

***Momordica charantia* (bitter melon) reduces plasma apolipoprotein B-100 and increases hepatic insulin receptor substrate and phosphoinositide-3 kinase interactions**

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Aqueous extracts or juice from unripened fruit of *Momordica charantia* (bitter melon) has traditionally been used in the treatment of diabetes and its complications. Insulin resistance is characterized by significant down-regulation of hepatic insulin signalling as documented by attenuated phosphorylation of insulin receptor (IR), IR substrates 1 and 2, phosphoinositide-3 kinase, protein kinase B, and over-expression of phosphotyrosine phosphatase 1B. We recently demonstrated that bitter melon juice (BMJ) is a potent inhibitor of apoB secretion and TAG synthesis and secretion in human hepatoma cells, HepG2, that may be involved in plasma lipid- and VLDL-lowering effects observed in animal studies. The aim of this study was to evaluate the effects of BMJ on plasma apoB levels and hepatic insulin signalling cascade in mice fed high-fat diet (HFD). Female C57BL/6 mice (4–6 weeks old) were randomized into three groups receiving regular rodent chow, HFD and HFD + BMJ. The data indicate that BMJ not only improves glucose and insulin tolerance but also lowers plasma apoB-100 and apoB-48 in HFD-fed mice as well as modulates the phosphorylation status of IR and its downstream signalling molecules. Investigating the biochemical and molecular mechanisms involved in amelioration of diabetic dyslipidaemia by BMJ may lead to identification of new molecular targets for dietary/alternative therapies.

Bitter melon: ApoB: Insulin receptor: Phosphorylation: Insulin receptor substrate: Phosphoinositide-3 kinase: Diabetes: Hyperlipidaemia

For the past two decades, obesity and associated metabolic disorders such as type 2 diabetes and CVD have been escalating worldwide. Major risk factors for CVD include insulin resistance, hypertension and diabetic dyslipidaemia. Hypertriglyceridaemia and hepatic overproduction of apoB and VLDL are among the most prevalent complications of insulin resistance and are associated with high risk of developing CVD. Currently, diabetes and dyslipidaemia treatments require a combination of drugs. There is no single drug treatment available that will treat both these disorders. Furthermore, available treatments are prone to various side-effects such as secondary weight gain, drug–drug interactions and secondary failure. It is therefore critical to identify new therapies that may influence lipid and glucose metabolism, have minimal side-effects and similar or more potent efficacies than conventional therapies.

Momordica charantia, also known as bitter melon (BM), is traditionally used in the treatment of diabetes and its complications⁽¹⁾. Animal studies also indicate additional effects of BM in regulating weight gain and lipid metabolism^(2–7).

Aqueous extracts of BM fruit and seeds have been demonstrated to reduce VLDL levels in normal control rats, and normalize elevated VLDL levels in diabetic rats⁽⁸⁾. The most fundamental molecular defect in insulin-resistant states is the resistance to cellular actions of insulin, particularly insulin-stimulated glucose uptake, which leads to hyperinsulinaemia, enhanced VLDL secretion and hypertriglycerolaemia. Assembly and secretion of VLDL is a complex process involving the interaction of apoB with both core and surface lipids to form a lipoprotein particle. Regulation of VLDL secretion by insulin is dependent on apoB synthesis and degradation, microsomal TAG transfer protein activity, apoB phosphorylation and/or apoB mRNA editing^(9,10). Insulin-mediated inhibition of apoB synthesis and secretion is further dependent on phosphoinositide-3 kinase (PI3K)-associated activation of protein kinase B (PKB/Akt)⁽¹¹⁾, while degradation of apoB in the endoplasmic reticulum/golgi is dependent only on PI3K phosphorylation⁽¹²⁾. Insulin resistance and dyslipidaemia are characterized by significant down-regulation of hepatic insulin signalling as documented by attenuated phosphorylation of IR, IR substrates

Abbreviations: Akt, protein kinase B; BM, bitter melon; BMJ, BM juice; HFD, high-fat diet; IR, insulin receptor; IRS-1, IR substrate 1; IRS-2, IR substrate 2; PI3K, phosphoinositide-3 kinase; PKB, protein kinase B; pTyr, tyrosine phosphorylation status.

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1 and 2 (IRS-1 and IRS-2), PI3K and/or PKB/Akt⁽¹³⁾. In fructose-fed, insulin-resistant hamster model, Taghibiglou *et al.*⁽¹⁴⁾ demonstrated a direct link between attenuated hepatic insulin signalling and synthesis and secretion of VLDL apoB.

Our recent *in vitro* studies demonstrated that BM juice (BMJ) significantly reduces apoB secretion under basal and lipid-rich conditions, in human hepatoma cells, HepG2⁽¹⁵⁾. Therefore, the aim of the present study was to evaluate the *in vivo* effects of BMJ on plasma apoB levels and the hepatic insulin signalling cascade in mice fed a high-fat diet (HFD). Our previous data indicate that BMJ lowers plasma apoB-100 and apoB-48 in HFD-fed mice and modulates phosphorylation status of IR and its downstream signalling molecules.

