Peroxisome Proliferator-Activated Receptor-γ Activators Monascin and Rosiglitazone Attenuate Carboxymethyllysine-Induced Fibrosis in Hepatic Stellate Cells through Regulating the Oxidative Stress Pathway but Independent of the Receptor for Advanced Glycation End Products Signaling

Wei-Hsuan Hsu,[†,‡] Bao-Hong Lee,[†,‡] Ya-Wen Hsu,[§] and Tzu-Ming Pan*,[‡]

[‡]Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan

[§]SunWay Biotechnology Company Limited, Number 139, Xing'ai Road, Neihu District, Taipei 11494, Taiwan

Ⓢ Supporting Information

ABSTRACT: Advanced glycation end products (AGEs) signaling through its receptors (RAGE) results in an increase in reactive oxygen species (ROS) and is thought to contribute to hepatic fibrosis via hyperglycemia. Carboxymethyllysine (CML) is a key AGE, with highly reactive dicarbonyl metabolites. We investigated the inhibitory effect of *Monascus*-fermented metabolite monascin and rosiglitazone on CML-induced RAGE signaling in hepatic stellate cells (HSCs) and its resulting antihepatic fibrosis activity. We found that monascin and rosiglitazone upregulated peroxisome proliferator-activated receptor-γ (PPAR-γ) to attenuate α-smooth muscle actin (SMA) and ROS generation in CML-treated HSCs in a RAGE activation-independent pathway. Therefore, monascin may delay or inhibit the progression of liver fibrosis through the activation of PPAR-γ and might prove to be a major antifibrotic mechanism to prevent liver disease.

KEYWORDS: carboxymethyllysine (CML), hepatic stellate cells, hepatic fibrosis, advanced glycation end products (AGEs), receptor for AGEs (RAGE), monascin, peroxisome proliferator-activated receptor-γ (PPAR-γ)

INTRODUCTION

Protein glycation is associated with hyperglycemia. Advanced glycation end products (AGEs) are generated by the non-enzymatic interaction between carbohydrates and proteins.[1−3] AGEs play an important role in metabolic syndrome and have recently been implicated in the development of liver fibrosis. Hepatic stellate cells (HSCs) express the receptor for AGEs (RAGE)[4] and also express many components of the NADPH oxidase complex, such as p47phox. Importantly, one study has implicated p47phox-derived reactive oxygen species (ROS) in HSCs activation.[5] To gain better insights into the role of AGEs in HSCs, we investigated the effect of AGEs on ROS production by HSCs. Carboxymethyllysine (CML) is a key AGE with highly reactive dicarbonyl metabolites (e.g., methylglyoxal) and promotes lipid peroxidation to generate malondialdehyde (MDA).[6]

Hyperglycemia is a common cause of chronic liver disease, which is strongly associated with insulin resistance, leading to non-alcoholic steatohepatitis (NASH) and hepatic fibrosis.[7] Interestingly, expression of the peroxisome proliferator-activated receptor-γ (PPAR-γ) protein is suppressed in HSCs during hepatic fibrosis.[8] In addition, one study found that leptin inhibits PPAR-γ and that this inhibition results in NASH in high-fat diet-induced mice.[9] These findings suggest that PPAR-γ is critical for maintaining the quiescent phenotype of HSCs. PPAR-γ ligands, such as rosiglitazone, have been reported to exert anti-inflammatory activity by inhibiting inflammatory gene expression.[10] Rosiglitazone has also been used to cure hepatic fibrosis in

mice.[11] Similarly, another PPAR-γ ligand, troglitazone, also exhibits antihepatic fibrosis activity.[12]

Yellow pigment monascin is a major anti-inflammatory compound initially identified from *Monascus*-fermented secondary metabolites. Monascin has been reported to possess cytotoxic/cytostatic,[13] anti-inflammatory activity,[14] and immunosuppressive activity on mouse T splenocytes.[15] We have previously demonstrated that monascin activates PPAR-γ and improves insulin resistance in C2C12 cells.[16] We also found that monascin lengthened the life span of *Caenorhabditis elegans* and that this effect was mediated by antioxidation.[17] We have also shown that monascin reduces hyperglycemia in streptozotocin-induced rats.[17] In a recent study, monascin was found to prevent the induced oxidative stress and hyperglycemia of glucose metabolites, methylglyoxal and AGEs, through upregulation of the expression of nuclear factor-erythroid 2-related factor 2.[18] In this study, we investigated the inhibitory effect of monascin on CML-induced RAGE signaling in HSCs and its resulting antihepatic fibrosis activity.

