22. Domanski P, Witte M, Kellum M, Rubinstein M, Hackett R, Pitha P, Colamonici OR (1995) Cloning and expression of a long form of the beta subunit of the interferon alpha beta receptor that is required for signaling. J Biol Chem 270:21606–21611
23. Yamaguchi Y, Hino K, Fujiwara D, Ren F, Katoh Y, Satoh Y, Okita K (2002) Expression of type I interferon receptor in liver and peripheral blood mononuclear cells in chronic hepatitis C patients. Dig Dis Sci 47:1611–1617
24. Zhao LJ, Hua X, He SF, Ren H, Qi ZT (2011) Interferon alpha regulates MAPK and STAT1 pathways in human hepatoma cells. Virol J 8:157
25. Yang CH, Murti A, Pfeffer LM (2005) Interferon induces NF-kappa B-inducing kinase/tumor necrosis factor receptor-associated factor-dependent NF-kappa B activation to promote cell survival. J Biol Chem 280:31530–31536
26. Tanabe Y, Nishibori T, Su L, Arduini RM, Baker DP, David M (2005) Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. J Immunol 174:609–613
27. Buller CL, Loberg RD, Fan MH, Zhu Q, Park JL, Vesely E, Inoki K, Guan KL, Brosius FC 3rd (2008) A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. Am J Physiol Cell Physiol 295:C836–C843
28. Yong VW, Moumdjian R, Yong FP, Ruijs TC, Freedman MS, Cashman N, Antel JP (1991) Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. Proc Natl Acad Sci USA 88:7016–7020
29. Rubio N, Torres C (1999) Interferon-gamma induces proliferation but not apoptosis in murine astrocytes through the differential expression of the myc proto-oncogene family. Brain Res Mol Brain Res 71:104–110
30. Satoh J, Paty DW, Kim SU (1996) Counteracting effect of IFN-beta on IFN-gamma-induced proliferation of human astrocytes in culture. Mult Scler 1:279–287
31. Pirisi M, Fabris C, Toniutto P, Falleti E, Tisminetzky SG, Gerotto M, Soardo G, Vitulli D, Forno MD, Baralle F, Bartoli E (1997) Endogenous interferon-alpha concentration and outcome of interferon treatment in patients with chronic hepatitis C. Dig Dis Sci 42:767–771
32. Uze G, Lutfalla G, Gresser I (1990) Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. Cell 60:225–234
33. Mogensen KE, Lewerenz M, Reboul J, Lutfalla G, Uze G (1999) The type I interferon receptor: structure, function, and evolution of a family business. J Interferon Cytokine Res 19:1069–1098
34. Yeh TC, Pellegrini S (1999) The Janus kinase family of protein tyrosine kinases and their role in signaling. Cell Mol Life Sci 55:1523–1534
35. Blank VC, Pena C, Roguin LP (2009) STAT1, STAT3 and p38MAPK are involved in the apoptotic effect induced by a chimeric cyclic interferon-alpha2b peptide. Exp Cell Res 316: 603–614
36. Tsuda M, Kohro Y, Yano T, Tsujikawa T, Kitano J, Tozaki-Saitoh H, Koyanagi S, Ohdo S, Ji RR, Salter MW, Inoue K (2011) JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. Brain 134:1127–1139
37. Caraglia M, Abbruzzese A, Leardi A, Pepe S, Budillon A, Baldassare G, Selleri C, Lorenzo SD, Fabbrocini A, Giuberti G, Vitale G, Lupoli G, Bianco AR, Tagliaferri P (1999) Interferon-alpha induces apoptosis in human KB cells through a stress-dependent mitogen activated protein kinase pathway that is antagonized by epidermal growth factor. Cell Death Differ 6:773–780
38. Yanase N, Hata K, Shimo K, Hayashida M, Evers BM, Mizuguchi J (2005) Requirement of c-Jun NH2-terminal kinase activation in interferon-alpha-induced apoptosis through upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in Daudi B lymphoma cells. Exp Cell Res 310:10–21
39. Barca O, Devesa-Peleteiro P, Seoane M, Senaris RM, Arce VM (2010) Bimodal effect of interferon-beta on astrocyte proliferation and survival: Importance of nuclear factor-kappaB. J Neuroimmunol 226:73–80
40. Al-Khalili L, Cartee GD, Krook A (2003) RNA interference-mediated reduction in GLUT1 inhibits serum-induced glucose transport in primary human skeletal muscle cells. Biochem Biophys Res Commun 307:127–132
41. Belanger M, Allaman I, Magistretti PJ (2010) Differential effects of pro- and anti-inflammatory cytokines alone or in combinations on the metabolic profile of astrocytes. J Neurochem 116:564–576
42. Hesselgesser J, Horuk R (1999) Chemokine and chemokine receptor expression in the central nervous system. J Neurovirol 5:13–26
43. Sun J, Zheng JH, Zhao M, Lee S, Goldstein H (2008) Increased in vivo activation of microglia and astrocytes in the brains of mice transgenic for an infectious R5 human immunodeficiency virus type 1 provirus and for CD4-specific expression of human cyclin T1 in response to stimulation by lipopolysaccharides. J Virol 82: 5562–5572

J Gastroenterol
DOI 10.1007/s00535-012-0546-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Carnosic acid (CA) prevents lipid accumulation in hepatocytes through the EGFR/MAPK pathway

Ting Wang · Yasuhiro Takikawa · Takahito Tabuchi · Takumi Satoh · Kunio Kosaka · Kazuyuki Suzuki

Received: 25 August 2011 / Accepted: 4 January 2012

Abstract
Background Carnosic acid (CA), found in rosemary, has been reported to have antioxidant and anti-adipogenic properties. We recently demonstrated that CA protects against steatosis in *ob/ob* mice. In the present report, we investigated the molecular mechanism by which CA inhibits lipids accumulation both in vivo and in vitro.
Methods In the in vivo study, *ob/ob* mice were fed a standard chow diet with or without CA for 5 weeks, then their hepatocyte lipid accumulation was determined. The serum concentrations of cytokines, the levels of lipid regulatory mediators, and the hepatic metabolic and signaling molecules were also evaluated. In the in vitro study, HepG2 cells were used to further clarify the effects of CA on cellular lipid accumulation and to confirm the signaling pathways involved in these effects.
Results CA significantly reduced hepatocyte lipid accumulation. This effect was associated with repressed levels of hepatic PPARγ, reduced expression of inflammatory cytokines such as IL-1β, IL-12, IL-17, IFN-γ, MCP-1, and MIP-1β, and increased ATP, acetyl CoA, NAD(P)$^+$, and NAD(P)H. Other signaling molecules, such as EGFR, MAPK, AMPK, and ACC, which regulate lipid metabolism, were activated in mice fed the CA diet. CA inhibited palmitate-induced cellular lipid accumulation and stimulated the phosphorylation of both EGFR and MAPK. Pretreatment with either the EGFR inhibitor AG1478 or the MEK-specific inhibitor U0126 abolished the effects of CA on cellular lipid accumulation and decreased both the protein expression and activity of PPARγ.
Conclusions EGFR/MAPK signaling plays an important role in the inhibitory effect of CA on hepatocyte lipid accumulation.

Keywords Carnosic acid · Cellular lipid accumulation · PPAR · EGFR/MAPK signaling

Abbreviations

ACC	Acetyl CoA carboxylase
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
CA	Carnosic acid
EGFR	Epithelium growth factor receptor
FAS	Fatty acid synthase
FFA	Free fatty acid
G-CSF	Granulocyte colony-stimulating factor
GMCSF	Granulocyte macrophage colony-stimulating factor
GSH	Glutathione
IFN	Interferon
IL	Interleukin
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
NAFLD	Nonalcoholic fatty liver disease
NF-κB	Nuclear factor-kappa B
PPAR	Peroxisome proliferation activator receptor
SREBP-1	Sterol regulatory element-binding protein-1

T. Wang · Y. Takikawa (✉) · K. Suzuki
Department of Gastroenterology and Hepatology, Iwate Medical University, Morioka, Iwate, Japan
e-mail: ytakikaw@iwate-med.ac.jp

T. Tabuchi · T. Satoh
Department of Welfare Engineering, Faculty of Engineering, Iwate University, Morioka, Iwate, Japan

K. Kosaka
Research and Development Center, Nagase Co., Ltd., Kobe, Japan

Published online: 18 February 2012

TG	Triglyceride
TGFβ1	Transforming growth factorβ1
TZDs	Thiazolidinediones
UCP2	Uncoupling protein 2
WAT	White adipose tissue

Introduction

Hepatic steatosis is an initial and critical step in the development of fatty liver diseases such as nonalcoholic fatty liver disease (NAFLD). Hepatocytes with an excessive triglyceride (TG) content may also present inflammation, insulin resistance, fibrosis, and even carcinogenesis [1]. Preventing the hepatocytes from accumulating fat is therefore the most effective way to block the progression of NAFLD to nonalcoholic steatohepatitis (NASH).

