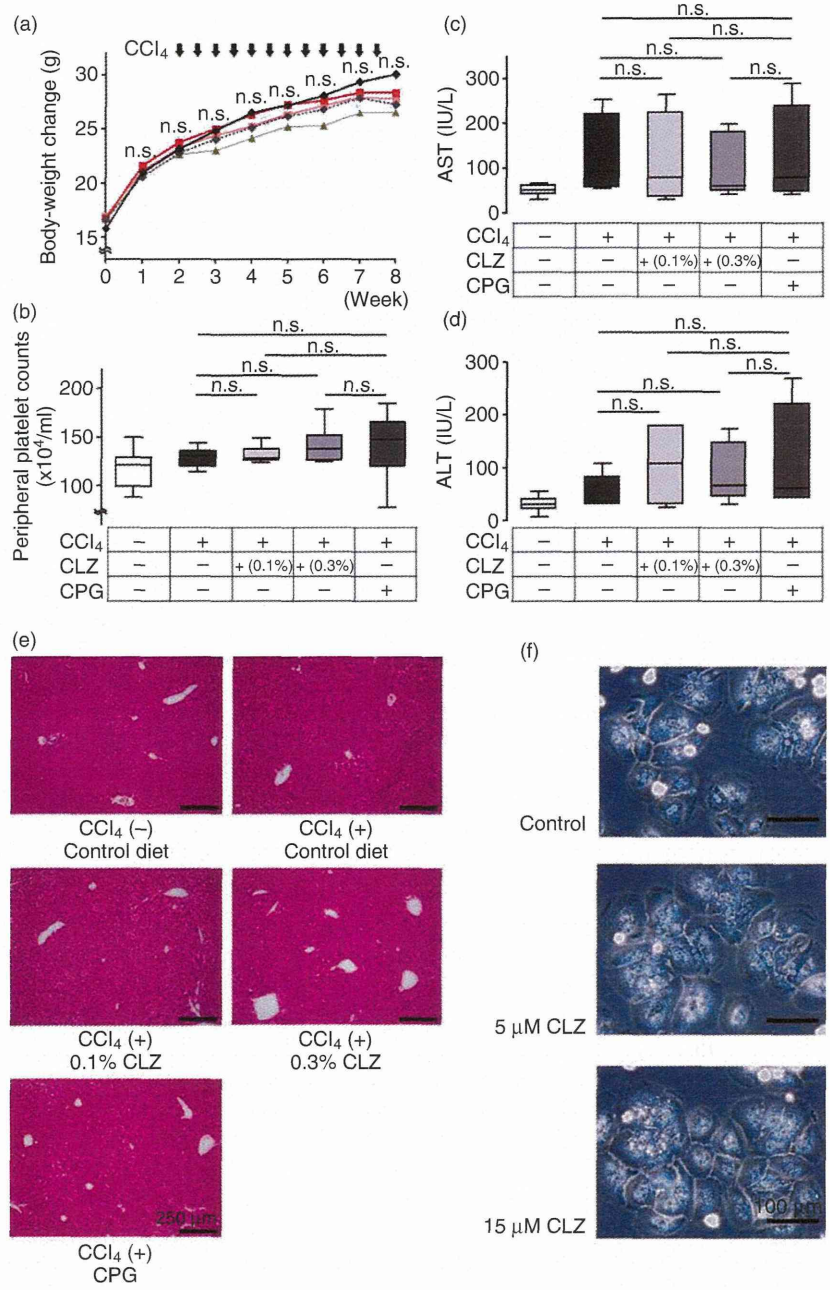


Figure 3 Bodyweight change, peripheral platelet counts, hepatocyte damage and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) did not differ among the CCl_4-treated groups. (a) Bodyweight change during the 8-week experimental duration. Mice without CCl_4 treatment showed the highest gain, however, no significant differences were observed among groups. (b) Peripheral platelet counts at death were not different among CCl_4-treated groups. (c,d) Serum AST and ALT levels at death were not different among CCl_4-treated groups. (e) Hematoxylin–eosin staining of liver sections from each group (original magnification ×100). No obvious differences in hepatocyte damage existed among CCl_4-treated groups. (f) Morphology of hepatocytes supplemented with cilostazol for 1 day was observed under a phase contrast microscope (original magnification ×200). The morphology was unaffected by cilostazol supplementation. On line plots, each plot represents the mean of measurements ($n = 10$). The box plots present the median and 25th–75th percentiles. Upper and lower lines represent the minimum and maximum values ($n = 10$). CCl_4, carbon tetrachloride; CLZ, cilostazol; CPG, clopidogrel. –◆–, CCl_4 (–) + control diet; –■–, CCl_4 (+) + 0.3% cilostazol; –■–, CCl_4 (+) + 0.1% cilostazol; –◆–, CCl_4 (+) + control diet; –▲–, CCl_4 (+) + clopidogrel.

the 0.3% cilostazol-administrated group (0.44%; 95% CI = 0.29–0.60) compared with that in the control group (1.14%; 95% CI = 0.55–1.72; $P < 0.05$; Fig. 4c). These results indicate that cilostazol has potent activity to attenuate HSC activation in the liver through unknown mechanisms.

