Regular Article

Caffeine Suppresses the Activation of Hepatic Stellate Cells cAMP-Independently by Antagonizing Adenosine Receptors

Momoka Yamaguchi, Shin-ya Saito, Ryota Nishiyma, Misuzu Nakamura, Kenichiro Todoroki, Toshimasa Toyooka, and Tomohisa Ishikawa

*Department of Pharmacology, School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan; and Laboratory of Analytical and Bio-analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan.

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Epidemiological reports have revealed that caffeine intake decreases the risk of liver disease. In this study, we investigated the effect of caffeine on the activation of primary HSCs isolated from mice. Caffeine suppressed the activation of HSC in a concentration-dependent manner. BAPTA-AM, an intracellular Ca2+ chelator, had no effect on the caffeine-induced suppression of HSC activation. None of the isoform-selective inhibitors of phosphodiesterase to affected changes in the morphology of HSC during activation, whereas CGS-15943, an adenosine receptor antagonist, inhibited them. Caffeine had no effect on intracellular cAMP level or on the phosphorylation of extracellular signal-regulated kinase (ERK)1/2. In contrast, caffeine significantly decreased the phosphorylation of Akt1. These results suggest that caffeine inhibits HSC activation by antagonizing adenosine receptors, leading to Akt1 signaling activation.

**Key words** hepatic stellate cell; transformation; caffeine; adenosine receptor; cAMP; Akt1

Liver fibrosis is a major consequence of chronic liver disease, in which the activation of hepatic stellate cells (HSCs) is a critical event. HSCs are located in the space of Disse and attach to sinusoidal endothelial cells, in a similar manner to pericytes on capillaries. Under physiological conditions, HSCs have long protrusions, forming a star-like shape, and are called quiescent HSCs. Quiescent HSCs contain lipid droplets, including vitamin A, and play a primary role in storing and supplying vitamin A. On the other hand, under conditions of liver injury, quiescent HSCs are activated by cytokines and transdifferentiate into myofibroblast-like activated HSCs.

Activated HSCs are characterized by increased proliferation, migration, and contraction, and by a decrease in vitamin A content. Activated HSCs secret cytokines such as hepatocyte growth factor for the regeneration of the liver, and produce collagen, which leads to liver fibrosis. HSCs are activated by oxidative stress and paracrine signaling from various neighboring cells, such as Kupffer cells, sinusoidal endothelial cells, hepatocytes, and platelets. Furthermore, the activation of HSCs is enhanced by autocrine signaling. Thus, HSCs play a critical role in the progression from liver fibrosis to hepatic cirrhosis and hepatoma, which makes them a suitable target for liver fibrosis therapy.

Caffeine is widely ingested in coffee, green tea, black tea, and chocolate. Several epidemiologic studies have suggested that intake of coffee and green tea is related to decreased liver disease. However, the mechanism of this caffeine effect has not been investigated in primary HSCs. In the present study, we investigated the underlying mechanism of the inhibition by caffeine of HSC activation induced by fetal bovine serum (FBS) in primary HSCs isolated from mice.

**Materials and Methods**

**Isolation and Culture of Hepatic Stellate Cells** HSCs were isolated from male ddY mice (body weight 40–60 g; SLC, Hamamatsu, Japan) by digestion with PRONASE® (Merck-Millipore, Tokyo, Japan) and collagenase (Yakult, Tokyo, Japan) and fractionated by 13% Histodenz (Sigma, St. Louis, MO, U.S.A.) according to the method described by Kojima et al. HSC was identified by autofluorescence derived from vitamin A, which was excited at 340 nm and emitted at 550 nm. The purity of HSC in each dish was confirmed to be greater than 90%. Isolated HSCs were seeded on culture dish or multi wall plates in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Gibco, Life Technologies) and incubated for 1 day, the time point before the first medium-change was taken as day 1 and the medium was changed every 2 d with or without reagents thereafter. The time point before 3rd medium-change was taken as day 7. Cells cultured for 2 and 7 d were regarded as quiescent and activated HSCs, respectively.

