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Dipeptidyl peptidase-4 inhibition ameliorates western diet-induced hepatic steatosis and insulin resistance through hepatic lipid remodeling and modulation of hepatic mitochondrial function

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Running Title: DPP-4 inhibitor improves hepatic function in western diet-induced obesity

ABSTRACT

Novel therapies are needed for treating the increasing prevalence of hepatic steatosis in western

populations. In this regard, dipeptidyl peptidase-4 (DPP-4) inhibitors have recently been reported

to attenuate the development of hepatic steatosis, but the potential mechanisms remain poorly

defined. In the current study, four week old C57Bl/6 mice were fed a high fat/high fructose western

diet (WD) or WD containing DPP-4 inhibitor, MK0626, for 16 weeks. The DPP-4 inhibitor

prevented WD-induced hepatic steatosis and reduced hepatic insulin resistance by enhancing insulin

suppression of hepatic glucose output. WD-induced accumulation of hepatic triacylglycerol (TAG)

and diacylglycerol (DAG) content was significantly attenuated with DPP-4 inhibitor treatment. In

addition, MK0626 significantly reduced mitochondrial incomplete palmitate oxidation and

increased indices of pyruvate dehydrogenase activity, TCA cycle flux, and hepatic TAG secretion.

Furthermore, DPP4-inhibition rescued WD-induced decreases in hepatic PGC-1α and CPT-1

mRNA expression and hepatic Sirt1 protein content. Moreover, plasma uric acid levels in WD fed

mice were decreased after MK0626 treatment. These studies suggest that DPP-4 inhibition

ameliorates hepatic steatosis and insulin resistance by suppressing hepatic TAG and DAG

accumulation through enhanced mitochondrial carbohydrate utilization and hepatic TAG

secretion/export with concomitant reduction of uric acid production.

Key Words: Lipidomics, NAFLD, obesity, hepatic insulin resistance, MK-0626 (DPP-4 inhibitor)

INTRODUCTION

Obesity is becoming an epidemic disease in western cultures, affecting more than one-third of the US adult population (1). Nonalcoholic fatty liver disease (NAFLD) progressing to steatohepatitis (NASH) and cirrhosis is also increasing in epidemic proportions concurrent with the obesity epidemic (2; 3). The dramatic rise in obesity and NAFLD appears to be due, in part, to consumption of a western diet (WD) containing high amounts of fat and fructose, and fructose consumption in the US has more than doubled in the last three decades (2; 4). Hepatic insulin resistance that develops with consumption of high-fat and high-fructose diets is closely linked to NAFLD and increases the risk for the development of type 2 diabetes (5; 6). Therefore, novel strategies targeting hepatic steatosis and insulin resistance have received considerable attention in recent years (7).

The gut-derived incretin hormones, glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotrophic peptide (GIP) play important roles in both postprandial and long-term glucose homeostasis by enhancing glucose-stimulated insulin secretion and suppressing glucagon release (8). The exopeptidase, dipeptidyl peptidase-4 (DPP-4), a serine protease found in the plasma and on the surface of diverse cells, rapidly degrades circulating GLP-1 and GIP, limiting the half-life of these hormones. Plasma DPP-4 activity and expression of DPP-4 on various inflammation-promoting immune cells is increased in obesity and diabetes, raising the possibility that its inhibition may reduce systemic and tissue inflammation (9; 10). In this regard, there is emerging evidence that DPP-4 inhibition may be a novel therapeutic strategy to prevent the development of hepatic insulin resistance and hepatic steatosis (11-13) . However, the precise mechanisms and mediators involved in this hepatic protection are not well understood.

Accumulating evidence suggests that hepatic insulin resistance is caused by dysfunction in three pathways of energy metabolism (14; 15). First, excess carbohydrate flux (glucose, fructose) is

associated with resistance to the suppressive effect of insulin on hepatic glucose production and

excess disposal of carbons via de novo lipogenesis (14; 16). Second, elevation in lipid synthesis (or

reduced lipid secretion/export) leads to accumulation of hepatic triacylglycerols (TAG) which are

inert but often track with increased levels of bioactive lipid intermediates diacylglycerols (DAG)

and ceramides that putatively lead to hepatic insulin resistance (17; 18). Third, the hepatic steatosis

linked to insulin resistance is associated with mitochondrial dysfunction and altered hepatic fatty

acid oxidation (15; 19; 20). The impact of DPP-4 inhibition on these metabolic processes has not

been previously examined.

MK0626 is a DPP-4 inhibitor closely related to sitagliptin with pharmacokinetics suited to

rodent model investigation (21; 22). Here we test the hypothesis that DPP-4 inhibition with

MK0626 will attenuate WD-induced hepatic steatosis and insulin resistance by reducing hepatic

lipid intermediate (DAGs and ceramides) accumulation with consequent improvement in hepatic

mitochondrial function and metabolism.

RESEARCH DESIGN AND METHODS

Animals and experimental design. C57Bl/6 mice were purchased from Charles River, Inc and cared for in accordance with National Institutes of Health guidelines. All procedures were approved in advance by the Institutional Animal Care and Use Committee of the University of Missouri. MK0626 was added to mouse chow to a final concentration of 33 mg/ kg chow to achieve a dose and plasma level of approximately 10 mg/ kg/ day and 300 nM, respectively (≥80% inhibition of plasma DPP4, Merck) based on previous pharmacology studies in rodents (21) and as previously published by our group and others (22-24). Male mice were divided into four groups (n=10-12 per group) to include C57Bl/6 control (CD), C57Bl/6 treated with MK0626 (CD-MK), Western Diet (WD) and WD treated with MK0626 (WD-MK) and put on diets at 4 wks of age for 16 weeks and sacrificed at 20 wks of age. Mice on the CD consumed product # 58Y2 (TestDiet, St. Louis, MO) providing 18.0% of energy as protein (16.9 g/100), 10.2% of energy as fat (4.3g/100g), and 71.8% of energy as carbohydrate (67.4 g/100g). Mice on the WD consumed product # 58Y1 (TestDiet, St. Louis, MO) providing 17.6% of energy as protein (20.5 g/100g), 46.4% of energy as fat (24g/100g), and 36.0% of energy as carbohydrate (41.8g/100g) with 17.5% fructose and 17.5% sucrose. The primary sources of fat in both diets were corn oil and lard. Mice were anesthetized [sodium pentobarbital (100 mg·kg–1)] following a 5 hr fast and killed by exsanguination by removal of the heart. Retroperitoneal and epididymal adipose tissue fat pads were removed from exsanguinated animals and weighed. For acute insulin stimulation studies (Additional n=6-7 per group), food was removed 5 hrs before mice were given an intraperitoneal injection of insulin (Humulin, 2.5 U/kg) and tissues were harvested under anesthesia 20 minutes post injection.

Biochemical parameters and DPP-4 activity. Plasma alanine aminotransferase (ALT) activity, cholesterol, nonesterified fatty acids (NEFA), uric acid, and TAG concentrations were determined

by automated analyzer. Plasma and liver DPP-4 activity was fluorometrically assessed as previously described by our group (22; 23).

Body composition. Percent body fat was measured by a nuclear magnetic resonance imaging whole-body composition analyzer (EchoMRI 4in1/1100, Echo Medical Systems, Houston, TX). This noninvasive measure was performed on conscious mice.

