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Bardoxolone methyl prevents insulin resistance and the development of hepatic steatosis in mice fed a high-fat diet

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Bardoxolone methyl prevents insulin resistance and the development of hepatic steatosis in mice fed a high-fat diet

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Abstract

High-fat (HF) diet-induced obesity is a major risk factor for the development of insulin resistance and hepatic steatosis. We examined the hypothesis that bardoxolone methyl (BM) would prevent the development of insulin resistance and hepatic steatosis in mice fed a HF diet. C57BL/6J male mice were fed a lab chow (LC), HF (40% fat), or HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Glucose metabolism was assessed using a glucose tolerance test (GTT) and insulin sensitivity test (IST). Signalling molecules involved in insulin resistance, inflammation, and lipid metabolism were examined in liver tissue via western blotting and RT-PCR. BM prevented HF diet-induced insulin resistance and alterations in the protein levels of protein tyrosine phosphatase 1B (PTP1B), forkhead box protein O1 (FOXO1) and BDNF, and expression of the *insulin receptor* (*IR)*, *IRS-1* and *glucose-6-phosphatase (G6Pase)* genes. Furthermore, BM prevented fat accumulation in the liver and decreases in the β-oxidation gene, *peroxisomal acyl-coenzyme A oxidase 1 (ACOX)* in mice fed a HF diet. In the livers of HF fed mice, BM administration prevented HF diet-induced macrophage infiltration, inflammation as indicated by reduced IL-6 and signal transducer and activator of transcription 3 (STAT3) protein levels and *TNFα* mRNA expression, and increased *nuclear factor-like 2 (Nrf2)* mRNA expression and nuclear protein levels. These findings suggest that BM prevents HF diet induced insulin resistance and the development of hepatic steatosis in mice fed a chronic HF diet through modulation of molecules involved in insulin signalling, lipid metabolism and inflammation in the liver.

1. Introduction

Obesity is a major risk factor for the development of insulin resistance, type 2 diabetes and hepatic steatosis (Kahn, Hull and Utzschneider, 2006,Forouhi and Wareham, 2010). It is widely accepted that high-fat (HF) diet-induced obesity causes increased fat accumulation, macrophage infiltration and chronic inflammation in peripheral tissues (Xu, Barnes, Yang et al., 2003,Weisberg, McCann, Desai et al., 2003). Increased fat accumulation and inflammation promotes insulin resistance and tissue injury in peripheral tissues involved in glucose and fat metabolism, such as the liver (Xu et al., 2003,Weisberg et al., 2003). A number of studies provide direct evidence demonstrating a link between obesity-associated inflammation and insulin resistance, and hepatic steatosis (Emanuela, Grazia, Marco de et al., 2012,Ginsberg, 2006,Qureshi and Abrams, 2007). However, there is a need to develop novel therapeutic approaches targeting hepatic inflammation and to improve obesity-induced insulin resistance and hepatic steatosis.

The activation of inflammatory molecules can promote the expression of the negative regulators of insulin signalling, protein tyrosine phosphatase B (PTP1B) and SOCS3 (Zabolotny, Kim, Welsh et al., 2008,Hong, Nguyen and Gao, 2001). PTP1B levels are increased in the liver of HF diet-induced obese mice, which contributes to the development of insulin resistance by reducing insulin signalling through inhibition of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) activation (Lam, Covey, Lewis et al., 2006,Goldstein, Bittner-Kowalczyk, White et al., 2000). SOCS3 is another important molecule which impairs insulin signal transduction in the liver through its inhibition of the binding of IR to IRS-1 (Ueki, Kondo and Kahn, 2004). Furthermore, activation of hepatic insulin signalling results in the inactivation of forkhead box protein O1 (FOXO1), which is a transcription factor inhibiting genes such as *glucose-6-phosphatase (G6Pase)* for endogenous glucose production via gluconeogenesis (Nakae, Kitamura, Silver et al., 2001,German, Kim, Schwartz et al., 2009). When insulin signalling is impaired, through inhibition by PTP1B or SOCS3, and activation of FOXO1, this leads to the promotion of glucose production and a reduction in glucose reuptake, leading to glucose intolerance and insulin

resistance in obesity (Nakae et al., 2001,German et al., 2009). Brain-derived neurotrophic factor (BDNF) also plays an import role in insulin action as it has been found to modulate hepatic glucose metabolism via its actions on glucokinse (GK) in obese insulin resistant rats (Kuroda, Yamasaki, Matsuhisa et al., 2003). In the liver, GK enhances glycolysis, resulting in reduced blood glucose levels (Hariharan, Farrelly, Hagan et al., 1997).

A HF diet is known to cause fat accumulation in the liver, which can progressively worsen to hepatic steatosis (Marchesini, Brizi, Bianchi et al., 2001). Hepatic lipid homeostasis is regulated by a number of genes that promote lipogenesis, including *ACC*, *FAS* and *SCD1*, and β oxidation, such as *ACOX* (Musso, Gambino and Cassader, 2009). Hepatic fat accumulation leads to macrophage infiltration which promotes the production of proinflammatory cytokines, such as interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα) and IL-1 β (McArdle, Finucane, Connaughton et al., 2013). Increased IL-6 has been found to enhance inflammatory signalling by increasing signal transducer and activator of transcription 3 (STAT3) levels, which promotes cytokine dependent signalling by increasing the expression of inflammatory genes such as IL-6 (Yang, Liao, Agarwal et al., 2007). In addition, inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) are important proinflammatory signalling molecules upstream of the transcription factor, nuclear factor kappa-B (NFκB), which promote PTP1B and SOCS3 activation (Zabolotny et al., 2008,Hong et al., 2001,Napetschnig and Wu, 2013).

The oleanolic acid synthetic derivative, bardoxolone methyl (BM) has attracted wide attention due to its anti-inflammatory effects (Liby and Sporn, 2012,Wang, Garvin, D'Ambrosio et al., 2011,Reisman, Chertow, Hebbar et al., 2012). Its ability to directly up-regulate the potent anti-inflammatory molecule, nuclear factor-like 2 (Nrf2), has demonstrated therapeutic benefits in human clinical trials for treating chronic kidney disease and advanced solid tumours (NIH, 2012,Pergola, Raskin, Toto et al., 2011,Liby, Yore and Sporn, 2007,Hong, Kurzrock, Supko et al., 2012). BM has also been found to directly influence the activity of proinflammatory signalling through IKKβ (Ahmad, Raina, Meyer et al., 2006).

Furthermore, a recent study demonstrated that 2 week administration of BM decreased hepatic inflammation in diet-induced obese mice (Saha, Reddy, Konopleva et al., 2010). In addition, previous studies have demonstrated that oral administration of a derivative of BM, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), prevents HF diet-induced obesity and attenuates diabetes in mice (Uruno, Furusawa, Yagishita et al., 2013,Shin, Wakabayashi, Yates et al., 2009). In this study, we investigated whether chronic oral BM administration in mice fed a HF diet for 21 weeks could prevent insulin resistance and liver injury in mice fed a HF diet. We also examined signalling molecules involved in insulin resistance, inflammation, and lipid metabolism in liver tissue.