Methods

Preparation of bitter melon juice

The Chinese variety of young BM (raw and green) was obtained from a local farmers' market, washed and deseeded. BMJ was extracted as published previously^(7,15). In brief, BMJ was extracted using a household juicer and centrifuged at 4500rpm at 4°C for 30 min. Supernatant BMJ was freeze-dried at -45°C for 72 h and stored at -80°C until used for feeding studies. Physical properties and mineral contents of Chinese BM were analysed at the Agriculture Diagnostic Service Center at the University of Hawaii at Manoa, and total carotenoids were analysed at the Analytical Laboratory of the Cancer Research Center of Hawaii (Table 1). Values represent means and standard deviations of five independent analyses of BM samples obtained from a local farmers' market throughout the year. The amounts of crude protein found in the extracts are similar to those reported previously⁽¹⁶⁾.

Table 1. Physical properties and mineral contents of Chinese bitter melon juice (BMJ)

(Mean values and standard deviations)

Analysis	BMJ (n 5)		Lyophilized BMJ (n 5)	
	Mean	SD	Mean	SD
DM (%)	3.0	0.08	75.0	0.5
Ash (%)	14.8	1.2	14.8	0.03
Crude protein (%)	24.3	0.8	24.7	0.4
Crude fat (%)	1.9	0.04	2.1	0.01
Minerals				
Phosphorus (%)	1.05	0.01	0.91	0.05
Potassium (%)	7.9	0.2	6.92	0.06
Calcium (%)	0.2	0.02	0.14	0.0
Magnesium (%)	0.06	0.002	0.52	0.003
Sodium (%)	0.8	0.05	0.3	0.002
Boron (ppm)	13.0	0.8	13.0	0.6
Copper (ppm)	21.0	0.02	19.0	1.2
Iron (ppm)	148.0	6.3	96.0	5.7
Manganese (ppm)	32.0	1.1	32.0	1.2
Molybdenum (ppm)	2.0	0.03	1.0	0.0
Zinc (ppm)	65.0	1.3	43.0	1.4
Total carotenoids (mg/100 g)	ND	ND	48.0	2.5

ND, not detected.

Animals and treatment

Female C57BL/6 mice (4–6 weeks old) were purchased from Jackson Laboratories (Ann Harbor, MI, USA) and randomized in three groups of seven to eight animals each: (1) control (n 7), (2) HFD (n 7) and (3) HFD + 1.5% freeze-dried BMJ (w/w, n 8). Control rodent chow contained 4.8 kJ% fat (17.03 kJ/g (4.07 kcal/g); #D12329; Research Diets, New Brunswick, NJ, USA), whereas HFD chow contained 58 kJ% fat (23.4 kJ/g (5.6 kcal/g); #D12331; Research Diets). Nutrient contents of the two diets are depicted in Table 2. Lyophilized BMJ powder was manually mixed with the diets daily to obtain a homogenous mixture and fed to the animals. These studies were approved by the Institutional Board of the University of Hawaii Institutional Animal Care and Use Committee. All procedures were conducted in accordance with guidelines established by the National Institutes of Health. Animals were housed individually and allowed to eat and drink *ad libitum* for 16 weeks. Food and water intake was measured daily, while body weights were measured on consecutive days. At the beginning of the study, tail vein blood was used to determine glucose levels with the OneTouch Ultra™ glucometer (Lifescan, USA). Two weeks before the end of the study, glucose and insulin tolerance tests were performed after an overnight 12 h fast according to published protocols⁽¹⁷⁾. In brief, tolerance tests were performed by an intraperitoneal injection of either glucose (1.5 mg/g body weight) or insulin (0.5 U/kg body weight). Tail blood was used to determine glucose levels at 0, 30, 60 and 120 min with the OneTouch Ultra™ glucometer. At the end of the study, after an overnight fast, mice in each group were anaesthetized by isoflurane, followed by blood draw by cardiac puncture for biochemical assays. For plasma preparation, whole blood was collected in heparinized tubes. Plasma TAG and cholesterol levels were measured using the Infinity TG Liquid Stable Reagent and Infinity Cholesterol Liquid Stable Reagent commercial kits (Thermo-DMA, St Louisville, CO, USA), according to the manufacturer's instructions, and absorbance was read at 540 nm using a Perkin-Elmer multiplate reader (Wallac Victor2; Perkin-Elmer Life Sciences). Plasma NEFA were determined using a commercial kit according to the manufacturer's protocol (Wako Chemicals Inc., Richmond, VA, USA). Liver function tests were analysed by measuring serum alanine amino transferase, aspartate aminotransferase (Biotron Diagnostics USA Inc., Hemet, CA, USA) and lactate dehydrogenase (Bioassays Systems, Hayward, CA, USA). All tissues were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Analysis of hepatic TAG

Hepatic TAG mass was analysed by the method of Scribner *et al.*⁽¹⁸⁾, with slight modifications. In brief, a 5% liver homogenate was prepared in sucrose buffer containing 0.3 mM-sucrose, 25 mM-2-mercaptoethanol and 10 mM-EDTA, pH 7.0, and mixed with hexane-isopropanol (3:2, v/v). The organic phase was dried, resuspended in 100 µl ethanol and TAG mass was enzymatically determined as mentioned previously⁽¹⁵⁾. TAG mass was normalized to liver weights (mg TAG/g tissue).

