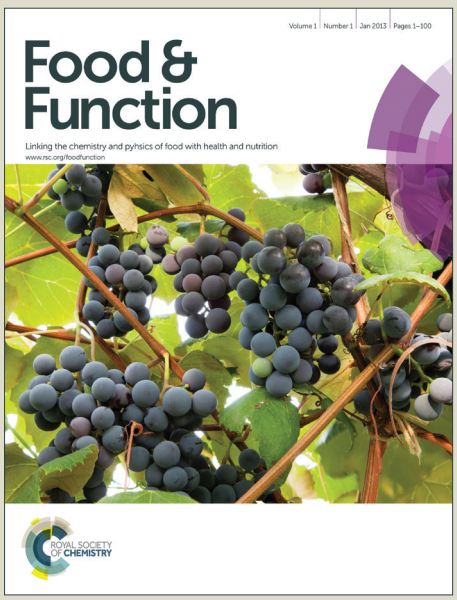
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Chalcones suppress fatty acid-induced lipid accumulation through LKB1/AMPK signaling pathway in HepG2 cells

Tianshun Zhang[1], Norio Yamamoto[2], Hitoshi Ashida[1]*

[1]*Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

[2]*Food Science Research Center, House Wellness Foods Corporation, Imoji 3-20, Itami, Hyogo, Japan*

*Corresponding author: Hitoshi Ashida, Ph.D.

Address: Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe 657-8501, Japan

E-mail: ashida@kobe-u.ac.jp Tel and Fax: +81-78-803-5878

Running title: Chalcones suppress lipid accumulation in hepatocytes

Keywords: chalcones; LKB1; AMPK; SREBP-1; PPARα

Abbreviations: 4HD, 4-hydroxyderricin; XAG, xanthoangelol; CAR, cardamonin; FKB, flavokawain B; FA, fatty acid; SREBP-1, sterol regulatory element-binding protein 1; PPAR, peroxisome proliferator-activated receptor; AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; NAFLD, nonalcoholic fatty liver disease; MAPKs, mitogen-activated protein kinases; C/EBP, CCAAT/enhancer-binding protein

Abstract

Excessive lipid accumulation in the liver has been proposed to cause hyperlipidemia, diabetes and fatty liver disease. 4-Hydroxyderricin (4HD), xanthoangelol (XAG), cardamonin (CAR) and flavokawain B (FKB) are chalcones that have exhibited various biological effects against obesity, inflammation, and diabetes; however, little is known about the inhibitory effects of these chalcones on fatty liver disease. In the present study, we investigated the ability of 4HD, XAG, CAR, and FKB to reduce lipid accumulation in hepatocytes. When HepG2 cells were treated with a mixture of fatty acids (FAs; palmitic acid:oleic acid = 1:2 ratio), significant lipid accumulation was observed. Under the same experimental conditions, addition of chalcones at 5 μM significantly suppressed the FA-induced lipid accumulation. We found the expression of sterol regulatory element-binding protein-1 (SREBP-1), a key molecule involved in lipogenesis, was decreased in these chalcone-treated cells. We also found that these chalcones increased the expression of peroxisome proliferator-activated receptor α (PPARα), which is involved in FA oxidation. Moreover, these chalcones increased phosphorylation of AMP-activated protein kinase (AMPK) and liver kinase B1 (LKB1), upstream regulators of SREBP-1 and PPARα. We confirmed that an AMPK inhibitor, compound C, reversed chalcone-induced changes in SREBP-1 and PPARα expression in the HepG2 cells. Collectively, we found that 4HD, XAG, CAR, and XAG attenuated lipid accumulation through activation of the LKB1/AMPK signaling pathway in HepG2 cells.

Introduction

The liver is an important organ that maintains whole-body energy homeostasis through metabolizing fatty acids (FAs) and glucose.[1] Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide, characterized by hepatic lipid accumulation in the absence of significant ethanol consumption. It encompasses a broad spectrum of liver diseases, ranging from hepatic steatosis to steatohepatitis, and later, progression to cirrhosis.[2] Several studies suggest that excessive intake of calories, visceral obesity, and insulin resistance burden liver function and are important risk factors for developing NAFLD.[3] Over-accumulation of lipids in hepatocytes is currently recognized as the most common cause of chronic liver disease and a factor for increased risk of other diseases.[4,5]

Lipid accumulation in the liver is caused by increased *de novo* lipogenesis and decreased lipid catabolism. Studies have shown that sterol regulatory element-binding protein-1 (SREBP-1) regulates lipid metabolism. SREBP-1 plays an essential role in the regulation of lipogenesis by stimulating FA and triglyceride synthesis.[6,7] It is also known that peroxisome proliferator-activated receptors (PPARs), which are ligand-activated nuclear receptors, mediate the critical transcriptional regulation of genes associated with lipid homeostasis.[8] PPARα is most highly expressed in the liver. It diminishes circulating triglycerides, prevents hepatic steatosis, and is involved in increasing hepatic FA oxidation.[9] Evidence indicates that AMP-activated protein kinase (AMPK) is involved in regulating hepatic lipogenesis.[10] Activation of hepatic AMPK attenuates FA synthesis and promotes FA oxidation.[11, 12] Recent studies revealed that liver kinase B1 (LKB1) is one of the important upstream kinases of AMPK.[13, 14] LKB1 can activate AMPK by phosphorylation at Thr172 in mammalian cells. This activation of AMPK leads to the phosphorylation and inhibition of acetyl-CoA carboxylase activity and an increase in FA oxidation.[15] It was also reported that

activated AMPK interacted with SREBP-1 and inhibited its transcriptional activity, leading to reduced lipogenesis and lipid accumulation.[16] Thus, AMPK should be a therapeutic target for treating fatty liver disease. Mitogen-activated protein kinase (MAPK) signaling occurs in response to almost any change in the extracellular or intracellular milieu that affects the metabolism of the cell, organ or the entire organism.[17] MAPKs play an important role in cellular metabolism, including regulating insulin signaling and adipocyte development, as well as hepatic lipid metabolism.[17-20]

Chalcones are present in various plants and have a variety of health-promoting effects. Several synthetic chalcones have also shown themselves biologically active.[22, 23] 4-Hydroxyderricin (4HD) and xanthoangelol (XAG) are two chalcones from *Angelica keiskei* Ashitaba, a Japanese herb that is used as a traditional medicine. The chalcones in this herb were reported to exert various biological effects against tumors,[24, 25] inflammation,[26] and diabetes.[27, 28] The chalcone cardamonin (CAR) is one of the main constituents of the seeds of *Alpinia katsumadai* Hayata, traditionally used in China as a herbal antiemetic and stomachic drug.[29] Flavokawain B (FKB), a 4'-*O*-methylated analog of CAR, is an active component of *Alpia pricei* Hayata and Kava. CAR and FKB also demonstrated antioxidant, anti-inflammatory, antidiabetic, and other important therapeutic activities.[30-32] The α,β-unsaturated carbonyl moiety in 4HD, XAG, CAR, and FKB might play an important role in their biological activity, because this moiety is known to regulate cellular functions, including the nuclear factor E2-related factor-2/Kelch-like ECH-associated protein system.[33] Different from hepatocytes, lipid accumulation in the adipocytes is deeply involved in balance between differentiation-dependent lipid accumulation and lipid catabolism such as lipolysis and lipid oxidation. Adipocyte-specific transcription factors, PPARγ and CCAAT/enhancer-binding proteins (C/EBPs) are known to be the master regulators of adipocyte differentiation.[34, 35] Our recent study showed that 4HD and XAG strongly inhibited

adipocyte differentiation by reducing expression of the adipocyte-specific transcription factors.[36] However, the inhibitory effects on hepatic steatosis are as yet unknown.

In this study, we first aimed to define an *in vitro* experimental model of hepatic steatosis without cytotoxicity using unsaturated and saturated FAs in human HepG2 cells. Palmitic and oleic acid mixture-induced steatosis causes apoptosis in hepatocyte cell cultures,[37] but monounsaturated FAs were less toxic in hepatocytes, as seen with other cell lines,[38] and were able to prevent/attenuate palmitic toxicity.[39, 40] Thus, we investigated the appropriate ratio of palmitic and oleic acid mixture to generate an experimental model of hepatic steatosis without cytotoxicity. We then used this model to investigate the preventive effect of 4HD, XAG, CAR, and FKB on FAs-induced lipid accumulation. To clarify the underlying molecular mechanisms, we further investigated the effects of chalcones (4HD, XAG, CAR, and FKB) on the expression of SREBP-1 and PPARα, and the phosphorylation of LKB1, AMPK, and MAPKs.