MATERIALS AND METHODS

Chemicals. Monascin was isolated from *Monascus*-fermented rice (red mold rice) (see Supplemental Figure 1 of the Supporting Information).[19]

Dichlorofluorescin diacetate (DCFH-DA), lysine, glucose, rosiglitazone (see Supplemental Figure 1 of the Supporting Information), GW9662,
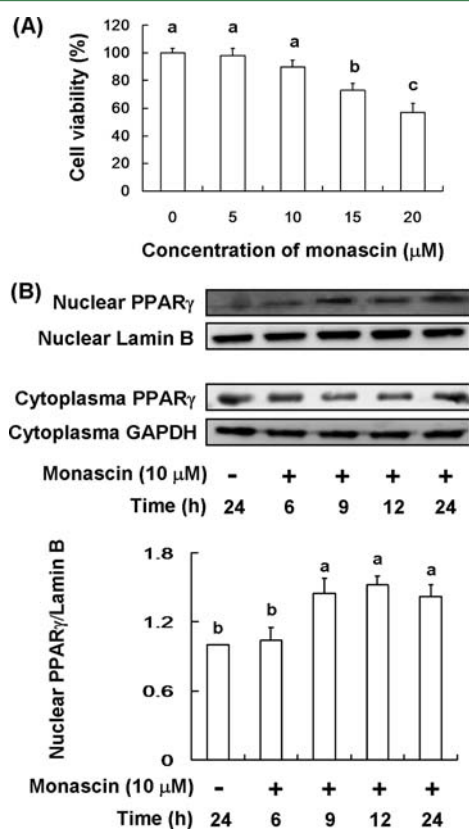

Figure 1. Effects of monascin on cell viability and PPAR-γ activation in HSCs. (A) HSCs were treated with various concentrations (5, 10, 15, and 20 μM) of monascin for 24 h, and the cell viability was measured using the MTT assay. (B) Monascin promoted PPAR-γ translocation from the cytoplasm to the nucleus after 9 h of treatment. Data were presented by the mean ± SD (n = 3). a, b, c, and d values of different letters are significantly different from each other (p < 0.05).

and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-p47phox antibody, anti-RAGE antibody, RAGE siRNA, anti-α-SMA antibody, anti-GAPDH antibody, and anti-lamin B antibody were purchased from Santa Cruz Biotechnology, Inc. (Burlingame, CA). Anti-PPARγ antibody was purchased from Cayman (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium pyruvate, and antibiotics (penicillin/streptomycin) were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). For CML preparation, lysine was for glycation, instead of bovine serum albumin (BSA), modified by Wang et al.[20]

Isolation and Culture of HSCs. Male C57BL6 mice (24 weeks old) were obtained from BioLASCO, Taiwan Co., Ltd. in this study. Animals were provided with food and water *ad libitum*. Animals were subjected to a 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C. The experiments were carried out in a qualified animal breeding room in the animal center at our institute [protocol complied with guidelines described in the "Animal Protection Law", amended on January 17, 2001, Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, Republic of China]. After sacrifice by CO₂, HSCs of mice were isolated by the pronase−collagenase perfusion *in situ* prior to density gradient centrifugation, as previously described.[21] HSCs were cultured in DMEM with 10% fetal bovine serum (FBS). For HSCs activation, CML (25−200 μg/mL) with monascin (5−20 μM) or rosiglitazone (10 μM) was treated in HSCs for 24 h.

Cell Viability. The cell viability was measured using the MTT reduction assay. HSCs were treated with monascin for 24 h. Incubation

was terminated by media aspiration and addition of 5 mg/mL MTT working solution to each well. Formazane formation was terminated after 3 h by removal of the MTT solution. Subsequently, appropriate amounts of dimethyl sulfoxide (DMSO) were added to each well to solubilize the formazane. The formazane-containing samples were transferred to a new 96-well plate, and the absorbance at 590 nm was measured with a spectrophotometer (U-2000, Hitachi, Tokyo, Japan).