Table 2. Nutrient composition of control and high-fat diets*

Contents	Control diet (#D12329)					High-fat diet (#D12331)				
	g%	kJ%	g	kJ	kcal	g%	kJ%	g	kJ	kcal
Protein	16.8	16.4				23.0	16.4			
Carbohydrate	74.3	73.1				35.5	25.5			
Fat	4.8	10.5				35.8	58.0			
Total		100.0					100.0			
kJ/g	17.03					23.26				
kcal/g	4.07					5.56				
Ingredients										
Casein, 80 Mesh			228	3816	912			228	3816	912
DL-Methionine			2	0	0			2	0	0
Maltodextrin 10			170	2845	680			170	2845	680
Maize starch			0	0	0			0	0	0
Sucrose			835	13975	3340			175	2929	700
Soyabean oil			25	941	225			25	941	225
Coconut oil, hydrogenated			40	1506	360			333.5	12558.2	3001.5
Mineral mix S10001			40	0	0			40	0	0
Sodium bicarbonate			10.5	0	0			10.5	0	0
Potassium citrate, 1 H ₂ O			4	0	0			4	0	0
Vitamin mix V10001			10	167	40			10	167	40
Choline bitartrate			2	0	0			2	0	0
FD&C Blue Dye no. 1			0.1	0	0			0	0	0
FD&C Blue Dye no. 40			0	0	0			0.1	0	0
Total			1366.6	23250.0	5557.0			1000.1	23256.2	5558.5

*Research Diets (New Brunswick, NJ, USA).

Analysis of plasma apolipoproteins

Plasma apoB-100 and apoB-48 levels were determined using Western blotting techniques⁽¹⁹⁾. In brief, 15 µg plasma proteins were separated on 7.5% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to nitrocellulose membrane at 80 V for 90 min, washed and blocked in 1% BSA. Membranes were incubated overnight with goat polyclonal anti-mouse apoB (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-mouse apoB 48/100 (BioDesign International, Saco, ME, USA) primary antibodies and with corresponding horseradish peroxidase-conjugated secondary antibodies for 2 h. apoB-100 and apoB-48 were detected using a commercially available electrochemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Protein bands were scanned using the Molecular Imager Gel Doc XR System (Bio-Rad) and intensities were analysed using the Discovery Series™ Quantity One 1-D Analysis Software (Bio-Rad).

Tyrosine phosphorylation status of insulin signalling proteins

Tyrosine phosphorylation status (pTyr) of IRβ, IRS-1 and IRS-2, and serine/threonine phosphorylation of Akt were analysed according to published protocols⁽⁹⁾. Liver homogenate (10%) was prepared on ice in a specialized buffer (150 mM-NaCl, 10 mM-tris(hydroxymethyl)aminomethane, pH 7.4, 1 mM-EDTA, 1 mM-ethyleneglycol-bis-(aminoethylether) N, N,N',N'-tetraacetic acid, 1% Triton X-100, 1% Nonidet P 40, 2 mM-phenylmethylsulphonyl fluoride, 100 mM-sodium fluoride, 10 mM-sodium pyrophosphate and 2 mM-sodium orthovanadate and protease inhibitors (Complete™; Boehringer Mannheim Corp., Indianapolis, IN, USA)). Protein concentrations were determined using Bradford protein assay reagent according to the manufacturer's instructions

(Bio-Rad Laboratories) and 0.5 mg proteins was subjected to overnight immunoprecipitation with specific polyclonal antibodies against either IRβ-subunit, IRS-1, IRS-2 or Akt (1 µg antibody/0.5 mg total cell lysate) and 10% protein A-Sepharose. Immunoprecipitates were then washed with ice-cold PBS containing 100 mM-sodium fluoride, 10 mM-sodium pyrophosphate, 2 mM-sodium orthovanadate, 0.1% Nonidet P 40 and 0.1% Triton X-100.

Immunoprecipitates were separated on 7.5% polyacrylamide gels (Bio-Rad) and were electrophoretically transferred on to nitrocellulose membranes. The membranes were blocked with 5% milk, incubated with either monoclonal antibody αpY (1:1000 dilution) or phosphor-serine/threonine antibody (Santa Cruz Biotechnology), washed and probed with a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Proteins were visualized with an enhanced electrochemiluminescence detection kit and band intensities were analysed as described earlier. The intensity of IR-β, IRS-1 and IRS-2 bands was initially normalized against β-actin and the normalized values of the protein of interest were used to adjust the ratio of the experimental phosphorylated bands.

Analysis of PPARα proteins

Nuclear fractions were isolated from frozen livers using the NXTRACT, CellLytic NuCLEAR Extraction Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Nuclear proteins (30 µg) were loaded on a 10% polyacrylamide gel, transferred to nitrocellulose membrane, blocked in 10% milk for 2 h, probed with anti-human PPARα primary antibody overnight at room temperature (Affinity Bioreagent, Golden, CO, USA), and followed with anti-mouse IgG2b conjugated to horseradish peroxidase, for 2 h (Santa Cruz

Biotechnology). Proteins were visualized by an electrochemiluminescence kit and band intensities were analysed as described earlier.