2. Materials and Methods

2.1. Animals and HF diet-induced obesity model

Male C57BL/6J mice (12 weeks old) were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained in the animal facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.* All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions (temperature 22 °C, 12hr light/dark cycle) and 1 week after acclimatisation were randomly divided into 3 groups (n=7 per group). For the next 21 weeks one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia), and mice in the treatment group were fed a HF diet for 21 weeks and an oral daily dose of BM (10 mg/kg) in their drinking water (Table 1). (Final average body weight after 21 weeks: LC, 27.15g; HF, 40.84g; BM, 28.13g). HOMA-IR was calculated using the formula, (Fasting Insulin x Fasting Glucose)/22.5.

Table 1 Composition of the high-fat and lab chow diets

	High-fat diet	Lab chow diet
Total energy (kcal/100g)		
Fat	40	5
Carbohydrate	45	75
Protein	15	20

Typical Ingredients;
High-fat diet: Casein (Acid), Sucrose, Lard, Sunflower Oil, Cellulose, Wheat Starch, Dextrinised Starch, Minerals, and Vitamins.
Lab chow diet: Cereal Grains, Meat Offal Meal, Fish Offal Meal, Whey Powder, Vegetable Oils, Soybean Protein, Cereal Offal, Corn Offal, Minerals, and Vitamins.

2.2. Glucose tolerance test

Mice were fasted overnight (16 hrs) before a glucose tolerance test (GTT) was performed to assess glucose clearance, following an intraperitoneal (i.p) injection of glucose (0.5 g/kg; Sigma-Aldrich, St Louis, MO). Blood samples were taken from the tail vein before and 30, 60 and 120 minutes following the injection of glucose. Blood glucose was measured using an Accu-Chek glucometer (Roche Diagnostics GmbH Mannheim, Germany).

2.3. Insulin sensitivity test

Mice were fasted for 5 hours before an insulin sensitivity test (IST) was performed to assess glucose clearance, following an i.p. injection of insulin (0.75 U/kg; Sigma-Aldrich, St Louis, MO). Blood samples were taken from the tail vein before and at 30, 60 and 120 minutes following the injection of insulin. Blood glucose was measured using an Accu-Chek glucometer (Roche Diagnostics GmbH Mannheim, Germany).

2.4. Tissue collection and sample preparations

For tissue analysis, mice were euthanised at week 21 of the experiment. Tissue was dissected from the mice and immediately frozen in liquid nitrogen before being stored at -80 °C.

2.5. Oil Red O staining

Oil Red O staining was used to examine hepatic lipid accumulation as described previously (Kudo, Tamagawa, Kawashima et al., 2007). Briefly, frozen liver sections (10 μm) were stained with 0.5% Oil Red O (Sigma-Aldrich) for 15 minutes and then washed. Three fields from three sections of each

mouse were viewed under a Leica microscope, and digital photographs were captured. Image J software (http://imagej.nih.gov/ij/download.html) was used to quantify the staining, which corresponds to the percentage of stained lipid droplets on an area of each slide (Mehlem, Hagberg, Muhl et al., 2013).

2.6. Haematoxylin and Eosin (H&E) staining

To determine the degree of liver damage fresh frozen liver sections (10 μm) were stained with Haematoxylin and Eosin for 30 seconds each. Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs were captured. The histological parameters of steatosis and ballooning were scored according to the method described by Kleiner and colleagues (Kleiner, Brunt, Van Natta et al., 2005). The steatosis grades were as follows: 0, <5%; 1, 5%–33%; 2, >33%–66%; 3, >66%. The ballooning classifications were grouped as: 0, no ballooning cells; 1, few ballooning cells; 2, many cells/prominent ballooning.

2.7. Immunohistochemistry

Liver sections fixed in 10% Formalin were embedded in paraffin before being sectioned (5 μm) onto Polylysine™ slides. Slides were incubated overnight at 4 °C with anti-rabbit F4/80 (1:150 Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer as described previously (Dinh, Szabo, Camer et al., 2015). Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs were captured. Image J software was used to quantify the area of F4/80 immunoreactivity on each slide.

2.8. Extraction of nuclear and cytosolic proteins

Nuclear and cytosolic proteins were extracted from liver tissue as described by Mobasher et al (Mobasher, Gonzalez-Rodriguez, Santamaria et al., 2013). Briefly, liver tissue was homogenised in a solution containing 10Mm HEPES-KOH (pH 7.9), 10mM KCL, 1.5mM $MgCl_2$, 0.5mM DTT, 0.2mM PMSF, and protease and phosphatase inhibitors (buffer A) before incubation on ice, vortexing and centrifugation. Following centrifugation, the supernatant containing the cytosolic fraction was collected

and frozen at -80 °C until use. The remaining pellet was resuspended in a solution containing 20mM HEPES-KOH (pH 7.9), 400mM NaCl, 1.5 mM $MgCl_2$ 0.2mM EDTA, 15% glycerol, 0.5mM DTT, 0.2mM PMSF and protease and phosphatase inhibitors (buffer B) before further centrifugation. Following multiple washes with buffer b and centrifugation of the pellet, the supernatant containing the nuclear fraction was collected and stored at -80 °C until use.

2.9. Western Blot analysis

For total protein extraction, the frozen liver tissue was homogenised in Nonidet P-40 lysis buffer. The following antibodies were used for western blotting: Nrf2 (sc-722), IL-1β (sc-7884), IL-6 (sc-7920) and BDNF (sc-546) (Santa Cruz Biotechnology, Dallas, TX); pIKK (#2697), STAT3 (#4904), FOXO1 (#2880), SOCS3 (#2932), and PTP1B (#5311) (Cell Signalling Technology, Beverly, MA). Both nuclear and cytosolic protein levels of Nrf2 were analysed. The bands corresponding to the proteins of interest were scanned and the band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad Laboratories, Hercules, California) as described in our previous study (Camer, Yu, Szabo et al., 2015). All quantitative analyses for total and cytosolic proteins were normalised to β-actin. Nuclear proteins were normalised to Lamin B.

2.10. Luminex Assay

Blood was collected in EDTA tubes from mice following euthanasia. Following centrifugation, plasma was extracted, collected and stored at -80 °C. Plasma insulin levels were measured using Luminex assay kits according to the manufacturer's instructions (Bio-Rad Diabetes Kit, Sydney).

2.11. RNA isolation and RT-PCR

Total RNA was extracted from mouse liver using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) before being reversed transcribed to complimentary first strand DNA with a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, California, USA) according to the manufacturer's directions. Quantitative real-time PCR (RT-PCR) was performed using a Lightcycler 480 real time PCR system (F.Hoffmann-La Roche Ltd, Switzerland). A 20ul final reaction volume containing

cDNA sample and SYBR green I master mix was used to perform the experiment. Briefly, amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The expression of mRNA was normalised to an internal control, GADPH. The degree of mRNA expression was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt =ΔCt sample - ΔCt reference) as described previously (Livak and Schmittgen, 2001).

2.12. Statistics

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality using a Kolmogorov-Smirnov normality test. Differences between mice fed a LC, HF, and HF plus BM diet were then determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as the mean ± SEM.