Materials and methods

Reagents

4HD and XAG were purified from "Ashitaba Chalcone Powder," a commercial product of Japan Bio Science Laboratory (Osaka, Japan). CAR was isolated from the seeds of *A. katsumadai* Hayata, and FKB was synthesized by direct aldol condensation of acetophenone with benzaldehyde, as previously described.[41] The chemical structures of these compounds are shown in Fig. 1. The substrate for the cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Mannheim, Germany). Antibodies for PPARα, horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), β-actin, p-AMPK, AMPK, p-LKB1, LKB, p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 and p38 were from Cell Signaling

Technology (Beverly, MA, USA), and SREBP-1 was from Abcam (Cambridge, MA, USA). Palmitic acid, oleic acid, linoleic acid, and linolenic acid were purchased from Wako (Osaka, Japan), and stearic acid was purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was from Nakarai Tesque (Kyoto, Japan).

Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma), 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were grown to 80% confluence and incubated in serum-free medium overnight before treatment. The cells were simultaneously exposed to FAs and treated with various concentrations of chalcones (4HD, XAG, CAR, or FKB) or vehicle (DMSO) for 24 h. A stock solution of each FA was dissolved in 99% MeOH and diluted in culture medium containing 1% BSA to obtain the desired final concentrations.

Cell viability assay

Cell viability was determined using a WST-1 assay. To detect the effects of FAs on HepG2 cell viability, the cells were treated with each FA at 0.5 or 1 mM for 24 h. To detect the effects of chalcones on HepG2 cell viability, the cells were treated with each chalcone at 1, 5, 10, and 20 μM or DMSO alone as a vehicle control (final concentration, 0.25%) for 24 h in culture medium. The cells were incubated with WST-1 reagent in DMEM medium for 2 h. The absorbance of the medium was measured at 450 nm with a reference wavelength of 630 nm using a Wallac multilabel counter (1420 ARVO Sx; PerkinElmer, Waltham, MA, USA).

Determination of intracellular lipid content

To detect the effects of FAs on lipid accumulation in HepG2 cells, the cells were treated with the each FA at 0.5 or 1 mM for 24 h, respectively. To detect the effects of chalcones on FAs-induced lipid accumulation in HepG2 cells, the cells were exposed to a mixture of FAs (palmitic acid:oleic acid = 1:2 ratio), and simultaneously treated with various concentrations of each chalcone as indicated in each figure or DMSO alone as a vehicle (final concentration, 0.25%) for 24 h. Sudan II straining was then carried out as previously described.[36] The cells were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 100 μL of 4% (*w/v*) paraformaldehyde in PBS for 1 h at 4°C, and stained with 100 μL of 0.5% (*w/v*) Sudan II solution in 60% (*v/v*) isopropanol for 1 h at room temperature. The cells were washed twice with distilled water to remove excess stain, and oil droplets present in the stained cells were dissolved in isopropanol containing 4% (*v/v*) Nonidet P-40. Absorbance of the dissolved solution was measured at a wavelength of 490 nm.

Western blotting analysis

Preparation of the cell lysate and western blotting were performed according to our previous report.[42] Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System (ATTO, Tokyo, Japan). The density of specific bands was calculated using ImageJ image analysis software.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Dunnett's test, and the statistical significance between with and without compound C treatment was analyzed using Student's *t*-test. The probability levels less than 0.05 were considered to indicate statistical significance.

Results

Effects of FAs on lipid accumulation and cell viability in HepG2 cells

Lipid accumulation (Fig. 2A) and cell viability (Fig. 2B) were investigated after treatment of HepG2 with palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid at 0.5 or 1 mM for 24 h. We found that all FAs significantly increased lipid accumulation in HepG2 cells (Fig. 2A). Under the same conditions, linoleic acid and linolenic acid showed cytotoxity at both 0.5 and 1 mM (Fig. 2B). Palmitic acid and stearic acid also showed cytotoxicity at 1 mM. Palmitic and oleic acids are the most abundant FAs in liver triglycerides in both normal subjects and patients with NAFLD.[43] Therefore, HepG2 cells were incubated with mixtures of varying proportions of palmitic acid and oleic acid to induce fat overloading of the liver. Results from lipid accumulation (Fig. 2C) and cell viability (Fig. 2D) assays showed that palmitic acid increased lipid accumulation but exhibited cytotoxicity at concentrations over 1.2 mM. Although lipid accumulation caused by oleic acid was less than that of palmitic acid, oleic acid did not exhibit cytotoxicity. To achieve maximal fat over-accumulation without cytotoxicity, a mixture of FAs consisting of a low proportion of palmitic acid (palmitic acid:oleic acid = 1:2 ratio) was selected for further experiments.

4HD, XAG, CAR, and FKB inhibited FAs-induced cellular lipid accumulation in HepG2 cells

We first investigated the effects of 4HD, XAG, CAR, and FKB on the cell viability of HepG2 cells using the WST-1 assay and found that these chalcones showed no cytotoxicity at concentrations up to 20 μM (Fig. 3A). To investigate the inhibitory effect of chalcones on the lipid accumulation in HepG2 cells, 5 μM of each chalcone and various concentrations of the FA mixture were simultaneously added to HepG2 cells for 24 h; the lipid contents were

then determined by Sudan II staining. As shown in Fig. 3B, the FA mixture increased lipid accumulation in HepG2 cells compared with DMSO-treated control cells in a dose-dependent manner. Simultaneous treatment with 4HD, XAG, CAR, and FKB at 5 μM decreased this lipid accumulation. CAR and FKB showed significant effects at FAs concentrations of 0.3, 0.6, and 1.2 mM; 4HD at 0.6 and 1.2 mM; and XAG at 1.2 mM. Thus, we selected the 1.2 mM FA mixture to investigate the dose-dependent effect of chalcones on FA mixture-induced lipid accumulation. As shown in Fig. 3C, the chalcones decreased lipid accumulation in a dose-dependent manner. All chalcones tested significantly inhibited the FA mixture-induced lipid accumulation at 5, 10, and 20 μM.

4HD, XAG, CAR, and FKB decreased SREBP-1 and increased PPARα expression in HepG2 cells

To determine the mechanism by which 4HD, XAG, CAR, and FKB decreased the FAs-induced lipid accumulation in HepG2 cells, western blotting analysis was performed to evaluate the expression levels of two important factors, SREBP-1 and PPARα. These proteins are involved in hepatic lipogenesis and FA oxidation, respectively.[6, 7, 9] As shown in Fig. 4, we confirmed that the FA mixture enhanced expression of SREBP-1 but did not affect expression of PPARα. 4HD and XAG decreased expression of SREBP-1 and increased expression of PPARα in a dose-dependent manner. Significant differences were observed at concentrations over 5 μM (Fig. 4A). The same trends were observed with CAR and FKB; i.e., they decreased expression of SREBP-1 and increased that of PPARα in a dose-dependent manner (Fig. 4B).

Effects of 4HD, XAG, CAR, and FKB on LKB1/AMPK and MAPK pathways in HepG2 cells

AMPK is a key regulator of lipogenesis and FA oxidation in metabolic tissues, including the liver. A change in AMPK activity in HepG2 cells is strongly associated with intracellular lipid metabolism.[10] To investigate the effects of chalcones on the phosphorylation of AMPK and its upstream kinase LKB1, HepG2 cells were treated with 1.2 mM FA mixture and 5 μM chalcones for 24 h. As shown in Fig. 5A and B, all chalcones significantly simulated phosphorylation of AMPK (4HD, 2.18-fold; XAG, 2.08-fold; CAR, 1.74-fold; and FKB, 1.85-fold) and LKB1 (4HD, 1.58-fold; XAG, 1.52-fold; CAR, 1.72-fold; and FKB, 1.64-fold). To confirm that AMPK activation was involved in the inhibitory effects of chalcones on the FA mixture-induced lipid accumulation, an AMPK inhibitor, compound C, was introduced (Fig. 6 A and B). Compound C decreased the AMPK activation resulting from treatment with chalcones. 4HD, XAG (Fig. 6A), CAR, and FKB (Fig. 6B) decreased expression of SREBP-1 (4HD, 67%; XAG 67%; CAR, 70%; and FKB, 74%) and increased PPARα (4HD, 1.80-fold; XAG, 1.69-fold; CAR, 1.56-fold; and FKB, 1.54-fold), the same as the results in Fig. 4. In the same series of cultures, these chalcone-induced effects were abolished by treatment with compound C. This indicated that the activation of LKB1/AMPK pathway by chalcones is involved in the FA mixture-induced lipid accumulation. MAPKs play an important role in various aspects of cellular metabolism, including regulation of hepatic lipid metabolism.[22] Thus, we investigated whether chalcones activate MAPKs. However, 4HD, XAG, CAR, and FKB did not alter the MAPKs activation under our experimental conditions (Fig. 7).

