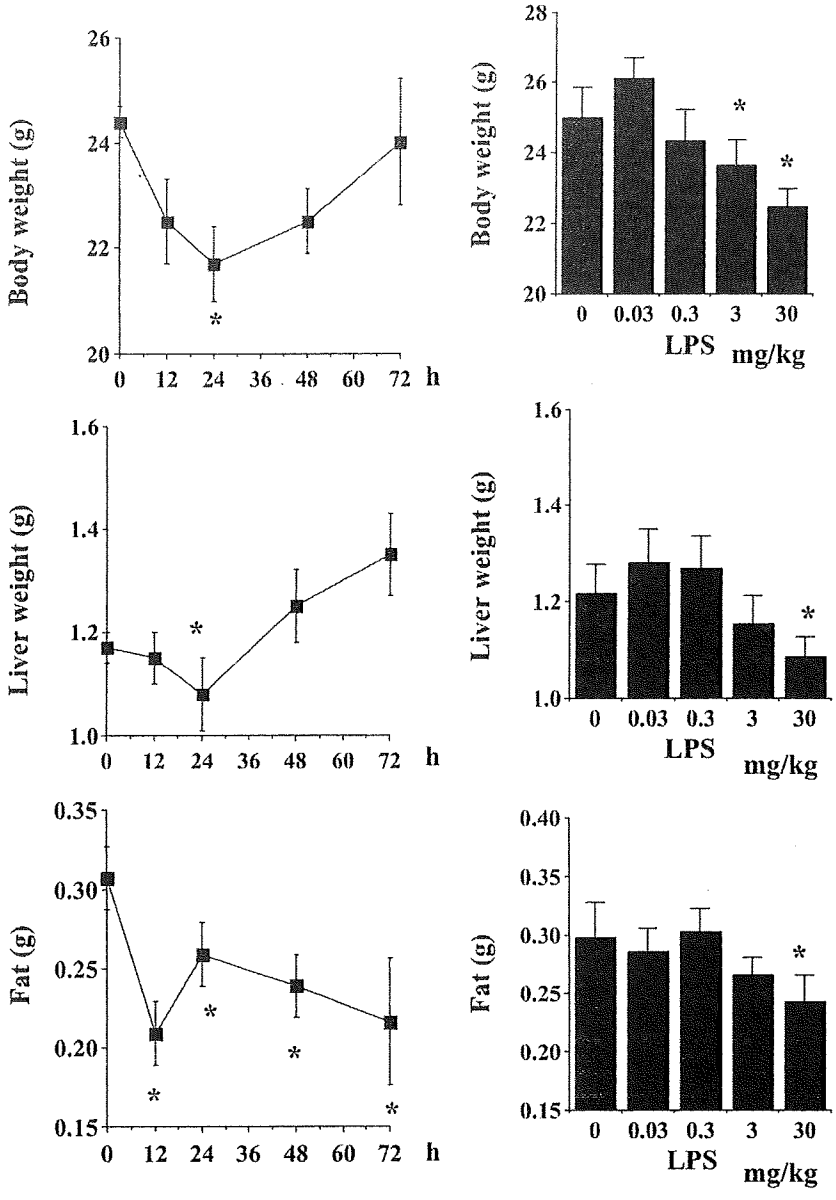


**Fig. 1.** Body, fat, and liver weight in mice injected intraperitoneally with lipopolysaccharide (*LPS*) and then killed at several time points. *Left panels* shows the time-course change in these parameters in mice administered 30 mg/kg of LPS. *Right panels* illustrates the dose–response effect of LPS on the parameters in mice 24 h after several injections of LPS. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

lation in the liver, we examined the effect of fasting for 24 h on the liver histology. Histological observation (Fig. 4) indicated that 24 h of fasting by itself did not induce lipid accumulation in the liver, strongly suggesting that reduced food intake was not a major factor causing liver steatosis. Oil red O staining confirmed that the lipid droplet-like structures seen in HE staining were indeed lipid droplets containing triglycerides (Fig. 3C, D). The time-course results with oil-red O staining showed that fat accumulation started within 12 h after injection of LPS, lipid deposition reached a maximum in the liver 24 h after LPS administration, and then the lipid content in the liver gradually decreased. Thus, a single intraperitoneal injection of LPS induced liver steatosis within 12 h, and fat accumulation disappeared by 48 h after LPS injection, establishing a novel transient liver steatosis model in mice by LPS.