Although there are a variety of conditions that increase the amount of hepatic lipids, de novo lipogenesis and fatty acid β-oxidation are important for maintaining the balance of hepatic lipid metabolism [2–4]. Molecules such as AMP-activated protein kinase (AMPK) and peroxisome proliferation activator receptor (PPAR) γ, which participate in regulating these two processes, have thus attracted a lot of interest. AMPK, a serine threonine kinase with a catalytic α subunit and regulatory β and γ subunits, has been identified to be a major regulator of glucose and lipid metabolism and represents an attractive target for therapeutic intervention in the treatment of hepatic disorders [5]. AMPK activation depends on the phosphorylation of a subunit on threonine-172 (Thr-172) by kinase LKB1 or CaMKKβ. The activation of hepatic AMPK leads to increased fatty acid oxidation and simultaneously inhibits hepatic lipogenesis, cholesterol synthesis, and glucose production [6]. The actions of AMPK are attributed to its direct phosphorylation and the inactivation of a number of metabolic enzymes, including acetyl CoA carboxylase (ACC), which is a key enzyme regulating both fatty acid synthesis and β-oxidation. In addition, AMPK activation reduces the expression of sterol regulatory element-binding protein-1 (SREBP-1) c, which is the predominant SREBP-1 isoform in the liver, and which transcriptionally regulates lipogenesis [5–7].

PPARγ is another target for the treatment of NASH because it plays a role in increasing insulin sensitivity [8]. PPARγ agonists, such as thiazolidinediones (TZDs), have been proposed to improve insulin sensitivity by increasing the expression and release of adiponectin, an adipokine that activates AMPK. However, as a lipogenic gene, PPARγ enhances fat accumulation in adipose tissue by stimulating adipocyte differentiation, and in the liver, hepatic PPARγ regulates triglyceride homeostasis and contributes to hepatic steatosis [8, 9]. PPARγ is phosphorylated and inactivated by the extracellular receptor kinase–mitogen-activated protein kinase (ERK–MAPK) pathway. The MAPK phosphorylation site was mapped to serine-82 of mouse PPARγ1, which corresponds to serine-112 of mouse PPARγ2. The treatment of macrophages with transforming growth factor (TGF) β1 increases PPARγ phosphorylation and decreases TZD-induced CD36 expression via activation of the ERK–MAPK pathway [10]. In addition, MAPK has been demonstrated to inhibit the differentiation of 3T3–L1 fibroblasts to adipocytes through repression of the transactivation function of PPARγ [11]. However, whether MAPK plays a role in regulating PPARγ in hepatocytes remains unclear.

We recently reported that carnosic acid (CA), an extract of rosemary leaves, prevented obesity, ameliorated steatosis of the liver, and improved the glucose tolerance in an NAFLD animal model [12]. In addition, rosemary extract is generally recognized as safe (GRAS) by the US Food and Drug Administration, and CA has been commercially used as the principal component of rosemary extract in various foods and supplements. These factors indicate that CA is a novel and potentially effective drug for the treatment of NAFLD and that it appears to be safe for human use.

Furthermore, the antioxidant effects of CA have been considered to be responsible for its biological activity [13]. It has been reported that CA protects neurons from free radicals and thereby shields the brain from ischemia. In addition, CA was found to stimulate Keap1/Nrf2 signaling, thus resulting in the production of antioxidants such as glutathione (GSH), thereby reducing oxidative stress [14, 15]. In addition, CA at 0.1–10 μM significantly inhibited preadipocyte 3T3L1 cells from differentiating into mature adipocytes through the same pathway [16].

In order to clarify the mechanism underlying the effects of CA on the lipid accumulation in hepatocytes, we first investigated the effects of CA on regulating inflammatory factors, lipid regulating mediators, and hepatic metabolic and signaling molecules in *ob/ob* mice. We then tested the possible signaling pathways involved in the activity of CA in vitro by using a cellular lipid accumulation model.

Materials and methods

Cell culture and treatment

Human hepatocellular liver carcinoma cells (HepG2) were grown in DMEM medium with 10% fetal bovine serum (FCS, Invitrogen). For cell treatment, a stock solution of CA (Nagase Co., Osaka, Japan) was prepared

in DMSO. A stock solution of sodium palmitate (Sigma-Aldrich Co., Japan) was dissolved in a bovine serum albumin/Hank's balanced salt solution (BSA/HBSS) mixture [17]. The in vitro model of cellular fat accumulation was established by treating HepG2 cells with palmitate as described previously [18]. Specific inhibitors of EGFR (AG1478), MEK (U0126), and AMPK (compound C) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Promega Corporation (Madison, WI, USA), and EMD Biosciences, Inc. (Darmstadt, Germany), respectively.

Animals and treatment

Four-week-old male obese leptin-deficient (*ob/ob*) mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were housed under a 12-h light–dark cycle in a temperature- and humidity-controlled environment. For the experiments, mice were randomly divided into two groups and were fed a standard chow diet with (CA+) or without (CA−) CA [0.05% (wt/wt), Nagase Co., Osaka, Japan] for 5 weeks. The mice were killed and tissues were sampled at the end of the experiment. Blood was collected by cardiac puncture, and serum samples were stored frozen at −20°C until the analysis by a Bioplex cytokine assay. Liver tissue was weighed and fixed in glutaraldehyde for an electron microscopy analysis. Frozen sections were used for the metabolome analysis (HMT com., Tsuruoka, Japan) and for the analyses of protein levels or activity. The use of mice in this study complied with the relevant guidelines based on the laws of Japan, and the study protocol was approved by the animal research committee of Iwate Medical University.

Electron microscopy

The livers were freshly isolated, perfused, and fixed with 2.5% glutaraldehyde. Tissue fragments were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Sections were cut for light microscopy (toluidine blue staining) and electron microscopy, which was performed on an electron microscope (H7100s, Hitachi, Tokyo, Japan). The total area of the lipid droplets in a randomly selected microscopic field was quantified by using the Image J software program (NIH, Washington D.C., USA). The average value for each group was then calculated.

Bioplex cytokine assay

The serum from mice was collected and analyzed by Bioplex cytokine assays (Bio-Plex Mouse Cytokine 18-Plex Panel, Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. We analyzed the serum for the concentration of interleukin (IL)-1, IL-2, IL-3, IL-5, IL-6, IL-9, IL10, IL-12, IL-13, IL-17, tumor necrosis factor (TNF) α, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GMCSF), interferon (IFN) γ, macrophage inflammatory protein (MIP)-1β, and monocyte chemoattractant protein 1 (MCP-1).

Western blot analysis

Total protein was isolated from liver tissue or HepG2 cells by a total protein extraction kit purchased from BioChain Institute, Inc. (Hayward, CA, USA). The nuclear fraction was isolated using the Nuclear/Cytosol Fraction Kit from Biovision Research Products (San Francisco, CA, USA). A total of 20 μg protein from each sample was separated by 10% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting was performed by using specific antibodies against PPARγ, SREBP-1, Nrf2, UCP2, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), FAS (Sigma Aldrich Japan Inc, Tokyo, Japan), phospho-AMPKα, phospho-ACC, phospho-EGFR, and phospho-MAPK (ERK1/2, Cell Signaling Technology, Charlottesville, VA, USA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

Oil Red O staining

HepG2 cells were washed once in PBS and fixed with freshly prepared 4% formaldehyde in PBS for 15 min. Cells were stained with Oil Red O as described elsewhere [19]. Spectrophotometric quantification of the staining was performed by dissolving the stained oil droplets in the cell monolayers with 4% Nonidet P-40 in isopropanol for 5 min. Then the absorbance was measured at 520 nm.

PPARγ transcription factor assay

After the treatment, the nuclear fraction of HepG2 cells was prepared, and the specific transcription factor DNA binding activity of PPARγ was detected by an enzyme-linked immunosorbent assay (ELISA; Cayman Chemical Company, Ann Arbot, MI, USA) according to the manufacturer's instructions.

Statistical analysis

The statistical analysis was carried out using Student's *t* test for unpaired comparisons. A p value less than 0.05