Hepatic histology and TAG, DAG, and ceramide content. At sacrifice, the liver was immediately removed, rinsed in chilled phosphate buffered saline, blotted dry, weighed, and either flash frozen in liquid nitrogen for storage at -80°C or placed in 10% NBF for formalin-fixation. Hematoxylin and eosin (H&E) staining was used for evaluation of steatosis by light microscopy as previously described by our group (19). Liver tissue was homogenized and lipids isolated by extraction into chloroform with appropriate internal standards included for each protocol. Extracted lipids were resuspended and diluted in methanol/chloroform (4:1, by volume) before analysis by electrospray ionization-mass spectrometry using a Thermo Electron TSQ Quantrum Ultra instrument (San Jose, CA). DAG molecular species were quantified as sodiated adducts using selected reaction monitoring as previously described with intensity of each species normalized to that of the internal standard di-20:0 DAG (25). TAG aliphatic groups were quantified by TAG fingerprinting techniques with neutral loss scanning for the loss of each fatty acid from the TAG species and comparisons to that of the neutral loss 268 which is derived from the internal standard Tri-17:1 TAG(26). Individual ceramide molecular species were quantified in negative ion mode using neutral loss 256 by comparing the ion intensity of individual molecular species to that of the internal standard (17:0 ceramide) after corrections for type I and type II ^{13}C isotope effects.

Western blot analyses. Western blots were performed as described earlier for oxidative phosphorylation (OXPHOS) electron transport chain complexes I through V (MitoProfile Total OXPHOS Rodent WB Antibody Cocktail; Abcam, Cambridge, MA.), NAD-dependent deacetylase

sirtuin-1 (Sirt1; Santa Cruz Biotechnology, Santa Cruz, CA), NAD-dependent deacetylase sirtuin-3 (Sirt3; Cell Signaling, Beverly, MA), microsomal triglyceride transfer protein (MTTP; Santa Cruz Biotechnology), apolipoprotein B100 (apoB10; Abcam), fatty acid synthase (FAS, Cell Signaling), acetyl-CoA carboxylase (ACC; Cell Signaling), sterol regulatory element binding protein (SREBP-1c; Santa Cruz Biotechnology), protein kinase B (Akt; Cell Signaling), and phospho-Akt Ser473 (Cell Signaling) (19; 27). Membranes stained with 0.1% amido-black (Sigma) were quantified to control for differences in protein loading or transfer of band densities as previously described (19).

Mitochondrial studies. *Palmitate and pyruvate oxidation*: Complete and incomplete oxidation of $[1-^{14}C]$ palmitate (American Radiochemicals; St. Louis, MO), $[1-^{14}C]$ pyruvate (PerkinElmer; Boston, MA), and $[2-^{14}C]$ pyruvate (PerkinElmer; Boston, MA) were measured in fresh isolated hepatic mitochondria preparations as previously described (27). Pyruvate ($[1-^{14}C]$ and $[2-^{14}C]$) were oxidized to $^{14}CO_2$ by isolated hepatic mitochondria in the appropriate reaction buffer. $[1-^{14}C]$ pyruvate oxidation was used as an index of pyruvate dehydrogenase activity (PDH) and $[2-^{14}C]$ Pyruvate oxidation as an index of tricarboxylic acid (TCA) cycle flux (28). *Mitochondrial respiration:* Mitochondrial respiration was assessed using high-resolution respirometry (Oroboros Oxygraph-2k; Oroboros Instruments; Innsbruck, Austria) as previously described (27). Briefly, oxygen flux was measured by addition of glutamate (5mM) and malate (2mM) to the chambers in the absence of ADP (GM-State 2) for assessment of State 2 respiration. Oxidative phosphorylation (OXPHOS) with electron flux through complex I was then quantified by titration of ADP (25-125 µM) (GM+ADP: State 3-Complex I) for assessment of State 3 respiration. Maximal ADP respiration with electron flux through both complex I and complex II was assessed by the addition of succinate (10 mM) (Succinate: State 3-Complex I+II). Finally, maximal capacity of the electron transport system was assessed by uncoupling with the addition of FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, 0.25 µM) (Uncoupled).

TAG secretion assay. In order to assess hepatic TAG secretion, five hour fasted mice were injected intraperitoneally with the lipase inhibitor Poloxamer 407 (P-407) (1 g/kg body weight) as a 75 mg/ml solution in saline as previously described (29). Blood samples were drawn into heparinized tubes at 0, 1 hr, 2 hr, and 3 hr after injection, plasma was separated and assayed for triglycerides as described above. Hepatic triglyceride production rates were calculated from the slope of the curve and expressed as mg/dL/hr.

mRNA expression. Total RNA was extracted from frozen liver using RNeasy kit and used for cDNA preparation and quantitative real time polymerase chain reaction (qPCR) with commercially available primers (19). Results were quantified by DdCT method relative to the housekeeping gene cyclophilin b, which relative gene expression did not differ among groups (p=0.6).

Hyperinsulinemic-euglycemic clamp. All procedures required for the hyperinsulinemic–euglycemic clamp were approved by the Vanderbilt University Animal Care and Use Committee. Catheters were implanted into a carotid artery and a jugular vein of mice for sampling and infusions respectively five days before the study as described by Berglund et al. (30). Hyperinsulinemic-euglycemic clamps were performed on mice fasted for 5 h using a modification of the method described by Ayala et al. (31). [3-^{3}H]-glucose was primed (2.4 μCi) and continuously infused for a 90 min equilibration and basal sampling periods (0.04 μCi/min). [3-^{3}H]-glucose was mixed with the non-radioactive glucose infusate (infusate specific activity of 0.4 μCi/mg) during the 2 h clamp period. Arterial glucose was clamped using a variable rate of glucose (plus trace [3-^{3}H]-glucose) infusion, which was adjusted based on the measurement of blood glucose at 10 min intervals. By mixing radioactive glucose with the non-radioactive glucose infused during a clamp, deviations in arterial glucose specific activity are minimized and steady state conditions are achieved. Baseline blood or plasma variables were calculated as the mean of values obtained in blood samples collected at −15 and −5 min. At time zero, insulin infusion (2.5 mU/kg of body weight per min)

was started and continued for 120 min. Mice received heparinized saline-washed erythrocytes from

donors at 5 μl/min to prevent a fall in hematocrit. Insulin clamps were validated by assessment of

blood glucose over time. Blood was taken at 80–120 min for the determination of [3-^{3}H]-glucose.

At the end of the clamps, animals were anesthetized and liver was taken and frozen immediately.

Rates of whole-body glucose appearance and uptake were determined as the ratio of the [^{3}H]-

glucose infusion rate to the specific activity of the plasma glucose during the final 40 minutes of the

clamps. Hepatic glucose production during the clamps was determined by subtracting the glucose

infusion rate from the whole-body glucose appearance. The glucose infusion rates across time have

been previously reported by our group (22).

Statistical analysis. Differences in outcomes among groups were determined using two-way

ANOVA and main effects (diet, drug) and interactions considered significant when $P<0.05$ (IBM

SPSS Statistics Version 22). Significant diet by drug interactions were followed up with Fisher

LSD post-hoc analyses.