Cilostazol directly and effectively inhibits the activation of HSC but not of Kupffer cells

To reveal the possible mechanisms underlying these *in vivo* observations, we performed *in vitro* studies in

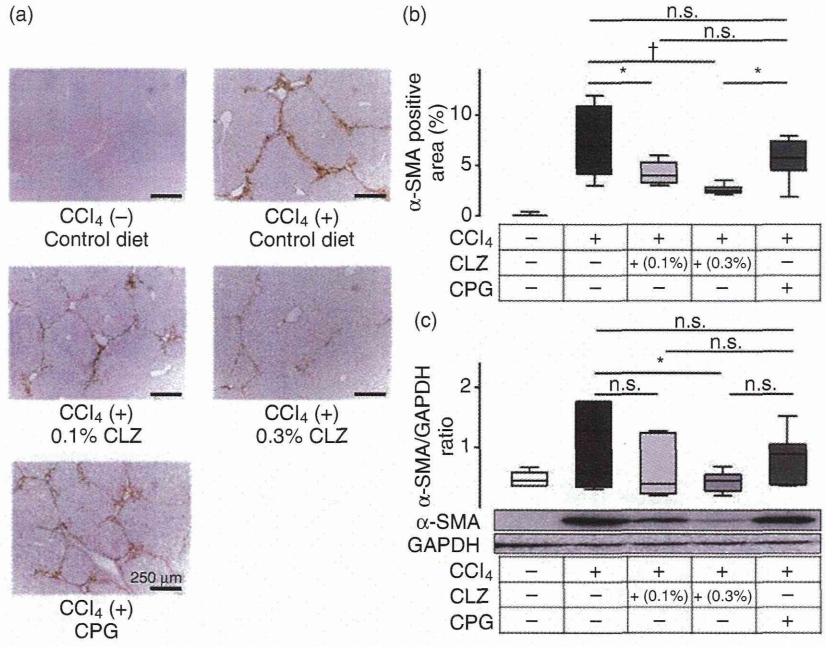


Figure 4 Cilostazol attenuated the expression of α-SMA protein in the liver. (a) α-SMA immunostaining of liver sections in each group. Treatment with CCl_4 for six weeks remarkably increased α-SMA expression. Among CCl_4-treated groups, the liver in cilostazol-administrated groups has a reduced α-SMA-positive area compared with that in the control diet or clopidogrel-administrated group (original magnification ×100). (b) Quantification of the α-SMA positive area in each group. Cilostazol-administrated groups had significantly decreased α-SMA positive areas compared with control diet and clopidogrel-administrated groups. (c) Measurement of α-SMA protein in the liver by immunoblotting. Administration of 0.3% cilostazol reduced α-SMA levels in CCl_4-treated mice. The box plots present the median and 25th–75th percentiles. Upper and lower lines represent the minimum and maximum values ($n = 10$). $^*P < 0.05$; $\dagger P < 0.001$ vs CCl_4-treated control diet group. α-SMA, α-smooth muscle actin; CCl_4, carbon tetrachloride; CLZ, cilostazol; CPG, clopidogrel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