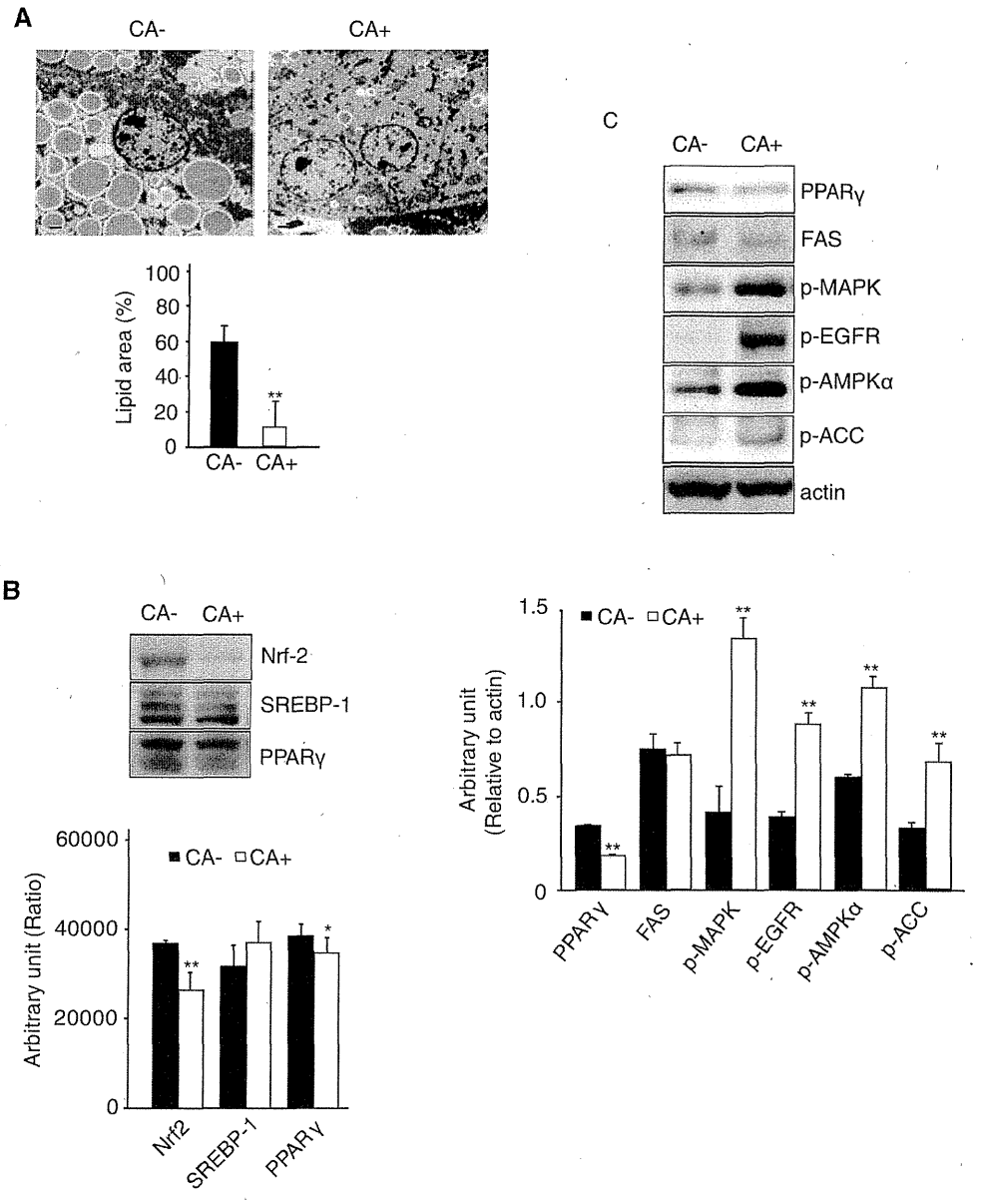

Fig. 1 Effects of CA treatment on hepatocyte lipid accumulation in *ob/ob* mice. **a** CA treatment inhibited hepatocyte lipid accumulation. Electron microscopy was performed and the results were quantified as described in the "Materials and methods". *Scale bar* 2 μm. **b** CA treatment significantly decreased the PPARγ protein level in the nuclear fraction of the liver. The nuclear fraction was extracted, and 20 μg of protein per sample was used for the Western blot analysis. **c** The effects of CA treatment on the protein levels of the signaling molecules involved in lipid metabolism. Tissue lysates (20 μg of total protein per sample) of liver samples were used for the Western blot analysis. The β-actin level was evaluated as a protein loading control. Representative results are shown. The quantitative data are presented as the means ± SD ($n = 3$ for **a** and $n = 5$ for **b**, **c**). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the CA group

was considered to be significant. The results are presented as the means ± SD.

Results

CA reduces hepatocyte lipid accumulation in *ob/ob* mice

We previously reported that CA effectively reduced the total hepatic lipid content in *ob/ob* mice. In this study, we first detected the lipid accumulation in hepatocytes by electronic microscopy. As shown in Fig. 1a, the hepatocytes from control mice showed profound lipid accumulation. In comparison, CA feeding significantly decreased the fat accumulation in hepatocytes. In addition, lipogenic genes such as SREBP-1, fatty acid synthase (FAS), and PPARγ in liver nuclear fractions and/or in total lysates were subsequently examined. We observed that the PPARγ levels were significantly reduced in the mice fed the CA diet in comparison to the controls. In contrast, neither the amount of cleaved SREBP-1 (60–70 kDa) nor the FAS levels were significantly different between the two groups (Fig. 1b, c).

We then focused on the liver metabolites that correlated with the process of fatty acid β-oxidation. Compared with control mice, mice fed the CA diet showed increased levels of liver metabolites, such as NADH (3.1 times that of the control), NAD^+ (3.3 times the control), acetyl CoA (1.7 times the control), ATP (2.6 times the control), and ADP (1.7 times the control), which are the major molecules that participate in the process of fatty acid β-oxidation (Table 1).

Table 1 Expression levels of hepatic metabolites involved in fatty acid β-oxidation and the NADPH-GSH system in *ob/ob* mice fed with or without CA

Metabolite	Relative area(/g)				Ratio
	CA−		CA+		CA+/CA−
FAD (divalent)	5.0 ± 0.27	10^{-1}	4.8 ± 0.52	10^{-1}	1.0
NADH	1.8 ± 0.70	10^{-1}	5.6 ± 0.29	10^{-1}	3.1
NAD^+	1.0 ± 0.8		3.3 ± 1.6		3.3
Acetyl CoA (divalent)	8.2 ± 0.48	10^{-2}	1.4 ± 0.31	10^{-1}	1.7
ATP	5.0 ± 0.38	10^{-1}	1.3 ± 0.21	10^{-2}	2.6
ADP	3.5 ± 1.6		5.8 ± 0.75		1.7
NADPH	2.1 ± 0.22	10^{-1}	4.1 ± 0.50	10^{-1}	2.0*
$NADP^+$	3.5 ± 0.96	10^{-1}	6.3 ± 1.4	10^{-1}	1.8
GSH	200 ± 9.8		200 ± 24		1.0
GSSG	34 ± 4.3		33 ± 4.4		1.0

*$p < 0.05$

Table 2 Serum concentrations of cytokines in *ob/ob* mice fed with or without CA

Cytokine	CA− (pg/mL)	CA+ (pg/mL)	Decrease (%)
IL-1α	1.3 ± 0.5	1.0 ± 0.3	23.1
IL-1β	176.0 ± 36.5	129.8 ± 32.4	26.3
IL-2	16.0 ± 3.4	13.7 ± 3.4	15.1
IL-3	41.7 ± 8.5	31.4 ± 6.2	24.7
IL-5	14.9 ± 4.2	10.6 ± 3.3	28.8
IL-6	4.1 ± 2.3	4.4 ± 6.0	−7.3
IL-9	373.1 ± 22.1	334.1 ± 33.4	10.5
IL-10	45.5 ± 8.6	40.1 ± 12.0	11.9
IL-12	152.0 ± 35.4	123.6 ± 35.7	18.7
IL-13	1160.8 ± 125.8	1047.6 ± 123.8	9.8
IL-17	55.3 ± 18.5	37.1 ± 7.5	32.9
IFN-γ	214.1 ± 25.9	161.7 ± 43.9	24.5
MCP-1	373.6 ± 39.7	309.7 ± 23.7	17.1
MIP-1β	46.4 ± 7.5	37.4 ± 11.9	19.4
TNF-α	271.9 ± 37.5	250.2 ± 50.5	8.0
G-CSF	60.3 ± 33.2	58.2 ± 43.8	3.5
GM-CSF	66.7 ± 10.6	53.1 ± 9.6	20.4

*$p < 0.05$, **$p < 0.01$

Activity of CA occurs independently of Nrf2/GSH signaling

In order to identify whether the Nrf2/GSH signaling pathway participates in the activity of CA, we evaluated the NADPH-glutathione (GSH) system in the livers of mice and detected the Nrf2 protein levels in the liver nuclear extracts. However, there were no significant differences in the levels of GSH in the present study, other than a significantly increased level of hepatic NADPH ($n = 3, p < 0.05$) in the mice fed the CA diet compared with controls (Table 2). Similarly, the CA treatment did not cause the expected increase in the Nrf2 levels in the liver nuclear fractions. On the contrary, the Nrf2 levels were decreased in the livers of mice fed the CA diet (Fig. 1b). Among the various molecules involved in lipid metabolism that were analyzed, both AMPKα and its downstream target, ACC, were activated in the livers of mice fed the CA diet in comparison to the controls. In addition, increased phosphorylation of both MAPK (ERK1/2) and its common upstream regulator, epithelial growth factor receptor (EGFR), was detected in the livers derived from the mice fed CA (Fig. 1c).

Reduction of lipid accumulation in HepG2 cells by CA is associated with EGFR and MAPK signaling

On the basis of the finding that CA feeding activated the phosphorylation of AMPK, EGFR, and MAPK, we examined the roles of these molecules in an in vitro model of cellular fat accumulation. As shown in Fig. 2a, HepG2 cells exhibited an excessive triglyceride accumulation after being induced by palmitate. The CA treatment significantly inhibited the effect of palmitate, however, co-treatment with a specific selective inhibitor of the EGFR (AG1478) or an inhibitor of the upstream effector of MAPK, MEK (U0126), recovered their cellular lipid accumulation (Fig. 2b). Furthermore, CA treatment stimulated the phosphorylation of both MAPK and EGFR in a time-dependent manner. AG1478 pretreatment (1 h prior to CA) effectively inhibited the phosphorylation of both MAPK and EGFR (Fig. 2c). Although CA treatment activated AMPK in HepG2 cells, there were no significant differences in the cellular triglyceride content between the cells co-treated with compound C, a specific selective inhibitor of AMPK, and the cells treated only with CA (Fig. 2b, c).

CA regulates both the expression level and activity of PPARγ through EGFR/MAPK signaling in HepG2 cells

We also determined whether CA-stimulated EGFR/MAPK signaling plays a role in regulating PPARγ in HepG2 cells. We observed that the protein level of PPARγ in palmitate-induced cells was significantly reduced after the CA treatment, but was rescued by the pretreatment with either AG1478 or U0126 (Fig. 3a). Furthermore, the specific transcription factor DNA binding activity of PPARγ was significantly reduced after the CA treatment, but was rescued by pretreatment with either AG1478 or U0126. Consistently, the decreased nuclear protein levels of PPARγ in CA-treated cells were strongly elevated by pretreatment with the above inhibitors (Fig. 3b, c).