ROS Measurement. The level of oxidative stress was monitored by the measurement of ROS. Collected cells were suspended in 500 μL of PBS and mixed with 10 μM (final concentration) DCFH-DA to incubate for 20 min at 37 °C. The cells were washed thrice with phosphate-buffered saline (PBS) to remove redundant DCFH-DA. The cell pellet was mixed with 500 μL of PBS, and the ROS level was assayed by flow cytometry (Becton-Dickinson, San Jose, CA).[22]

Real-Time Polymerase Chain Reaction (PCR). Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. cDNA from 3 μg of RNA was generated using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) according to the instructions of the manufacturer. The reverse-transcription product was diluted in water, and a volume corresponding to 30 ng of original RNA was used for real-time PCR. Real-time PCR amplification and detection were performed using the SYBR Green qPCR SuperMix-UDG with ROX (Life Technologies) in a fluorescence thermal cycler (StepOne Real-Time PCR system, Life Technologies) according to the protocol of the manufacturer. Primers: MMP-13 sense, 5′-CCT CTG GTC TTC TGG CAC AC-3′ and anti-sense, 5′-GGC TGG GTC GTC ACA CTT CTC TGG-3′; GAPDH sense, 5′-GGC AAA TTC AAC GGC ACA GT-3′ and anti-sense, 5′-AGA TGG TGA TGG GCT TCC C-3′; and TIMP-1 sense, 5′-CAT CTG GCA TCC TCT TGT TG-3′; and anti-sense, 5′-CTC GTT GAT TTC TGG GGA AC-3′.

Nuclear Protein Extraction. Nuclear protein extraction from HSCs was obtained according to the kit protocol supplied by the manufacturer (Fermentas, Life Sciences, Canada).

RAGE Knockdown of HSCs by Specific siRNA. For RAGE silence, RAGE interference of HSCs was performed with lipofectamine RNAiMAX transfection reagent followed by the protocols provided (Invitrogen, Carlsbad, CA). The sequence of specific small interfering RNA (siRNA) for RAGE was transfected into HSCs for 48 h, and the control group was transfected with scramble siRNA. Cell lysates were subjected to western blotting.

Western Immunoblotting. Cells were lysed in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 500 μM sodium vanadate, and 10 μg/mL aprotinin overnight. Then, the cell extract was centrifuged (12000g for 10 min) to recover the supernatant. The supernatant was taken as the cell extract. The cell protein was resolved on 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel and transferred to polyvinyldiene fluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk solution for 1 h and incubated overnight with primary antibodies for 4 h; subsequently, the membrane was washed 3 times each for 5 min in phosphate-buffered saline with Tween 20 (PBST), shaken in a solution of horseradish peroxidase (HRP)-linked secondary antibody for 1 h, and washed 3 more times each for 5 min in PBST. The expressions of proteins were detected by enhanced chemiluminescent (ECL) reagent (Millipore, Billerica, MA).

Statistical Analysis. Experimental results were averaged in triplicate analysis. The data were recorded as the mean ± standard deviation (SD) and analysis by a statistical analysis system (SAS Inc., Cary, NC). One-way analysis of variance (ANOVA) was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests. Results were considered statistically significant at p < 0.05.

■ RESULTS

Effects of Monascin on HSC Viability and PPAR-γ Activation. HSCs were treated with various concentrations (5, 10, 15, and 20 μM) of monascin for 24 h, at which point the
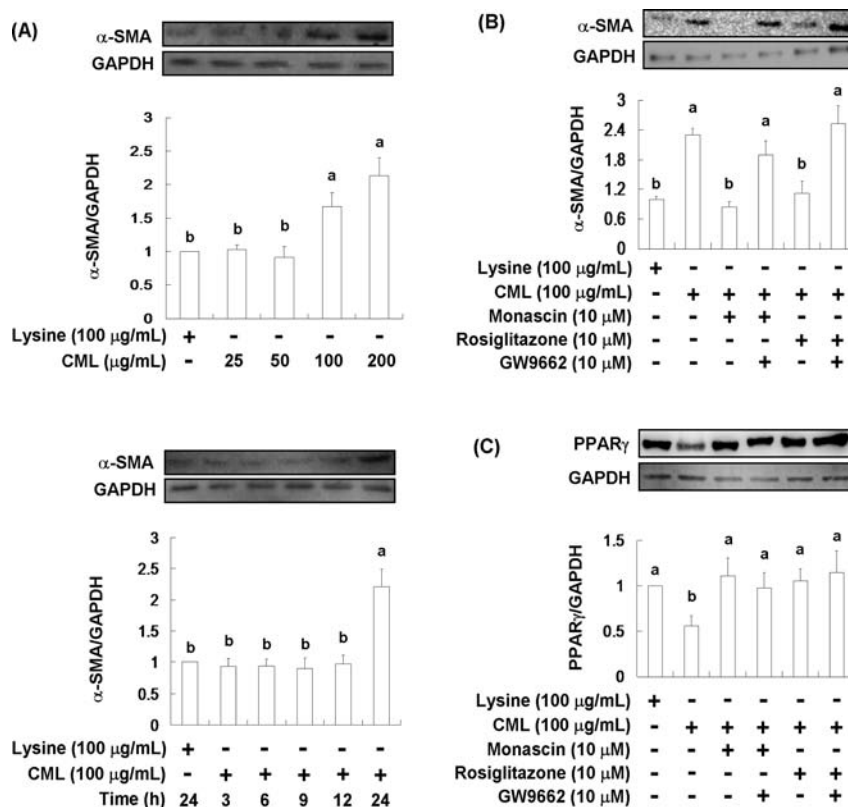
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dx.doi.org/10.1021/jf402082g | J. Agric. Food Chem. 2013, 61, 6873−6879