primary cultures of HSC. Generally, isolated HSC undergo autonomous activation in culture, and the activation is associated with a depletion of retinoid droplets, morphological change, cell proliferation, and expression of several activation markers such as α-SMA and collagen α1 (I). During 6 days of culture, control HSC gradually lost retinoid droplets and showed myofibroblast-like activated morphology, whereas cilostazol-supplemented HSC maintained retinoid droplets and retained quiescent morphology (Fig. 5a). In addition, cilostazol suppressed HSC proliferation in a dose-dependent manner, without showing cell toxicity (Fig. 5b). The expression of α-SMA protein was dose-dependently suppressed in the presence of cilostazol (Fig. 5c). Because Kupffer cells have been shown to be implicated in liver fibrosis as well as HSC,[11,38–40] we examined the effect of cilostazol on Kupffer-cell activation *in vivo* and *in vitro*. Pathological examination revealed a weak tendency for decrease in the F4/80 positive (Kupffer cell) area in the liver of cilostazol-administrated mice, however, we could not detect significant changes in our experimental setting (Fig. 5d,e), and no significant change was observed in mRNA levels of tissue TNF-α and TGF-β1 in the liver by cilostazol treatment (Fig. 5f). In fact, cilostazol did not affect the mRNA expression of F4/80 in isolated Kupffer cells (Fig. 5g), suggesting the minimal effect of cilostazol *in vivo* may be simply explained by the secondary effect of the resolution of fibrosis. Likewise, cilostazol exhibited an insignificant effect on the Kupffer cell production of TNF-α, IL-1β, MCP1 and TGF-β1 (Fig. 5g). These data together propose the notion that the *in vivo* therapeutic efficacy of cilostazol is mediated, at least in part, by its direct effects on HSC. If so, why did HSC respond well to cilostazol? One possible explanation is that HSC are more sensitive to cilostazol than other cell types (e.g., Kupffer cells). Actually, cilostazol-induced cAMP accumulation, which is an indicator for cilostazol