3. Results

3.1. Bardoxolone methyl treatment prevented HF diet-induced insulin resistance

To explore the role of BM in glucose homeostasis and insulin sensitivity, glucose tolerance tests (GTTs) and insulin sensitivity tests (ISTs) were performed (Figures 1A and C). HF diet fed mice had significantly higher blood glucose levels during fasting (0 minute) and 120 minutes following an i.p. injection of glucose compared to LC fed mice. However, administration of BM normalised blood glucose levels at 120 minutes in the GTT test in HF diet fed mice (-18.07%, p = 0.015), with significance confirmed with area under the curve (AUC) analysis (Figure 1B). However, BM did not prevent HF diet-induced increases in fasting blood glucose levels (p>0.05). Consistent with the effect of BM on improving glucose clearance, BM treatment also reduced blood glucose levels during the IST in mice fed a HF diet (Figure 1C). HF diet fed mice had significantly higher blood glucose levels at fasting and 30, 60 and 120 minutes following insulin injection compared to LC fed mice. BM treatment significantly

decreased blood glucose levels at 30 and 60 minutes post i.p. insulin injection (Figure 1C) in the mice fed a HF diet, with significance confirmed with area under the curve (AUC) analysis (Figure 1D) (Blood glucose levels 30 minutes following i.p. insulin injection: -34.23%, p = <0.001; Blood glucose levels 60 minutes following i.p. insulin injection: -25.92%, p = 0.048).

Fasting plasma insulin levels were examined to determine if BM could prevent HF diet-induced hyperinsulemia. As expected, mice fed a HF diet for 21 weeks had significantly elevated plasma insulin levels compared to LC fed mice, which was attenuated by BM administration (Figure 1E). To determine if BM treatment could prevent HF diet-induced insulin resistance, HOMA-IR was calculated. HF diet-fed mice were found to have a significantly elevated HOMA-IR compared to LC group (Figure 1F). However, BM administration in HF diet fed mice significantly prevented this increase in HOMA-IR. These results suggest that BM can prevent hyperinsulemia and insulin resistance induced by a chronic HF diet.

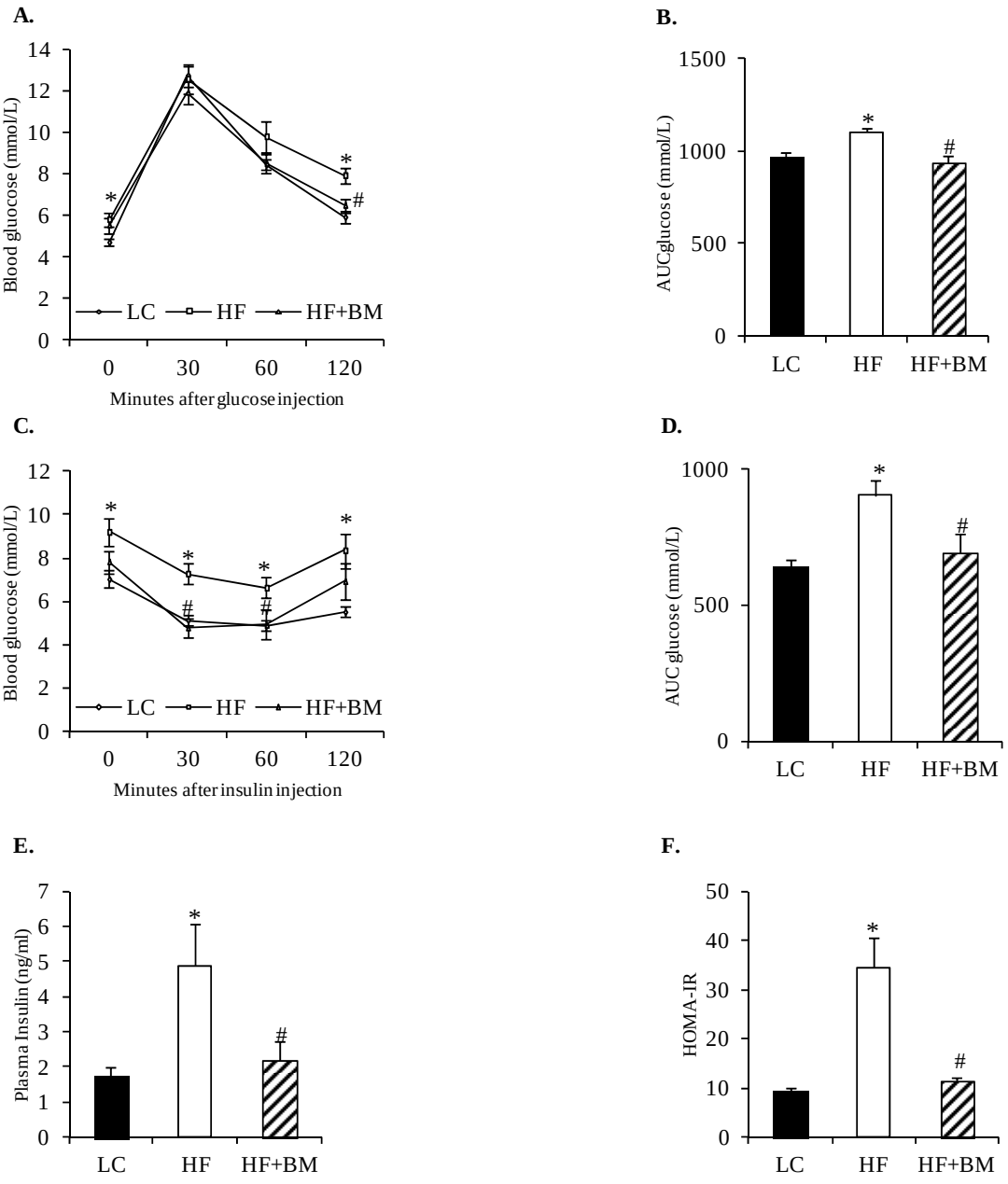

Figure 1. Effect of chronic administration of bardoxolone methyl (BM) on glucose tolerance (A, B), insulin sensitivity (C, D), hyperinsulemia (E) and HOMA-IR (F) in mice fed a high-fat (HF) diet for 21 weeks (n=7 per group). *, p = <.0.05 vs. lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM.

We evaluated the effect of BM on the expression of molecules involved in insulin resistance and glucose metabolism in the liver using western blotting and RT-PCR analysis. Western blot showed that a HF diet elevated hepatic PTP1B and FOXO1, and reduced BDNF protein expression, which was significantly reversed by BM treatment (Figure 2A). No significant differences in protein expression of SOCS3 were found between any groups (Figure 2A). RT-PCR analysis found that a HF diet

significantly reduced *IR* and *IRS-1* mRNA expression and significantly increased *G6Pase* and *GK* mRNA expression (Figure 2B). BM treatment significantly prevented HF diet induced decreases in *IR* and *IRS-1* and increases in *G6Pase* mRNA expression (Figure 2B). However, BM was unable to prevent HF diet induced decreases in the mRNA expression of *GK* (Figure 2B). These data suggest that BM prevents the development of HF diet induced hepatic insulin resistance by regulating the insulin signalling proteins, FOXO1, PTP1B and BDNF, and *IR*, *IRS-1* and *G6Pase* genes to promote insulin signalling, and reduce glucose production.