Discussion

In this study, we found that treating HepG2 cells with an FA mixture consisting of palmitic acid and oleic acid (1:2 ratio) provides a suitable *in vitro* model for hepatic steatosis (Fig. 2). Using this model, we found four chalcones, 4HD, XAG, CAR, and FKB, that

significantly decreased the FA mixture-induced lipid accumulation in HepG2 cells without causing cytotoxicity (Fig. 3). These results indicate that 4HD, XAG, CAR, and FKB will be effective compounds to prevent hepatic steatosis. We conducted further experiments and found that the inhibitory effect of these chalcones on the FA mixture-induced lipid accumulation is involved in down-regulation of SREBP-1 and up-regulation of PPARα (Fig. 4), consequences of LKB1/AMPK activation (Figs. 5 and 6) but not MAPKs (Fig. 7).

Intracellular lipid accumulation is the main pathological characteristic of a human liver with NAFLD.[44] FAs are the main substrates for synthesis of triglycerides in hepatocytes. It is known that 25% of liver triglycerides are derived from increased *de novo* lipogenesis.[45] *De novo* lipogenesis is mediated by SREBP-1, a key lipogenic transcription factor, and is nutritionally regulated by glucose and insulin.[46, 47] SREBP-1 regulates the lipogenic process by activating genes involved in FA and triglyceride synthesis that contribute to hepatic steatosis.[7] SREBP-1 plays a considerable role in the pathogenesis of NAFLD.[5] Increased SREBP-1 levels have been found in patients with histologically diagnosed NAFLD,[48] and in the fatty livers of obese (ob/ob) mice[5] and rats fed a high-fat diet.[49] We found that 4HD, XAG, CAR, and FKB decreased the FA mixture-induced SREBP-1 expression in HepG2 cells (Fig. 4). The results in this study suggest that 4HD, XAG, CAR, and FKB prevent hepatic steatosis by decreasing expression of SREBP-1. Moreover, we also found that 4HD, XAG, CAR, and FKB increased expression of PPARα in HepG2 cells. PPARα is expressed at high levels in tissues with high rates of FA oxidation, such as brown fat, liver, and heart.[9] PPARα-mediated responses have been well studied in the liver. It has been reported that a PPARα agonist normalized fatty livers in fat-fed rats,[50] and markedly improved lipid accumulation in the livers of mice.[51] Curcumin, another plant-based chemical, also decreases oleic acid-induced lipid accumulation through enhancing the expression of PPARα.[52] Our findings, together with these previous results, suggest that 4HD, XAG, CAR, and FKB prevent hepatic steatosis by

increasing expression of PPARα, which is involved in FA oxidation. Collectively, 4HD, XAG, CAR, and FKB may modulate the FA mixture-induced hepatic lipid accumulation by down-regulating *de novo* lipogenesis and up-regulating FA oxidation.

To determine the signaling mechanism underlying the decrease of SREBP-1 expression and the increase of PPARα expression induced by 4HD, XAG, CAR, and FKB, we investigated the effects of these chalcones on LKB1/AMPK activation in HepG2 hepatocytes. It is known that AMPK plays a key role in regulating carbohydrate and fat metabolism, serving as a metabolic master switch that responds to alterations in cellular energy charge.[10, 53] AMPK is expected to be a therapeutic target for treating fatty liver disease because it is involved in regulating hepatic lipogenesis.[10] Activation of hepatic AMPK turns off FA synthesis by decreasing the expression of SREBP-1.[11] Our findings showed that 4HD, XAG, CAR, and FKB increased AMPK activation (Fig. 5). When cells were treated with an AMPK inhibitor, compound C, the expression of SREBP-1 was increased and the inhibitory effect of the chalcones on SREBP-1 expression was lost (Fig. 7). AMPK also simulates FA oxidation by increasing expression of proteins involved in FAs oxidation, including PPARα.[54] Our finding showed that 4HD, XAG, CAR, and FKB simultaneously increased both the expression of PPARα (Fig. 4) and the phosphorylation of AMPK (Fig. 5). Compound C partly canceled out the increase in the expression of PPARα (Fig. 6). These results suggest that 4HD, XAG, CAR, and FKB have the ability to activate AMPK, then reduce SREBP-1 expression and enhance PPARα expression, leading to inhibition of hepatic steatosis. AMPK activity is regulated by phosphorylation at Thr172 by the upstream serine/threonine kinase LKB1.[13, 14, 55] Consistent with phosphorylation of AMPK α at Thr172, 4HD, XAG, CAR, and FKB caused phosphorylation of LKB1 at Ser428 in HepG2 cells (Fig. 5). These results indicate that 4HD, XAG, CAR, and FKB inhibit the FA mixture-induced lipid accumulation by activation of LKB1/AMPK pathway. MAPKs are involved in regulating lipid metabolism,[17] and it was

reported that SREBP-1 expression is linked to the MAPK cascade.[20] However, we found that 4HD, XAG, CAR, and FKB did not alter the phosphorylation levels of several MAPKs (ERK, JNK, and p38) under our experimental conditions (Fig. 7). Therefore, 4HD, XAG, CAR, and FKB inhibit the FA mixture-induced lipid accumulation without involving the MAPK pathways. In the case of adipocytes, we previously found that 4HD and XAG inhibited lipid accumulation and adipocytes differentiation effectively by down-expression of the adipocyte-specific transcription factors, PPARγ and C/EBPα in 3T3-L1 cells. The effects are not only involved in activation of AMPK but also MAPKs.[36] Different from the adipocytes, in the present study, we found that chalcones inhibited lipid accumulation by modulating expression of SREBP1 and PPARα, which relating to activation of AMPK but not MAPKs in hepatocytes. Our results indicate that these chalcones should be effective food factors to modulate lipid metabolism in both adipocytes and hepatocytes with different mechanisms.

Conclusion

We established an *in vitro* model of hepatic steatosis using a mixture of palmitic acid and oleic acid. Using this model, we found that four chalcones, 4HD, XAG, CAR, and FKB, inhibit the FA mixture-induced lipid accumulation in HepG2 cells. The inhibitory mechanism is, at least in part, dependent on decreased expression of SREBP-1 and increased expression of PPARα through the activation of the LKB1/AMPK signaling pathway, but not MAPK signaling pathways. Our findings suggest that 4HD, XAG, CAR, and FKB might be valuable for preventing NAFLD and NAFLD-related metabolic syndrome.

Acknowledgement

Part of this study was supported by Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary

320 Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.

321

References

1 O. Cheung, A. J. Sanyal, *Semin. Liver Dis.,* 2008, **28**, 351-359.

2 C. A.Matteoni, Z. M. Younossi, T. Gramlich, N.Boparai, Y. C. Liu, A. J. McCullough, *Gastroenterology*, 1999, 1**16**, 1413-1419.

3 J. D. Browning, J.D. Horton, *J. Clin. Invest.,* 2004, **114**, 147-152.

4 G. Targher, L. Bertolini, S. Rodella, R. Tessari, L. Zenari, G. Lippi, G. Arcaro, *Diabetes. Care,* 2007, **30**, 2119-2121.

5 M. H. Ahmed, C. D. Byrne, *Diabetes Obes. Metab.,* 2009, **11**, 188-195.

6 M. S. Brown, J. L. Goldstein, *Cell*, 1997, **89**, 331-340.

7 A. J. Sanyal, *Nat. Clin. Pract. Gastroenterol. Hepatol.,* 2005, **2**, 46-53.

8 S. E. Lee, H. T. Shin, H. J. Hwang, J. H. Kim, *Phytother. Res.* 2003, **17**, 1041-1047.

9 O. Braissant, W. Wahli, *Endocrinology*, 1998, **139**, 2748-2754.

10 G. Schimmack, R. A. Defronzo, N. Musi, *Diabetes. Obes. Metab.* 2006, **8**, 591-602.

11 D. G. Hardie, *Nat. Rev. Mol. Cell Biol,.* 2007, **8**, 774-785.

12 J. D. McGarry, N. F. Brown, *Eur. J. Biochem.,* 1997, **244**, 1-14.

13 S. A. Hawley, J. Boudeau, J. L. Reid, K. J. Mustard, L. Udd, T. P. Makela, D. R. Alessi, D. G. Hardie, *J. Biol.,* 2003, **2**, 28.