RESULTS

Effects of WD and MK0626 on body weight, percent body fat, liver weight, and biochemical

parameters. WD-induced increases in body weight (+60% compared with CD, *P*<0.001, **Table 1**),

adiposity (+2.5 fold compared with CD groups, P<0.0001, **Table 1**), and food intake (**Table 1**)

were not affected by MK0626. However, MK0626 administration suppressed liver weights in the

WD-fed mice (*P*<0.001) (**Table 1**). WD feeding increased serum ALT levels by 40% (main effect,

P=0.01) but MK0626 treatment did not affect serum ALT in either diet group. In addition,

MK0626 increased serum TAGs (main effect, P=0.04) and WD increased serum cholesterol (main

effect, P=0.03), but serum NEFA were not affected by either diet or drug treatment (**Table 1**). We

have recently reported increased levels of plasma DPP-4 activity in WD-fed mice and suppression

of DPP-4 activity by MK0626 (22; 23). In the present study, DPP-4 activity was not increased in

liver homogenates of WD-fed (non-drug treated) mice, but MK0626 treatment caused significant

(*P*<0.01) inhibition of DPP-4 activity in both CD and WD groups (**Table 1**). In addition, WD

significantly increased plasma uric acid levels (main effect, P=0.003, Table 1), with MK0626

treatment significantly (P=0.03) lowering uric acid levels in both CD and WD groups, similar to

what we have previously reported (22).

MK0626 ameliorates WD-induced hepatic insulin resistance. Euglycemia was maintained in all

groups during the 2-hour clamp procedure and did not differ significantly among groups (**Figure**

1A). In addition, as we have previously shown (22), WD-fed mice displayed significant whole-

body insulin resistance with ~50% lower glucose infusion rates during the hyperinsulinemic clamp

compared to CD mice; the whole-body insulin sensitivity was not improved by MK0626 treatment

(**Figure 1A and 1B**). Not surprising, plasma insulin during basal and insulin clamp conditions was

significantly higher in WD vs CD mice (P<0.05, **Figure 1C**), but did not differ within each diet

between drug and non-drug treated groups. More importantly, MK0626 treatment rescued hepatic

insulin resistance induced by WD-feeding as assessed by insulin-mediated suppression of hepatic glucose production during the hyperinsulinemic-euglycemic clamp (**Figure 1D**). Given the chronic nature of the hyperinsulinemia during the clamp and the activation of multiple kinases and phosphatases, hepatic insulin signaling was assessed following acute insulin stimulation studies. The blunted insulin suppression of hepatic glucose output in WD animals was associated with impaired hepatic insulin signaling at the phosphorylation of Akt (Ser473), which was increased with MK0626 treatment (**Figure 1E**, main effect for diet and drug, $P<0.05$ for each).

Effects of WD and MK0626 on hepatic TAG, DAG, and ceramide content. MK0626-treatment largely ameliorated the marked accumulation of lipid droplets induced by the WD (Representative H&E staining shown in **Figure 2A**). This observation was confirmed with mass spectrometric analysis of hepatic TAG content (**Figure 2B**), with total hepatic TAG (insert) and each major fatty acid species of TAG dramatically elevated in WD fed mice. Normalization to the CD values was observed with MK0626 treatment. Analysis of the fatty acid composition (mol%) revealed that feeding the corn oil-rich diet resulted in the expected increase in 18:2 in WD-fed animals, while MK0626 reduced the 18:2 percentage in this group. Analysis of hepatic liver DAG concentrations (**Figure 3A**) revealed that in WD-fed mice, DAG fatty acids were more unsaturated, similar to the TAG composition. All fatty acid species assessed (34:3, 34:2, 34:1, 36:3, 36:2, 36:1, 38:6, and 38:4) were significantly elevated by WD (main effect, $P<0.01$, **Figure 3A**). More importantly, MK0626 treatment significantly reduced the content of these DAG species and total hepatic DAG content in WD-fed mice (diet by drug interaction, $p<0.01$). In contrast, total hepatic ceramide content did not differ among groups (**Figure 3C**), but 16:0, 18:0, and 20:0 were significantly ($p<0.05$, **Figure 3B**) increased by WD feeding, and 24:1 and total unsaturated ceramides was significantly ($p<0.05$, **Figure 3B and 3C**) decreased by WD feeding.

Effects of MK0626 on hepatic TAG synthesis and secretion markers. To determine whether increased accumulation of hepatic TAG was due to elevations in de novo lipogenesis, we determined hepatic mRNA levels and protein content of ACC, FAS, and SREBF (SREBP-1c). WD feeding significantly reduced ACC and FAS mRNA and protein levels and SREBF mRNA expression (P<0.05, **Figure 4A and 4B**). In addition, MK0626 significantly lowered ACC and FAS mRNA expression in CD mice (diet and drug interaction, P<0.05, **Figure 4A**). Hepatic TAG secretion was assessed after the administration of the lipase inhibitor Poloxamer 407 (P-407). Hepatic TAG secretion was reduced by 40% in WD mice (P<0.001, **Figure 4C and 4D**), a reduction which was partially rescued by MK0626 treatment (diet by drug interaction, P=0.02, **Figure 4C and 4D**). Hepatic TAG secretion rates corresponded to WD feeding induced reductions in apoB mRNA expression (**Figure 4E**) but not directly to apoB100 protein content (**Figure 4F**). In addition, MK0626 significantly increased hepatic MTTP mRNA expression (**Figure 4E**), but MTTP protein content did not differ among group (**Figure 4F**).

MK 0626 improves indices of hepatic mitochondrial PDH activity and TCA cycle flux and decreases incomplete fatty acid oxidation. To evaluate the effects of DPP-4 inhibition on hepatic mitochondrial function, we assessed several indices of carbohydrate and fatty acid oxidation and mitochondrial respiration. The mitochondrial oxidation of $1\text{-}^{14}C$ pyruvate and $2\text{-}^{14}C$ pyruvate, indices of PDH activity and TCA cycle flux, respectively, did not differ between CD and WD mice (**Figure 5A and 5B**). However, MK0626 significantly increased $1\text{-}^{14}C$ pyruvate oxidation and $2\text{-}^{14}C$ pyruvate oxidation in both CD-fed mice and WD-fed mice (main effect for drug for each, $P<0.05$; **Figure 5B and 5B**). Examination of mitochondrial fatty acid oxidation revealed a dramatic suppression of complete $1\text{-}^{14}C$ palmitate oxidation to CO_2 in WD-fed animals, a suppression not corrected by MK0626 administration (**Figure 5C**). However, MK0626 treatment significantly decreased incomplete $1\text{-}^{14}C$ palmitate oxidation captured as acid soluble metabolites

(P<0.05, **Figure 5D**). Hepatic mitochondrial state 2, state 3, and maximal uncoupled respiration

was not affected by WD-feeding or MK0626 administration (**Figure 5E**).

MK0626 partially rescues WD-induced down-regulation in genes and proteins regulating

hepatic mitochondrial function. We examined gene expression and protein content of several

markers of hepatic mitochondrial biogenesis and content (**Figure 6**). Hepatic PGC-1α and CPT-1

were significantly reduced in WD vs. CD mice, reductions which were completely prevented by

MK0626 (diet by drug interaction, *P*<0.05, **Figure 6A**). In addition, although mRNA levels of

TFAM and PPARα were not decreased in WD-fed mice, the levels were significantly increased

after MK0626 treatment (*P*<0.05, **Figure 6A**). We also evaluated hepatic protein levels of

OXPHOS subunits, Sirt1, and Sirt3 by Western blot. As shown in **Figure 6B**, hepatic protein levels

of OXHOS subunits I-V remained unaltered either in WD-fed mice or WD-mice fed mice treated

with MK0626. However, the WD-induced reduction in protein deacetylase Sirt1 was completely

prevented in the WD-MK treatment group (**Figure 6C**). Moreover, MK0626 treatment increased

hepatic Sirt3 protein content in isolated mitochondria in WD-fed mice (diet by drug interaction,

P=0.03; **Figure 6D**).