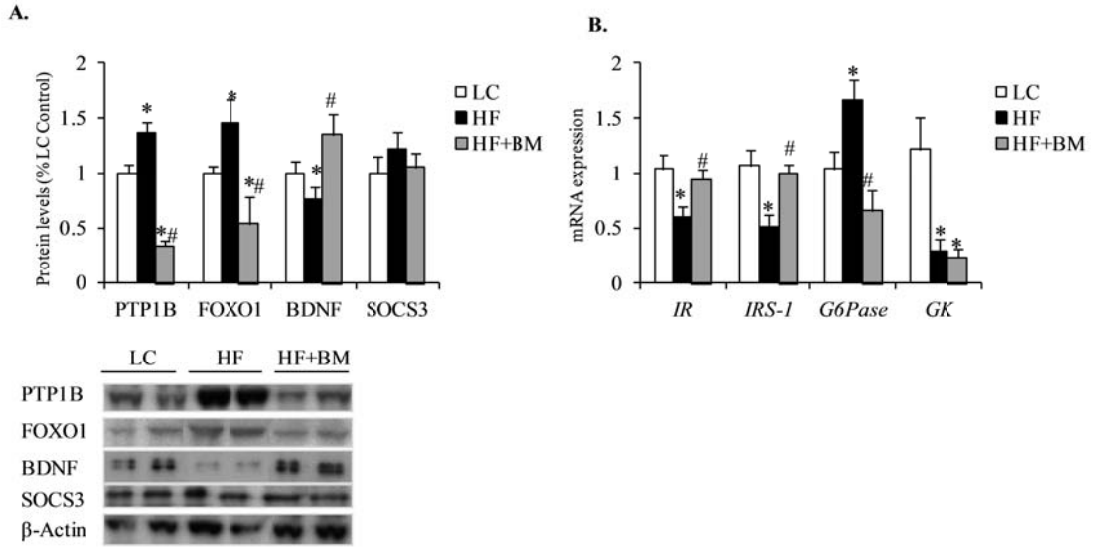

Figure 2. Effect of chronic bardoxolone methyl (BM) treatment on the hepatic insulin signalling proteins (A) FOXO1, PTP1B, BDNF and SOCS3, and genes (B) *IR*, *IRS-1*, *G6Pase* and *GK*, in the livers of mice fed a high-fat (HF) diet for 21 weeks (n= 7 per group). *, p = <0.05 vs. lab chow (LC), #, p= <0.05 vs. HF group, values are means ±SEM.

3.2. Bardoxolone methyl prevented HF diet-induced hepatic fat accumulation and alterations in fatty acid metabolism-related genes

Hepatic steatosis is a severe fatty liver disease caused by the accumulation of fat deposits in hepatocytes in liver tissue (Marchesini et al., 2001). On a histological level, the diagnostic criteria for hepatic steatosis includes the presence of steatosis and ballooning (Neuschwander-Tetri and Caldwell, 2003). Upon gross examination, we found that the BM treated livers weighed less and were visibly less steatotic than the livers from the HF diet fed group (Final liver weight -23.18%, p = <0.001, Figures 3A and B). We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of

BM on hepatic lipid content, ballooning and steatosis (Figure 3C). Histological examination revealed that the hepatocytes of HF diet fed mice were enlarged and contained large cytoplasmic lipid droplets compared to LC fed mice (Lipid content area (%) difference: -61.94%, p = <0.001; ballooning difference: -72.78%, p = <0.001; steatosis difference: -58.65%, p = <0.001). This change in hepatic cellular morphology was prevented by BM treatment, where the percentage of hepatic lipid area, ballooning, and steatosis were significantly lower compared to the HF diet fed group (Lipid content area (%) difference: -61.94%, p = <0.001; ballooning difference: -72.78%, p = <0.001; steatosis difference: 62.02%, p = <0.001) (Table 2).

Fatty acid metabolism-related genes in the liver were measured using RT-PCR in order to assess if these markers were responsible for BM's ability to prevent HF diet induced hepatic fat accumulation (Figure 3D). The results showed that BM prevented HF diet-induced decreases in the β oxidation gene, *ACOX* (HF vs. LC difference: -98.84%, p = <0.001; HF vs. BM difference: -94.02%, p = <0.001). However the expression of *ACOX* was still significantly higher in the LC group compared to HF diet fed mice treated with BM (LC vs. BM difference: -80.61%, p = <0.001). Furthermore, the levels of the lipogenic genes *SCD1* and *FAS* were significantly lower in the BM group compared to the untreated HF diet group. However, *FAS* expression in the LC group was significantly higher than both the HF diet group and BM group, and there were no significant differences between the LC and HF diet group in *SCD1* mRNA expression. In addition, there were no significant differences between *ACC* mRNA expression in the HF and BM groups. However, *ACC* mRNA expression was significantly lower in BM treated mice compared to LC fed mice. These results suggest that BM prevents HF diet-induced fat accumulation in the liver by increasing β oxidation and inhibiting genes involved in lipogenesis.

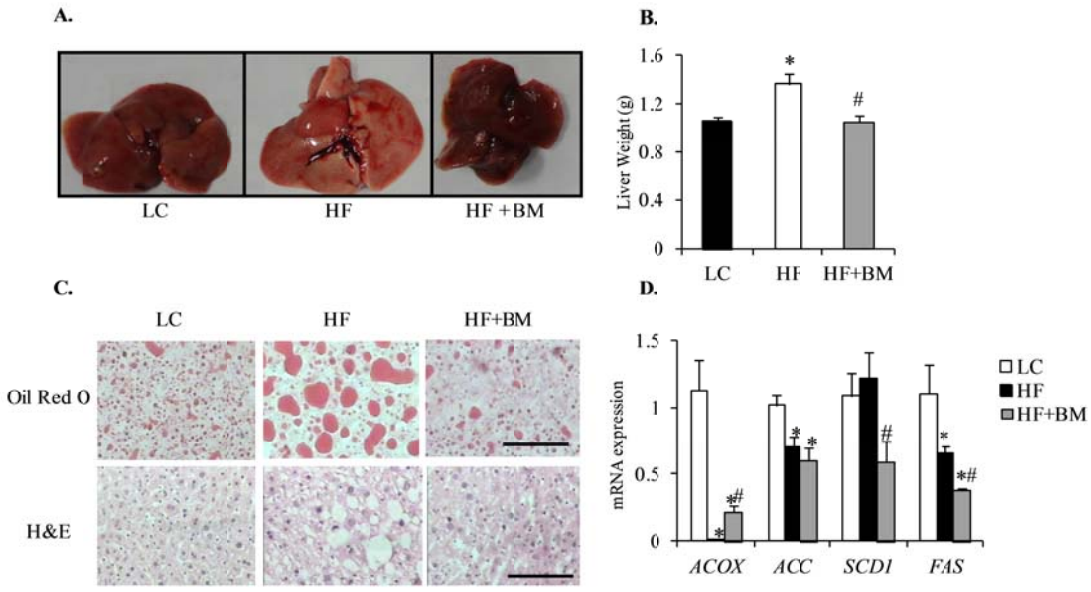

Figure 3. Effect of chronic administration of bardoxolone methyl (BM) on liver appearance (A), weight (B), lipid content (C), and expression of fatty acid metabolism-related genes (D) *FAS*, *SCD1*, *ACC* and *ACOX*, in the livers of mice (n= 7 per group) fed a high-fat (HF) diet for 21 weeks. *, p = <0.05 vs lab chow (LC) group, #, p= <0.05 vs. HF group, values are means ±SEM. Scale bar= 50μm.