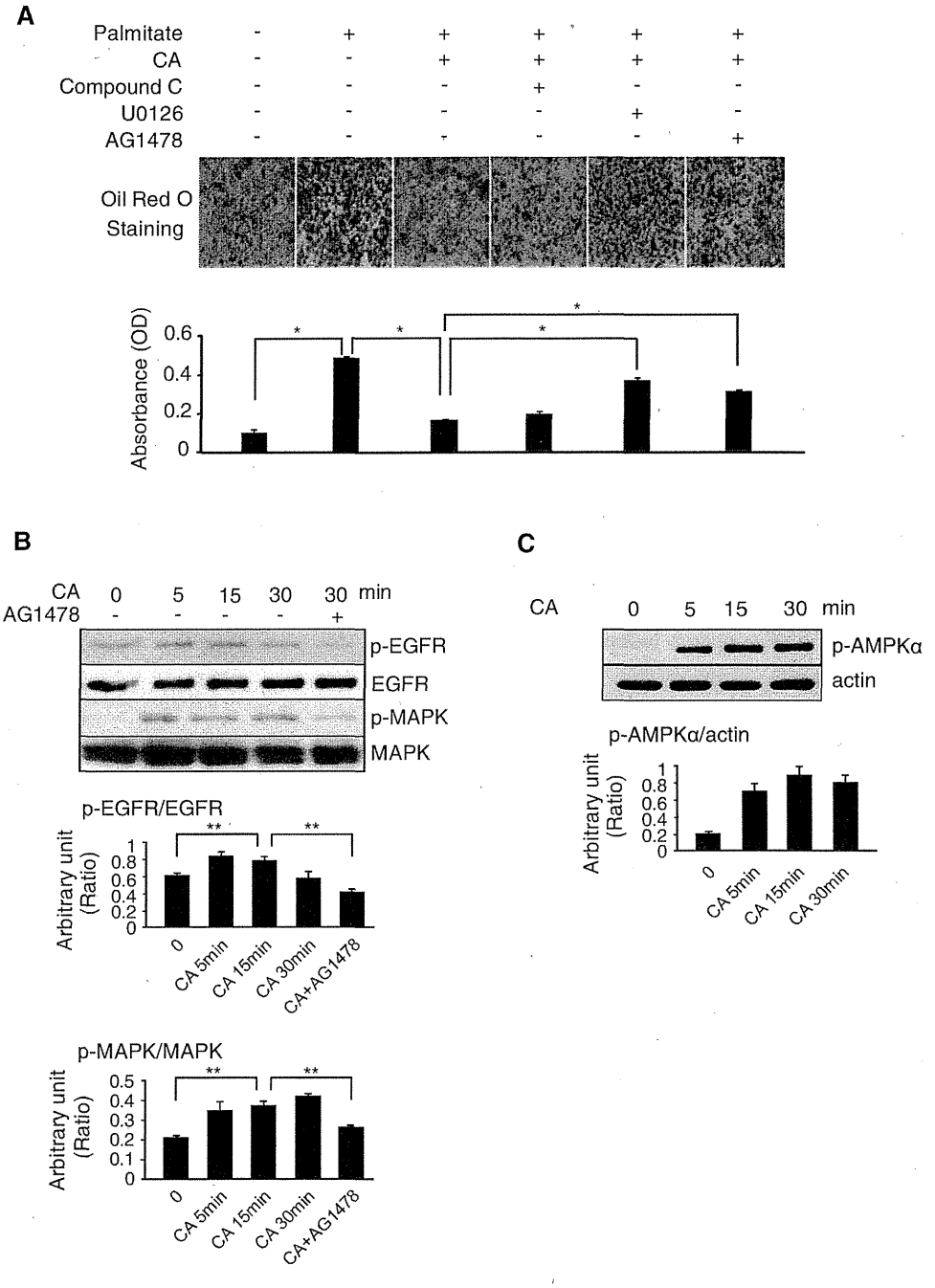

Fig. 2 Reduction of lipid accumulation in HepG2 cells by CA is associated with activation of MAPK and EGFR. **a** Both the EGFR and MAPK contribute to the inhibitory effects of CA on the cellular lipid accumulation induced by palmitate. HepG2 cells were induced by palmitate as described in the "Materials and methods". A concentration of 10 μM CA was added 1 day before the palmitate treatment. Then, 10 μM AG1478, 10 μM U0126, or 15 μM compound C was added, together with CA, as indicated. The cells were incubated for 18 h before the Oil Red O staining. **b** CA activated the EGFR/MAPK pathway in HepG2 cells. The 80% confluent HepG2 cells were starved in medium without FCS for 4 h. The cells were then treated with 20 μM CA for the indicated times. A concentration of 10 μM AG1478 was added 1 h prior to the CA treatment. **c** CA stimulated the phosphorylation of AMPKα in HepG2 cells. Cells were treated with CA for the indicated times as described in **b**. A 20-μg aliquot of total protein from each sample was used for the Western blot analysis. β-actin was evaluated as a protein loading control. All of the above experiments were repeated three times and representative results are shown. The quantitative data are presented as the means ± SD. $*p < 0.05$, $**p < 0.01$

Effects of CA on serum cytokine levels in *ob/ob* mice

Inflammatory cytokines are known to enhance the extent of liver injury [20, 21]. In the present study, a variety of cytokines such as IL-1β, IFN-γ, IL-12, IL-17, MCP-1, and MIP-1β were detected in the serum of control mice. In contrast, the serum of the mice fed the CA diet showed a significant decrease in the levels of these inflammatory cytokines. There were no significant differences in the levels of anti-inflammatory cytokines such as IL-10. The levels of TNF-α were also not significantly different between the CA and control groups (Table 1).

Discussion

The aim of the present study was to investigate the molecular and cellular mechanisms underlying the role of CA in lipid accumulation in hepatocytes. Oxidative stress contributes to the progression of steatosis to NASH by producing lipid peroxidation, inflammation, and fibrosis [22, 23]. Recently, Nrf2 was recognized as a target for the treatment of NAFLD [24, 25]. On the basis of the finding of our previous study, we thus hypothesized that CA exerts its effect on NAFLD by activating the Keap1/Nrf2 pathway. However, neither the protein levels of Nrf2 in the

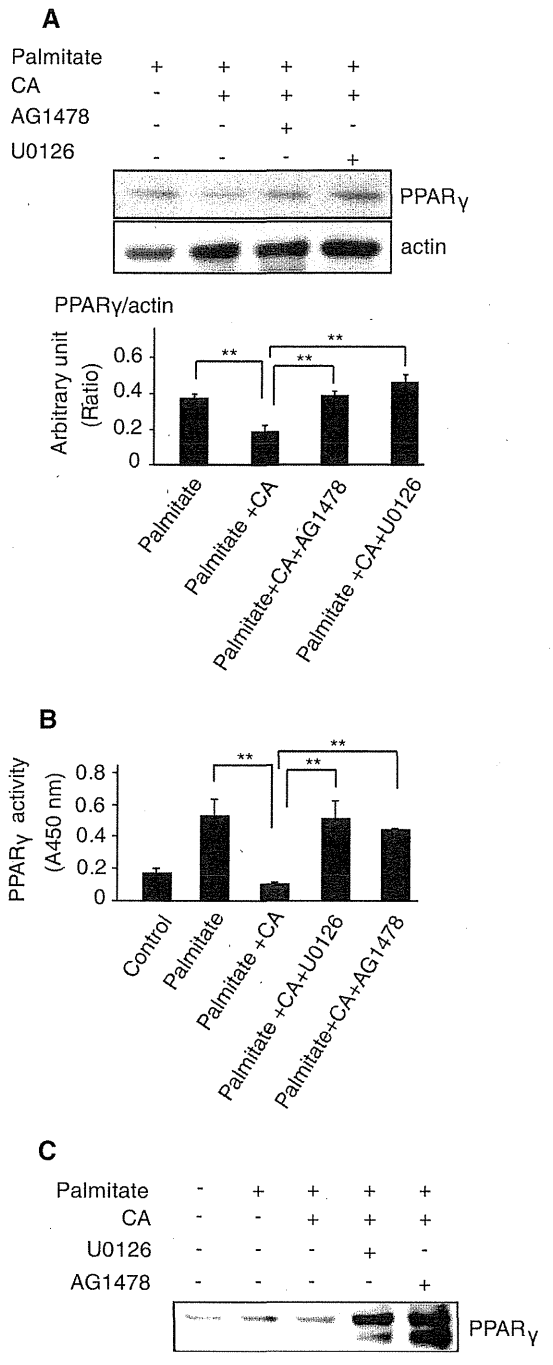

Fig. 3 CA decreased both the protein levels and activity of PPARγ through the EGFR/MAPK signaling pathway. Palmitate and CA were added to the cells as described in Fig. 2a. Then, 10 μM AG1478 or 10 μM U0126 was added as indicated. **a** The CA-stimulated EGFR/MAPK cascade decreased the total protein level of PPARγ. A 20-μg aliquot of total protein from each sample was used for the Western blot analysis. β-actin was evaluated as a protein loading control. **b**, **c** The CA-stimulated EGFR/MAPK cascade inhibited the activity of PPARγ. Analyses of both the protein levels and the activity of PPARγ were performed by using the nuclear fractions of the samples. PPARγ transcription factor assay was performed as described in the "Materials and methods". All of the above experiments were repeated three times and representative data are shown. The quantitative data are presented as the means ± SD. $^{**}p < 0.01$

nuclear fraction nor the production of GSH in the liver of CA-treated mice was elevated in comparison to control mice. Our present data therefore do not support the hypothesis that CA inhibits fat accumulation in the liver through the Nrf2/GSH pathway. However, because *ob/ob* mice present simple hepatic steatosis but not advanced stages of NAFLD such as NASH [26], further studies are needed to clarify the effect of CA on advanced NAFLD and to determine whether Keap1/Nrf2 signaling participates in the function of CA.