14 A. Woods, S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, *Curr. Biol.,* 2003, **13**, 2004-2008.

15 S. H. Park, S. R. Gammon, J. D. Knippers, S. R. Paulsen, D. S. Rubink, W. W. Winder, *J. Appl. Physiol.*, 2002, **92**, 2475-2482.

16 Y. Li, S. Xu, M. M. Mihaylova, B. Zheng, X. Hou, B. Jiang, O. Park, Z. Luo, E. Lefai, J. Y. Shyy, B. Gao, M. Wierzbicki, T. J. Verbeuren, R. J. Shaw, R. A. Cohen, M. Zang, *Cell Metab.,* 2011, **13**, 376-388.

17 H. Gehart, S. Kumpf, A. Ittner, R. Ricci, *EMBO Reports*, 2010, **11**, 834-840.

18 M. Deak, A. D. Clifton, J. M. Lucocq, D. R. Alessi, *EMBO J*., 1998, **17**, 4426-4441.

19 Y. Xiong, Q. F. Collins, J. An, E. Lupo, H. Y. Liu, D. Liu, J. Robidoux, Z. Liu, W. Cao, *J. Biol. Chem.,* 2007, **282**, 4975-4982.

20 J. Kotzka, D. Müller-Wieland, G. Roth, L. Kremer, M. Munck, S. Schürmann, B. Knebel, W. Krone, *J. Lipid Res*., 2000, **41**, 99-108.

21 A. Koteish, A. M. Diehl, *Liver. Dis*., 2001, **21**, 89-104.

22 J. R. Dimmock, D. W. Elias, M. A. Beazely, N. M. Kandepu, *Curr. Med. Chem.,* 1999, **6**, 1125-1149.

23 B. P. Bandgar, S. S. Gawande, R. G. Bodade, N. M. Gawande, C. N. Khobragade, *Bioorg. Med. Chem*., 2009, **17**, 8168-8173.

24 Y. Kimura, M. Taniguchi, K. Baba, *Planta. Med.,* 2004, **70**, 211-219.

25 T. Akihisa, T. Motoi, A. Seki, T. Kikuchi, M. Fukatsu, H. Tokuda, N. Suzuki, Y. Kimura, *Chem. Biodivers.,* 2012, **9**, 318-330.

26 N. Ohkura, Y. Nakakuki, M. Taniguchi, S. Kanai, A. Nakayama, K. Ohnishi, T. Sakata, T. Nohira, J. Matsuda, K. Baba, G. Atsumi, *Biofactors*, 2011, **37**, 455-461.

27 K. Kawabata, K. Sawada, K. Ikeda, I. Fukuda, K. Kawasaki, N. Yamamoto, H. Ashida, *Mol. Nutr. Food Res.*. 2011, **55**, 467-475.

28 T. Enoki, H. Ohnogi, K. Nagamine, Y. Kudo, K. Sugiyama, M. Tanabe, E. Kobayashi, H. Sagawa, I. Kato, *J. Agric. Food Chem.,* 2007, **55**, 6013-6017.

29 M. Kuroyanagi, T. Noro, S. Fukushima, R. Aiyama, A. Ikuta, I. Itokawa, M. Morita, *Chem. Pharm. Bull.,* 1983, **31**, 1544-1550.

30 C. H. Lee, P. Olson, R. M. Evans, *Endocrinology*, 2003, **144**, 2201-2207.

31 Y. S. Yu, C. L. Hsu, G. C. Yen, *J. Agric. Food Chem.,* 2009, **57**, 7673-7680.

32 C. T. Lin, K. J. Senthil Kumar, Y. H. Tseng, Z. J. Wang, M. Y. Pan, J. H. Xiao, S. C. Chien, S. Y. Wang, *J. Agric. Food Chem.,* 2009, **57**, 6060-6065.

372 33 E.H. Kim, Y. J. Surh, *Biochem. Pharmacol.*, 2006, **72**, 1516-1528.

373 34 P. Tontonoz, E. Hu, B. M. Spiegelman, *Cell*, 1994, **79**, 1147-1156.

374 35 M. I. Lefterova, M. A. Lazar, *Trends Endocrinol Metab.*, 2009, **20**,107-114.

375 36 T. Zhang, K. Sawada, N. Yamamoto, H. Ashida, *Mol. Nutr. Food Res.,* 2013, **57**,
376 1729-1740.

377 37 A.E. Feldstein, A. Canbay, M.E. Guicciardi, H. Higuchi, S.F. Bronk, G.J. Gores, *J.*
378 *Hepatol.,* 2003, **39**, 978-983.

379 38 H. Malhi, S.F. Bronk, N.W. Werneburg, G.J. Gores, *J. Biol. Chem.,* 2006, **281**,
380 12093-12101.

381 39 Y. Wei, D. Wang, F. Topczewski, M.J. Pagliassotti, *Am. J. Physiol. Endocrinol. Metab.*,
382 2006, **291**, E275-E281.

383 40 M. Ricchi, M.R. Odoardi, L. Carulli, C. Anzivino, S. Ballestri, A. Pinetti, L.I. Fantoni, F.
384 Marra, M. Bertolotti, S. Banni, A. Lonardo, N. Carulli, P. Loria., *J. Gastroenterol.*
385 *Hepatol*., 2009, **24**, 830-840.

386 41 N. Yamamoto, K. Kawabata, K. Sawada, M. Ueda, I. Fukuda, K. Kawasaki, A. Murakami,
387 H. Ashida, *Phytother. Res.,* 2011, **25**, 1218-1224.

388 42 T. Furuyashiki, H. Nagayasu, Y. Aoki, H. Bessho, T. Hashimoto, K. Kanazawa, H. Ashida,
389 *Biosci. Biotechnol. Biochem.,* 2004, **68**, 2353-2359.

390 43 J. Araya, R. Rodrigo, L. A. Videla, L. Thielemann, M. Orellana, P. Pettinelli, *J. Clin. Sci.*,
391 2004, **106**, 635-643.

392 44 X. Wu, L. Zhang, E. Gurley, E. Studer, J. Shang, T. Wang, C. Wang, M. Yan, Z. Jiang, P.
393 B. Hylemon, A. J. Sanyal, W. M. Pandak, H. Zhou, *Hepatology*, 2008, **47**, 1905-1915.

394 45 K. L. Donnelly, C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, E. J. Parks, *J.*
395 *Clin. Invest*., 2005, **115**, 1343-1351.

396 46 J. L. Goldstein, M. S. Brown,. *J. Clin. Invest.,* 2008, **118**, 1220-1222.

47 G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Centre T. Doebber, N. Fujii, N. Musi, M. F. Hirshman, L. J. Goodyear, D. E. Moller, *J. Clin. Invest.,* 2001, **108**, 1167-1674.

48 M. Kohjima, N. Higuchi, M. Kato, K. Kotoh, T. Yoshimoto, T. Fujino, M. Yada, R. Yada, N. Harada, M. Enjoji, R. Takayanagi, M. Nakamuta, *Int. J. Mol. Med.,* 2008, **21**, 507-511.

49 L. Madsen, R. K. Petersen, M. B. Sorensen, C. Jorgensen, P. Hallenborg, L. Pridal, J. Fleckner, E. Z. Amri, P. Krieg, G. Furstenberger, R. K. Berge, K. Kristiansen, *Biochem. J.,* 2003, **375**, 539-549.

50 J. M. Ye, M. A. Iglesias, D. G. Watson, B. Ellis, L. Wood, P. B. Jensen, R. V. Sorensen, P. J. Larsen, G. J. Cooney, K. Wassermann, E. W. Kraegen, *Am. J. Physiol. Endocrinol. Metab.,* 2003, **284**, E531-E540.

51 M. Fischer, M. You, M. Matsumoto, D. W. Crabb, *J. Biol. Chem.,* 2003, **278**, 27997-28004.

52 O. H. Kang, S. B. Kim, Y. S. Seo, D. K. Joung, S. H. Mun, J. G. Choi, D. Y. Kwon, *Eur. Rev. Med. Pharmacol. Sci.,* 2013, **17**, 2578-2586.

53 W. W. Winder, D. G. Hardie, *Am. J. Physiol.,* 1999, **277**, E1-E10.

54 W. J. Lee, M. Kim, H. S. Park, H. S. Kim, M. J. Jeon, K. S. Oh, E. H. Koh, J.C.Won, M.S. Kim, G. T. Oh, M. Yoon, K. U. Lee,J. Y. Park, *Biochem. Biophys. Res. Commun.,* 2006, **340**, 291-295.