Figure 2. Monascin attenuated CML-induced hepatic fibrosis in HSCs. (A) CML enhanced the expression of α-SMA at doses of 100 and 200 μg/mL in 24 h treatments. (B) Both monascin and rosiglitazone (10 μM) inhibited α-SMA expression in CML-induced HSCs, whereas this inhibitory effect was abolished by PPAR-γ antagonist GW9662. (C) Total PPAR-γ levels were reduced with CML treatment for 24 h but could be rescued by monascin/rosiglitazone treatment with or without GW9662 in HSCs. Data were presented by the mean $\pm$ SD ($n = 3$). a and b values of different letters are significantly different from each other ($p < 0.05$).

cell viability was investigated. The results indicated that 15 and 20 μM monascin lowered HSCs viability (Figure 1A). Therefore, we used 10 μM monascin to treat HSCs for the subsequent experiments. Recent studies have indicated that monascin acts as a PPAR-γ agonist in C2C12 cells, resulting in enhanced glucose uptake.[18] We also discovered that monascin significantly promoted nuclear PPAR-γ translocation after 9 h of treatment, suggesting that monascin maintains PPAR-γ translocation in HSCs ($p < 0.05$) (Figure 1B).

Monascin Attenuates CML-Induced Hepatic Fibrosis in HSCs. CML markedly enhanced the expression of α-smooth muscle actin (α-SMA) in a dose-dependent (25, 50, 100, and 200 μg/mL) manner in 24 h treatments. Specifically, 100 μg/mL CML significantly increased the α-SMA level after 24 h (Figure 2A). Additionally, monascin (10 μM) and rosiglitazone (10 μM) both inhibited α-SMA production in CML-induced HSCs; however, this inhibitory effect was abolished by GW9662 (PPAR-γ antagonist) treatment (Figure 2B). Total HSCs PPAR-γ levels were reduced with CML treatment (at 24 h) but could be rescued by monascin/rosiglitazone treatment with or without GW9662 (Figure 2C). These findings indicated that activation of PPAR-γ plays an important role in preventing α-SMA production and hepatic fibrosis of HSCs.

Effect of Monascin on Matrix Metalloproteinase-13 (MMP-13) and Tissue Inhibitor of Metalloproteinase (TIMP) in CML-Treated HSCs. MMP-13 can induce hepatic fibrosis and regression through extracellular matrix (ECM) degradation.[23,24] TIMP is a key regulator of fibrogenic events in the liver and suppresses ECM degradation by forming an

inhibitory complex with MMP.[25] Activated HSCs are the major cellular source of TIMP in the injured liver.[26] Secretion of abundant levels of TIMP by activated HSCs leads to decreased hepatic collagenase activity and thereby promotes a net ECM increase. TIMP also promotes hepatic fibrosis by inhibiting the apoptosis of activated HSCs, thus perpetuating the fibrogenic process. The hypothesis that progression of liver fibrosis is associated with inhibition of liver matrix degradation is strongly supported by previous studies that examined MMP and TIMP and used cultured HSCs.[27−30] CML treatment for 24 h markedly increased MMP-13 and TIMP mRNA levels in HSCs, but this increase was suppressed by monascin and rosiglitazone treatment. In addition, GW9662 abolished the inhibitory effects of monascin and rosiglitazone on MMP-13 and TIMP in CML-treated HSCs (Figure 3).