Our data indicate that CA-induced PPARγ reduction is involved in the prevention of lipid accumulation in hepatocytes. This is supported by a previous report indicating that liver-specific inhibition of PPARγ results in decreased hepatic steatosis [27]. We also demonstrated that the EGFR/MAPK signaling activated by CA is responsible for the suppression of both the protein expression and transcriptional activity of PPARγ. The EGFR is one of the potential upstream regulators of MAPK. It was reported that the ligand-dependent formation of EGFR homodimers or heterodimers with one of its homologs (ErbB2, ErbB3, and ErbB4) results in opposing effects on preadipocyte proliferation and differentiation [28]. In addition, activation of the EGFR/MAPK cascade plays an important role in the anti-obesity activity of evodiamine, a major component of traditional Chinese medicine [29, 30]. We herein demonstrated that the EGFR/MAPK pathway plays an important role in preventing lipid accumulation in the liver. Although the molecular mechanism by which CA activates the EGFR was not elucidated in the present study, an in vitro study showed that CA activated EGFR as early as 5 min post-stimulation, which suggests that CA possibly directly targets the EGFR through oligomerization or autophosphorylation, rather than an indirect mechanism. It is worth noting that EGFR activation is usually associated with an increased risk of cancer because it promote cell proliferation. However, so far, there have not been any reports of carcinogenic activity for CA. On the contrary, CA has been demonstrated to induce apoptosis in cancer cell lines [31, 32]. However, considering the possibility of the clinical application of CA, it is necessary to carefully determine whether there is any risk of cancer related to CA treatment.

In spite of the increased EGFR/MAPK signaling, our data from the present study showed that the phosphorylation of both AMPK and ACC was enhanced in the livers of mice fed a CA diet, and that this was accompanied by elevated levels of various hepatic metabolic molecules that participate in fatty acid β-oxidation. These data suggest that CA-stimulated AMPK plays a role in promoting fat consumption in the liver. However, the SREBP-1 levels in the nuclear fraction of the liver were not changed in the CA-treated mice. Although CA stimulated the phosphorylation of AMPK in HepG2 cells, a specific inhibitor of

AMPK did not block the inhibitory effects of CA on the cellular lipid accumulation induced by palmitate. These findings indicate that CA-induced AMPK activation does not directly suppress the short-term lipid accumulation induced by palmitate loading. On the other hand, the liver AMPK level regulates glucose homeostasis, in addition to regulating hepatic lipogenesis [6]. We previously reported that CA treatment improved the glucose intolerance in *ob/ob* mice [12]. Therefore, it is possible that CA indirectly blocks hepatocyte lipid accumulation in the long term by restoring insulin sensitivity, although future studies will be needed to determine whether this is the case.

Our previous data showed that CA treatment did not change the food intake of mice in comparison to controls [12]. These data suggest that CA protects against hepatic steatosis through a leptin-independent pathway, although further proof using other NAFLD animal models, such as MCD diet mice, is needed [33]. Leptin-deficient mice produce low levels of cytokines [34]. In the present study, the CA-treated mice showed even lower serum levels of many cytokines in comparison to the control mice. In particular, the serum levels of inflammatory cytokines like IFN-γ, MCP-1, MIP-1β, and GM-CSF were significantly lower in the CA-treated mice than in controls. So far, we do not know whether or not CA-induced PPARγ reduction relates to the changes of inflammation. It is also possible that the reduction of inflammatory cytokines in the in vivo experiments may occur due to the secondary effects of CA through body weight suppression, and this possibility should be examined in future studies. Our data above indicate that CA has potent anti-inflammatory activities even in *ob/ob* mice. Therefore, CA is expected to prevent the development of steatosis and to decrease the transition from NAFLD to steatohepatitis.

Acknowledgments This study was supported by a Grant-in-Aid for Strategic Medical Science Research Center (S1001001) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Ms. A. Watanabe and Mr. K. Ishida for their valuable technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Liu Q, Bengmark S, Qu S. The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD). Lipids Health Dis. 2010;9:42.
2. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Prog Lipid Res. 2009;48(1):1–26.
3. Tessari P, Coracina A, Cosma A, Tiengo A. Hepatic lipid metabolism and non-alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis. 2009;19(4):291–302.
4. Lelliott C, Vidal-Puig AJ. Lipotoxicity, an imbalance between lipogenesis de novo and fatty acid oxidation. Int J Obes Relat Metab Disord. 2004;28(Suppl 4):S22–8.
5. Viollet B, Guigas B, Leclerc J, Hebrard S, Lantier L, Mounier R, et al. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol (Oxf). 2009;196(1):81–98.
6. Zhang BB, Zhou G, Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab. 2009;9(5): 407–16.
7. Yang YM, Han CY, Kim YJ, Kim SG. AMPK-associated signaling to bridge the gap between fuel metabolism and hepatocyte viability. World J Gastroenterol. 2010;16(30):3731–42.
8. Gavrilova O, Haluzik M, Matsusue K, Cutson JJ, Johnson L, Dietz KR, et al. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. J Biol Chem. 2003;278(36): 34268–76.
9. Kallwitz ER, McLachlan A, Cotler SJ. Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. World J Gastroenterol. 2008; 14(1):22–8.
10. Burns KA, Vanden Heuvel JP. Modulation of PPAR activity via phosphorylation. Biochim Biophys Acta. 2007;1771(8):952–60.
11. Bost F, Aouadi M, Caron L, Binetruy B. The role of MAPKs in adipocyte differentiation and obesity. Biochimie. 2005;87(1): 51–6.
12. Wang T, Takikawa T, Satoh T, Yoshioka Y, Kosaka K, Tatemichi Y, et al. Carnosic acid prevents obesity and hepatic steatosis in *ob/ob* mice. Hepatol Res. 2011;40(6):605–12.
13. Aruoma OI, Halliwell B, Aeschbach R, Loligers J. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. Xenobiotica. 1992;22(2):257–68.
14. Satoh T, Kosaka K, Itoh K, Kobayashi A, Yamamoto M, Shimojo Y, et al. Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. J Neurochem. 2008;104(4):1116–31.
15. Tamaki Y, Tabuchi T, Takahashi T, Kosaka K, Satoh T. Activated glutathione metabolism participates in protective effects of carnosic acid against oxidative stress in neuronal HT22 cells. Planta Med. 2009;76(7):683–8.
16. Takahashi T, Tabuchi T, Tamaki Y, Kosaka K, Takikawa Y, Satoh T. Carnosic acid and carnosol inhibit adipocyte differentiation in mouse 3T3–L1 cells through induction of phase2 enzymes and activation of glutathione metabolism. Biochem Biophys Res Commun. 2009;382(3):549–54.
17. Vock C, Gleissner M, Klapper M, Doring F. Identification of palmitate-regulated genes in HepG2 cells by applying microarray analysis. Biochim Biophys Acta. 2007;1770(9):1283–8.
18. Gomez-Lechon MJ, Donato MT, Martinez-Romero A, Jimenez N, Castell JV, O'Connor JE. A human hepatocellular in vitro model to investigate steatosis. Chem Biol Interact. 2007;165(2): 106–16.
19. Kasturi R, Joshi VC. Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3–L1 cells. J Biol Chem. 1982;257(20):12224–30.
20. Gao B. Cytokines, STATs and liver disease. Cell Mol Immunol. 2005;2(2):92–100.
21. Nanji AA, Jokelainen K, Rahemtulla A, Miao L, Fogt F, Matsumoto H, et al. Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat. Hepatology. 1999;30(4):934–43.
22. Lewis JR, Mohanty SR. Nonalcoholic fatty liver disease: a review and update. Dig Dis Sci. 2010;55(3):560–78.

23. Musso G, Gambino R, Cassader M. Non-alcoholic fatty liver disease from pathogenesis to management: an update. Obes Rev. 2010;11(6):430–45.
24. Kathirvel E, Chen P, Morgan K, French SW, Morgan TR. Oxidative stress and regulation of anti-oxidant enzymes in cytochrome P4502E1 transgenic mouse model of non-alcoholic fatty liver. J Gastroenterol Hepatol. 2010;25(6):1136–43.
25. Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, et al. Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. Free Radic Biol Med. 2010;48(2): 357–71.
26. Anstee QM, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol. 2006; 87(1):1–16.
27. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest. 2003;111(5):737–47.
28. Harrington M, Pond-Tor S, Boney CM. Role of epidermal growth factor and ErbB2 receptors in 3T3–L1 adipogenesis. Obesity. 2007;15(3):563–71.
29. Wang T, Wang Y, Yamashita H. Evodiamine inhibits adipogenesis via the EGFR–PKCalpha–ERK signaling pathway. FEBS Lett. 2009;583(22):3655–9.
30. Wang T, Wang Y, Kontani Y, Kobayashi Y, Sato Y, Mori N, et al. Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. Endocrinology. 2008;149(1): 358–66.
31. Sharabani H, Izumchenko E, Wang Q, Kreinin R, Steiner M, Barvish Z, et al. Cooperative antitumor effects of vitamin D3 derivatives and rosemary preparations in a mouse model of myeloid leukemia. Int J Cancer. 2006;118(12):3012–21.
32. Tsai CW, Lin CY, Lin HH, Chen JH. Carnosic acid, a rosemary phenolic compound, induces apoptosis through reactive oxygen species-mediated p38 activation in human neuroblastoma IMR-32 cells. Neurochem Res. 2011;36(12):2442–51.
33. Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. J Clin Invest. 2000;105(8):1067–75.
34. Siegmund B, Sennello JA, Lehr HA, Batra A, Fedke I, Zeitz M, et al. Development of intestinal inflammation in double IL-10 and leptin-deficient mice. J Leukoc Biol. 2004;76(4):782–6.