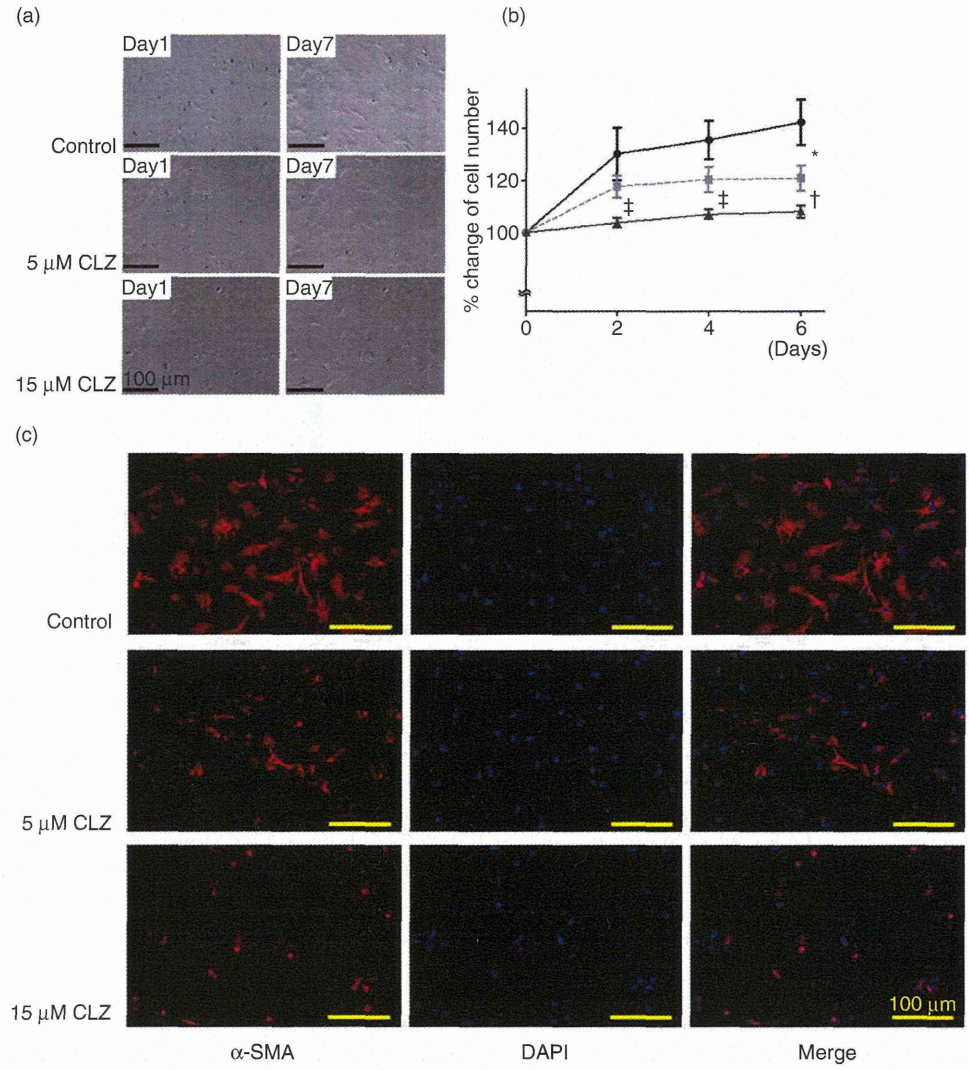


Figure 5 Cilostazol suppressed the proliferation and activation of HSC, but did not affect Kupffer-cell activation. (a) Morphological changes in HSC from 0–6 days were viewed on a phase contrast microscope (original magnification ×200). HSC supplemented with 15 μM cilostazol resulted in visible short cytoplasmic dendritic processes and perinuclear vacuoles containing retinoids. (b) HSC proliferation was determined by direct count of the cell numbers. Cilostazol supplementation slowed the increase in cell numbers compared with control. (c) Immunofluorescent staining of α-SMA (red) in HSC on the second day of culture (original magnification ×200). The protein expression of α-SMA was decreased in cilostazol-supplemented HSC in a dose-dependent manner. (d) F4/80 immunostaining of liver sections in each group. (original magnification ×100). (e) Quantification of the F4/80-positive area in each group. Cilostazol-administrated groups tended to show fewer F4/80-positive areas than the control, but no significant differences were observed among CCl_4-treated groups. (f) mRNA expression levels of TNF-α and TGF-β1 in the liver were not affected by cilostazol. (g) Expression of Kupffer cell marker (F4/80) and inflammation-related genes (TNF-α, IL-1β, MCP1 and TGF-β1) in primary Kupffer cells on the second day of culture was not altered by cilostazol. (h) Accumulation of cAMP in primary cultured HSC and Kupffer cells. Cilostazol supplementation significantly elevated the cAMP level only in HSC. The box plots present the median and 25th–75th percentiles. Upper and lower lines represent the minimum and maximum values ($n = 4$). *$P < 0.05$; †$P < 0.001$; ‡$P < 0.01$ vs control group. α-SMA, α-smooth muscle actin; cAMP, cyclic adenosine monophosphate; CCl_4, carbon tetrachloride; CLZ, cilostazol; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; HSC, hepatic stellate cell; IL-1β, interleukin-1β; MCP1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1. –●–, control; -■-, 5 μM CLZ; –▲–, 15 μM CLZ.