DISCUSSION

In this investigation, we provide mechanistic insight by which DPP-4 inhibition protects against development of hepatic insulin resistance and steatosis in a mouse model fed a high-fat and high-fructose WD. These potential mechanisms are physiologically inter-related and, as shown in **Figure 7**, independent lines of evidence demonstrated that the MK0626 compound resulted in a multi-faceted improvement in liver insulin sensitivity and fatty acid metabolism. Specifically, DPP-4 inhibition ameliorated hepatic DAG accumulation independent of changes in body weight or adiposity. It also increased hepatic TAG export/secretion, enhanced indices of mitochondrial carbohydrate utilization, and decreased incomplete hepatic mitochondrial fatty acid oxidation, with concomitant reduction of uric acid production.

NAFLD is now considered an integral component of obesity related metabolic syndrome and this condition is a risk factor for the progression of cardiovascular and renal disease (6). Hepatic steatosis is strongly linked to the development of hepatic insulin resistance (14; 19). Emerging evidence suggests that DPP4 inhibition or augmentation of GLP-1 utilizing GLP-1 receptor agonists may be useful in suppressing hepatic insulin resistance and/or steatosis (11-13; 32; 33). Interestingly, GLP-1 receptor activation with exendin-4 has been shown to reverse hepatic steatosis (32; 33), in part by decreasing hepatic lipogenesis but not through enhancing hepatic VLDL production (33) as we have shown with DPP-4 inhibition in the current study. The strategy of DPP-4 inhibition is especially intriguing given the observation that T-helper cell surface expression of DPP-4 and serum levels of DPP-4 are elevated in obesity, insulin resistance, and diabetes (9; 10). In addition, hepatic DPP-4 expression is elevated in NAFLD patients and both serum DPP-4 and hepatic expression are related to NAFLD severity (34; 35). Our current findings are in support of the DPP-4 inhibition strategy in the management of liver disease, as administration of MK0626 dramatically suppressed development of WD-induced hepatic steatosis in concert with

enhanced insulin suppression of hepatic glucose production. Previous in vitro hepatocyte studies demonstrate direct effects of GLP-1 and GLP-1 receptor activation on increasing Akt phosphorylation, induction of genes controlling fatty acid oxidation (36; 37), and direct in vitro effects of DPP-4 inhibition on hepatic stellate cell activation (38). Therefore, the reported improvements with MK0626 shown herein are likely due to both changes in GLP-1 and GLP-1 receptor activation as well as direct effects of DPP-4 inhibition on hepatocyte metabolism.

The mechanisms underlying obesity-related hepatic insulin resistance and fatty liver disease are under intense investigation. Additionally, the hepatic protective effects of DPP-4 inhibitors are not well understood, but likely go beyond mere improvement of glycemic control through enhanced glucose-stimulated insulin secretion (10). Hepatic insulin action to regulate hepatic glucose output is mediated through activation of the insulin receptor, insulin receptor substrates (IRS-1 & -2), phosphatidylinositol 3-kinase, and Akt pathway (14). Under normal insulin-sensitive conditions, insulin inhibits glycogenolysis and gluconeogenesis, suppressing glucose production (39). However, in the insulin-resistant state, defects in hepatic insulin signaling impair insulin-suppression of hepatic glucose production, leading to hyperglycemia and compensatory hyperinsulinemia (40). Increased accumulation of lipid metabolites/intermediates, such as DAGs and ceramides, are thought to be initiators in the development of insulin resistance (14; 41). Numerous studies have implicated hepatic DAGs in potentially causing hepatic insulin resistance (14; 19); although, recent studies did not support the importance of hepatic accumulation of DAGs (42). In addition, polyunsaturated fatty acids in DAG are thought to be responsible for PKC activation (43). Lipidomic analysis revealed significant accumulation of polyunsaturated fatty acids in DAG in WD-fed mice and DPP-4 inhibition with MK0626 completely abrogated this accumulation. Previous studies have also implicated hepatic ceramides in hepatic insulin resistance (44); however, in this investigation, total hepatic ceramide content was unchanged with WD feeding

or MK0626 administration. This is in agreement with recent reports showing a lack of correlation

between hepatic insulin resistance and ceramide accumulation (44; 45), and collectively, these data

support a stronger role for hepatic DAG accumulation in WD-induced hepatic insulin resistance in

the present model.

Our group has previously demonstrated a significant role for de novo fatty acid synthesis in

liver TAG accumulation in NAFLD patients (20). In addition, increased fat and sucrose

consumption can induce lipogenic genes, and this phenomenon is suppressed in rodents by DPP-4

inhibitors (12; 46). However, in the present study, hepatic mRNA expression and protein content

for lipogenic markers ACC, FAS, and SREBP were down-regulated with the WD, with no further

reduction seen with MK0626. Direct suppression of these lipogenic genes is a likely consequence

of the high saturated fatty acid content in the WD diet (47). This notion is further supported by

recent observations that lipogenic genes are suppressed with a high-fat and high-fructose

combination diet but not with high-fat or high-fructose alone (46). Our findings suggest that the

protective effects of DPP-4 inhibition in this model may not be mediated through direct suppression

of hepatic de novo lipogenesis.

The export of hepatic TAG is dependent on VLDL-TAG packaging, a process requiring

MTTP and apo B (48). Hepatic MTTP expression is decreased in the setting of insulin resistance

and NAFLD (49) and marked accumulation of hepatic TAG is not always accompanied by

increased secretion of VLDL (44). Here we demonstrate a dramatic reduction in hepatic TAG

secretion in WD-fed mice, a finding which likely is contributing to the hepatic steatosis in the

model. In addition, we demonstrate for the first time that DDP-4 inhibition partially rescued

hepatic TAG secretion in WD mice. Circulating plasma TAGs were also increased in the MK0626

treated mice. Interestingly, hepatic TAG secretion was actually blunted with MK0626 in the CD

fed mice, a finding likely related to the minimal hepatic TAG present in the low fat fed mice. We

also observed WD-induced reductions in hepatic apoB mRNA expression and MK0626 induced

increases in MTTP and apoB100 mRNA expression in WD-fed mice. These data indicate that

DPP4 inhibition with MK0626 may be alleviating hepatic TAG accumulation, in part, through

increased hepatic TAG secretion/export.

Recent reports suggest that hepatic mitochondrial dysfunction may be an initial event in

liver lipid accumulation (15; 19) and intimately linked to the development of hepatic insulin

resistance (50). Sirt1 is involved in mitochondrial biogenesis and mitochondrial metabolism and

regulates the mRNA levels of PPARα, PGC-1α, CPT-1α, and TFAM (51; 52). Studies have shown

that fructose can suppress Sirt1 in hepatocytes (53), and GLP-1 agonism has been shown to increase

Sirt1 levels in high fat fed mice (37). Results of the current investigation demonstrate that WD-

induced suppression of Sirt1 level was completely prevented with DPP-4 inhibition. Furthermore,

although not significantly decreased with WD-feeding, Sirt3, a mitochondrial protein deacetylase

known to regulate mitochondrial function (52), was increased by 25% with DPP-4 inhibition. The

WD-induced suppression in Sirt1 expression was accompanied by WD-induced impairment in

complete mitochondrial palmitate oxidation that was not rescued by MK0626 treatment. This could

be due to the effects of fructose, as fructose administration is associated with inhibition of fatty acid

oxidation (54), raising the possibility of a fructose-mediated inhibition of fatty acid oxidation that is

not entirely relieved by MK0626. However, MK0626 administration prevented WD-induced

suppression of hepatic PGC-1α and CPT-1 mRNA expression and significantly increased TFAM

and PPARα mRNA expression in the WD-fed mice, suggesting potentially better maintenance of

mitochondrial biogenesis with DPP-4 inhibition. The examination of the direct role of DPP-4

inhibition on mitochondrial biogenesis is warranted in future investigations.