CML-Induced Hepatic Fibrosis Is Not Dependent upon RAGE Signals in HSCs. AGEs have been shown to activate HSCs and hepatic fibrosis via the RAGE signaling pathway.[31,32] We investigated whether CML treatment promotes RAGE and p47phox (NADPH oxidase subunit), a downstream target of RAGE. Our results suggest that CML does not affect the total p47phox and RAGE levels in HSCs; similarly, membrane translocations of p47phox and RAGE did not occur with CML treatment. This finding suggests that CML-mediated hepatic fibrosis was not dependent upon RAGE signaling. In addition, monascin and rosiglitazone treatments did not affect the total or membrane p47phox and RAGE protein levels (panels A and B of Figure 4). In contrast, ROS levels of HSCs were markedly elevated after CML treatment for 24 h, and monascin treatment attenuated
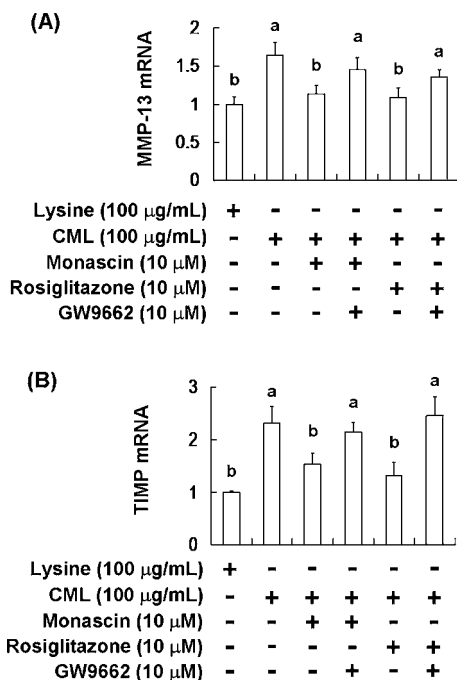
Figure 3. Monascin decreased MMP-13 and TIMP expression in CML-treated HSCs. (A) MMP-13 and (B) TIMP mRNA levels were decreased after 24 h of treatment of monascin in CML-treated HSCs, and GW9662 abolished the inhibitory effects of monascin and rosiglitazone on MMP-13 and TIMP in CML-treated HSCs. Data were presented by the mean $\pm$ SD ($n = 3$). a and b values of different letters are significantly different from each other ($p < 0.05$).

ROS production (Figure 4C). These findings indicate that oxidative stress plays a key role in CML-mediated hepatic fibrosis and that ROS generation is not associated with RAGE activation.

To validate the hypothesis that CML does not affect RAGE, we used siRNA to silence RAGE expression in HSCs. The results indicated that treatment with RAGE siRNA for 48 h inhibits HSCs RAGE expression (Figure 5A). However, CML-induced ROS production was not attenuated in siRNA-

treated HSCs, and monascin and rosiglitazone both suppressed ROS levels in RAGE-silenced HSCs (Figure 5B). Similarly, CML enhanced α-SMA in RAGE-silenced HSCs, while monascin and rosiglitazone both inhibited CML-induced α-SMA production (Figure 5C).

As shown in Figure 6, HSC MMP-13 and TIMP mRNA levels were markedly elevated by CML treatment for 24 h, and this elevation was not affected by RAGE siRNA treatment. In addition, monascin and rosiglitazone attenuated MMP-13 and TIMP mRNA levels in HSCs stimulated with CML and treated with RAGE siRNA.

In addition, total PPAR-γ levels were reduced after CML induction in RAGE siRNA-treated HSCs (Figure 7), suggesting that CML treatment resulted in hepatic fibrosis mediated by oxidative stress and PPAR-γ suppression and not the RAGE pathway. Monascin and rosiglitazone both protected against the CML-induced reduction of PPAR-γ.