Table 2 Liver histopathology in mice following 21 weeks of LC, HF, or HF and BM diet

Parameter	LC	HF	HF+BM	F value	P value
Lipid content area (%)	12.46±1.92[b]	32.74±3.32[a]	12.46±1.88[b]	23.144	<0.001
Ballooning	0.43±0.17[b]	1.58±0.15[a]	0.43±0.14[b]	17.6	<0.001
Steatosis	0.86±0.23[b]	2.08±0.19[a]	0.79±0.21[b]	10.793	<0.001

Lipid content area (%): The percentage of lipid present on a viewed field on a microscope. Hepatocyte ballooning: 0, none; 1, few ballooning cells; 2, many cells/prominent ballooning. Liver steatosis: 0: <5%; 1: 5%–33%; 2: >33%–66%; 3: >66%.Values are means ±SEM. LC, lab chow diet, HF, high-fat diet, HF+BM, high-fat diet and bardoxolone methyl treatment. [a]p<0.05 vs LC, [b]p<0.05 vs HF.

3.3. Bardoxolone methyl prevented HF diet-induced hepatic macrophage infiltration and inflammation

Along with fat accumulation, hepatic steatosis is characterised by a proinflammatory state in liver tissue (Marchesini et al., 2001). To investigate the effect of BM on macrophage accumulation in HF diet fed mouse livers, we performed immunohistochemistry with anti-F4/80 antibody (Figure 4A). We found that macrophage numbers increased in the livers of HF diet fed mice as indicated by accumulation of F4/80 positive cells. BM administration significantly prevented an increase in the number of F4/80 positive cells in the livers of HF diet fed mice (Figure 4B). The hepatic levels of the proinflammatory cytokine, IL-6, and signalling molecule, STAT3, were significantly increased in HF diet fed mice. However, this HF

diet-induced increase in IL-6 and STAT3 was prevented by BM treatment (Figure 4C). There were no significant differences in the expression of hepatic pIKK or IL-1β between the groups. In addition, nuclear protein levels of Nrf2 were significantly reduced in the livers of HF diet fed mice. However, this reduction was significantly prevented by BM treatment (Figure 4D). There were no significant differences in hepatic cytosolic Nrf2 protein levels among any of the groups. Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IL-6* mRNA expression, and decrease in *Nrf2* mRNA expression in mice fed a HF diet (Figure 4E). The alterations in *TNFα* and *Nrf2* mRNA levels were significantly prevented by BM administration. However, BM treatment was unable to prevent HF diet-induced elevations in *IL-6* mRNA expression. No significant differences were found in the mRNA expression of *IKKβ* and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet induced hepatic macrophage infiltration by regulating proinflammatory signalling molecules and activating Nrf2 in the liver.

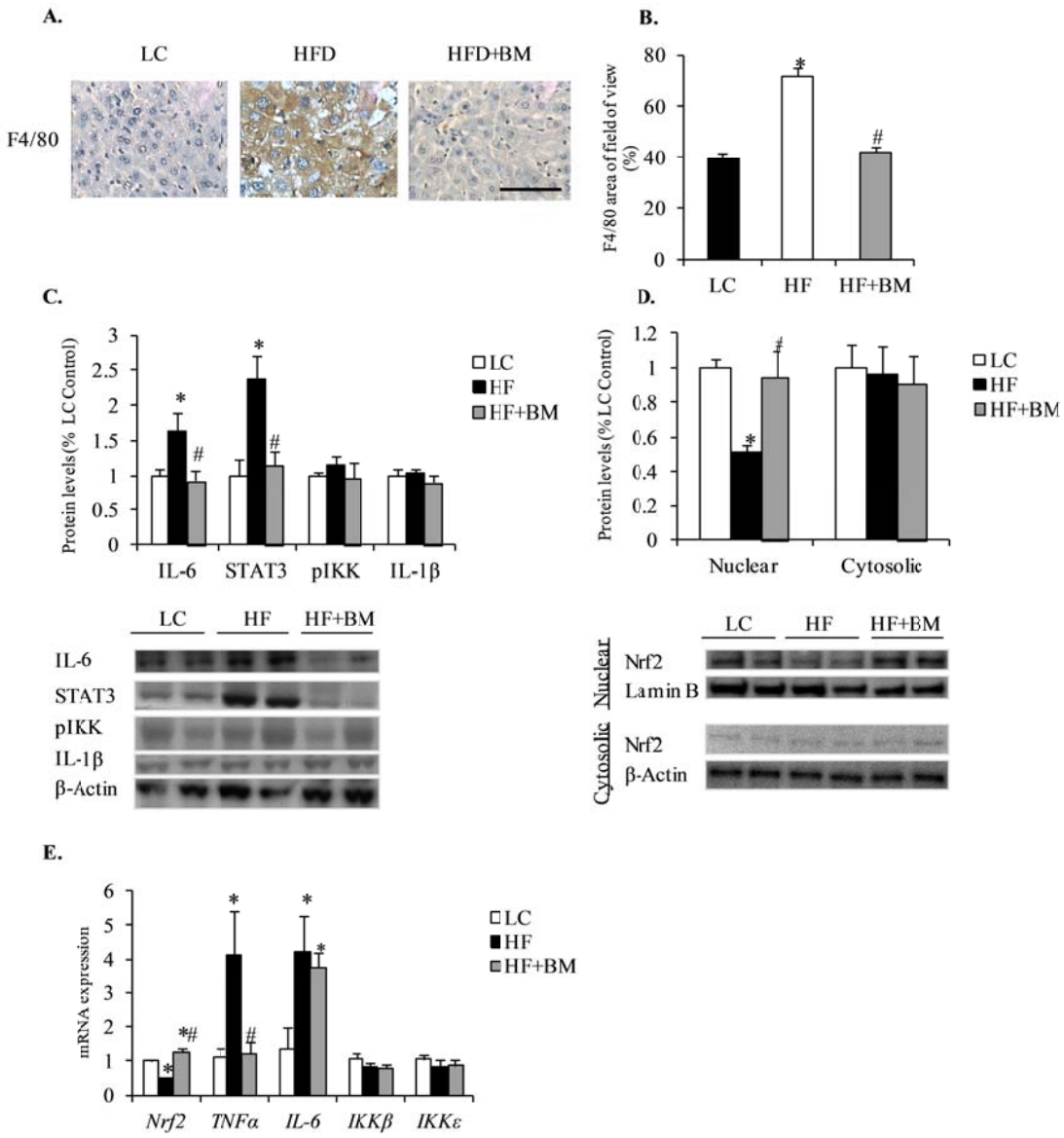

Figure 4. Effect of chronic administration of bardoxolone methyl (BM) on macrophage infiltration (A) and area of F4/80 immunoreactivity (B), inflammatory proteins (C) IL-6, STAT3, pIKK, and IL-1β, (D) nuclear and cytosolic protein expression of Nrf2, and genes (E) *Nrf2*, *TNFα*, *IL-6*, *IKKβ* and *IKKε*, in the livers of mice (n= 7 per group) fed a high-fat (HF) diet for 21 weeks. *, p = <0.05 vs lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM. Scale bar= 50μm.

4. Discussion

Rodents fed a HF diet show fat accumulation, low grade inflammation and insulin insensitivity in peripheral tissues, including the liver (Xu et al., 2003,Weisberg et al., 2003). BM has recently received considerable attention because of its anti-inflammatory, anti-oxidant, and blood glucose lowering effects (Saha et al., 2010). Although a recent study has investigated the acute anti-inflammatory effects of BM in diet-induced obese mice (Saha et al., 2010), the actions of BM on insulin signalling, fat accumulation and inflammation in the livers of mice fed a chronic HF diet have not been examined previously. In the

current study, we found that BM not only prevents HF diet-induced hepatic insulin resistance and inflammation, but it also reduces liver injury by preventing the development of fat accumulation and progression to hepatic steatosis.