Statistical analysis

Statistical analysis was conducted using SAS software (SAS Institute, Cary, NC, USA). A one-way ANOVA model was used to compare means between the three animal groups (control, HFD and HFD + BMJ). For repeated assays of plasma concentrations of various blood analytes, a repeated measure ANOVA was used to compare means adjusted for the time interval at which samples were obtained. For each sample, duplicate determinations were performed. Appropriate transformation of the data was performed to normalize distributions and stabilize variances. *Post hoc* pair-wise multiple comparisons were evaluated using the Ryan–Einot–Gabriel–Welsch multiple range test. All *P* values were based on two-sample tests. Results were considered significant at $P < 0.05$.

Results

Effects of bitter melon juice on body weight and plasma chemistry

BMJ is not a part of the traditional rodent chow and has an extremely bitter taste. It is well-known that rodents are sometimes very sensitive to inclusion of 'unusual' taste in their diet that can reduce food intake. As observed in Table 3, supplementation with BMJ had no significant effect on the overall daily food intake, measured as kJ/d. HFD-fed mice started gaining weight after 4 weeks and as expected gained 110% more weight than control mice receiving regular chow ($P < 0.05$). Although supplementation with BMJ resulted in 21% lower weight gain than HFD-fed mice, the body weights were still significantly higher than the control group (Table 3, $P < 0.05$). As previously reported, HFD significantly increased plasma glucose,

TAG, total cholesterol and hepatic TAG mass, while BMJ normalized the plasma glucose and lipids (Table 3)⁽²⁰⁾. Similarly, BMJ normalized liver weight and lowered hepatic TAG levels by 30% in mice-fed HFD (Table 3, $P < 0.05$). BMJ was observed to significantly lower increased serum aspartate aminotransaminase, alanine amino transferase and lactate dehydrogenase in HFD-fed mice (Table 3, $P < 0.05$) and normalize the glucose (Fig. 1(A) and insulin tolerance tests (Fig. 1(B)).

Effects of bitter melon juice on plasma apoB

To confirm our *in vitro* observations⁽¹⁵⁾, plasma apoB-100 and apoB-48 were analysed by Western blotting in mice fed control rodent chow (control), HFD and HFD + BMJ. As observed in Fig. 2, HFD significantly increased plasma apoB-100 levels by 103% and apoB-48 by 52% ($P < 0.05$), while BMJ normalized the plasma levels of both apolipoproteins.

Effects of bitter melon juice on tyrosine phosphorylation of insulin receptor β and insulin receptor substrate 1 and 2 proteins

Neither HFD nor BMJ treatment had any significant effects on the abundance of hepatic IR β (Fig. 3). Feeding of 58% fat diet for 16 weeks significantly reduced p-Tyr of IR β by 42% as compared to control ($P < 0.05$), while BMJ significantly increased the p-Tyr levels of IR β by 56% above that of control and 270% above that of HFD-fed mice (Fig. 3, $P < 0.05$). Similarly, HFD significantly reduced p-Tyr IRS-1 by 45% as compared to control, while BMJ significantly increased it by 84% above control and 334% above HFD-fed mice (Fig. 4(A), $P < 0.05$). In contrast, HFD had no effect on IRS-2 phosphorylation, while BMJ-treated mice demonstrated a significant increase by 55% above that of control (Fig. 5(A), $P < 0.05$). Both, IRS-1 and IRS-2 mass were unaffected by HFD or HFD + BMJ (Figs. 4 and 5).

Table 3. Metabolic parameters in C57BL/6 female mice fed three different diets* (Mean values and standard deviations)

Variables	Control (n 7)		HFD (n 7)		HFD + BMJ (n 8)	
	Mean	SD	Mean	SD	Mean	SD
Food intake (kJ/d)	72.0 ^a	3.8	70.7 ^a	5.0	66.1 ^a	6.3
Food intake (kcal/d)	17.2 ^a	0.9	16.9 ^a	1.2	15.8 ^a	1.5
Final body weight (g)	19.9 ^a	0.9	41.8 ^b	1.9	33.3 ^c	1.3
Plasma glucose (mg/l)	1121 ^a	76	1666 ^b	171	1221 ^a	157
Plasma TAG (mg/l)	390 ^a	116	1711 ^b	411	585 ^a	140
Plasma cholesterol (mg/l)	851 ^a	89	1774 ^b	261	1180 ^a	182
Plasma NEFA (mEq/l)	0.4 ^a	0.02	0.7 ^b	0.04	0.6 ^a	0.03
Liver weight (g)	1.0	0.1	1.2 ^b	0.08	1.1 ^b	0.19
Liver TAG (mg/g tissue)	110.5 ^a	2.4	180.5 ^b	3.7	123.1 ^a	3.2
Serum AST (U/l)	8.2 ^a	0.25	75.3 ^b	5.7	25.4 ^a	5.9
Serum ALT (U/l)	10.3 ^a	1.4	48.2 ^b	9.6	18.2 ^a	3.3
Serum LDH (U/l)	95.1 ^a	15.2	273.2 ^b	45.8	120.7 ^a	17.0

ALT, alanine amino transferase; AST, aspartate aminotransaminase; BMJ, bitter melon juice; HFD, high-fat diet; LDH, lactate dehydrogenase.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of procedures and diets, see Methods and Tables 1 and 2.