To further clarify whether LPS transiently induced liver steatosis, expression of ADRP, a protein that coats lipid droplets, was evaluated. ADRP was expressed in the liver 12 and 24 h after 30 mg/kg LPS administration (Fig. 5A). High-power observation of a liver specimen stained with ADRP antibody showed that ADRP coated the lipid droplets in the hepatocytes (Fig. 5B). Moreover, ADRP protein was expressed transiently, in good agreement with the HE and oil red O staining results. LPS induced ADRP expression in a dose-dependent manner (Fig. 5C). Obvious induction of ADRP expres-

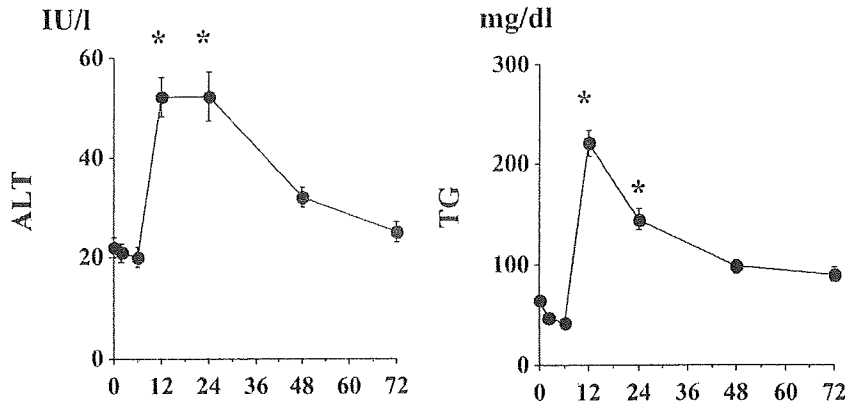


**Fig. 2.** Time-course change of biochemical markers alanine aminotransferase (*ALT*) and triglycerides (*TG*) in serum of mice administered 30 mg/kg of LPS and killed at several time points. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

sion was observed in mice injected with LPS at doses of 3 mg/kg or larger. We also examined the time-course effect of LPS on ADRP mRNA expression in the mouse liver (Fig. 5D). Real-time PCR demonstrated that ADRP mRNA expression in the liver increased by 6 h after a 30 mg/kg LPS injection. ADRP mRNA expression was gradually upregulated from 6 to 12 h after LPS administration, and then decreased to the baseline level, supporting our results on the time-course change of ADRP expression after LPS administration.

LPS also increased mRNA expression of TNFα in the liver within 2 h (Fig. 6).

PPARα and γ and RXRα are transcription factors that may be involved in accumulation of lipids in the liver.[8] PPARα, PPARγ, and RXRα mRNAs in the liver were drastically downregulated within 2 h after injection of 30 mg/kg LPS (Fig. 7). In contrast, SREBP-1 mRNA expression was not changed by LPS (Table 1).

To clarify whether fatty acid synthesis or lipid oxidation or both were involved in the accumulation of lipids in hepatocytes after LPS administration, we analyzed mRNA expression of several molecules that play a role in fatty acid synthesis or lipid oxidation in the liver (Fig. 8). LPS potently inhibited mRNA expression of the enzymes that play a key role in fatty acid synthesis, fatty acid synthase, stearoyl-CoA desaturase, and acetyl-CoA carboxylase α, suggesting that the lipid accumulation does not result from increased de novo synthesis of fatty acid. In contrast, mRNA expression of three enzymes involved in fatty acid oxidation, enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, was downregulated within 2 h after LPS administration.

## Discussion

Although a number of studies have demonstrated that LPS affects biochemical markers of lipid metabolism in which the liver plays a key role,[1] it has not been known whether LPS induced distinct pathological changes in the liver. The present study demonstrated for the first time that intraperitoneal injection of LPS is capable of inducing transient lipid accumulation in the mouse liver. We therefore propose that LPS and inflammation should be listed as factors inducing liver steatosis.