A number of studies suggest that obesity-induced inflammation plays an important role in the development of insulin resistance (Emanuela et al., 2012,Boden and Shulman, 2002). A HF diet can promote insulin resistance by elevating proteins levels of the negative regulator, PTP1B, which impairs hepatic insulin signalling (Zabolotny et al., 2008). In the liver, insulin signal transduction suppresses hepatic glucose production through the inhibition of gluconeogenesis (Saltiel and Kahn, 2001). The hepatic insulin signalling cascade results in the inhibition of FOXO1, a transcription factor that promotes the expression of gluconeogenic genes such as *G6Pase* (Schmoll, Walker, Alessi et al., 2000,Yeagley, Guo, Unterman et al., 2001). Mice lacking the hepatic *FOXO1* gene display reductions in gluconeogenic gene expression, resulting in reduced glucose production and improved glucose clearance (Matsumoto, Pocai, Rossetti et al., 2007). BDNF is also an important molecule for preventing insulin resistance that increases glucose metabolism, resulting in reduced blood glucose levels (Kuroda et al., 2003,Hariharan et al., 1997). Our study demonstrated that BM treatment in mice fed a HF diet for 21 weeks reduced hepatic PTP1B and FOXO1, and increased BDNF protein levels, which was coupled with a reduction in plasma insulin levels. In addition, the HF diet-induced decreases in *IR* and *IRS-1*, and increase in *G6Pase* mRNA expression was significantly prevented by BM treatment. Overall, our results suggest that the action of BM in preventing HF diet-induced glucose intolerance and insulin resistance was through, at least partially, inhibiting FOXO1/*G6Pase* mediated hepatic glucose production.

Obesity from a HF diet is known to result in the accumulation of fat into the liver (Marchesini et al., 2001). A HF diet results in an increase in the expression of the hepatic lipogenic genes, *ACC*, *FAS* and *SCD1* and fat accumulation in the livers of mice fed a HF diet (Choi, Um, Ahn et al., 2014). Moreover, hamsters fed a HF diet have an increase in hepatic lipidemia that is coupled with a decrease in mRNA

expression of the β fatty oxidation gene, *ACOX* (Choi, Gwon, Ahn et al., 2013). We found that chronic HF diet feeding resulted in an accumulation of fat and hepatocyte injury in the liver and was associated with an increase in *SCD1* and *FAS*, and a decrease in *ACOX* genes. These alterations were prevented by BM administration. These results suggest that HF diet-induce fat accumulation in the liver and associated decreases in a β oxidation gene can be prevented by BM administration.

Obesity-induced fat accumulation is associated with the infiltration of macrophages into adipose tissue, where they promote the release of proinflammatory cytokines such as TNFα and IL-6 (Wellen and Hotamisligil, 2005). IL-6 has been found to increase STAT3 levels to promote inflammatory signalling in human mammary epithelial (hTERT-HME1) cells (Yang et al., 2007). We found that there was increased macrophage infiltration, coupled with an increase of the proteins IL-6, and STAT3, and the *IL-6* and *TNFα* genes in the livers of mice chronically fed a HF diet. Furthermore, our results demonstrated that HF diet-induced macrophage infiltration, along with IL-6 and STAT3 protein levels and *TNFα* mRNA expression could be prevented by BM administration. However, although BM prevented HF diet induced increases in IL-6 protein expression, it failed to prevent HF diet induced increases in *IL-6* mRNA expression, attributed to the post transcription of IL-6. One of the possible mechanisms for the anti-inflammatory effects of BM in the liver includes preventing the activation of IL-6, resulting in reduced STAT3 and preventing *TNFα* mRNA expression which all contribute to attenuating the proinflammatory response.

BM has been reported to be one of the most potent known activators of Nrf2 in several peripheral tissues including the eyes and kidneys (Camer, Yu, Szabo et al., 2014). In the livers of mice, Nrf2 activation causes reduced expression of the inflammatory cytokines, TNFα and IL-6 (Liu, Wu, Lu et al., 2013,Wang, Cui, Li et al., 2013). Nrf2 deletion is associated with increased liver weight gain, and hepatic steatosis in mice fed a HF diet (Wang et al., 2013). Furthermore, mice deficient in Nrf2 and fed a HF diet have been reported to show rapid development of hepatic steatosis and associated increases in the fatty acid lipogenic genes *ACC*, *FAS* and *SCD1* (Okada, Warabi, Sugimoto et al., 2013) and

reduction in the β oxidation gene, *ACOX* (Tanaka, Ikeda, Yamamoto et al., 2012). In addition, HF diet-induced hepatic steatosis in mice can be improved through regulation of Nrf2 (Yang, Li, Liu et al., 2014). In our study, BM administration prevented HF diet induced decreases in Nrf2 protein levels in the nucleus and *Nrf2* gene expression. This suggests the ability of BM to prevent HF diet induced elevations in proinflammatory signalling molecules and fat accumulation in the liver may be due to its ability to regulate the expression of the *Nrf2* gene and Nrf2 nuclear protein levels.

In conclusion, our findings suggest that chronic supplementation with BM may play an important role in preventing the actions of a HF diet in the development of inflammation, insulin resistance and hepatic steatosis in mice. A proposed model of the potential molecular mechanisms targeted by BM in mice fed a HF diet is presented in Figure 5. Since obesity-induced inflammation and insulin resistance has been implicated in the progression of liver disease, BM may have beneficial effects in preventing the progression of HF diet induced liver steatosis. With further research and eventual human clinical trials, the possibility of using BM for the prevention of insulin resistance and associated development of hepatic steatosis appears promising.