55 R. J. Shaw, M. Kosmatka, N. Bardeesy, R. L. Hurley, L. A. Witters, R. A. DePinho, L. C. Cantley, *Proc. Natl. Acad. Sci. U S A,* 2004, **101**, 3329-3335.

Figure legends

Fig. 1. Chemical structures of 4HD, XAG, CAR, and FKB.

Fig. 2. Effects of FA on lipid accumulation and cell viability in HepG2 cells. (A, B) Effects of FA on lipid accumulation and cytotoxicity in HepG2 cells. The cells were incubated with each FA at 0.5 or 1 mM for 24 h. MeOH was used as a vehicle control. (C, D) Effects of different ratios of palmitic acid and oleic acid on lipid accumulation and cytotoxicity in HepG2 cells. The cells were incubated with palmitic acid and oleic acid mixtures with varying ratios (1:0, 2:1, 1:1, 1:2 and 0:1) for 24 h. Intracellular lipid accumulation was determined by Sudan II staining (A, C). Cell viability was measured by WST-1 assays (B, D). The results are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01 *vs.* MetOH-treated control cells (Dunnett's test).

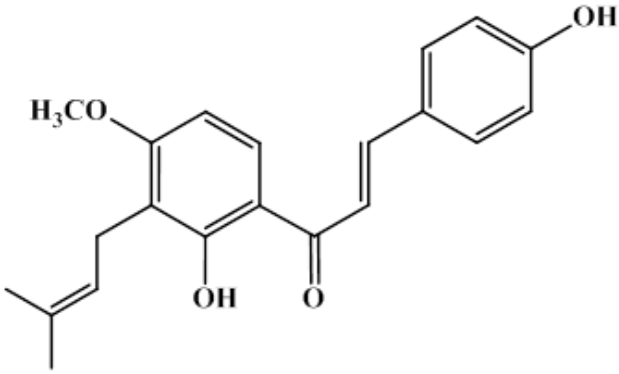
Fig. 3. 4HD, XAG, CAR, and FKB suppressed FA mixture-induced lipid accumulation in HepG2 cells. (A) The cells were treated with each chalcone at concentrations of 1, 5, 10, or 20 μM for 24 h. Cell viability was measured by WST-1 assays. (B) The cells were exposed to 0, 0.3, 0.6, 1.2, and 2.4 mM FA mixture (palmitic acid:oleic acid =1:2 ratio) with or without 5 μM of individual chalcones for 24 h. (C) The cells were exposed to 1.2 mM FA mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without each chalcone at 1, 5, 10, or 20 μM for 24 h. In these experiments, DMSO was used as a vehicle control. Intracellular lipid content was determined by Sudan II staining. The results are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01 *vs.* DMSO-treated control cells at each dose of FA (Dunnett's test).

Fig. 4. 4HD, XAG, CAR, and FKB decreased SREBP-1 expression and increased PPARα

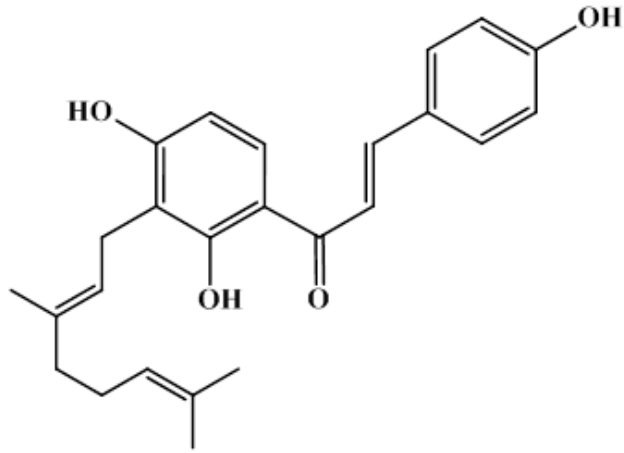
447 expression in HepG2 cells. The cells were exposed to 1.2 mM FA mixture consisting of 0.4
448 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or
449 FKB at 5 μM for 24 h. DMSO was used as a vehicle control. The protein expression levels of
450 SREBP-1 and PPARα were determined by western blotting. A typical representative result
451 was shown from three independent experiments. Densitometric analysis of specific bands was
452 also shown after normalization by β-actin expression. The results are presented as the mean ±
453 SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells
454 (Dunnett's test).
455
456 Fig. 5 4HD, XAG, CAR, and FKB simulated the phosphorylation of AMPK and LKB1 in
457 HepG2 cells. The cells were exposed to 1.2 mM FA mixture consisting of 0.4 mM palmitic
458 acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM
459 for 24 h. DMSO was used as a vehicle control. The phosphorylation levels of AMPK and
460 LKB1, and the expression levels of AMPK, LKB1, and β-actin, were determined by western
461 blotting. A typical representative result was shown from three independent experiments.
462 Densitometric analysis of specific bands for pAMPK and pLKB1 was also shown after
463 normalization by corresponding protein expression. The results are presented as the mean ±
464 SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells
465 (Dunnett's test).
466
467 Fig. 6. The AMPK inhibitor compound C reversed the effects of 4HD, XAG, CAR, and FKB
468 on SREBP-1 and PPARα expression. The cells were exposed to 1.2 mM FA mixture
469 consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG,
470 and (B) CAR or FKB at 5 μM for 24 h. Compound C at 20 μM was simultaneously added to
471 the cells. DMSO was used as a vehicle control. The phosphorylation levels of AMPK, and the

expression levels of AMPK, SREBP-1, PPARα, and β-actin, were determined by western blotting. A typical representative result was shown from three independent experiments. Densitometric analysis of specific bands for pAMPK was shown after normalization by AMPK expression, and that of SREBP-1 and PPARα was also shown after normalization by β-actin expression. The results are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01 *vs*. DMSO-treated cells in each group with or without compound C treatment (Dunnett's test). ##P<0.01 *vs*. DMSO-treated cells, between with and without compound C treatment (Student's *t*-test).

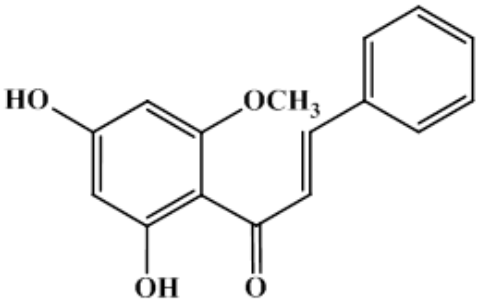
Fig. 7. Effects of 4HD, XAG, CAR, and FKB on the phosphorylation of MAPKs in HepG2 cells. The cells were exposed to 1.2 mM FA mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM for 24 h. DMSO was used as a vehicle control. The phosphorylation and total levels of ERK, JNK, and p38 were determined by western blotting. A typical representative result was shown from three independent experiments.



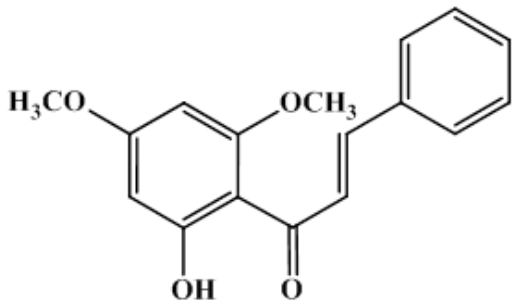
4-hydroxyderricin (4HD)



xanthoangelol (XAG)