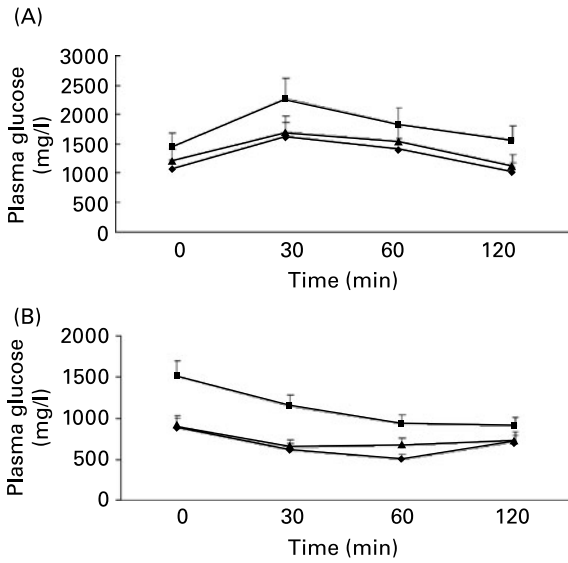


Fig. 1. Effects of bitter melon juice (BMJ) on plasma glucose tolerance (A) and insulin tolerance (B) in mice fed control rodent chow (◆), high-fat diet (HFD; ■) and HFD + BMJ (▲). Values are means with standard deviations depicted by vertical bars (n 6). Mean values were significantly different from those of the control and HFD + BMJ group: * P <0.05.

Effects of bitter melon juice on interactions between insulin receptor substrate and phosphoinositide-3 kinase

To evaluate the interactions between IRS-1/IRS-2 and PI3K, the IRS immunoprecipitates were separated on 7.5% SDS gels and the transferred proteins were probed with antibody against p85 and p110 subunits of PI3K. HFD significantly reduced the interactions between IRS-1 and p110 subunits of PI3K by 56%, while BMJ increased it by 280% above HFD and 23% above control animals (Fig. 4 (B), P <0.05). In contrast, HFD did not affect the IRS-2 and p110 interaction, while BMJ increased it by 60% above both groups (Fig. 5 (B),

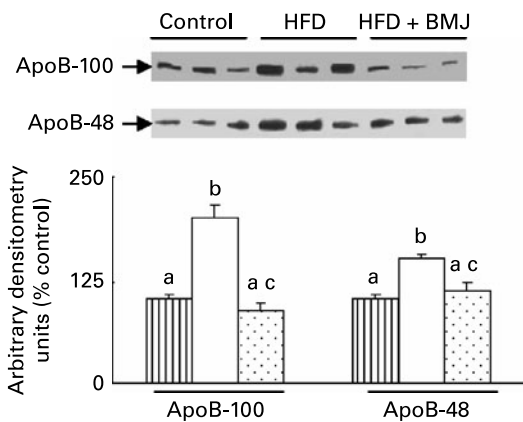


Fig. 2. Plasma apoB-100 and apoB-48 protein levels in mice fed high-fat diet (HFD, □) and HFD + bitter melon juice (BMJ, ▨; control, ▨). The graph represents the densitometry scans of 220 kDa (apoB-48) and 514 kDa (apoB-100) band intensities. Data are expressed as a percentage of control values, set at 100%. Values are means with standard deviations depicted by vertical bars (n 6). ^{a,b,c}Mean values with unlike letters were significantly different (P <0.05).

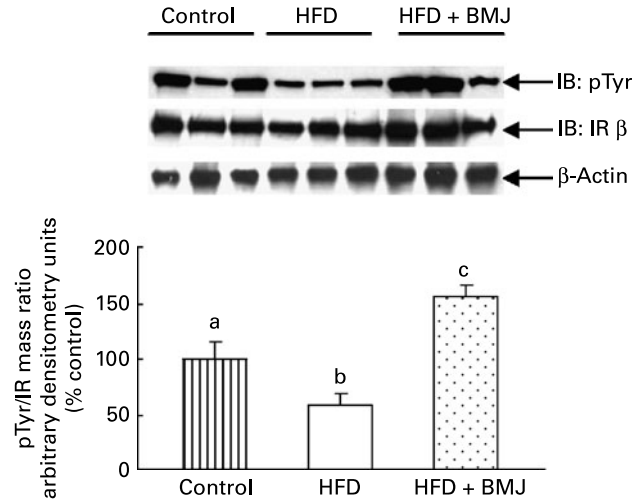


Fig. 3. Tyrosine phosphorylation (pTyr) of hepatic insulin receptor β (IR β) in mice fed high-fat diet (HFD) and HFD + bitter melon juice (BMJ). The graph represents the densitometry scans of 95 kDa band intensities. The β-actin band demonstrates equal amounts of protein loaded on the gel. Data are expressed as a percentage of control values, set at 100%. Values are means with standard deviations depicted by vertical bars (n 6). ^{a,b,c}Mean values with unlike letters were significantly different (P <0.05). IB, immunoblotting.