If β-oxidation is not matched with enhanced activity of the TCA cycle, this will lead to the

incomplete oxidation of lipids and the accumulation of acetyl-CoA metabolites (acetyl-carnitine)

(55). Lipid intermediates from incomplete oxidation of fatty acids are also linked to hepatic insulin

resistance and mitochondrial dysfunction (56). These metabolites may also be directed to fatty acid

biosynthesis pathways and are implicated in tissue insulin insensitivity and mitochondrial oxidative

stress (55). To our knowledge, we report for the first time that DPP-4 inhibition decreased

incomplete palmitate oxidation in WD-fed mice, thereby suggesting the possible role of suppressed

incomplete fatty acid oxidation by MK0626 in improving hepatic insulin resistance.

DPP-4 inhibition with MK0626 also enhanced indices of mitochondrial carbohydrate

oxidation, including PDH activity and TCA cycle flux, which was not accompanied by significant

alterations in hepatic mitochondrial respiration or in changes in electron transport chain protein

expression. These novel results suggest that DPP-4 inhibition enhances the oxidation and disposal

of glucose/fructose carbon intermediates, thereby potentially reducing the concentrations of these

intermediates for lipogenesis. The direct examination of fructose metabolism is warranted in future

investigations.

High fructose consumption is known to increase liver uric acid production, deplete high

energy phosphates and elevated glucose flux (57). In addition, uric acid has been shown to cause

hepatic steatosis in cultured hepatocytes by enhancing lipogenesis; whereas, suppression of uric

acid production has been shown to ameliorate hepatic steatosis (4; 58; 59). MK0626 seems to be

unique among DPP-4 inhibitors, as it decreased plasma uric acid in WD mice, suggesting additional

hepatic protective effects of MK0626 through enhancing fructose metabolism and shunting away

from the uric acid pathway.

In summary, the current investigation highlights newly described pleiotropic protective

effects of DDP-4 inhibition on hepatic metabolism and lipid accumulation. Our findings indicate

that DPP-4 inhibition with MK0626 ameliorated high-fat/high-fructose induced hepatic insulin

resistance, hepatic steatosis and hepatic DAG accumulation independent of changes in body weight

or adiposity. DPP-4 inhibition also increased hepatic TAG secretion, enhanced indices of

mitochondrial carbohydrate utilization with concomitant reduction of uric acid production, and

decreased incomplete hepatic mitochondrial fatty acid oxidation. These findings collectively

demonstrate a strong potential clinical utility for DPP-4 inhibition in the prevention of hepatic

insulin resistance and development of hepatic steatosis.

DISCLOSURE STATEMENT

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AUTHOR CONTRIBUTIONS

Involved in the study concept and design (ARA, JH, GL, CM, JPT, JRS, RSR); acquisition of data (ARA, DAF, GL, CM, MMD, KDF, JPT, EJP, JRS, RSR); analysis and interpretation of data (ARA, DAF, JPT, EJP, JRS, RSR); drafting of the manuscript (ARA, EJP, JRS,RSR); critical revision of the manuscript for important intellectual content (ARA, JH, DAF, RN, GL, CM, MMD, KDF, JPT, EJP, JRS,RSR); statistical analysis (ARA, EJP, RSR); obtained funding (JH, RN, DAF, JPT, JRS, RSR).

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Table 1. Animal, liver, and plasma characteristics

	CD	CD-MK	WD	WD-MK
Body weight (g)	27.5 ± 0.6	27.7 ± 0.7	39.0 ± 1.4*	37.2 ± 1.4*
Liver weight (g)	1.14 ± 0.04	1.02 ± 0.05#	1.46 ± 0.08*	1.16 ± 0.04*#
Fat pad mass (g)	0.76 ± 0.09	0.84 ± 0.11	2.80 ± 0.14*	2.52 ± 0.21*
% body fat	14.5 ± 2.2	17.7 ± 0.6	35.1 ± 0.8*	36.7 ± 1.7*
Food intake (kcal/wk)	84.0 ± 3.4	81.7 ± 4.9	89.7 ± 3.6*	95.2 ± 5.1*
Plasma ALT (U/L)	12.3 ± 1.0	14.2 ± 0.8	22.4 ± 2.8*	18.3 ± 1.9*
Plasma total cholesterol (mg/dl)	112.9 ± 7.4	111.9 ± 7.0	137.7 ± 8.1*	126.1 ± 7.8*
Plasma triglycerides (mg/dl)	54.1± 3.1	59.6 ± 4.4#	61.1 ± 5.3	67.9 ± 3.8#
Plasma NEFA (nmol/L)	0.70 ± 0.06	0.70 ± 0.08	0.83 ± 0.08	0.76 ± 0.07
Plasma uric acid (mg/dL)	0.45 ± 0.04	0.40 ± 0.03#	0.72 ± 0.08*	0.49 ± 0.04*#
Liver DPP-4 activity (RLUs)	28391 ± 1551	9729 ± 1362#	27567 ± 1931	7336 ± 679#

Values are means ±SE [n=8-12 per group except percent body fat (n=6-7 per group) and food intake (n=6-8 per group)]. CD, control diet; CD-MK, control diet with MK0626; WD, western diet; WD-MK, western diet plus MK0626. Fat pad mass is the sum of epididymal and retroperitoneal fat pads. * P<0.05, main effect of diet; # P<0.05, main effect of MK compound. RLUs, relative light units.

Figure Legends

Figure 1. DPP-4 inhibition attenuated WD-induced hepatic insulin resistance. Blood glucose levels and glucose infusion rate to maintain euglycemia (A), glucose infusion rate (GIR) during the final 40 minutes (steady-state) of a hyperinsulinemic-euglycemic clamp (B), plasma insulin during basal and steady state insulin clamp conditions (C), hepatic glucose production during the basal and insulin-stimulated condition and percent insulin suppression of hepatic glucose output during the clamp (D), and hepatic insulin signaling at the liver of Akt (Ser473 phosphorylation) from acute insulin stimulation studies (E). CD, Control diet; CD-MK, Control diet plus MK0626; WD, Western diet; WD-MK, Western diet plus MK0626. n = 5 per group for clamp data and n=6 per group for acute insulin stimulation studies; * P<0.05, main effect of diet; # P<0.05, main effect of MK compound.