**Assessment of HSC Activation** HSCs were cultured on a 96-well plate for 7 d. Cells were fixed with 2% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 45 min, permeabilized with 0.1% Triton X-100 for 1 h, and blocked with 3% bovine serum albumin fraction V (BSA, Roche, Mannheim, Germany) for 30 min in phosphate-buffered saline (PBS; 137 mM NaCl, 8.10 mM Na2HPO4·12H2O, 2.68 mM KCl, 1.47 mM KH2PO4). The cells were then incubated with mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (1:1000, Sigma) in PBS containing 1% BSA overnight at 4°C, followed by incubation with Alexa Fluor® 546 conjugated goat anti-mouse immunoglobulin G (IgG) antibody
leupeptin, and 250 mM sucrose) on ice. The cell lysates were 

phenylmethylsulfonyl fluoride, 10 mM Tris, (Nacalai Tesque, Kyoto, Japan) in TBS-T buffer (10 mM sodium phosphate, 150 mM NaCl, 0.1% Tween 20). The concentration of cAMP in the supernatant was measured using a Cyclic AMP EIA kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). cAMP level was normalized by DNA content for each sample. The DNA content was measured by spectrophotometer.

**Western Blot** HSCs treated with caffeine (3 mM) or CGS-15943 (1 μM) from day 2 for 24 h were collected and disrupted with a sonicator (UH-50, SMT Co., Ltd., Tokyo, Japan) in a homogenization buffer (20 mM Tris-HCl (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 50 μg/mL aprotinin, 10 μg/mL leupeptin, and 250 mM sucrose) on ice. The cell lysates were loaded with one-volume load of a sampling buffer (125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, and 0.001% bromophenol blue) and boiled at 95°C for 5 min. After SDS-polyacrylamide gel electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane, and blocked with Blocking One-P® (Toyobo, Osaka, Japan) overnight at 4°C. After washing with 1% BSA for 1 h at room temperature. Signals were detected using ImmunoStar LD reagents (Wako).

**cAMP Level** On the 2nd day after cell seeding, HSCs were treated with forskolin or caffeine. After 15 min, the reaction was terminated with 0.1 N HCl, and the sample was collected by cell scraper. Precipitation was removed by centrifugation (1000 × g, 10 min). The concentration of cAMP in the supernatant was measured using a Cyclic AMP EIA kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). cAMP level was normalized by DNA content for each sample. The DNA content was measured by spectrophotometer.

**RESULTS**

The area of α-SMA-positive staining, which is a marker of activated HSC, was markedly increased, and the autofluorescence of vitamin A, which is a marker of quiescent HSC, disappeared due to the culturing of the primary HSCs in DMEM containing FBS for 7 d (Figs. 1A, B). Caffeine decreased the α-SMA-positive area in a concentration-dependent manner (Figs. 1C, D). In the presence of 10 mM caffeine, the autofluorescence of vitamin A was still observed at day 7 (Fig. 1B). These results suggest that caffeine suppressed the activation of HSCs induced by FBS.

Caffeine is well known to function as an agonist of ryanodine receptor Ca2+ release channels, as an inhibitor of phosphodiesterase (PDE), and as a non-selective adenosine receptor antagonist. To elucidate the mechanism of the inhibitory