P <0.05). Neither HFD nor BMJ demonstrated any significant change in interactions between IRS-1/2 and p85 proteins as compared to controls (Figs. 4 (C) and 5 (C).

Effects of bitter melon juice on protein kinase B expression and serine 473/threonine 308 phosphorylation

Both, HFD and BMJ had no effect on either the Akt1 mass or serine 473/threonine 308 phosphorylation as compared to mice fed a control diet (data not shown). Similarly, HFD and BMJ did not affect Akt2 expression and its phosphorylation (data not shown).

Effects of bitter melon juice on hepatic PPARα protein expression

Hepatic PPARα proteins were analysed by Western blotting to test the hypothesis that BMJ normalizes the increased NEFA flux to the liver due to increased PPARα expression in HFD-fed mice. As observed in Fig. 6, HFD significantly increased PPARα protein expression by 156% above that of control, while feeding of BMJ significantly reduced it to control levels (P <0.05).

Discussion

Increased VLDL production and apoB secretion in insulin-resistant states confer a high risk of CVD and is likely a result of reduced sensitivity to insulin⁽²¹⁾. Since BM normalizes whole-body insulin sensitivity in animal studies^(7,22) and reduces apoB secretion in HepG2 cells⁽²³⁾, we tested the hypothesis that BMJ-associated apoB regulation *in vivo* was associated with modulation of the hepatic insulin signalling pathway. We present novel data indicating that BMJ can normalize plasma apoB-100 and apoB-48 in mice fed

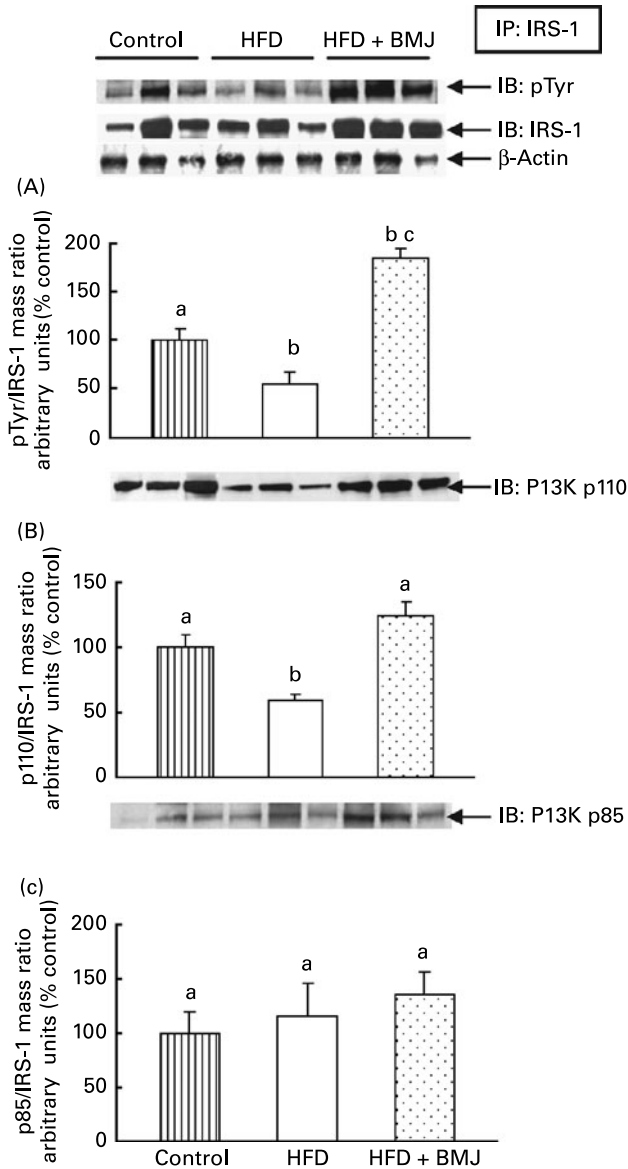


Fig. 4. Tyrosine phosphorylation (pTyr) of insulin receptor substrate 1 (IRS-1) and its interactions with p85 and p110 subunits of phosphoinositide-3 kinase (PI3K), in mice fed high-fat diet (HFD) and HFD + bitter melon juice (BMJ). The graph represents the densitometry scans of 180 kDa (pTyr, IRS-1; A), 110 kDa (p110/PI3K; B) and 85 kDa (p85/PI3K; C) band intensities. The β -actin band demonstrates the amount of protein loaded on the gel. Data are expressed as a percentage of control values, set at 100%. Values are means with standard deviations depicted by vertical bars (n 6). ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). IP, immunoprecipitation; IB, immunoblotting.

a HFD for 16 weeks, possibly through modulation of the insulin signalling pathway.