■ DISCUSSION

HSCs are fat-storing cells that are activated after liver injury. Importantly, HSCs activation can lead to a myofibroblastic phenotype and contribute to the fibrotic process. NASH is a common but often silent chronic liver disease that clinically and histologically resembles alcoholic liver disease but occurs in persons who drink little or no alcohol.[33,34] NASH is part of a spectrum of non-alcoholic fatty liver diseases that ranges from pure fatty liver (steatosis) to steatohepatitis and cirrhosis.[35] Hyperglycemia, which facilitates AGE formation, is a feature of diabetes. Type 2 diabetes mellitus is commonly accompanied by NASH and can lead to hepatic fibrosis. HSCs activation is critical to the early phase of liver fibrosis and is accompanied by high expression of the α-SMA protein.[23,24] HSCs express many components of the NADPH oxidase complex, and one report suggests a role for NADPH oxidase-derived ROS in HSCs activation. Importantly, AGEs have been found to induce HSCs activation and result in a biomarker of hepatic fibrosis release *in vitro*.[32,36] However, high-blood glucose levels can also cause hepatic fibrosis,[37] indicating that AGEs may not be required for this process *in vivo*.
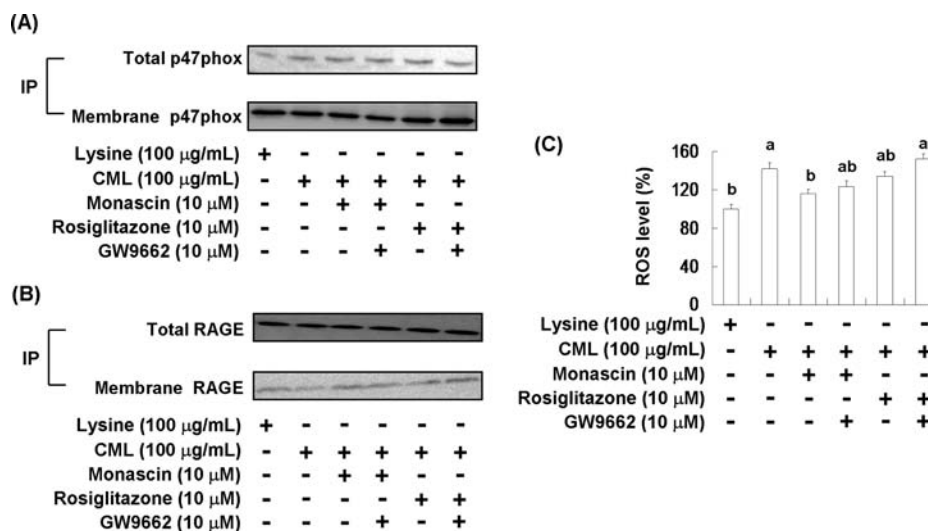