Figure 2. DPP-4 inhibition reduces WD-induced hepatic steatosis and hepatic TAG accumulation. Hematoxylin and eosin staining of hepatocytes for lipid droplets (A). Hepatic lipids were extracted and analyzed by LC/MS and hepatic TAG content was determined as fatty acid content of TAG (B). Distribution of fatty acid species in TAG (C). CD, Control diet; CD-MK, Control diet plus MK0626, WD, Western diet; WD-MK, Western diet plus MK0626. n = 5 per group; * P<0.01, main effect of diet; # P<0.01, diet by drug interaction, WD-MK vs WD.
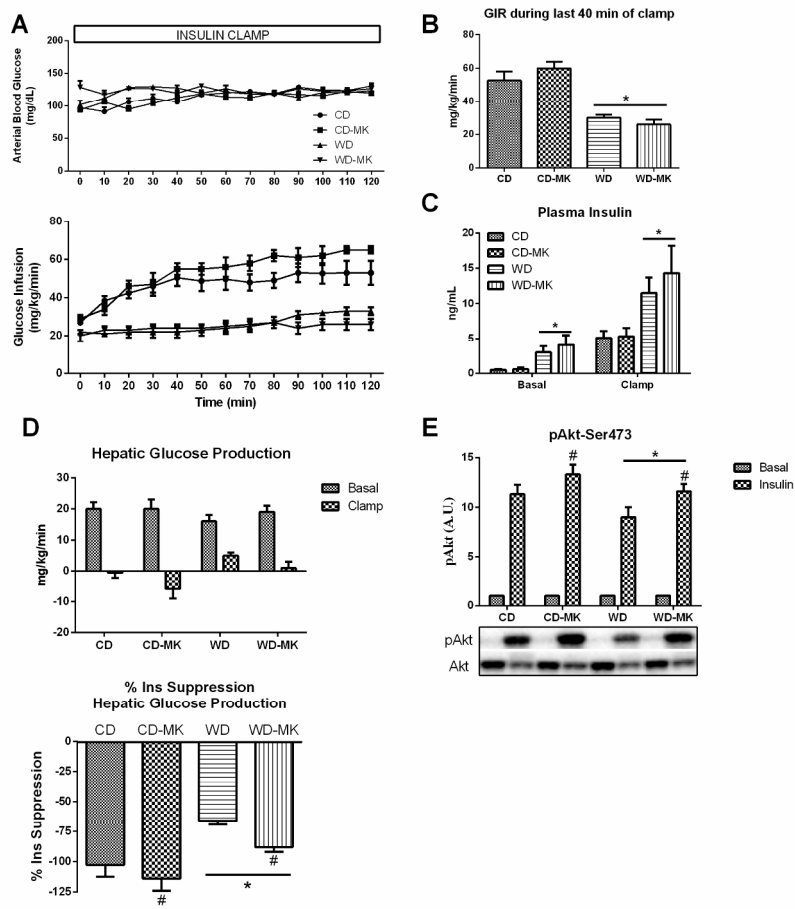
Figure 3. DPP-4 inhibition lowers WD-induced hepatic DAG accumulation. Hepatic lipids were extracted and analyzed by LC/MS. Fatty acid species and total fatty acid content in DAG (A), fatty acid species and total fatty acid content in ceramides (B). CD, Control diet; CD-MK, Control diet plus MK0626; WD, Western diet; WD-MK, Western diet plus MK0626. n = 5 per group * P<0.01, main effect of diet; # P<0.01, diet by drug interaction, WD-MK vs WD.

Figure 4. Effects of DPP-4 inhibition on hepatic de novo lipogenesis markers and hepatic TAG secretion. Hepatic mRNA expression for ACC, FAS and SREBF (A), protein content for ACC, FAS and SREBP-1c (B), hepatic TAG secretion time course (C) and rate (D), mRNA expression for MTTP and apoB (E), and protein content for MTTP and apoB100 (F). CD, Control diet; CD-MK, Control diet plus MK0626; WD, Western diet; WD-MK, Western diet plus MK0626. n = 8-10 per group for gene expression and protein content, n = 6-7 per group for TAG secretion studies. * main effect of diet, P<0.01; $^{\&}$ significantly different than CD (interaction, P<0.05); $^{\$}$ significantly different than WD (interaction, P<0.05).
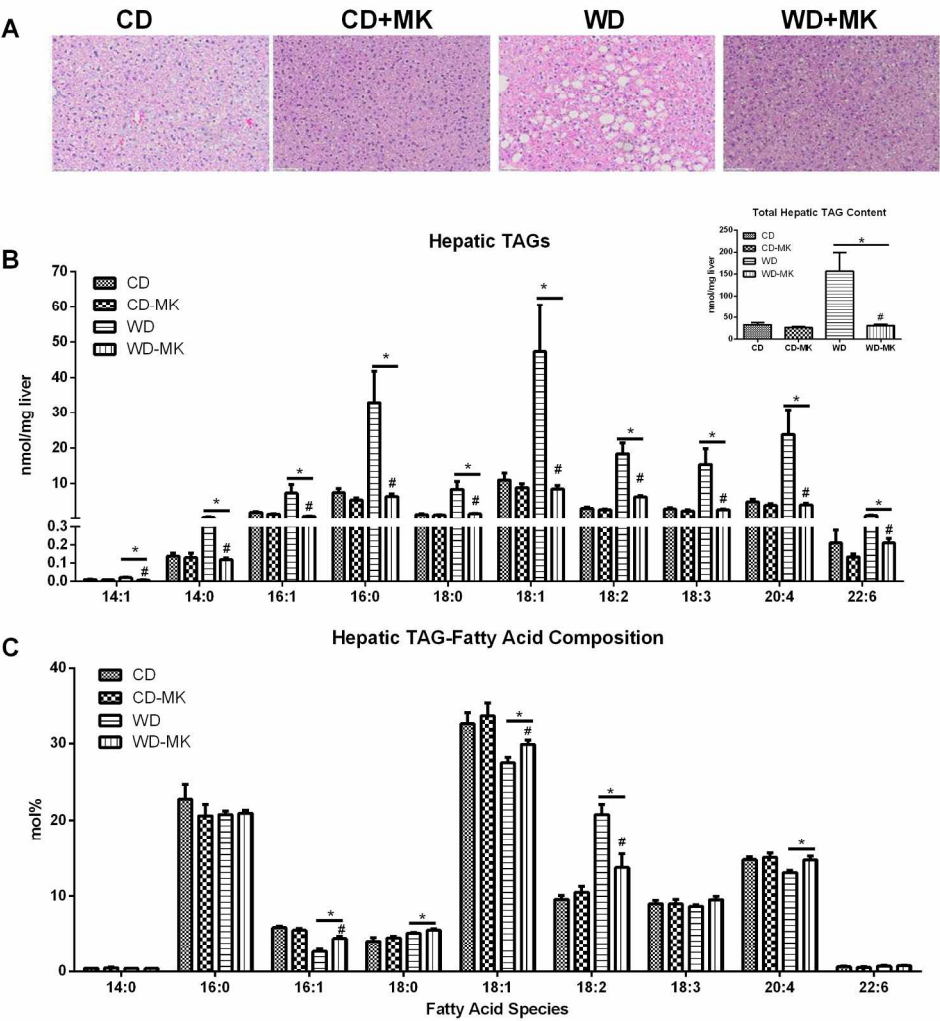
Figure 5. Effects of DPP-4 inhibition on hepatic [1-^{14}C]-pyruvate oxidation to CO_2 (A), [2-^{14}C]-pyruvate oxidation to CO_2 (B), [1-^{14}C]-palmitate oxidation to CO_2 (C), incomplete [1-^{14}C]-palmitate oxidation (D), and mitochondrial respiration (E) in isolated mitochondria. CD, Control diet; CD-MK, Control diet plus MK0626; WD, Western diet; WD-MK, Western diet plus MK0626. n = 6-10 per group *significant main effect for diet ($P<0.05$); # significant main effect for MK compound ($P<0.05$).