Insulin is known to suppress apoB secretion *via* increasing PI3K activity and its association with IRS proteins. PI3K consists of a regulatory subunit, p85, and catalytic domain, p110, and plays a critical role not only in glucose, but also in apoB metabolism⁽²⁴⁾. In HFD-fed mice, hepatic insulin resistance was evident by reduced phosphorylation of hepatic IR β and IRS-1, as well as the decreased association between IRS-1 and the catalytic domain of PI3K, p110, and elevated plasma glucose. BMJ significantly reduced plasma apoB-100

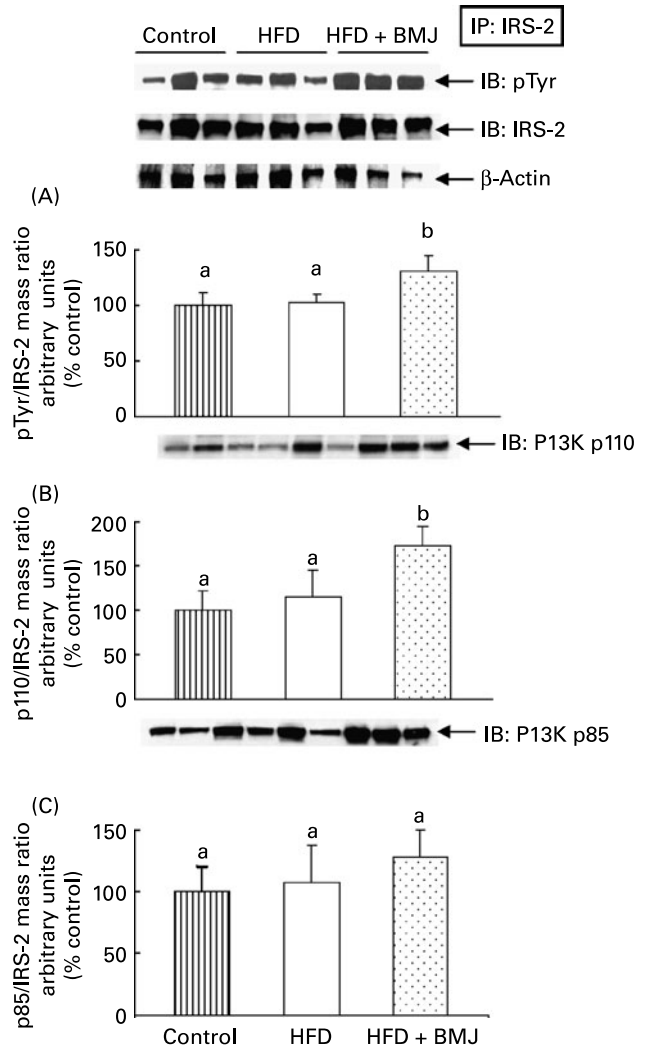


Fig. 5. Tyrosine phosphorylation (pTyr) of insulin receptor substrate 2 (IRS-2) and its interactions with p85 and p110 subunits of phosphoinositide-3 kinase (PI3K), in mice fed high-fat diet (HFD) and HFD + bitter melon juice (BMJ). The graph represents the densitometry scans of 180 kDa (pTyr, IRS-2; A), 110 kDa (p110/PI3K; B) and 85 kDa (p85/PI3K; C) band intensities. The β -actin band demonstrates the amount of protein loaded on the gel. Data are expressed as a percentage of control values, set at 100%. Values are means with standard deviations depicted by vertical bars (n 6). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). IP, immunoprecipitation; IB, immunoblotting.

and apoB-48 in HFD-fed mice treated with BMJ. In our previous *in vitro* study, BMJ-induced reduction in apoB secretion may have been caused by a lack of lipid bioavailability and/or impaired lipidation process during lipoprotein assembly⁽¹⁵⁾. In the current study, feeding of BMJ increased tyrosine phosphorylation of IR β and activated the downstream signalling cascade as evident by increased tyrosine phosphorylation of IRS-1 and IRS-2 and their increased association with the catalytic subunit of PI3K, p110, due to its insulin-mimetic activity⁽¹⁾. Under normal physiological conditions, p85 exists in stoichiometric excess of p110 subunit. However, mice lacking various isoforms of the p85 α or p85 γ subunit of PI3K paradoxically demonstrate increased insulin sensitivity, due to improved PI3K signalling downstream of IRS proteins⁽²⁵⁾. Studies suggest that the free p85 monomers bind to