Figure 4. CML-induced ROS generation was not associated with RAGE activation in HSCs. CML, monascin, or rosiglitazone treatment for 24 h did not affect the total and the membrane (A) RAGE downstream target p47phox and (B) RAGE levels in HSCs. (C) HSC ROS levels were increased after CML treatment for 24 h, and monascin treatment decreased the ROS production. Data were presented by the mean $\pm$ SD ($n = 3$). a and b values of different letters are significantly different from each other ($p < 0.05$).
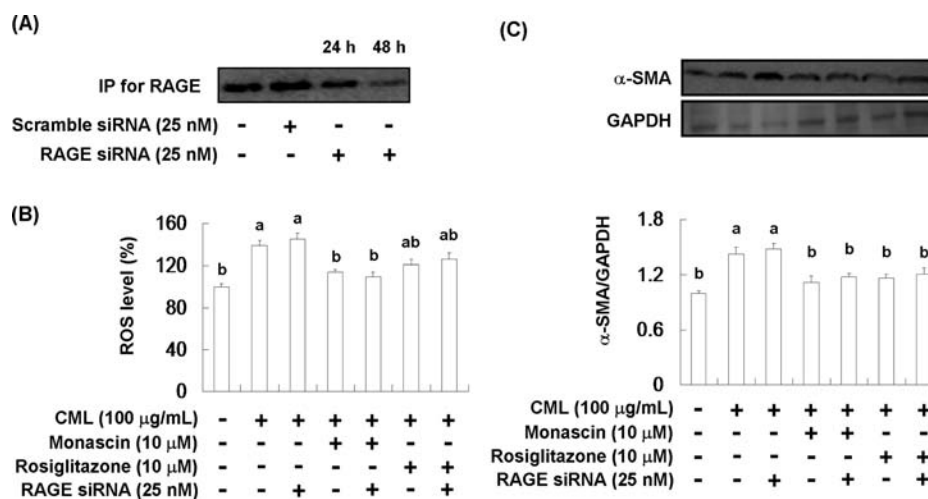
Figure 5. CML-induced hepatic fibrosis is not dependent upon RAGE signals in HSCs. (A) RAGE siRNA treatment for 48 h inhibits HSCs RAGE expression. (B) CML-induced ROS production was not attenuated in siRNA-treated HSCs, and monascin and rosiglitazone both suppressed ROS levels in RAGE-silenced HSCs after 24 h of treatment. (C) CML enhanced α-SMA in RAGE-silenced HSCs, while monascin and rosiglitazone both inhibited CML-induced α-SMA production after 24 h of treatment. Data were presented by the mean ± SD (n = 3). a and b values of different letters are significantly different from each other ($p < 0.05$).
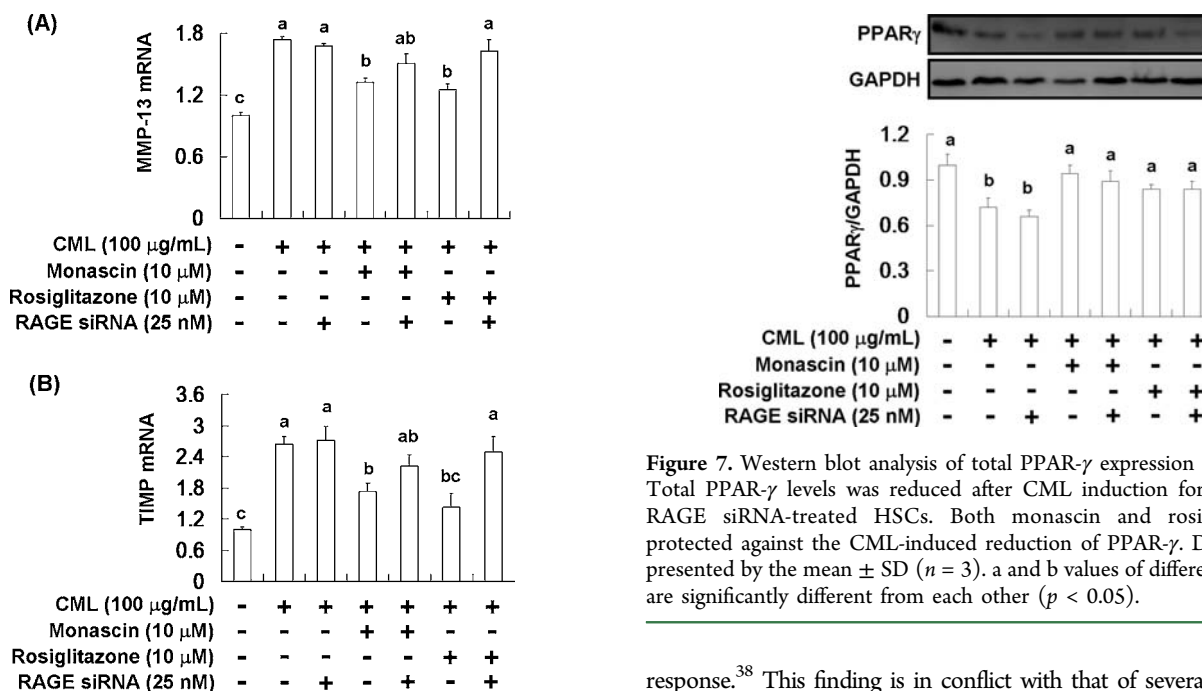

Figure 6. Inhibition of monascin on MMP-13 and TIMP expression was not associated with RAGE activation in HSCs. (A) MMP-13 and (B) TIMP mRNA levels were elevated by CML treatment for 24 h in HSCs, and this elevation was not affected by RAGE siRNA treatment. Monascin and rosiglitazone decreased MMP-13 and TIMP mRNA levels in HSCs stimulated with CML and treated with RAGE siRNA. Data were presented by the mean ± SD (n = 3). a, b, and c values of different letters are significantly different from each other ($p < 0.05$).