**PDE1A**

| Gene   | Forward sequences (5'→3') | Reverse sequences (5'→3') |
|--------|--------------------------|--------------------------|
| PDE1A  | CTAAAGATGAACCTGGAGGACCTCCTCGGAAAC | TGGAGAAAGTGAAGGCCTAATTACAGC |
| PDE1B  | CCACCAACTAAGCAGTCTGTT | GGTGGCTCACAAGTGAAGT |
| PDE1C  | ATGGTGGGCTGAGCTATCCACC | CCGTTTGGCCTACCTGTCTTATAAGGAGG |
| PDE2A  | GCATGTGCTACAGCTGGAC | AGCATGCGCTGACATCTTGAG |
| PDE3A  | TGCTATAACAGCAGCTTCT | ATGACGAAAGCCGAGGTG |
| PDE3B  | ACCATGAAACGCAACAA | GTGATCGTGCAATCGCTG |
| PDE4A  | GGCGGACACTGAAGAATTTCCC | CAGGGTGTCACATCGTGG |
| PDE4B/C | CGCTGGAGGAGAATAGACTGAGT | GACAGACCTGGTCCCTGAGT |
| PDE4D  | CCTCTATGTGTTATGATCAGCCACCC | GATGCACATCATGTATGTCACG |
| PDE5A  | GGCTCTCCATGAGGAAATA | ATTCACCTGGCAGAGGTCTT |
| PDE7A  | CCGAATCTGGAGAATAGCAGCA | GACAGCGGCTCTGAGGAAATA |
| PDE7B  | CAAATGGTGGGAGAATAGGICTG | GGTCGCTTGGCGTGGTAAATC |
| RyR1   | TACCTCCAGACAACCCACA | ACATCTCCAGAGGAGAAGAG |
| RyR2   | GCGAGCTGCTACTTACGACC | CGTTGCTACTGCGACAG |
| RyR3   | AACGTGCTGTCTGAGGAA | ATGTTCCTCAGCAGTCG |
| GAPDH  | ACCAGACCCCTCTCTTGAC | TCGACGACATCCAGCAC |
| β-Actin| CTTCAGCTGCTGGTGGTAA | AGCCATGTACCTGACATCC |

**Table 1. Sequences of Primers Used for RT-PCR**

PDE: phosphodiesterase, RyR: ryanodin receptor, GAPDH: glyceraldehydes-3-phosphate dehydrogenase.
effect of caffeine on HSC activation, we first investigated the possible involvement of ryanodine receptors in the caffeine effect. We confirmed that both quiescent and activated HSCs expressed the mRNAs of RyR1 and RyR2. The contribution of Ca\(^{2+}\) to the inhibitory effect of caffeine on the activation of HSCs was examined using an intracellular Ca\(^{2+}\) chelator BAPTA-AM. BAPTA-AM (5 \(\mu\)M) with 0.05% cremophor EL was loaded into quiescent HSCs 1.5 h before the addition of 3 mM caffeine. After 7-d culturing, there was no difference in cell morphology or \(\alpha\)-SMA-positive area between cells treated with or without BAPTA-AM (Figs. 2B, C), suggesting that Ca\(^{2+}\) release through ryanodine receptors contributed little to the suppressive effect of caffeine on HSC activation.

We next examined the contribution of PDE inhibition to the suppressive effect of caffeine on HSC activation. Using RT-PCR, we confirmed the mRNA expression of PDE1A, 1B, 1C, 2A, 3A, 3B, 4A, 4B/C, 4D, 5A, 7A, and 7B in both quiescent and activated HSCs (Fig. 3A). The effect of PDE inhibition on the activation of HSCs was investigated with subtype-specific PDE inhibitors, i.e., the PDE1 inhibitor 8-Br-cGMP (30 \(\mu\)M), the PDE2 inhibitor EHNA (10 \(\mu\)M), the PDE3 inhibitor cilogstamide (1 \(\mu\)M), the PDE4 inhibitor rolipram (10 \(\mu\)M), and the PDE5 inhibitor zaprinast (3 \(\mu\)M). None of the PDE inhibitors had a significant effect on morphology or on the \(\alpha\)-SMA-positive area of the HSCs (Figs. 3B, C). We further measured intracellular cAMP level using an enzyme-linked immunosorbent assay (ELISA) kit. In quiescent HSCs, forskolin (10 \(\mu\)M), an adenylate cyclase activator, drastically increased intracellular cAMP levels, whereas caffeine (3 mM) had no significant effect on these levels under the current experimental conditions (Fig. 3D).