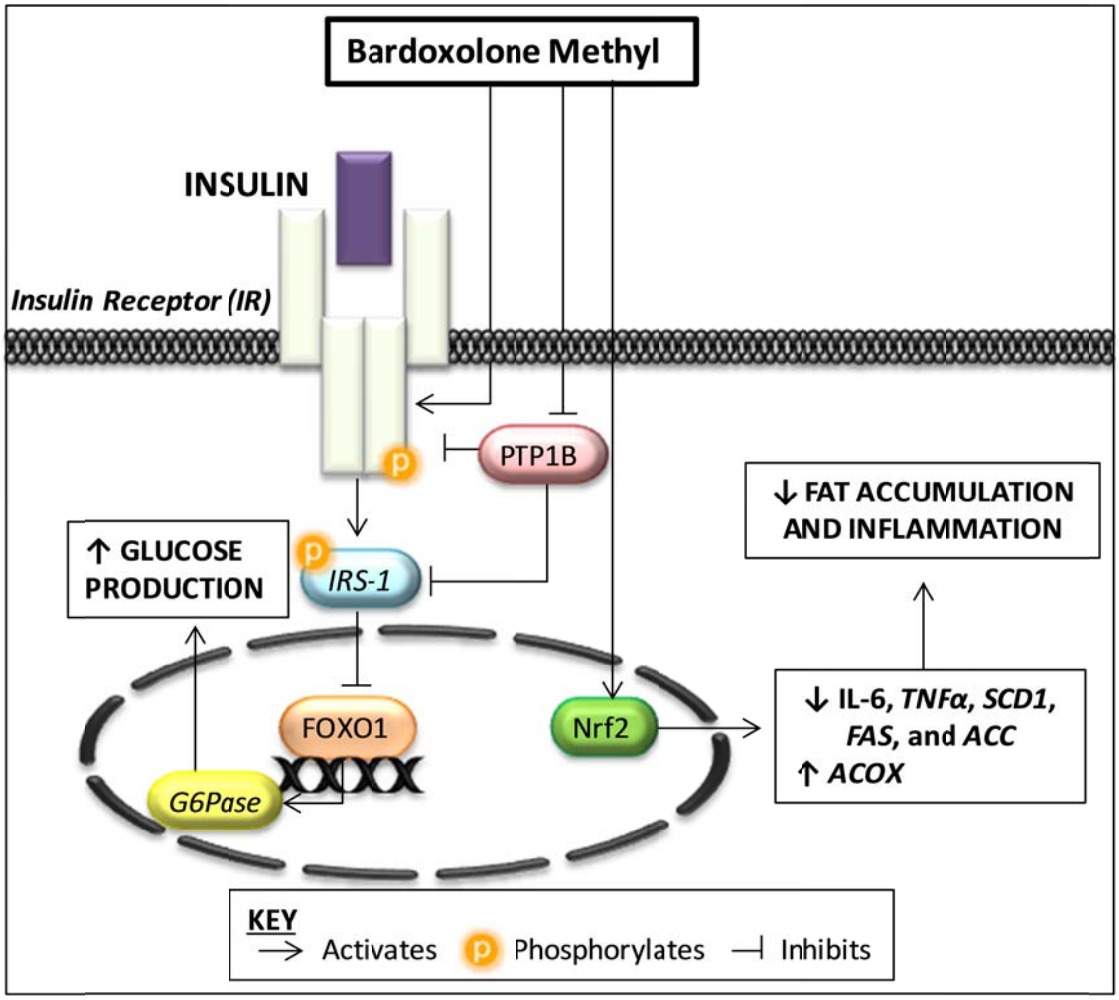

Figure 5. A proposed model of molecular targets of bardoxolone methyl in preventing HF diet-induced insulin resistance and the development of hepatic steatosis.

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References

[1] Kahn, S.E., Hull, R.L. and Utzschneider, K.M., 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes, Nature. 444, 840-6.

[2] Forouhi, N.G. and Wareham, N.J., 2010. Epidemiology of diabetes, Medicine. 38, 602-606.

[3] Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A. and Chen, H., 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance, J Clin Invest. 112, 1821-30.

[4] Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. and Ferrante, A.W., Jr., 2003. Obesity is associated with macrophage accumulation in adipose tissue, J Clin Invest. 112, 1796-808.

[5] Emanuela, F., Grazia, M., Marco de, R., Maria Paola, L., Giorgio, F. and Marco, B., 2012. Inflammation as a Link between Obesity and Metabolic Syndrome, J Nutr Metab. 2012, 476380.

[6] Ginsberg, H.N., 2006. Is the slippery slope from steatosis to steatohepatitis paved with triglyceride or cholesterol?, Cell Metab. 4, 179-81.

[7] Qureshi, K. and Abrams, G.A., 2007. Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease, World J Gastroenterol. 13, 3540-53.

[8] Zabolotny, J.M., Kim, Y.B., Welsh, L.A., Kershaw, E.E., Neel, B.G. and Kahn, B.B., 2008. Protein-tyrosine phosphatase 1B expression is induced by inflammation in vivo, J Biol Chem. 283, 14230-41.

[9] Hong, F., Nguyen, V.A. and Gao, B., 2001. Tumor necrosis factor alpha attenuates interferon alpha signaling in the liver: involvement of SOCS3 and SHP2 and implication in resistance to interferon therapy, FASEB J. 15, 1595-7.

[10] Lam, N.T., Covey, S.D., Lewis, J.T., Oosman, S., Webber, T., Hsu, E.C., Cheung, A.T. and Kieffer, T.J., 2006. Leptin resistance following over-expression of protein tyrosine phosphatase 1B in liver, J Mol Endocrinol. 36, 163-74.

[11] Goldstein, B.J., Bittner-Kowalczyk, A., White, M.F. and Harbeck, M., 2000. Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein, J Biol Chem. 275, 4283-9.

[12] Ueki, K., Kondo, T. and Kahn, C.R., 2004. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms, Mol Cell Biol. 24, 5434-46.

[13] Nakae, J., Kitamura, T., Silver, D.L. and Accili, D., 2001. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression, J Clin Invest. 108, 1359-67.

[14] German, J., Kim, F., Schwartz, G.J., Havel, P.J., Rhodes, C.J., Schwartz, M.W. and Morton, G.J., 2009. Hypothalamic leptin signaling regulates hepatic insulin sensitivity via a neurocircuit involving the vagus nerve, Endocrinology. 150, 4502-11.

[15] Kuroda, A., Yamasaki, Y., Matsuhisa, M., Kubota, M., Nakahara, I., Nakatani, Y., Hoshi, A., Gorogawa, S., Umayahara, Y., Itakura, Y., Nakagawa, T., Taiji, M., Kajimoto, Y. and Hori, M., 2003. Brain-derived neurotrophic factor ameliorates hepatic insulin resistance in Zucker fatty rats, Metabolism. 52, 203-8.

[16] Hariharan, N., Farrelly, D., Hagan, D., Hillyer, D., Arbeeny, C., Sabrah, T., Treloar, A., Brown, K., Kalinowski, S. and Mookhtiar, K., 1997. Expression of human hepatic glucokinase in transgenic mice liver results in decreased glucose levels and reduced body weight, Diabetes. 46, 11-6.

[17] Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A.J., Natale, S., Forlani, G. and Melchionda, N., 2001. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome, Diabetes. 50, 1844-50.

[18] Musso, G., Gambino, R. and Cassader, M., 2009. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD), Prog Lipid Res. 48, 1-26.

[19] McArdle, M.A., Finucane, O.M., Connaughton, R.M., McMorrow, A.M. and Roche, H.M., 2013. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies, Front Endocrinol (Lausanne). 4, 52.

[20] Yang, J., Liao, X., Agarwal, M.K., Barnes, L., Auron, P.E. and Stark, G.R., 2007. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB, Genes Dev. 21, 1396-408.

[21] Napetschnig, J. and Wu, H., 2013. Molecular basis of NF-kappaB signaling, Annu Rev Biophys. 42, 443-68.

[22] Liby, K.T. and Sporn, M.B., 2012. Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease, Pharmacol Rev. 64, 972-1003.

[23] Wang, H., Garvin, J.L., D'Ambrosio, M.A., Falck, J.R., Leung, P., Liu, R., Ren, Y. and Carretero, O.A., 2011. Heme oxygenase metabolites inhibit tubuloglomerular feedback in vivo, Am J Physiol Heart Circ Physiol. 300, H1320-6.

[24] Reisman, S.A., Chertow, G.M., Hebbar, S., Vaziri, N.D., Ward, K.W. and Meyer, C.J., 2012. Bardoxolone methyl decreases megalin and activates nrf2 in the kidney, J Am Soc Nephrol. 23, 1663-73.

[25] NIH, 2012. Bardoxolone Methyl Evaluation in Patients With Chronic Kidney Disease and Type 2 Diabetes (BEACON).