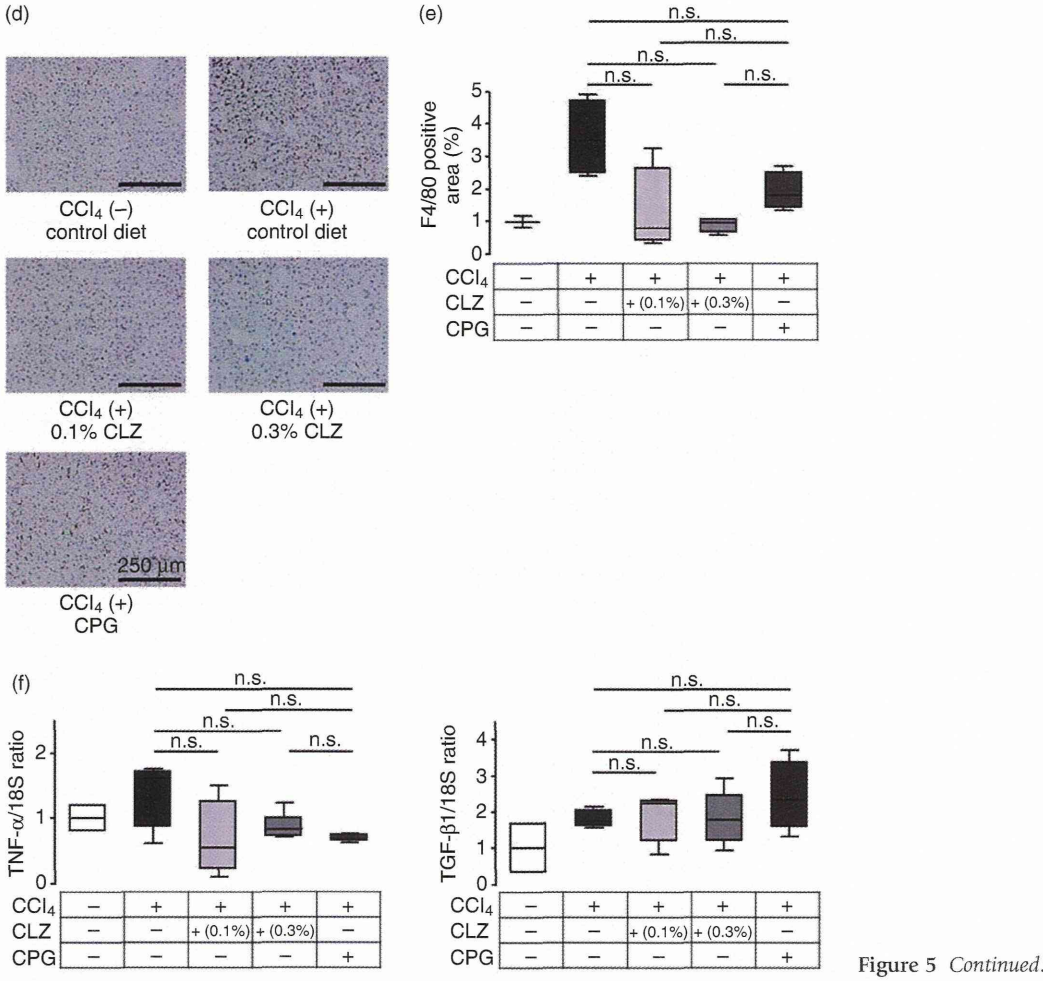
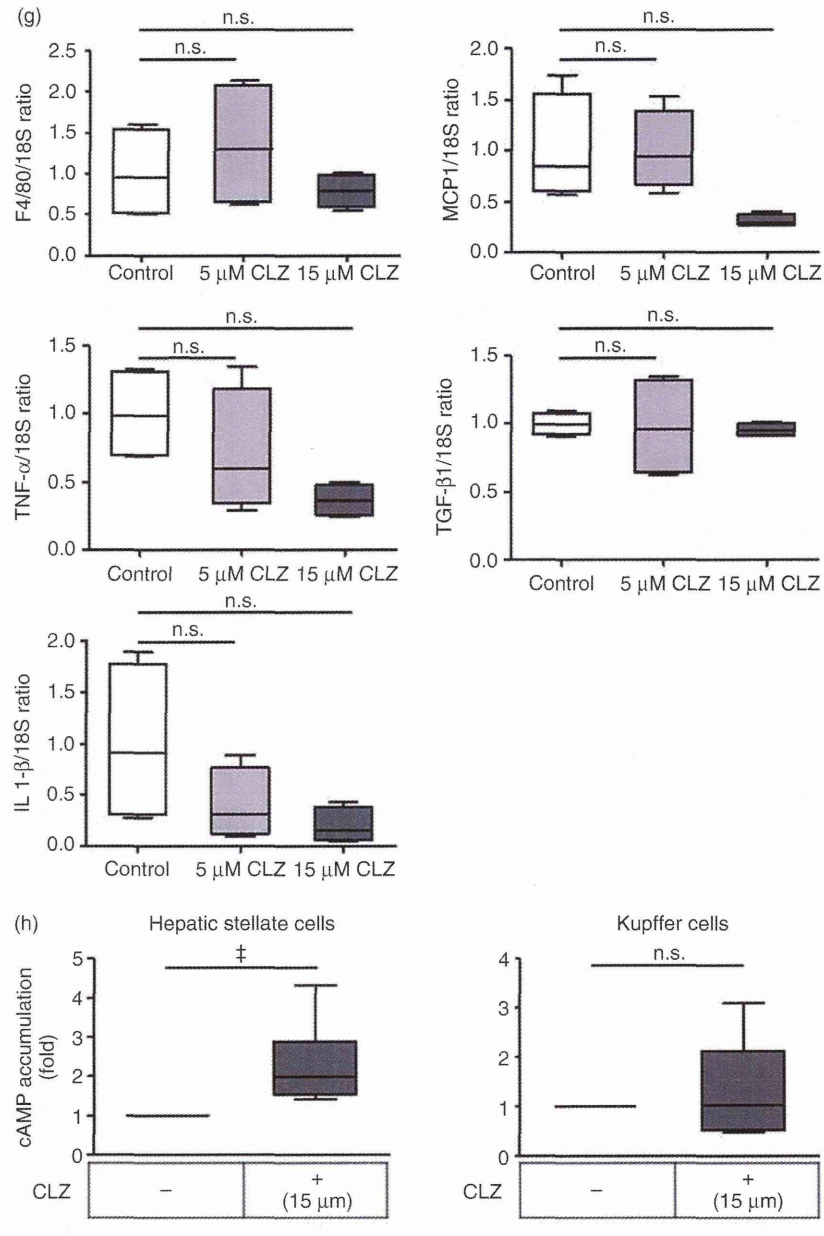


Figure 5 *Continued.*

inhibition of the PDE3 enzyme, was significantly higher only in HSC supplemented with cilostazol (2.283; 95% CI = 1.45–3.12; $P < 0.01$) but not in Kupffer cells (1.363; 95% CI = 0.4374–2.289; Fig. 5 h).