Finally, we investigated whether the adenosine receptor antagonistic effect of caffeine is also involved in caffeine’s suppressive effect on HSC activation. CGS-15943 (1 \(\mu\)M), a subtype-nonselective adenosine receptor antagonist, significantly decreased the area of \(\alpha\)-SMA positive staining (Figs. 4A, B). It is well known that adenosine A\(_1\) and A\(_2A\) receptors inhibit cAMP production via \(\mathrm{Gi}\), whereas adenosine A\(_2A\) and A\(_2B\) receptors stimulate it via \(\mathrm{Gs}\). However, we did not find any changes in intracellular cAMP levels in the HSCs due to caffeine. Therefore, we explored the possible involvement of other signaling pathways. Caffeine (3 mM) and CGS-15943 (1 \(\mu\)M) significantly decreased the phosphorylation of Akt1 (Figs. 4C, E). Concomitantly, caffeine tended to inhibit and CGS-15943 significantly inhibited expression level of Akt1 (Figs. 4C, E). In contrast, both inhibitors showed significant
effect on neither the phosphorylation level nor the total level of ERK1/2 (Figs. 4D, F).

DISCUSSION

The activation of HSCs in primary culture can be induced by FBS, which includes various growth factors. In the present study, we have clearly demonstrated that the activation of mouse primary HSCs by FBS was suppressed by caffeine: Caffeine decreased the area of α-SMA positive staining, which is a marker of activated HSCs, and resulted in the retention of vitamin A autofluorescence, which is a marker of...
quiescent HSCs. These results support epidemiological studies which have shown the suppression of liver fibrosis due to the intake of caffeine or caffeine-containing beverages. We have further investigated the mechanism underlying this caffeine effect. Among the well-known functions of caffeine, we suggest that its antagonistic effects on adenosine receptors are involved in the suppressive effect on HSC activation, which is likely to be mediated via the inhibition of Akt1.

Caffeine is known to function as an adenosine receptor antagonist. In the present study, we found that the adenosine receptor antagonist CGS-15943, like caffeine, decreased the area of α-SMA-positive staining in mouse HSCs cultured in the presence of FBS for 7d, which indicates the suppression of HSC activation. On the other hand, the other functions of caffeine, i.e., agonistic effects on ryanodine receptors or inhibition of PDE, are unlikely to be involved, as discussed below. We confirmed using LC/MS that adenosine was present in the culture medium under our experimental conditions (data not shown). This suggests that caffeine inhibits the activation of HSCs by antagonizing adenosine receptors. Several studies have suggested the contribution of adenosine to the development of hepatic fibrosis: Ethanol, CCl₄, and thioacetamide induce extracellular generation of adenosine by ecto-5'-nucleotidase (CD73) in hepatic slices, which contributes to the development of alcohol-induced fatty liver and hepatic fibrosis. Ethanol has also been shown to stimulate adenosine release from the hepatoma cell line HepG2.

Some G protein-coupled receptors (GPCRs) have been shown to activate the Akt pathway. There are several reports suggesting that the Akt pathway mediates responses to adenosine receptor stimulation: The phosphorylation of Akt and ERK1/2 via the activation of adenosine A₂₃ receptors regulates the function of glioblastoma stem cells, and the activation of adenosine A₁ receptors induces the activation of Akt in melanoma cells. The focal adhesion kinase-phosphatidylinositol 3 (PI3)-kinase-Akt signaling pathway has been suggested to be critical for HSC proliferation and type I collagen expression in activated rat HSCs. In the present study, caffeine and CGS-15943 significantly inhibited the phosphorylation of Akt1 in mouse HSCs. The phosphorylation of ERK1/2 seemed also to lead to the activation of HSCs, and several GPCRs, including adenosine receptors, can activate ERK1/2. However, the present study shows that caffeine and CGS-15943 had no effect on the phosphorylation of ERK1/2 in mouse HSCs. This suggests that adenosine receptors induce the activation of Akt1, thereby leading to the activation of HSCs, and that caffeine suppresses this mechanism by antagonizing adenosine receptors. Interestingly, similarly to the present results in mouse HSCs, caffeine and CGS-15943 have been shown to suppress proliferation of human hepatic cancer cells by inhibiting the PI3 kinase-Akt pathway, independently of ERK1/2. As the present study has determined neither the adenosine receptor subtype involved in the caffeine effect nor the detailed signaling components of the Akt pathway, a more detailed analysis is needed to confirm this notion.