[26] Pergola, P.E., Raskin, P., Toto, R.D., Meyer, C.J., Huff, J.W., Grossman, E.B., Krauth, M., Ruiz, S., Audhya, P., Christ-Schmidt, H., Wittes, J. and Warnock, D.G., 2011. Bardoxolone methyl and kidney function in CKD with type 2 diabetes, N Engl J Med. 365, 327-36.

[27] Liby, K.T., Yore, M.M. and Sporn, M.B., 2007. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer, Nature Reviews Cancer. 7, 357-369.

[28] Hong, D.S., Kurzrock, R., Supko, J.G., He, X., Naing, A., Wheler, J., Lawrence, D., Eder, J.P., Meyer, C.J., Ferguson, D.A., Mier, J., Konopleva, M., Konoplev, S., Andreeff, M., Kufe, D., Lazarus, H., Shapiro, G.I. and Dezube, B.J., 2012. A phase I first-in-human trial of bardoxolone methyl in patients with advanced solid tumors and lymphomas, Clin Cancer Res. 18, 3396-406.

[29] Ahmad, R., Raina, D., Meyer, C., Kharbanda, S. and Kufe, D., 2006. Triterpenoid CDDO-Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on Cys-179, J Biol Chem. 281, 35764-9.

[30] Saha, P.K., Reddy, V.T., Konopleva, M., Andreeff, M. and Chan, L., 2010. The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid methyl ester has potent anti-diabetic effects in diet-induced diabetic mice and Lepr(db/db) mice, J Biol Chem. 285, 40581-92.

[31] Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., Sugawara, A., Kensler, T.W. and Yamamoto, M., 2013. The Keap1-Nrf2 system prevents onset of diabetes mellitus, Mol Cell Biol. 33, 2996-3010.

[32] Shin, S., Wakabayashi, J., Yates, M.S., Wakabayashi, N., Dolan, P.M., Aja, S., Liby, K.T., Sporn, M.B., Yamamoto, M. and Kensler, T.W., 2009. Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide, Eur J Pharmacol. 620, 138-44.

[33] Kudo, T., Tamagawa, T., Kawashima, M., Mito, N. and Shibata, S., 2007. Attenuating effect of clock mutation on triglyceride contents in the ICR mouse liver under a high-fat diet, J Biol Rhythms. 22, 312-23.

[34] Mehlem, A., Hagberg, C.E., Muhl, L., Eriksson, U. and Falkevall, A., 2013. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease, Nat Protoc. 8, 1149-54.

[35] Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., Yeh, M., McCullough, A.J., Sanyal, A.J. and

Clin, N.S., 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease, Hepatology. 41, 1313-1321.
[36] Dinh, C.H., Szabo, A., Camer, D., Yu, Y., Wang, H. and Huang, X.F., 2015. Bardoxolone methyl prevents fat deposition and inflammation in the visceral fat of mice fed a high-fat diet, Chem Biol Interact. 229, 1-8.
[37] Mobasher, M.A., Gonzalez-Rodriguez, A., Santamaria, B., Ramos, S., Martin, M.A., Goya, L., Rada, P., Letzig, L., James, L.P., Cuadrado, A., Martin-Perez, J., Simpson, K.J., Muntane, J. and Valverde, A.M., 2013. Protein tyrosine phosphatase 1B modulates GSK3beta/Nrf2 and IGFIR signaling pathways in acetaminophen-induced hepatotoxicity, Cell Death Dis. 4, e626.
[38] Camer, D., Yu, Y., Szabo, A., Fernandez, F., Dinh, C.H. and Huang, X.F., 2015. Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory, Prog Neuropsychopharmacol Biol Psychiatry. 59, 68-75.
[39] Livak, K.J. and Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2â^'Î"Î"CT Method, Methods. 25, 402-408.
[40] Neuschwander-Tetri, B.A. and Caldwell, S.H., 2003. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference, Hepatology. 37, 1202-19.
[41] Boden, G. and Shulman, G.I., 2002. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction, Eur J Clin Invest. 32 Suppl 3, 14-23.
[42] Saltiel, A.R. and Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism, Nature. 414, 799-806.
[43] Schmoll, D., Walker, K.S., Alessi, D.R., Grempler, R., Burchell, A., Guo, S., Walther, R. and Unterman, T.G., 2000. Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity, J Biol Chem. 275, 36324-33.
[44] Yeagley, D., Guo, S., Unterman, T. and Quinn, P.G., 2001. Gene- and activation-specific mechanisms for insulin inhibition of basal and glucocorticoid-induced insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase transcription. Roles of forkhead and insulin response sequences, J Biol Chem. 276, 33705-10.
[45] Matsumoto, M., Pocai, A., Rossetti, L., Depinho, R.A. and Accili, D., 2007. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver, Cell Metab. 6, 208-16.
[46] Choi, W.H., Um, M.Y., Ahn, J., Jung, C.H. and Ha, T.Y., 2014. Cooked rice inhibits hepatic fat accumulation by regulating lipid metabolism-related gene expression in mice fed a high-fat diet, J Med Food. 17, 36-42.
[47] Choi, W.H., Gwon, S.Y., Ahn, J., Jung, C.H. and Ha, T.Y., 2013. Cooked rice prevents hyperlipidemia in hamsters fed a high-fat/cholesterol diet by the regulation of the expression of hepatic genes involved in lipid metabolism, Nutr Res. 33, 572-9.
[48] Wellen, K.E. and Hotamisligil, G.S., 2005. Inflammation, stress, and diabetes, J Clin Invest. 115, 1111-9.
[49] Camer, D., Yu, Y., Szabo, A. and Huang, X.F., 2014. The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer and derivatives for type 2 diabetes and associated complications, Mol Nutr Food Res. 58, 1750-9.
[50] Liu, J., Wu, K.C., Lu, Y.F., Ekuase, E. and Klaassen, C.D., 2013. NRF2 Protection against Liver Injury Produced by Various Hepatotoxicants, Oxid Med Cell Longev. 2013, 305861.
[51] Wang, C., Cui, Y., Li, C., Zhang, Y., Xu, S., Li, X., Li, H. and Zhang, X., 2013. Nrf2 deletion causes "benign" simple steatosis to develop into nonalcoholic steatohepatitis in mice fed a high-fat diet, Lipids Health Dis. 12, 165.

[52] Okada, K., Warabi, E., Sugimoto, H., Horie, M., Gotoh, N., Tokushige, K., Hashimoto, E., Utsunomiya, H., Takahashi, H., Ishii, T., Yamamoto, M. and Shoda, J., 2013. Deletion of Nrf2 leads to rapid progression of steatohepatitis in mice fed atherogenic plus high-fat diet, J Gastroenterol. 48, 620-32.

[53] Tanaka, Y., Ikeda, T., Yamamoto, K., Ogawa, H. and Kamisako, T., 2012. Dysregulated expression of fatty acid oxidation enzymes and iron-regulatory genes in livers of Nrf2-null mice, J Gastroenterol Hepatol. 27, 1711-7.

[54] Yang, Y., Li, W., Liu, Y., Sun, Y., Li, Y., Yao, Q., Li, J., Zhang, Q., Gao, Y., Gao, L. and Zhao, J., 2014. Alpha-lipoic acid improves high-fat diet-induced hepatic steatosis by modulating the transcription factors SREBP-1, FoxO1 and Nrf2 via the SIRT1/LKB1/AMPK pathway, J Nutr Biochem.