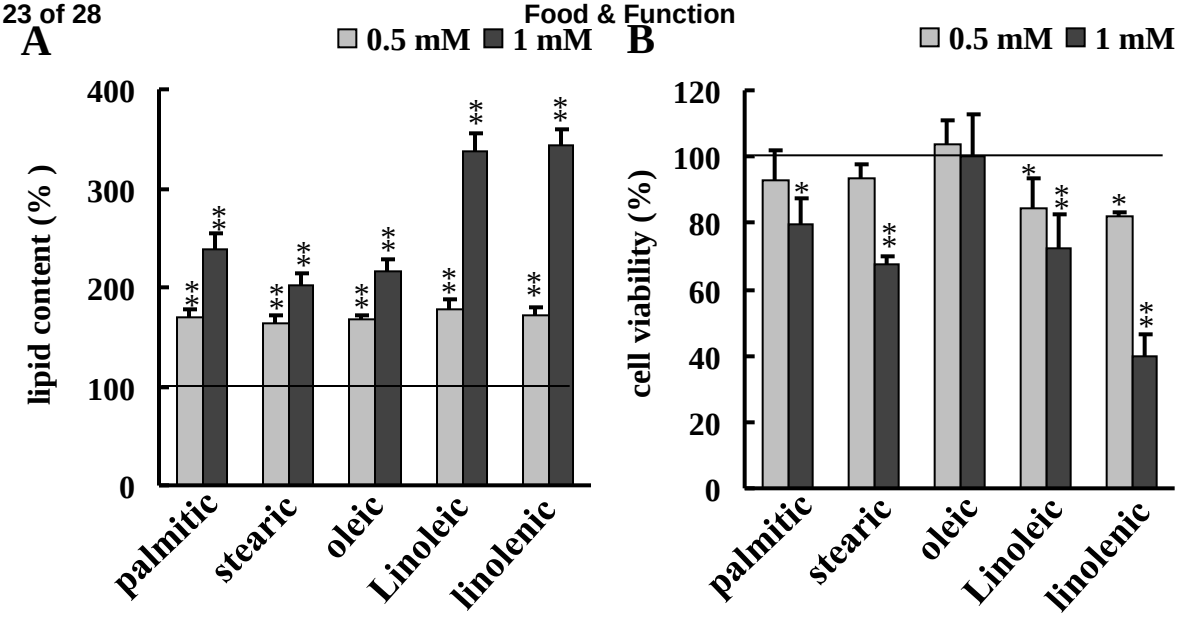


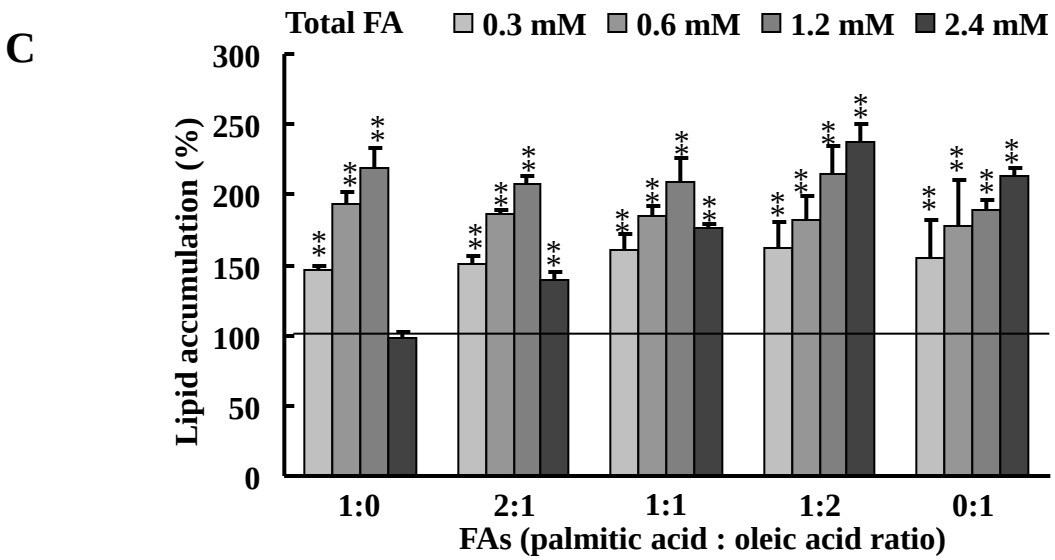
cardamonin (CAR)



flavokawain B (FKB)

Fig.1 Zhang *et al.*





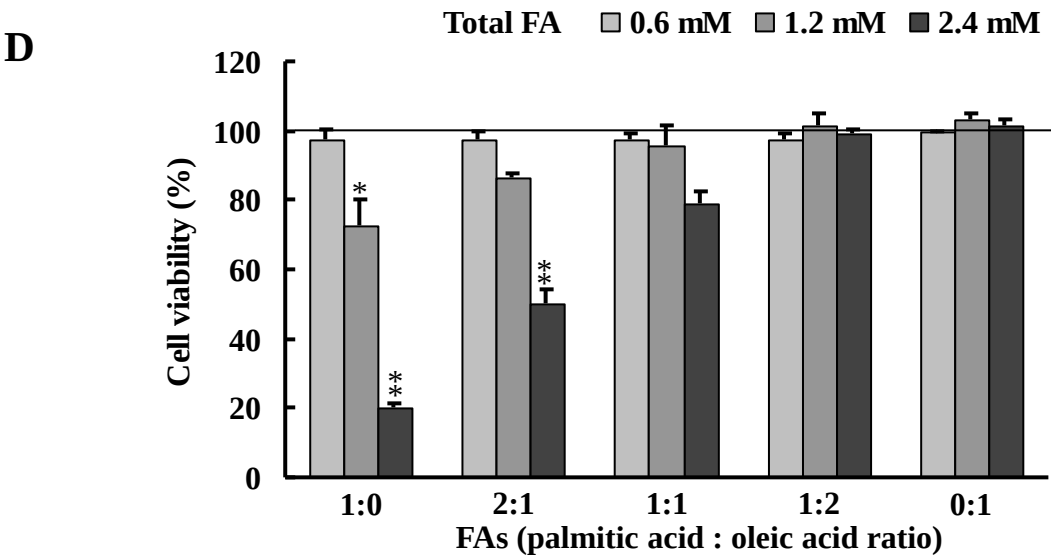


Fig.2 Zhang *et al.*

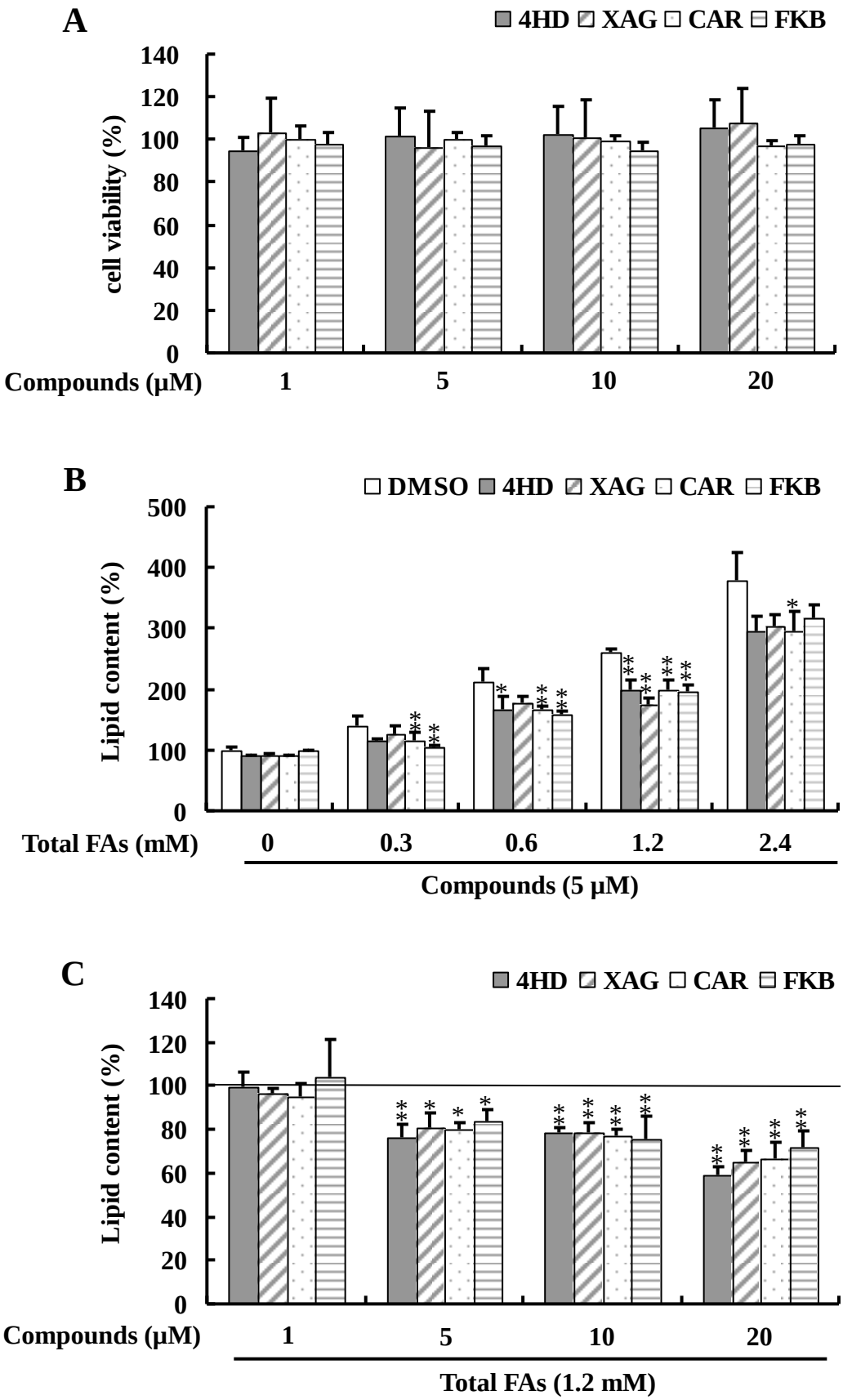
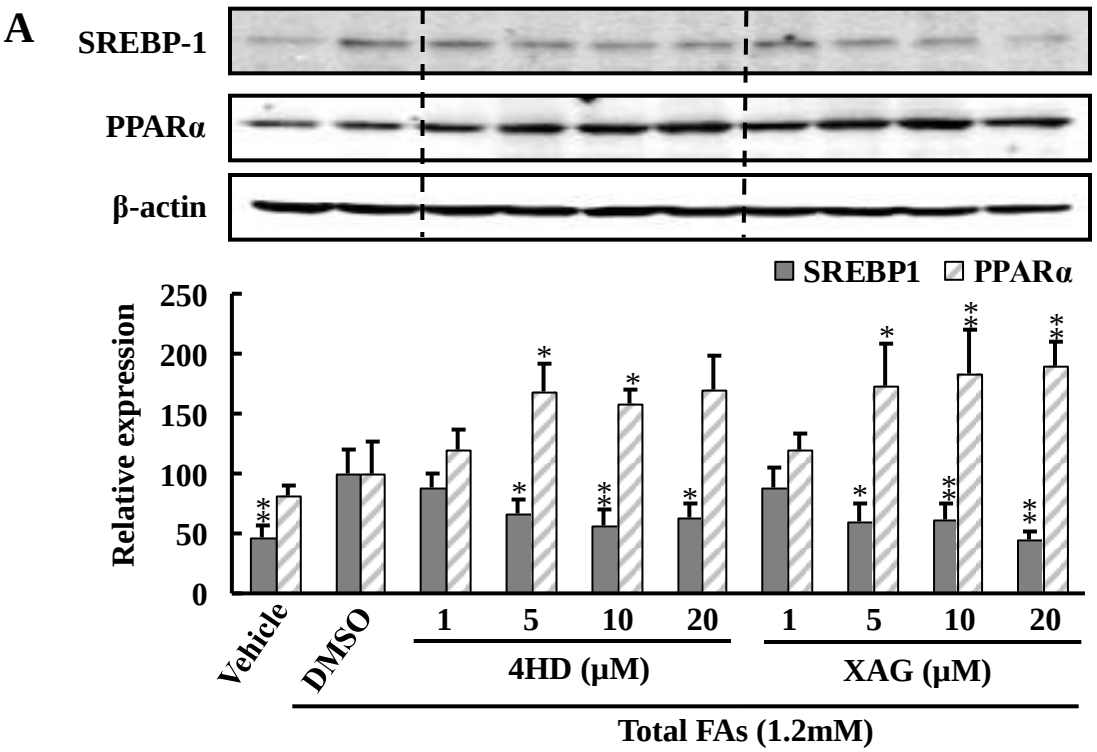