The present results using BAPTA-AM exclude the possible...
involvement of intracellular Ca\(^{2+}\) in the caffeine effect on HSC activation. There are several reports that Ca\(^{2+}\) influences proliferation and apoptosis in activated HSCs.\(^{22,23}\) Caffeine at high concentrations causes a pronounced increase in the sensitivity of ryanodine receptors to Ca\(^{2+}\), thereby inducing Ca\(^{2+}\) release even at basal cytosolic Ca\(^{2+}\) concentrations. In addition, we have confirmed the expression of RyR1 and RyR2 in HSCs. However, BAPTA-AM, a chelator of intracellular Ca\(^{2+}\), did not affect the suppressive effect of 3 mM caffeine on HSC activation. It is plausible that Ca\(^{2+}\) is not involved in the effect of caffeine, at least at the 3 mM level, on HSC activation.

The present study also provides evidence against the involvement of cAMP elevation through PDE inhibition in the caffeine effect. Several studies have suggested that cAMP mediates the inhibition of HSC activation. Kawada et al. reported that dibutyryl cAMP inhibited the myofibroblastic transformation of rat HSCs in vitro.\(^{24}\) Moreover, ATP and adenosine have been shown to suppress the activation of HSCs via cAMP elevation.\(^{25,26}\) In agreement with these findings, Shim et al. reported that caffeine decreased fibrosis and \(\alpha\)-SMA expression in human HSC line LX-2 cells cultured in DMEM supplemented with FBS, and that these decreases were accompanied by an elevation in cAMP level.\(^{27}\) However, other studies have reported contradictory results: The inhibition by caffeine of acetaldehyde-induced activation of HSC-T6 cell lines\(^{28}\) and rat HSCs\(^{29}\) was accompanied by a decrease in cAMP level by antagonizing Gs-coupled adenosine A\(_2\) receptors. Therefore, the involvement of cAMP in the caffeine effect on HSC activation is still controversial. In the present study, no selective PDE inhibitors suppressed the activation of HSCs, even though the concentration of each PDE inhibitor used was 10 times higher than IC\(_{50}\).\(^{30,32}\) Moreover, caffeine did not increase intracellular cAMP level in HSCs. We also confirmed that the adenylate cyclase inhibitor SQ22,536 (10 \(\mu\)M) did not affect the decrease in the area of \(\alpha\)-SMA-positive staining induced by 3 mM caffeine (data not shown).

Taken together, these results suggest that cAMP elevation through PDE inhibition is unlikely to be involved in the suppressive effect of caffeine on the HSC activation, at least under the experimental conditions of this study. The production of cAMP in isolated mouse HSCs in this study may have been at too small a volume to cause any effects even after the inhibition of PDE.

In a clinical study, plasma caffeine levels have been 0.01–0.2 mM by intake of a cup of coffee (caffeine 100 mg/150 mL coffee).\(^{33}\) It is important to point out that caffeine contacts to HSCs before metabolizing by hepatocytes, and the plasma caffeine level in the portal vein should be higher than that in whole body. It is, therefore, possible to say that caffeine acts on HSCs at mM range. Moreover, the plasma caffeine concentration in liver disease patients has been shown to be 7 times higher than that in healthy subjects.\(^{33}\) This also supports the clinical use of caffeine.

In summary, in the present study, isolated mouse HSCs were transformed into activated HSCs by culturing in a medium supplemented with FBS. We found a suppressive effect of caffeine on the activation of HSCs by FBS and investigated the signaling mechanism. These results suggest that caffeine suppresses the activation of HSCs by inhibiting the adenosine receptor-mediated signaling pathway involving Akt1. They also offer new insight into the role of the adenosine receptors that stimulate the Akt1 pathway in the pathogenesis of liver fibrosis. These findings may help to develop a rationale for the use of adenosine receptor antagonists to prevent the progression of liver fibrosis.

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Conflict of Interest The authors declare no conflict of interest.

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