肝性脳症

鈴木 一幸

ポイント

- ☆肝硬変による肝性脳症は，門脈－大循環短絡(シャント)の因子が強いタイプ(シャント型あるいは慢性再発型)と肝細胞障害の強いタイプ(肝細胞障害型あるいは末期昏睡型)に分かれ，予後は後者で悪い．
- ☆肝性脳症の誘因・増悪因子を検索する．
- ☆潜在性肝性脳症の存在にも留意して診療を行う．
- ☆精神神経機能異常の程度により昏睡度(I～V度)を判定し，合わせて肝病態(肝障害の程度)を把握する．

肝性脳症の概念・臨床病型

肝性脳症とは，急性および慢性肝疾患の経過中に出現する意識障害をはじめとする多彩な精神神経症状の総称(症候群)である．意識障害は軽度のものから深昏睡に至るものまで幅広く含まれる．したがって，わが国では肝性脳症をきたす基礎肝病変により，急性型，慢性型，特殊型の３つの臨床病型に分類される．

急性型は劇症肝炎に代表されるが，一部の症例では慢性肝疾患の急性増悪例(acute on chronic)も含まれる．一方，慢性型は肝硬変あるいは肝硬変合併肝癌が主な基礎肝病変であり，通常，門脈大循環短絡(シャント)の因子が強いタイプ(シャント型あるいは慢性再発型)と肝細胞障害の強いタイプ(肝細胞障害型あるいは末期昏睡型)とに分かれる[1]．さらに最近は，画像診断学の進歩により，明らかな慢性肝疾患の存在がなくても門脈－大循環短絡路(肝内，肝外シャント)の形成によって肝性脳症を生じる症例も報告されてきている．特発性門脈圧亢進症(idiopathic portal hypertension)では，食道静脈瘤破裂による大量出血後に脳症を生じる可能性がある．

表１に最近欧米で提唱されている肝性脳症の分類[2]を示すが，サブカテゴリーについては今後の検証が必要と考えられる．

潜在性肝性脳症

近年，理学的に精神神経機能が一見正常と判断される(日常診療の際には普通に会話し，異常の存在を疑うことが少ない)例において定量的精神神経機能検査(表２)を行うと，少なからず異常を認める例が存在し，このような病態を潜在性肝性脳症あるいはミニマル肝性脳症と呼ぶことが提唱されている．いわゆる昏睡ゼロの範疇に入る病態である．潜在性肝性脳症を顕性肝性脳症の前段階と捉えるか，あるいは肝硬変自体による中枢神経系の形態あるいは機能異常

すずき かずゆき：岩手医科大学附属病院消化器・肝臓内科 〒020-8505 盛岡市内丸19-1

0025-7699/12/¥500/論文/JCOPY

【表 1】肝性脳症の新しい分類(文献 2 より引用改変)

型	名称
A(Acute)型	急性肝不全(劇症肝炎など)でみられる脳症
B(Bypass)型	門脈－大循環系バイパスによる脳症で，肝硬変などの肝疾患を伴わない
C(Cirrhosis)型	肝硬変と門脈圧亢進症/門脈－大循環短絡路バイパスでみられる脳症
	エピソード(間欠)型脳症
	1．誘因あり型
	2．誘因なし型
	① 再発型(2 回以上/年)
	② 非再発(特発)型
	持続型脳症
	1．軽症型(grade I)
	2．重症型(grade II～IV)
	3．治療依存型
	ミニマル脳症
	潜在性脳症といわれたもの

：サブカテゴリー

による変化と捉えるかについては未だ結論は得られておらず，標準的な診断法もコンセンサスも得られていない．また，わが国では潜在性肝性脳症について十分に議論されておらず，臨床実地家においても認知度が低いのが現状である[3]．

発生機序

肝性脳症の発生には，以下の 2 つの因子が相互に関連している．

・**肝性因子**：脳内神経伝達の障害に関与する肝由来の因子で，高度の肝細胞機能障害や肝細胞壊死により生じる中毒性物質が増加，あるいは肝で合成される必須物質が不足する．

・**門脈因子**：腸管由来の中毒物質が門脈－大循環短絡路より直接大循環に流入し，これが脳に達して神経伝達を障害する．

上記 2 因子のどちらの比重が高いかによって治療の反応性と予後が異なってくる．中毒物質にはアンモニア，低級脂肪酸，インドール，フェノール，ベンゾジアゼピン(末梢性)などが挙げられるが，なかでもアンモニアは肝性脳症の発生機序に最も深く関与する因子として古くから重視されている[4]．

【表 2】潜在性肝性脳症の診断に用いられる検査法

定量的精神神経機能検査
- WAIS 成人知能診断検査
- そのほかの定量検査：数字追跡・光や音反応時間など
- コンピュータによる機能検査

電気生理学的検査
- 脳波(脳波 mapping)
- 大脳誘発電位(聴覚・視覚)
- 事象関連電位 P300
- 臨界フリッカー頻度(CFF-test)

肝性脳症の発生機序としては多因子説，アミノ酸代謝異常説，偽性神経伝達物質説，γアミノ酪酸(GABA)/ベンゾジアゼピン受容体複合体異常説などの説があるが，単一では明確に肝性脳症の発生機序を説明できず，いずれの説にもアンモニア因子が関与している[4]．

肝性脳症の誘因・増悪因子

代表的な誘因として，食事蛋白量の過剰摂取，消化管出血，便秘，感染症，鎮静薬・鎮痛薬の過剰投与，利尿薬の過剰投与による脱水・電解質異常などがあり，われわれの経験では慢性型において約 70%の例に何らかの誘因を認める．近年は消化管出血による肝性脳症例は減少しており，誘因不明例が多い．大きな門脈－大循環短絡を有する例では，食事蛋白量の過剰摂取や便秘などにより容易に脳症を繰り返すのが特徴である．

脳症の増悪因子として低酸素血症，循環不全，低血糖，低血圧，血清電解質(特にナトリウム，カリウム，マグネシウム)異常，血漿蛋白(アルブミン)減少などが挙げられる．

【表3】肝性脳症の昏睡度分類(犬山シンポジウム，1982年)

昏睡度	主な精神症状・神経症状	Japan Coma Scale (3-3-9方式)
Ⅰ	睡眠—覚醒リズムの逆転，多幸気分，時に抑鬱状態，だらしなく，気に留めない態度，retrospectiveにしか判定できない場合が多い	1
Ⅱ	指南力(時，時間)障害，物を取り違える(confusion) 異常行動(例：お金をまく，化粧品をゴミ箱に捨てるなど) 時に傾眠状態(普通の呼びかけで開眼し，会話ができる)，無礼な言動があったりするが，医師の指示に従う態度を見せる 興奮状態がない，尿・便失禁がない，羽ばたき振戦あり	2,3,10
Ⅲ	しばしば興奮状態またはせん妄状態を伴い，反抗的態度を見せる，嗜眠状態(ほとんど眠っている)，外的刺激で開眼しうるが，医師の指示に従わない，または従えない(簡単な命令には応じる) 羽ばたき振戦あり(患者の協力が得られる場合) 指南力は高度の障害	20,30
Ⅳ	昏睡(完全な意識の消失) 痛み刺激に反応する 刺激に対して払いのける動作，顔をしかめるなどが見られる	100,200
Ⅴ	深昏睡 痛み刺激にもまったく反応しない	300

診断法

鑑別疾患

最初に意識障害が先行する肝疾患によるものか，あるいは肝疾患以外の疾患によって生じたか否かを鑑別する必要がある．特に中枢神経系疾患，糖尿病ケトアシドーシス，低血糖による意識障害，肝硬変では硬膜下血腫やアルコール性離脱症候群との鑑別が重要である．

昏睡度の判定

次いで昏睡度の判定を行うが，わが国では犬山シンポジウムの昏睡度分類(**表3**)にしたがって行うのが通例である．昏睡Ⅱ度の判定は明らかな羽ばたき振戦，失見当識，異常行動などを認めるため比較的容易であるが，昏睡Ⅰ度の判定は難しいことも多い．外来での患者表情あるいは言葉使いなどを注意深く観察し，あるいは家族からの日常生活状況(食欲，睡眠，行動パターン，活動量など)の聴取を行って精神神経異常の存在を早期に疑うことがきわめて重要である．

検査

・**定量的精神神経機能検査**(**表2**)：数字追跡試験を行う．

・**脳波検査**：高振幅徐派の出現(典型例では三相波がみられる)をみる．

・**血液生化学検査**：一般肝機能検査，肝予備能(プロトンビン時間)とともに，血液アンモニア値の測定は必須である．血液ガス分析，血中アミノ酸濃度(分岐鎖アミノ酸/芳香族アミノ酸比：Fischer比，分岐鎖アミノ酸/チロシン比：BTR)も可能であれば行う．

以上の検査結果は肝病態の把握に必要であるのみならず，治療反応性や最終予後の判断にも有力な情報をもたらす．

・**画像検査**：腹部超音波あるいはCT検査を行い，肝硬変の存在あるいは門脈－大循環短絡路の有無，腹水の有無などを観察する．可能であ

れば脳 CT 検査(脳萎縮・血腫の有無)や MRI 検査(T1 強調画像による淡蒼球高信号の確認)を施行する[5].