Cilostazol suppressed PDGFR expression in HSC

To further delineate the effect of cilostazol on the activation of HSC, we characterized the cilostazol-affected gene expression profiles during the activation phase of HSC. First, to confirm the direct effects of cilostazol on the gene activation mechanism of HSC, we examined the α-SMA and collagen α1 (I) gene induction. As suggested by the previous data (Figs 2,4,5), mRNA induction of α-SMA was lower in cells supplemented with 5 μM cilostazol (0.555; 95% CI = 0.085–1.024) and 15 μM cilostazol (0.221; 95% CI = 0.086–0.356) when compared with the control (2.53; 95% CI = 1.01–4.05; $P < 0.01$; Fig. 6a). Similarly, collagen α1 (I) mRNA expression was lower in cells supplemented with 5 μM cilostazol (0.411; 95% CI = 0.010–0.833) and 15 μM cilostazol (0.059; 95% CI = 0.042–0.159) as compared with the control cells (2.20; 95% CI = 0.31–4.08; $P < 0.01$; Fig. 6b). Then, to gain further mechanistic insight into the action of cilostazol on HSC, the mRNA expression of PDGF-B, PDGFR-β and TGF-βR1, an important cytokine and cytokine receptors for HSC activation, was determined. The expression of PDGF-B, one of the most important mitogens for HSC, was unaffected by cilostazol treatment (Fig. 6c), however, PDGFR-β mRNA expression in the 5 μM cilostazol-supplemented cells (0.282; 95% CI = 0.104–0.460) and



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15 μM cilostazol-supplemented cells (0.336; 95% CI = 0.036–0.636) was significantly decreased compared with that in control cells (0.749; 95% CI = 0.290–1.210; $P < 0.001$; Fig. 6d). In contrast, TGF-βR1 mRNA expression was not affected by cilostazol treatment (Fig. 6e). These results indicate the possibility that cilostazol attenuates the activation-induced proliferation of HSC through the abrogation of PDGF–autocrine signaling by limiting the receptor (PDGFR-β) signaling regardless of the ligand (PDGF) availability.

DISCUSSION

THE P.O. ADMINISTRATION of cilostazol effectively prevents the development of CCl_4-induced liver fibrosis in mice. In agreement with the previous study,