Fig.3 Zhang *et al.*



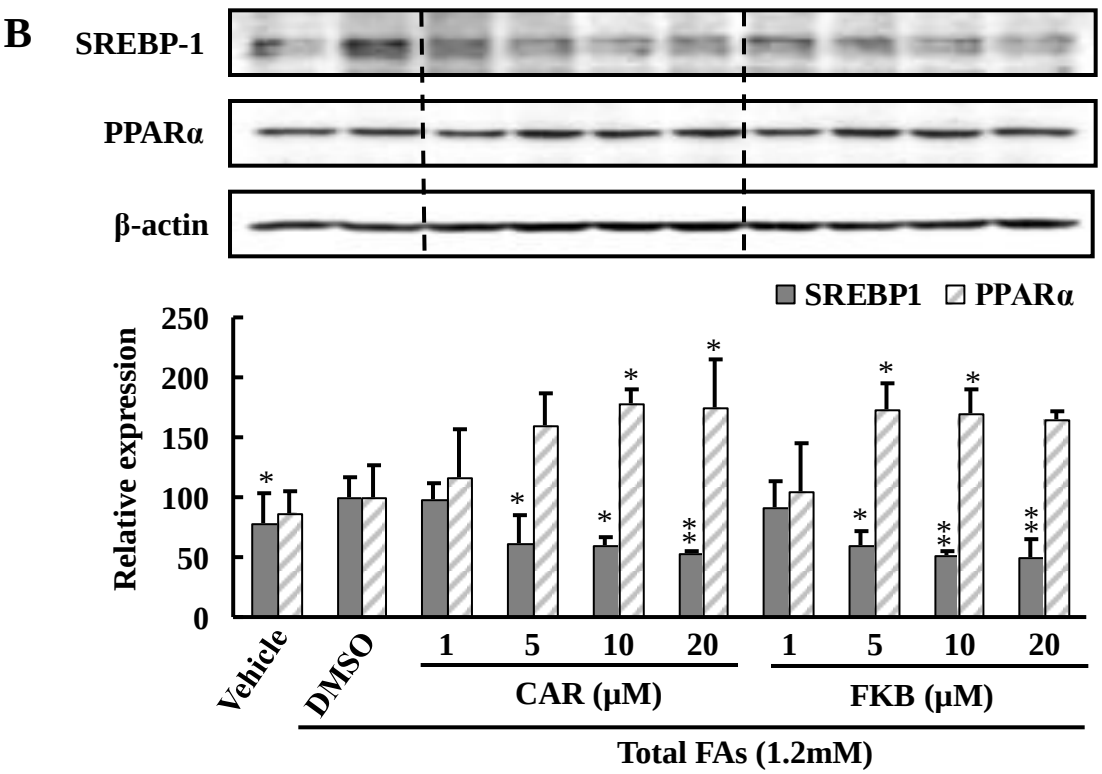


Fig.4 Zhang *et al.*

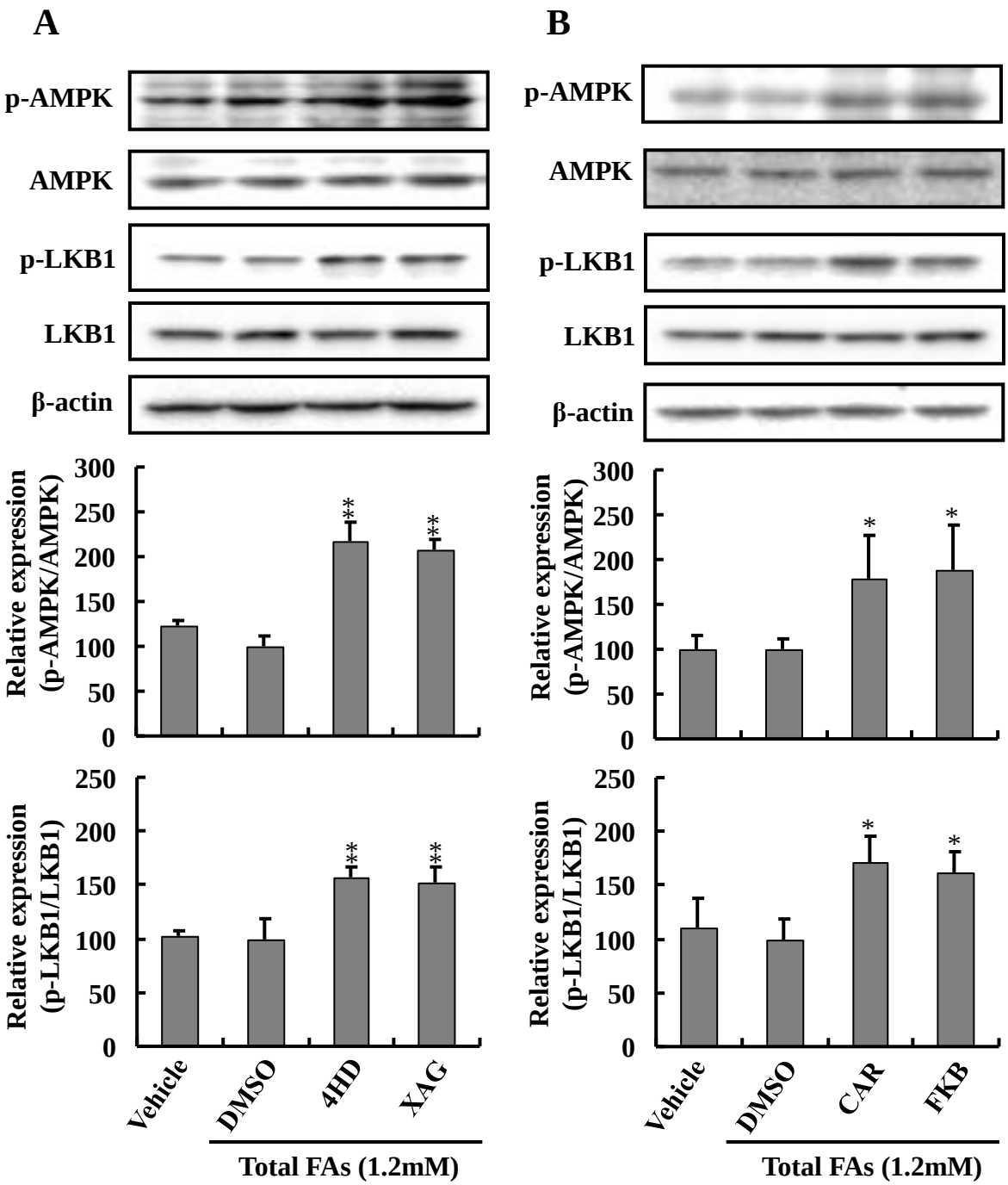