文献
1) 佐藤俊一, 鈴木一幸：慢性肝不全, 山村雄一, 吉利和(監修), 最新内科学体系 肝・胆道疾患 1—肝機能不全, 中山書店, pp174-191, 1991
2) Ferenci P, et al：Hepatic encephalopathy-difinition, nomenclature, diagnosis and quantification；Final report of the working party at the 11th World Congress of Gastroenterology, Vienna, 1998. Hepatology 35：716-721：2002
3) 鈴木一幸：肝性脳症治療の up-date. 日消誌 107：14-21, 2010
4) Butterworth RF：Pathogenesis of hepatic encephalopathy；New insights from neuroimaging and molecular studies. J Hepatol 39：278-285, 2003
5) 加藤章信, 鈴木一幸：肝性脳症—診断・検査. 日消誌 104：344-351, 2007

劇症肝炎（急性肝不全）

滝川康裕* 宮本康弘*

索引用語：肝性脳症，プロトロンビン時間，人工肝補助，肝移植

1 はじめに

劇症肝炎あるいは急性肝不全は頻度が少ないため，日米ともに多施設共同で臨床統計を行っている．一方，急性発症から急激に進行する致命率の高い疾患であるため，介入試験が困難であり，質の高いエビデンスに乏しいのが現状である．

本稿では，臨床統計を中心に急性肝不全の現状を解説する．

2 劇症肝炎および急性肝不全の概念，定義

1. 急性肝不全症候群の概念

急性肝不全（Acute hepatic failure: ALF）とは急激かつ高度の肝細胞機能障害に基づいて肝性脳症をはじめとする肝不全症状をきたす予後不良の疾患群である[1]．多くの場合，ウイルスや薬物による広汎あるいは亜広汎肝細胞死によって引き起こされるが，Reye症候群や急性妊娠性脂肪肝のように壊死・炎症がほとんどない病態でも起こりうる．

肝細胞死の機序は，これまで炎症を主体とした場合（ウイルス性，薬物アレルギー性，自己免疫性肝炎）とその他（薬物中毒，循環障害など）の場合に分けて考えられてきたが，発端が非炎症性の肝細胞死であっても組織障害に伴う炎症機構が障害を増幅することもあり，両者は明瞭に区別できないこともある．

2. わが国における「劇症肝炎」の概念とその変遷

わが国では急性肝障害の原因の大多数がウイルス性と考えられてきたことから，肝炎（ウイルス性肝炎，薬剤アレルギー性肝炎，急性発症自己免疫性肝炎）による急性肝不全を「劇症肝炎」とし，急性肝不全の代表として扱ってきた経緯がある．したがって，米国や英国で急性肝不全の成因の第1位を占めるアセトアミノフェン中毒や循環障害などはわが国の劇症肝炎からは除外されてきた．一方で，劇症肝炎の成因のひとつとして，その多くを占める「成因不明（あるいは非A非B型肝炎）」例が本当に「肝炎」であるのかは明確ではなかった．

Yasuhiro TAKIKAWA et al: Fulminant hepatitis（Acute liver failure）
*岩手医科大学消化器・肝臓内科［〒020-8505 岩手県盛岡市内丸 19-1］

表1　急性肝不全の定義(厚生労働省「難治性の肝・胆道疾患に関する調査研究」班，文献2より引用

正常肝ないし肝予備能が正常と考えられる肝に肝障害が生じ，初発症状出現から8週以内に，高度の肝機能障害に基づいてプロトロンビン時間が40%以下ないしはINR値1.5以上を示すものを「急性肝不全」と診断する．急性肝不全は肝性脳症が認められない，ないしは昏睡度がⅠ度までの「非昏睡型」と，昏睡Ⅱ度以上の肝性脳症を呈する「昏睡型」に分類する．また，「昏睡型急性肝不全」は初発症状出現から昏睡Ⅱ度以上の肝性脳症が出現するまでの期間が10日以内の「急性型」と，11日以降56日以内の「亜急性型」に分類する．

(注1) B型肝炎ウイルスの無症候性キャリアからの急性増悪例は「急性肝不全」に含める．また，自己免疫性で先行する慢性肝疾患の有無が不明の症例は，肝機能障害を発症する前の肝機能に明らかな低下が認められない場合は「急性肝不全」に含めて扱う．

(注2) アルコール性肝炎は原則的に慢性肝疾患を基盤として発症する病態であり，「急性肝不全」から除外する．ただし，先行する慢性肝疾患が肥満ないしアルコールによる脂肪肝の症例は，肝機能障害の原因がアルコール摂取ではなく，その発症前の肝予備能に明らかな低下が認められない場合は「急性肝不全」として扱う．

(注3) 薬物中毒，循環不全，妊娠脂肪肝，代謝異常など肝臓の炎症を伴わない肝不全も「急性肝不全」に含める．ウイルス性，自己免疫性，薬物アレルギーなど肝臓に炎症を伴う肝不全は「劇症肝炎」として扱う．

(注4) 肝性脳症の昏睡度分類は犬山分類(1972年)に基づく．ただし，小児では「第5回小児肝臓ワークショップ(1988年)による小児肝性昏睡の分類」を用いる．

(注5) 成因分類は「難治性の肝疾患に関する研究班」の指針(2002年)を改変した新指針に基づく．

(注6) プロトロンビン時間が40%以下ないしはINR値1.5以上で，初発症状出現から8週以降24週以内に昏睡Ⅱ度以上の脳症を発現する症例は「遅発性肝不全」と診断し，「急性肝不全」の類縁疾患として扱う．

表2　世界の代表的な急性肝不全の定義(診断基準)と臨床病型分類

	定義	臨床病型	文献
日本	急性肝不全：初発症状発現から8週以内に昏睡Ⅱ度以上，PT<40%，INR>1.5	急性型：0～10日 亜急性型：11～56日 遅発性肝不全：8～24週(類縁疾患)	2
英国 (King's College)	Acute liver failure：発黄から12週以内に昏睡	Hyperacute：0～7日 Acute：8～28日 Subacute：29～12週	4
フランス (Clity)	Acute liver failure：PTまたはFV≦50% Fulminant, subfulminant hepatic failure：昏睡	Fulminant hepatic failure：0～2週 Subfulminant hepatic failure：2～12週	5, 6
米国	発症から24週以内に意識障害，PT INR>1.5	分類なし	3

PT: プロトロンビン時間，FV: 凝固第5因子

これらの問題点を解消し，欧米との概念の整合性を得る目的から，わが国でも急性肝不全の概念の導入が検討され，2011年厚生労働省科学研究費補助金(難治性疾患克服事業)「難治性の肝・胆道疾患に関する調査研究」班において診断基準が定められた(表1)[2]．

3. 定義および診断基準

急性肝不全についてはTreyらの定義[1]が基本となっている．すなわち，重篤な肝障害の結果，発症から8週以内に脳症を発現した状態で，基本的には回復する可能性のある状態をいう．世界的にこの定義が基本となり，これに加えて肝機能障害の客観的指標とし

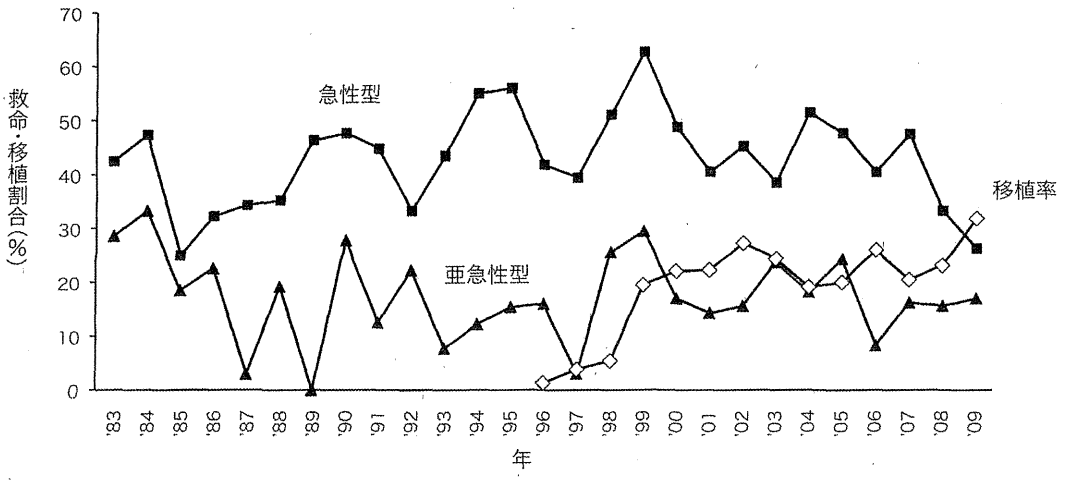

図1　わが国における劇症肝炎の内科的救命率と肝移植率の変遷(文献11)

て，プロトロンビン時間（PT）を採用している場合が多い．表2に各国の急性肝不全の定義あるいは診断基準を示す[2~6]．

わが国の定義では，「高度の肝機能障害」の客観的指標として，蛋白合成能を表すPTを採用し，PT40％以下またはPT INR1.5以上を示す急性肝障害を急性肝不全と定めている．従来の劇症肝炎は，肝炎による昏睡型急性肝不全に相当する．