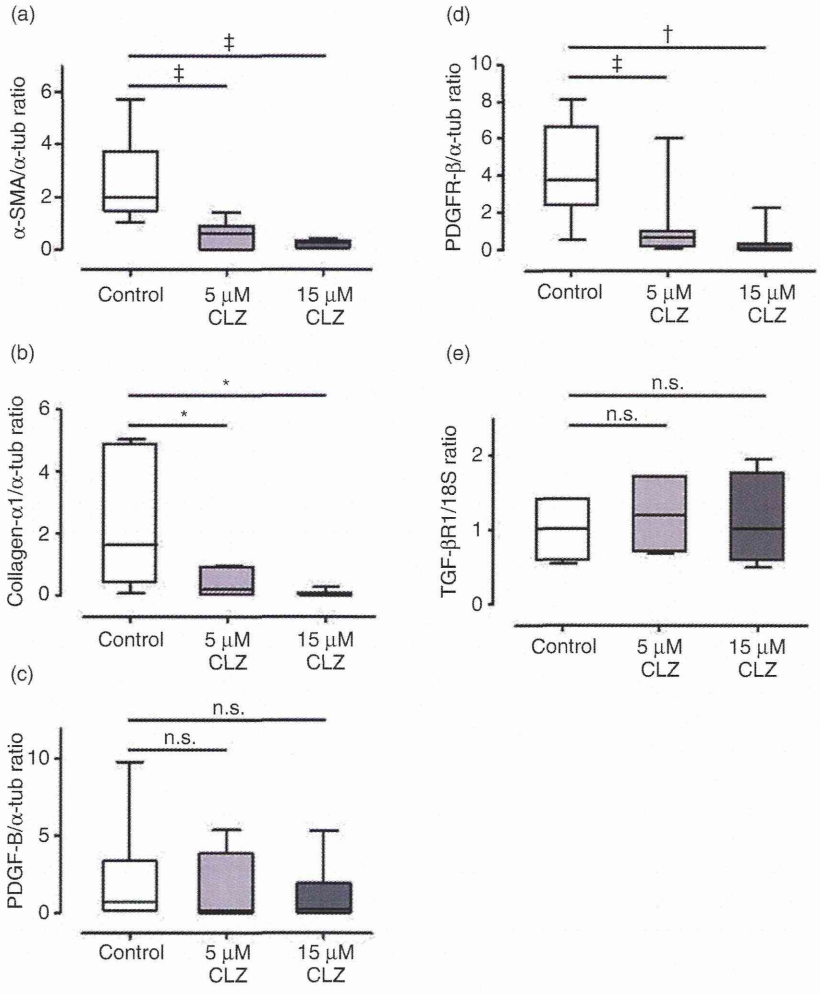

Figure 6 Expression of genes associated with HSC activation was assessed by reverse transcription quantitative polymerase chain reaction. (a) α-SMA, (b) collagen α1 (I) and (d) PDGFR-β expression levels in HSC supplemented with 5 μM and 15 μM cilostazol for 2 days were significantly suppressed compared with control (*$P < 0.05$; ‡$P < 0.01$; †$P < 0.001$ vs control, respectively). The expression of (c) PDGF-B and (e) TGF-βR1 had no difference between control and cilostazol-supplemented HSC. The box plots present the median and 25th–75th percentiles. Upper and lower lines represent the minimum and maximum values ($n = 7$). α-SMA, α-smooth muscle actin; CLZ, cilostazol; HSC, hepatic stellate cell; PDGF-B, platelet growth factor-B; PDGFR-β, platelet growth factor receptor-β; TGF-βR1, transforming growth factor-β receptor 1.

cilostazol was not toxic to HSC as indicated by the morphology and proliferation of the cells (Fig. 5a,b).[25] It is noteworthy that unlike many other candidate medications, cilostazol is already widely used as an antiplatelet agent in clinical practice with proven long-term safety. For this reason, cilostazol holds potential to become an antifibrotic agent for chronic liver diseases in humans.

In the present study, we employed clopidogrel as an alternative antiplatelet agent for the comparison. Although both cilostazol and clopidogrel showed minimal side-effects (Fig. 3), only cilostazol attenuated liver fibrosis (Figs 2,4), suggesting that cilostazol may have distinct antifibrotic mechanisms apart from its antiplatelet action. This notion is consistent with the results of the present *in vitro* study in which the treatment of primary HSC with cilostazol attenuated the HSC proliferation (Fig. 5b) and the expression of α-SMA and collagen α1 (I) (Figs 5c,6), indicating the direct effect of cilostazol on HSC. On the other hand, our *in vitro* and *in vivo* studies showed only a minimal effect of cilostazol on Kupffer cells (Fig. 5d,e,g,h), and no significant change was detected in inflammatory and fibrogenic genes (such as TNF-α and TGF-β1) by cilostazol administration (Fig. 5f). These results lend support to the concept that cilostazol exerts its antifibrotic effect(s) via the suppression of HSC activation *in vivo*.

As reported,[6,39,41] PDGFR-β was absent in quiescent HSC, but was upregulated in an early stage of liver injury. Activating factors from autocrine or paracrine sources such as TGF-β1 stimulate the transcriptional induction of PDGFR-β in quiescent HSC, thereby

rendering them responsive to PDGF-B chain molecules. Among several activating pathways, the autocrine loop exerted by PDGF–PDGFR signaling is regarded as one of the most potent mitogenic pathways for HSC.[42] Although PDGF itself seemed unaffected in our study, our quantitative analyses showed that cilostazol significantly suppressed PDGFR-β in HSC (Fig. 6c,d). Because the PDGF–PDGFR signaling not only promotes myofibroblast proliferation but also participates in other fibrogenic actions, including stimulation of collagen production and promotion of cell adhesion, it has been speculated that the activated PDGF–PDGFR signaling pathway is a candidate target for antifibrotic therapy in liver diseases.[43] Indeed, focusing on PDGFR, recent studies have shown attenuation of hepatic fibrosis by a PDGFR tyrosine kinase inhibitor.[44–46] In the same sense, a blockade of the autocrine loop of PDGF–PDGFR signaling by cilostazol may also have multiple benefits for preventing the development of hepatic fibrosis.

Cilostazol is a selective inhibitor of PDE3, and PDE3 inhibition in platelets exhibits antithrombotic effects by preventing platelet aggregation. Recently, increased intracellular cAMP levels and activation of protein kinase A (PKA) were reported to reduce PDGF-stimulated cellular proliferation.[47,48] Interestingly, cilostazol has been shown to be effective against the development of non-alcoholic fatty liver disease through the activation of the cAMP/PKA signaling pathway *in vivo*.[26] Although the exact mechanism remains to be determined, there may be a link between PDGFR downregulation and cAMP/PKA signaling in HSC.

In conclusion, orally bioavailable cilostazol attenuates HSC activation, possibly through the suppression of PDGFR expression in HSC, and thereby alleviates hepatic fibrogenesis. Further studies may yield a future intervention strategy against liver diseases.