Fig.5 Zhang *et al.*

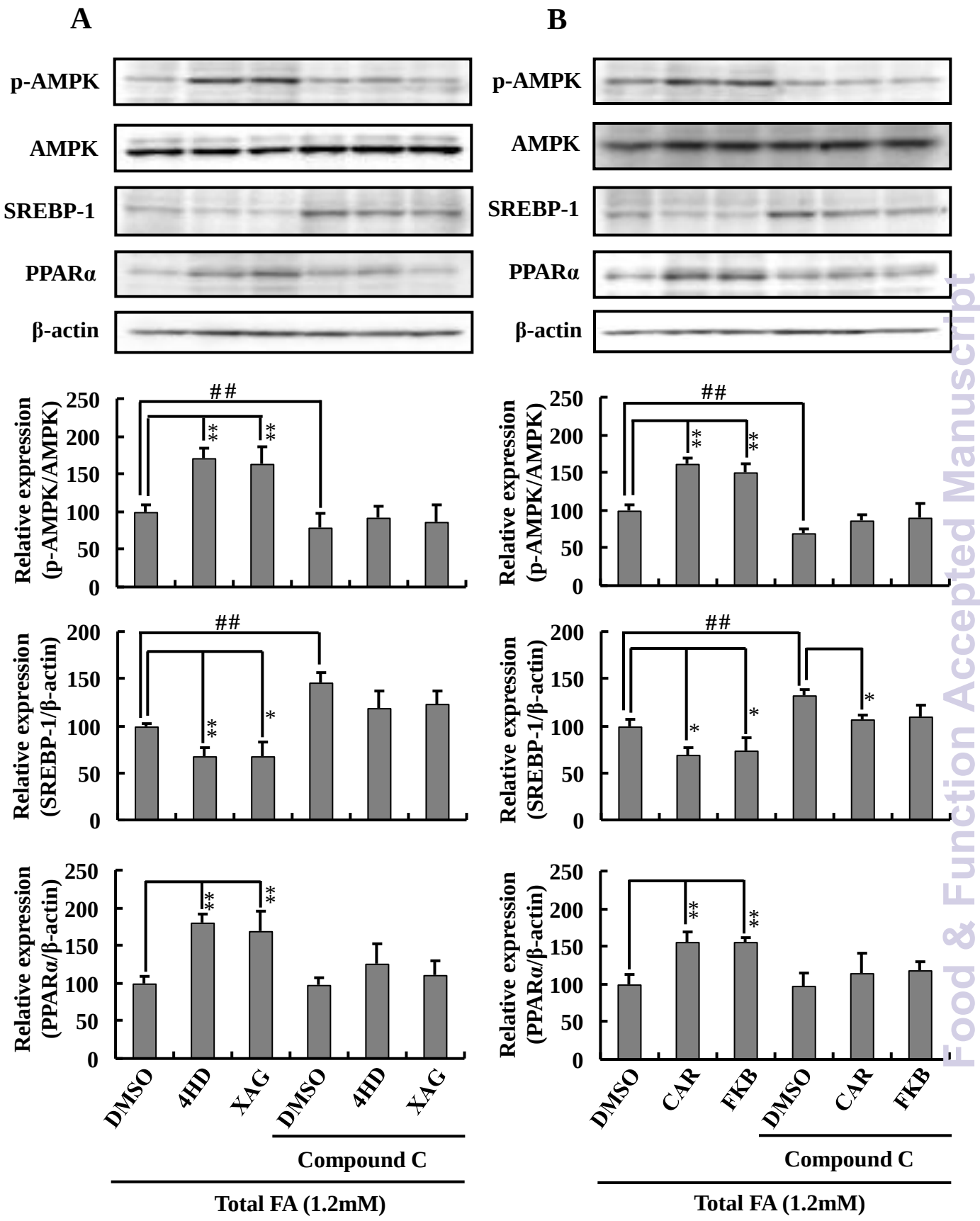


Fig.6 Zhang *et al.*

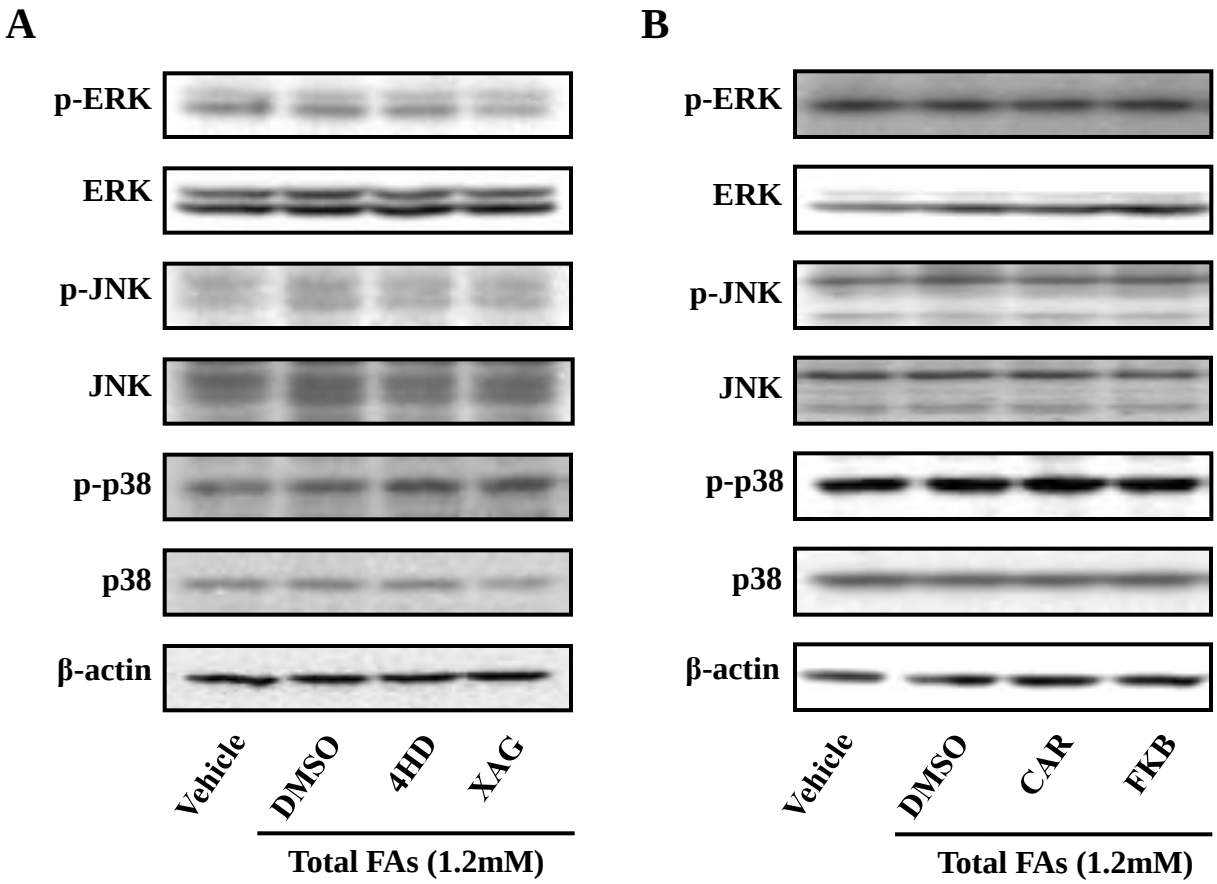


Fig.7 Zhang *et al.*