4. PTの扱い

PTは肝機能の指標として古くから用いられているが，その測定法は標準化されていない．経口抗凝固薬（ワーファリン）服用患者の管理に関しては，国際標準比(international normalized ratio: INR)が設定され，世界的に普及しているが，これを肝疾患患者に対してそのまま適用すると，試薬間，施設間差が大きいことがすでに指摘されている[7,8]．しかし，米国ではINRを急性肝不全の定義に加え，移植適応の優先順位の指標（Model for Endstage Liver Disease: MELD）にも採用しており，広く普及しているという現実がある[9]．

5. 臨床病型の考え方：発症－昏睡期間の扱い

昏睡型の急性肝不全の予後は発症あるいは黄疸の発現から肝性昏睡の発現までの期間により異なることが知られており，この期間によっていくつかの臨床病型に分けられている．この病型の設定は，表2に示したように各基準まちまちである．わが国では劇症肝炎の全国集計を基に，初発症状から昏睡までの期間が10日以内の急性型と11日以上の亜急性型に分類している（表1）．発症-昏睡期間のさらに長い遅発性肝不全は，極めて予後不良で，急性肝不全の類縁疾患とされる．わが国における劇症肝炎の内科的救命率の変遷と肝移植率を図1に示す．

米国では，予後を規定しているのは成因であり，発症－昏睡期間（臨床病型）が本質ではないとしている．臨床病型が一見予後と関連しているようにみえるのは，各病型に含まれる成因に偏りがあるからだと明言している[10]．

3 急性肝不全の成因

わが国では急性肝不全全体としての全国的

表3　わが国における劇症肝炎の成因(厚生労働省難治性の肝・胆道疾患調査研究班，2005～2009，文献11より改変)

	全症例 (n=488)	劇症肝炎急性型 (n=277)	劇症肝炎亜急性型 (n=233)	遅発性肝不全 (n=28)
A型	2.9	5.7	0.4	0
B型	39.8	54.2	26.6	32.1
C型	1.0	0.9	1.3	0
E型	0.8	0.9	0.9	0
その他のウイルス	0.8	0.9	0.9	0
自己免疫性	9.6	2.2	14.2	32.1
薬物性	14.8	13.7	15.5	17.9
成因不明	28.9	19.4	39.5	17.9
評価不能	1.4	2.2	0.9	0

%

表4　急性肝不全の成因(全国救急施設へのアンケート調査，2008年)

(症例数) %	非昏睡型 (79)	急性型 (58)	亜急性型 (34)	遅発性肝不全 (2)	Acute-on-Chronic 肝不全 (44)	計 (217)
ウイルス性	32.9	32.8	32.4	0	25.0	30.9
薬物性	5.1	13.8	17.6	50.0	2.3	9.2
自己免疫性	0	8.6	11.8	0	0	4.1
成因不明	11.4	8.6	17.6	50.0	4.5	10.6
分類不能	0	3.4	0	0	0	0.9
中毒性	5.1	5.2	0	0	2.3	3.7
アルコール性	17.7	12.1	8.8	0	43.2	19.8
循環障害	15.2	5.2	0	0	6.8	8.3
悪性腫瘍浸潤	3.8	0	2.9	0	9.1	3.8
代謝性	0	0	2.9	0	0	0.5
術後肝不全	0	1.7	0	0	2.3	0.9
その他	8.9	8.5	5.9	0	4.5	7.4

な集計はない．上述の理由から，「劇症肝炎(肝炎によると考えられる昏睡型急性肝不全)の成因調査」が1983年以降続けられている．近年の成因を表3に示す[11]．B型肝炎ウイルスが最も多く，次いで，成因不明，薬物，自己免疫性の順である．急性型ではB型が半数以上を占め，亜急性型では成因不明が最も多い．成因不明例のクラスター分析により，既知成因との類似性をみると，その約40％が自己免疫性肝炎か薬物性に類似した群に分類される[12]．このことから，既知の薬物性と併せると，推定薬物性肝障害による劇症肝炎は，B型に次ぐ頻度と推定される．

非肝炎の急性肝不全の成因に関しては，新定義策定のための資料として，2008年に全国の救急施設へのアンケート調査により集計されたものがある[13]．その結果を表4に示す．この時点ではアルコール性が含まれてい

表5　米国における急性肝不全(1998～2008, 1,321例)の成因(文献14より改変)

	頻度(%)
アセトアミノフェン	46
成因不明	14
薬物性肝障害	12
B型肝炎	7.7
自己免疫性肝炎	5.9
循環障害	4.6
A型肝炎	2.6
Wilson病	1.4
Budd-Chiari症候群	0.9
妊娠関連*	0.8
その他	4.8

*：HELLP症候群および急性妊娠性脂肪肝

表6　わが国における劇症肝炎の治療法とその施行率(2004～2009, 文献11より改変)

	全症例 (n=488)	劇症肝炎急性型 (n=277)	劇症肝炎亜急性型 (n=233)	遅発性肝不全 (n=28)
ステロイド	73.4	68.3	76.4	89.3
グルカゴン－インスリン療法	14.4	13.7	14.7	17.9
特殊組成アミノ酸	20.2	14.3	23.6	39.3
血漿交換	89.8	92.5	89.3	71.4
血液濾過透析	74.0	75.1	74.9	57.1
プロスタグランジンE1	6.8	6.7	7.3	3.6
インターフェロン	13.9	15.4	12.9	10.7
サイククロスポリンA	10.0	7.0	12.9	10.7
核酸アナログ	38.6	50.9	27.5	32.1
抗凝固療法	46.7	43.2	51.1	39.3
肝移植	23.2	15.9	30.9	17.9

%

るが，アルコールは本質的に慢性肝障害であり，多くはacute-on-chronic hepatic failureの病型をとるため，急性肝不全の定義からは除外されている．循環障害によるものが最も多く，次いで中毒性(毒キノコ：アマニチン，アセトアミノフェンなど)，代謝性(甲状腺機能亢進症など)があげられている．救急施設ではアルコールを除くと，肝炎と非肝炎はほぼ同等だが，昏睡型に限ると肝炎が多くなっている．

米国の集計結果を表5に示す．アセトアミノフェンが約半数を占め，次いで成因不明となっている．この傾向は英国も同様である[14]．

4　急性肝不全の予知・予防

昏睡発現後の急性肝不全の内科的救命率は非常に低いことから，昏睡発現を予知し，早

期に治療開始することによってこれを阻止あるいは予後改善に繋げる試みがなされている[15]．劇症化予知式の作成とこれに基づいた広域の患者搬送システムによりプロスペクティブな検討が続けられている．

5 急性肝不全の治療法

表6に現在わが国で行われている劇症肝炎の内科的治療法とその施行率を示す[11]．わが国ではほとんどのすべての症例に人工肝補助療法(ALS，血液濾過透析＋血漿交換)が行われているのが特徴である．ALSにより肝機能の一部を代償し，意識レベルを維持し，合併症を予防しながら肝の再生を待つのが基本的な治療方針である．そして，内科治療の限界を適切な時期に判断し，肝移植の適応を検討する．わが国では，劇症肝炎の約25％に肝移植が行われている．しかし，いずれの治療法も，無作為化比較試験(RCT)などによるエビデンスが得られてはいない．

急性肝不全は稀な疾患であり，1施設での症例数が限られている．しかも急激な経過で生命が危険な状況に陥るため，根本的な治療法についてのRCTが行い難い．このため，肝移植はもちろんのこと，人工肝補助療法などの主要な治療法に関するRCTは，わが国では全くなく，欧米でもほとんど行われていないのが現状である．一般的な管理法や合併症予防に関しても同様であるが，欧米を中心に症数例の臨床研究が報告されている．

1. 一般的管理法

昏睡型の急性肝不全は，肝移植可能な専門施設の集中治療室(ICU)で管理するのが一般的で，内科的治療の死亡率を低下させると古くからいわれている[16,17]．薬物による肝不全の可能性あるいは相加的・相乗的肝障害の悪化の可能性を考えて，すべての薬物を中止するのが原則である[18]．

急性肝不全の水分・電解質管理，栄養療法に関するRCTは国内外ともに存在しないが，意識障害あるいは人工呼吸管理のため，経腸または静脈栄養管理を行う．わが国では窒素負荷を避けるため，蛋白・アミノ酸制限をする場合が多い[19]が，欧米では極端な窒素制限は行っていない[20]．

また，欧米では，脳浮腫誘発の危険性を考慮し，脳圧モニタリングのうえ輸液を管理し，マンニトール，高張食塩水輸液，過換気，低体温など種々の治療法を試みている[10～19]が，わが国では，血液濾過透析により体液・電解質管理を行うため脳浮腫に対するこのような対策は一般的ではない[11]．

2. 糖質コルチコイドおよびN-アセチルシステイン

劇症肝炎が過剰な免疫反応により起こると考えられていることから，抗炎症治療として糖質コルチコイド(ステロイド)が用いられ，わが国でも保険適応が認められている．しかし，欧米ではRCTでその有効性は認められなかった[21～23]ことより，現在は使用されていない．わが国では，高サイトカイン血症に対する対策として，現在でも広く使用されている．かつて大量長期投与により感染症や消化管出血を誘発した反省と，血中サイトカイン動態の研究成果を基に，発症早期に短期大量[23]あるいは肝局所投与[25]が行われている．

N-アセチルシステイン(NAC)は，アセトアミノフェンによる肝障害に対して有効であることが示され，一般的に用いられているのは周知の事実である[26]．さらに，アセトアミノフェン以外の成因による急性肝不全でも，昏睡度がI～II度の初期の段階で投与すると，内科的救命率が向上することが米国のRCTで示されている[27]．わが国ではアセト