As demonstrated in this study, LPS dose-dependently reduced body weight within 24 h. Although we did not examine the effect of LPS on food intake in this study, LPS-induced anorexia[9–11] is considered to be a factor in the body weight loss caused by LPS. One might speculate that reduced feeding by itself might be implicated in the lipid accumulation in the liver after LPS administration. However, that is unlikely because 24 h of fasting by itself failed to induce lipid accumulation in mouse liver in this study.

Histological observations indicated that lipid accumulation in the liver after LPS administration is stronger around the portal vein than around the central veins. We have recently demonstrated that a high fat diet causes lipid droplets to form in hepatocytes around the central veins in mice.[6] It is known that steatosis around the central veins is strong in fatty liver resulting from obesity or alcohol.[12] The observation that lipid accumulation occurred mainly in hepatocytes around the portal vein rather than the central veins as a result of LPS administration suggests that the mechanism of LPS-induced liver steatosis differs from that of well-established causative factors such as obesity or alcohol.

Lipid droplets are cytoplasmic organelles that serve as storage sites for neutral lipids. ADRP is intrinsically associated with the surfaces of lipid droplets and is believed to function in the intracellular mobilization and storage of neutral lipids.[13–15] ADRP abundance is directly proportional to the level of intracellular lipid found within a cell, and ADRP is increased in specific diseases involving fat accumulation.[13,16,17] Although there is little evidence for whether ADRP expression is indeed increased in fatty liver, in which the liver stores