Figure 6. Effects of DPP-4 inhibition on hepatic mitochondrial genes PGC-1α, TFAM, CPT-1 and PPARα (A), oxidative phosphorylation (OXPHOS) complex I-V protein content (B), Sirt1 protein content (C), and Sirt3 protein content measured in isolated mitochondria (D). CD, Control diet; CD-MK, Control diet plus MK0626 WD, Western diet; WD-MK, Western diet plus MK0626. n = 6-10 per group for Western blot analyses and n=8-10 per group for gene expression. *significant main effect for diet ($P<0.05$); # significant main effect for MK compound ($P<0.05$); $^{\&}$ significantly different than CD (interaction, P<0.05); $^{\$}$ significantly different than WD (interaction, P<0.05).
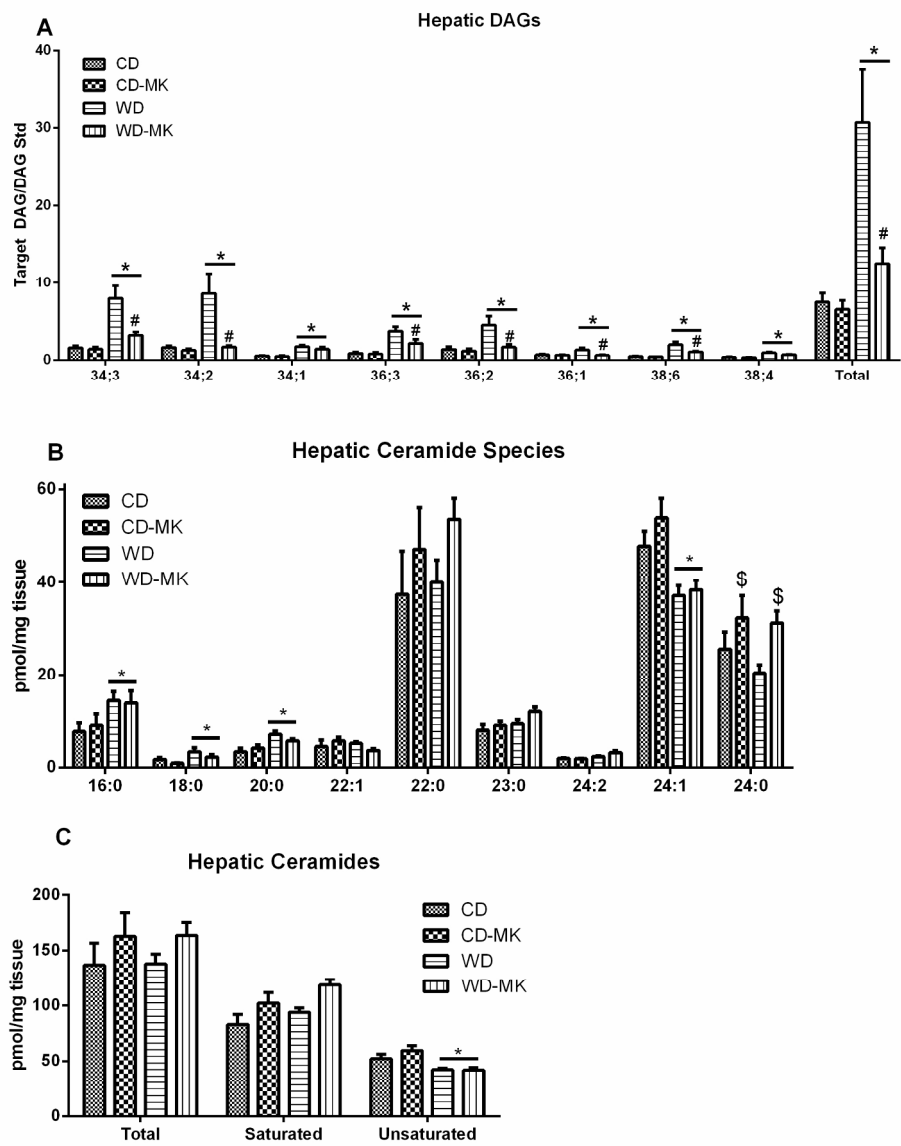
Figure 7.   Mechanistic schematic of DDP-4 inhibition with MK0626 on hepatic metabolism and hepatic insulin resistance. Improvement in hepatic insulin resistance by DPP-4 inhibitor MK0626 (1) resulted from decreased levels of DAG and TAG (2), increasing hepatic TAG secretion (3), enhancing select genes and proteins involved in