In this study, we found that CML markedly induced HSCs activation and increased the expression of hepatic fibrogenetic biomarkers, including MMP-13, TIMP, and α-SMA (Figures 2 and 3), independent of RAGE activation. This hypothesis was confirmed in RAGE-silenced HSCs in Figures 5 and 6. Recent investigations have focused on identifying potential mechanisms that link RAGE with CML. Buetler et al. have found that CML is unable to bind to RAGE and activate the inflammatory

Figure 7. Western blot analysis of total PPAR-γ expression in HSCs. Total PPAR-γ levels was reduced after CML induction for 24 h in RAGE siRNA-treated HSCs. Both monascin and rosiglitazone protected against the CML-induced reduction of PPAR-γ. Data were presented by the mean ± SD (n = 3). a and b values of different letters are significantly different from each other ($p < 0.05$).

response.[38] This finding is in conflict with that of several reports that claim that CML is a major RAGE ligand.[39,40] However, our results are in agreement with the studies that show that CML does not activate RAGE.[41,42] Our data indicated that CML treatment markedly induced oxidative stress and resulted in hepatic fibrosis.

A recent study demonstrated that PPAR-γ expression decreases with HSCs activation. This indicates that PPAR-γ is critical for maintaining the quiescent phenotype of HSCs.[24] Reduction in PPAR-γ expression and enhanced α-SMA expression has also been observed in NASH,[24] suggesting that PPAR-γ might be a potential target for preventing hepatic fibrosis. Importantly, these data further suggest that selective PPAR-γ agonists may be effective therapeutic agents for liver fibrosis. In agreement with this, it has been reported that the RAGE-mediated effects of AGEs, namely, increased oxidative stress, cell growth, and inflammation, resulting in fibrosis, can be inhibited by curcumin in HSCs through PPAR-γ activation.[43] PPAR-γ is a key transcriptional regulator that

suppresses HSCs activation and proliferation and inhibits several transcription factors associated with α-SMA expression and hepatic fibrosis, such as activated protein-1 (AP-1), nuclear factor-κB (NF-κB), and specific protein-1 (Sp1).[44] Notably, monascin and rosiglitazone (PPAR-γ agonist) suppressed α-SMA expression and hepatic fibrosis induced by CML as well as elevated PPAR-γ activation and expression in HSCs (Figures 1 and 7).

Monascin has been reported to show an antioxidative effect by upregulating nuclear factor-erythroid 2-related factor 2 in THP-1 monocytes and in diabetic animals in our recent studies, suggesting that monascin could attenuate oxidative stress *in vitro* and *in vivo*.[17,18] Recently, we have found that oxidative stress is able to result in PPAR-γ degradation, thereby attenuating PPAR-γ activity.[45] However, monascin is able to lower the ROS level to protect PPAR-γ, hence resulting in antihepatic fibrosis effects in CML-treated HSCs. Our data indicate that monascin and rosiglitazone dramatically upregulate PPAR-γ, which, in turn, attenuates α-SMA and ROS generation in CML-treated HSCs. Importantly, these effects were independent of RAGE activation. Therefore, monascin may delay or inhibit the progression of liver fibrosis through the activation of PPAR-γ and might prove to be a major antifibrotic mechanism to prevent liver disease (Figure 8).
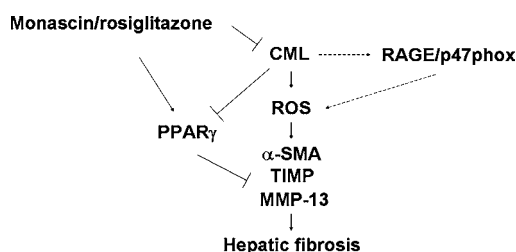

Figure 8. Potential mechanism of monascin on antifibrosis in HSCs. Monascin and rosiglitazone upregulated PPAR-γ to attenuate fibrotic biomarker expression and ROS generation in CML-treated HSCs.

Taken together, monascin activated PPAR-γ and exerted antioxidation effects against HSCs activation induced by CML, and this effect was greater than rosiglitazone, suggesting that monascin is a novel protective agent for antihepatic fibrosis. The bioavailability of monascin remains unknown *in vivo*, including absorption, degradation, and metabolism, but the protection of monascin on antimetabolic syndromes, such as diabetes and dyslipidemia, have been reported *in vivo*.[18,19,46]

ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical structures of (A) monascin and (B) rosiglitazone (Supplemental Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
*Telephone: +886-2-33664519, ext. 10. Fax: +886-2-33663838. E-mail: tmpan@ntu.edu.tw.

Author Contributions
†Wei-Hsuan Hsu and Bao-Hong Lee contributed equally to this work.

Notes
The authors declare no competing financial interest.

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