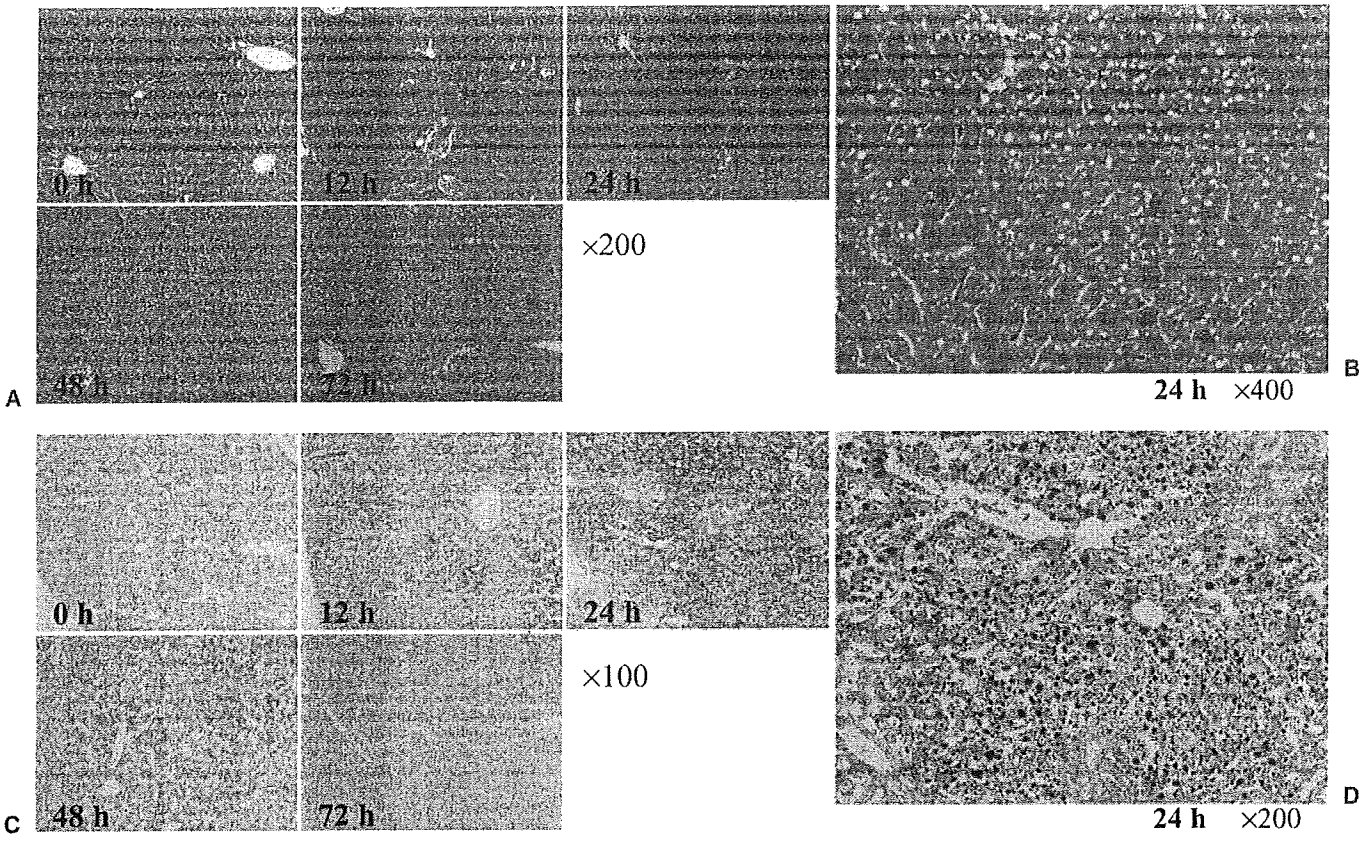


**Fig. 3.** **A** Time-course change of liver tissues [hematoxylin and eosin (HE) staining] in mice injected with LPS. Mice were administered several doses of LPS intraperitoneally and then killed at several time points. **B** High-power view of liver tissue (HE staining) 24 h after LPS (30 mg/kg) injection. **C** Time-course change in liver tissue (oil red O staining) in mice injected with LPS. **D** High-power view of liver tissue (oil red O staining) 24 h after LPS (30 mg/kg) injection

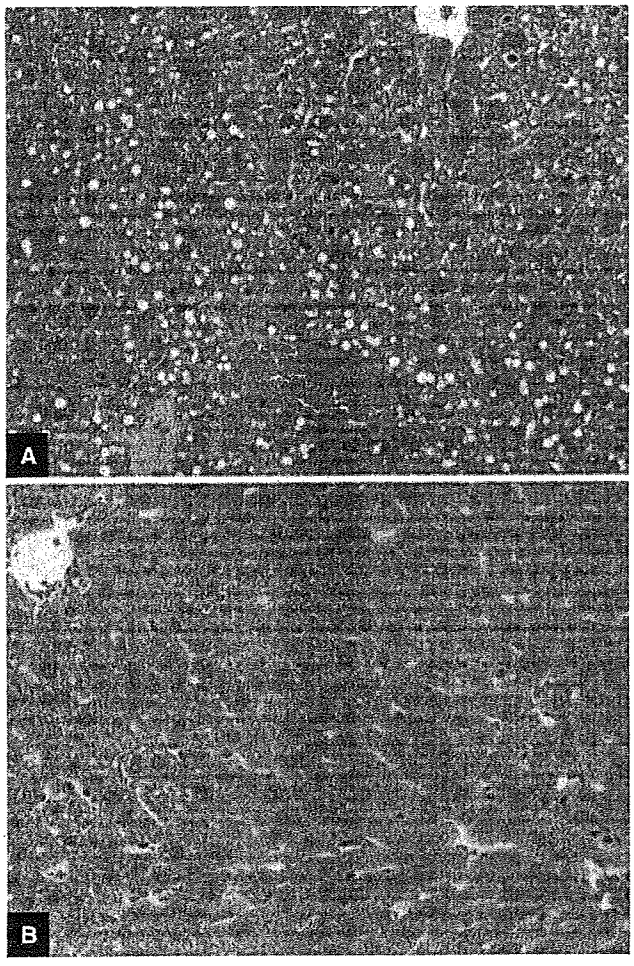


**Fig. 4A,B.** Effect of a 24-h fast on induction of lipid accumulation in the liver. Mice were deprived of food and then injected with LPS (30 mg/kg) (**A**) or not (**B**), and then killed after 24 h (HE staining)