ACKNOWLEDGMENTS

WE ARE GRATEFUL for technical assistance provided by the Central Laboratory of Osaka City University Medical School. We also thank Kimie Takafuji (Hamanaka), Eri Hikawa, Tadashi Mizutani, Kenta Aohara, Kazushi Yamasato and Marika Horiuchi. This work was supported in part by a Grant-in-Aid for Scientific Research (Grant Numbers 20372435 [M. A.], 21689035 [K. H.], 24689056 [M. A.]) from the Japan Society for the Promotion of Science; a grant from the Japanese Ministry of Health, Labour and Welfare (K. Iwaisako., K. Ikeda, M. A and S. U.); and grants from Otsuka Pharma (K. H and S. U.), the Uehara Memorial Foundation (M. A.) and Kanae Foundation for the Promotion of Medical Science (M. A.).

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's web-site:

Video Clip S1–S3 Time-lapse motion pictures of cultured hepatic stellate cells (HSC) with or without cilostazol treatment.

Nutrition 30 (2014) 1195–1207

Contents lists available at ScienceDirect

Nutrition

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Basic nutritional investigation

Whey-hydrolyzed peptide-enriched immunomodulating diet prevents progression of liver cirrhosis in rats

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ARTICLE INFO

Article history:
Received 14 September 2013
Accepted 5 February 2014

Keywords:
Whey-hydrolyzed peptide
Immunomodulating diet
Liver fibrosis
Cirrhosis
Antifibrotic
Hepatocytes-protective

ABSTRACT

Objective: Liver fibrosis and subsequent cirrhosis is a major cause of death worldwide, but few effective antifibrotic therapies are reported. Whey-hydrolyzed peptide (WHP), a major peptide component of bovine milk, exerts anti-inflammatory effects in experimental models. A WHP-enriched diet is widely used for immunomodulating diets (IMD) in clinical fields. However, the effects of WHP on liver fibrosis remain unknown. The aim of this study was to investigate the antifibrotic effects of WHP in a rat cirrhosis model.

Methods: Progressive liver fibrosis was induced by repeated intraperitoneal administration of dimethylnitrosamine (DMN) for 3 wk. Rats were fed either a WHP-enriched IMD (WHP group) or a control enteral diet (control group). The degree of liver fibrosis was compared between groups. Hepatocyte-protective effects were examined using hepatocytes isolated from rats fed a WHP diet. Reactive oxygen species and glutathione in liver tissue were investigated in the DMN cirrhosis model.

Results: Macroscopic and microscopic progression of liver fibrosis was remarkably suppressed in the WHP group. Elevated serum levels of liver enzymes and hyaluronic acid, and liver tissue hydroxyproline content were significantly attenuated in the WHP group. Necrotic hepatocyte rates with DMN challenge, isolated from rats fed a WHP-enriched IMD, were significantly lower. In the DMN cirrhosis model, reactive oxygen species were significantly lower, and glutathione was significantly higher in the WHP group's whole liver tissue.

Conclusion: A WHP-enriched IMD effectively prevented progression of DMN-induced liver fibrosis in rats via a direct hepatocyte-protective effect and an antioxidant effect through glutathione synthesis.

Introduction

Liver cirrhosis is the end stage of chronic liver injury resulting from various causes, such as viral or alcoholic hepatitis, and non-alcoholic steatohepatitis [1]. It is histopathologically characterized as the loss of hepatocytes with interstitial fibrosis [2]. Progression of fibrosis and subsequent cirrhosis lead to life-threatening liver failure and carcinogenesis [1,3]. Despite extensive research on liver cirrhosis, there are few medications (without adverse side effects) proven to be clinically useful for prevention or slowing the progression of liver fibrosis [4,5]. Therefore, new antifibrotic agents with less toxicity are needed for the management and prevention of liver fibrosis [6].

Continuous hepatocellular damage caused by virus and alcohol introduces an inflammatory response with release of inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-10. These inflammatory cytokines promote remodeling and macrophage phagocytosis of necrotic hepatocytes. Subsequent activation of hepatic stellate cells (HSCs) by transforming growth factor (TGF)-β promotes

KJ, TK, and KI designed the research. TK, TH, and SU conducted the research. KJ, TK, TH, KI, KE, and YU analyzed the data. KJ wrote the initial draft. KJ, TK, TH, and KI reworked further drafts, and TK and SU had primary responsibility for the final content. All authors read and approved the final manuscript.

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0899-9007/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nut.2014.02.005