mitochondrial content and function (4), suppressing incomplete oxidation of fatty acids (5), enhancing utilization of metabolites through enhanced PDH activity (6) and TCA cycle flux (6), presumably decreasing carbon intermediates available of lipogenesis, and lowering uric acid levels (7) and thereby suppressing the lipogenic effects of uric acid.
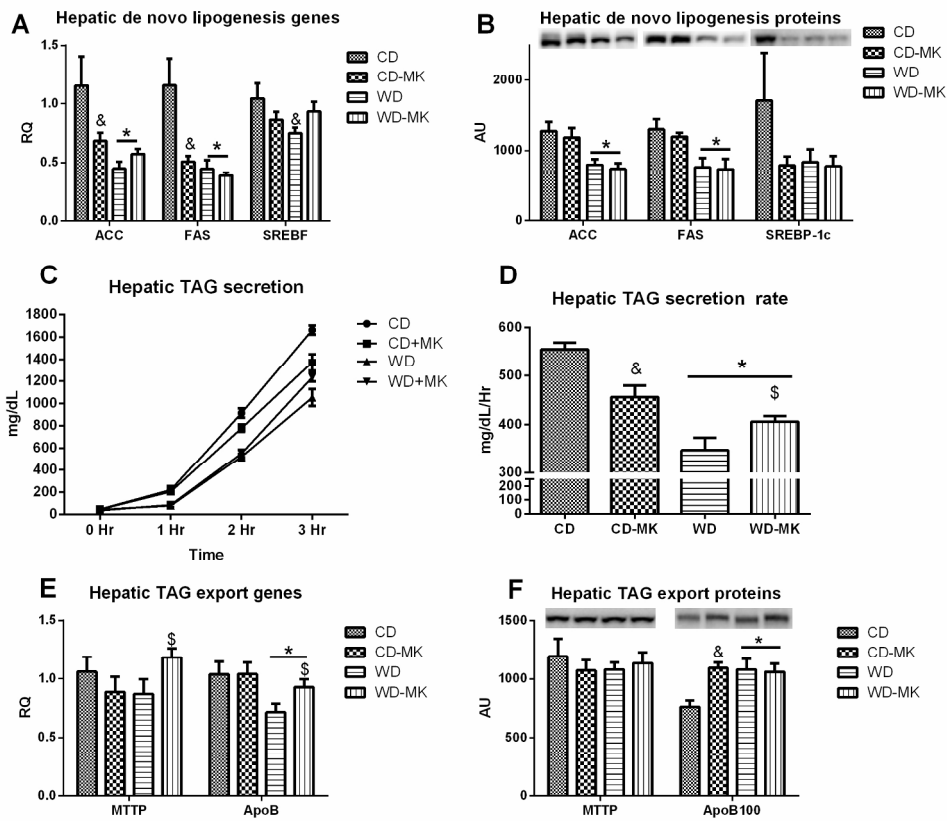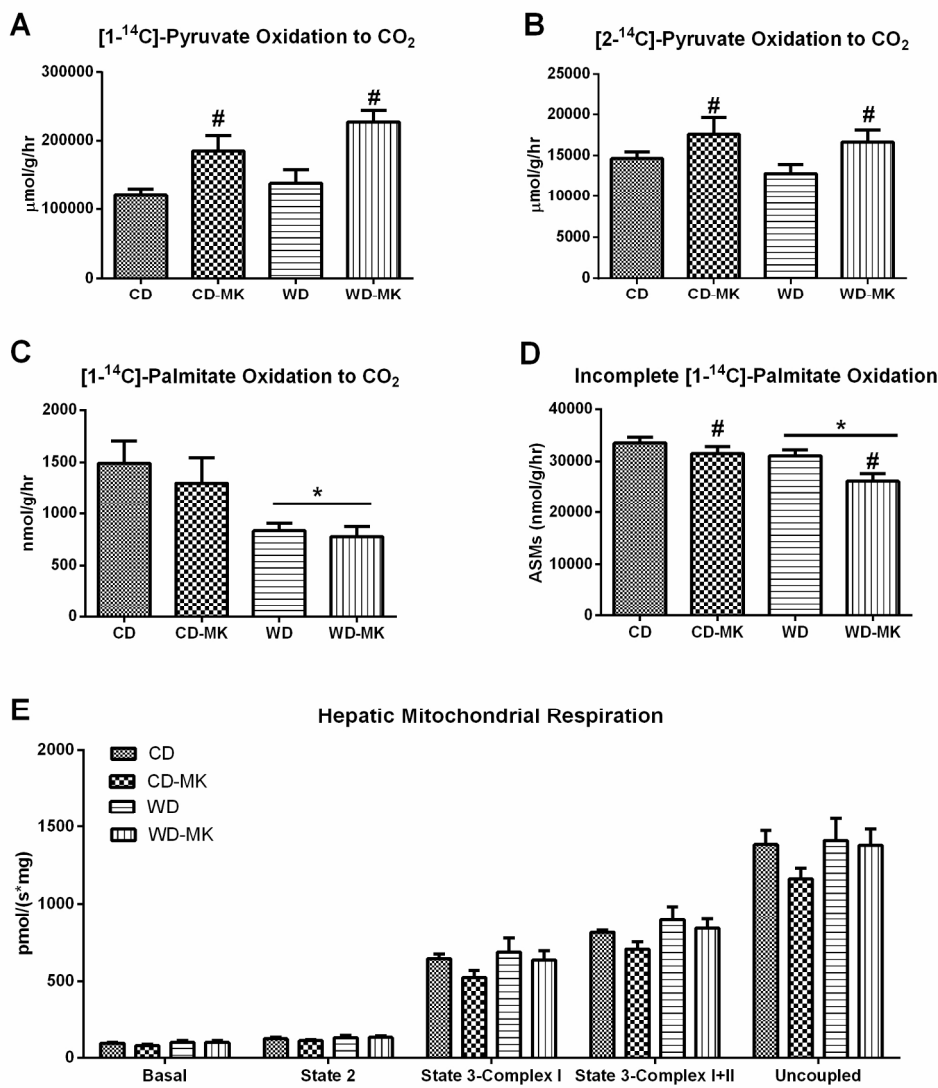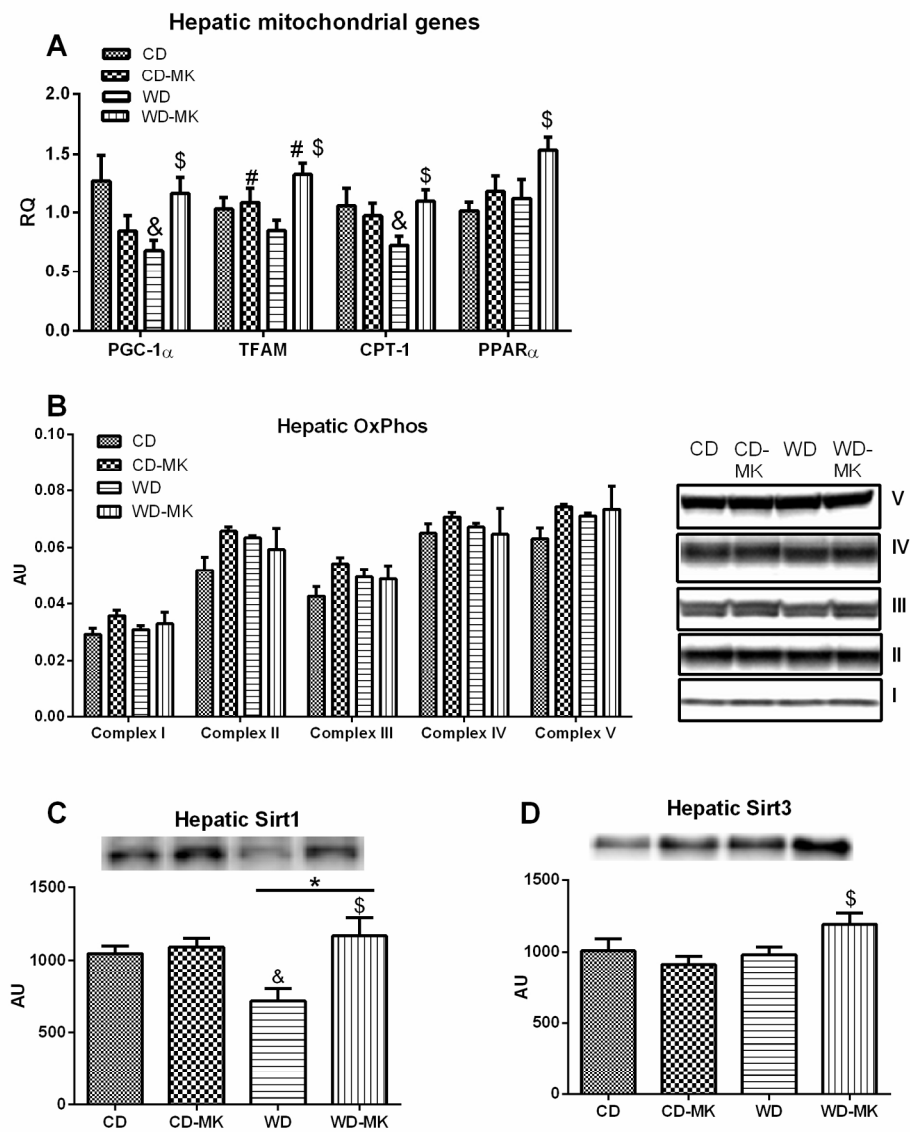
183x263mm (300 x 300 DPI)

193x206mm (300 x 300 DPI)

A **Hepatic DAGs**

B **Hepatic Ceramide Species**

C **Hepatic Ceramides**

207x251mm (300 x 300 DPI)

211x184mm (300 x 300 DPI)

A $[1-{}^{14}C]$-Pyruvate Oxidation to CO_2

B $[2-{}^{14}C]$-Pyruvate Oxidation to CO_2

C $[1-{}^{14}C]$-Palmitate Oxidation to CO_2

D Incomplete $[1-{}^{14}C]$-Palmitate Oxidation

E Hepatic Mitochondrial Respiration

183x208mm (300 x 300 DPI)

A Hepatic mitochondrial genes

Legend:
- CD
- CD-MK
- WD
- WD-MK

B Hepatic OxPhos

C Hepatic Sirt1

D Hepatic Sirt3

164x199mm (300 x 300 DPI)