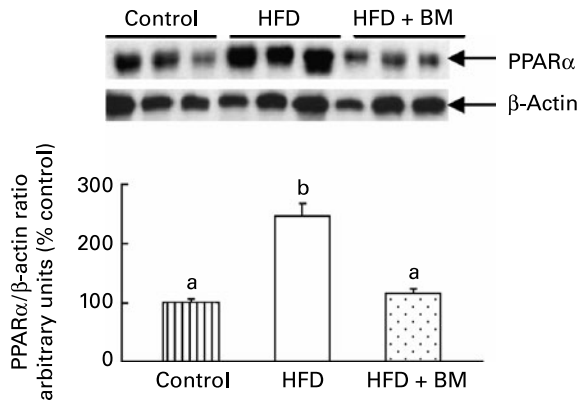


Fig. 6. Hepatic PPAR α protein levels in mice fed high-fat diet (HFD) and HFD + bitter melon juice (BMJ). The graph represents the densitometry scans of 52 kDa band intensities. The β -actin band demonstrates the amount of protein loaded on the gel. Data are expressed as a percentage of control values, set at 100%. Values are means with standard deviations depicted by vertical bars (n 6). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

phosphorylated IRS proteins thereby blocking access to p85–p110 heterodimers. Thus a delicate balance between p85 monomers and p85–p110 heterodimers dictates PI3K activity⁽²⁶⁾. The present results indicate that BMJ significantly increased the amount of p110 associated with IRS-1 and IRS-2, but had no effect on p85 binding, suggesting activation of PI3K activity. BMJ had no effect on Akt tyrosine phosphorylation. However, we did not explore the phosphorylation of Akt at serine 473 and threonine 308 residues. BMJ-associated increased interactions between IRS and PI3K subunits could involve apoB degradation or reduction in the synthesis and secretion of apoB, since BMJ reduces plasma apoB in HFD-fed mice as well as its secretion in HepG2 cells⁽¹⁵⁾. Future studies will investigate the role of BMJ-associated activation of AMP-activated protein kinase and lipoprotein secretion.

Besides apoB-100, BMJ also normalized plasma apoB-48 in HFD-fed mice. Intestinal over-production of apoB48-containing lipoproteins occurs in response to HFD leading to post-prandial lipidaemia. Although, unlike man and hamsters, mice produce apoB-48-containing particles from both the liver and intestine, one cannot rule out the possibility that reduction of plasma apoB-48 observed in the present study may involve intestinal regulation of apoB-48 by BMJ.

HFD-fed mice in the present study demonstrate increase in plasma NEFA, which is attenuated by BMJ. The data support and extend the previous observations by Patsouris *et al.*⁽²⁷⁾, which demonstrated that nuclear transcription factor PPAR α is significantly increased in HFD-fed mice possibly in response to an increased NEFA flux to the liver. Patsouris *et al.*⁽²⁷⁾ further demonstrate that insulin suppresses PPAR α activation in hepatocytes and that hepatic up-regulation of PPAR α by HFD may be associated with attenuation of insulin signalling-associated increases in plasma NEFA. Recent studies by Ameen *et al.*⁽²⁸⁾ demonstrated that in mice, Wy 14,643, a PPAR α agonist, increased apoB-100 secretion despite decreased TAG synthesis, specifically through activation of microsomal TAG transfer protein mRNA transcription and protein expression. We have previously demonstrated that reduction in apoB secretion was in part associated

with reductions in TAG synthesis as well as microsomal TAG transfer protein mRNA gene expression in HepG2 cells treated with BMJ. In the current study, although we did not analyse hepatic microsomal TAG transfer protein expression, reduction in PPAR α expression paralleled the reduction in hepatic and plasma TAG as well as plasma apoB-100 secretion in mice fed BMJ as compared to those receiving HFD alone. The present results are, however, contradictory to those of Chao & Huang⁽²⁹⁾ who demonstrated that 72 h treatment of H4IIEC3 rat liver cells with BMJ extract (100 and 150 mg/l) activated transcriptional factors such as PPAR α and PPAR γ , possibly contributing to its hypolipidaemic and hypoglycaemic effects. Nevertheless, BMJ-associated reductions in PPAR α protein levels were not below those of untreated control animals. Alternatively, the differences in PPAR α could arise due to the differences in the models employed and the fact that BMJ was tested in conjunction with HFD as well as the differences in the *in vitro* and *in vivo* metabolism of BMJ.

Although HFD significantly increased liver weight and hepatic TAG levels, BMJ had no effect on liver weight, but significantly lowered hepatic TAG mass in HFD-fed mice. Similar BMJ-associated reduction in hepatic TAG mass without a change in liver weight has been observed in rats fed BM pulp⁽³⁰⁾ or BMJ^(4,22,31) and has been attributed to increased clearance of hepatic TAG by BM through increased β -oxidation of NEFA.

Traditionally, diabetic subjects are recommended to consume about 30–60 g fresh BMJ twice a day. However, there are no clinical studies that demonstrate an effective physiological or pharmacological dosage of BM consumption by man. Nevertheless, BMJ preparations from independent laboratories have demonstrated comparable beneficial effects not only on glucose metabolism, but also on plasma and hepatic lipids^(5,30). Earlier studies by Chen *et al.*⁽⁷⁾ and Chen & Li⁽²²⁾ did not observe any dose-dependent effects of BMJ on body weight and metabolic parameters of glucose and lipid metabolism. With HFD, the maximum effects on metabolic parameters were observed at a dose of 1.5% of lyophilized BMJ (w/w) fed *ad libitum*⁽⁷⁾. The BM dose of 1.5% lyophilized juice was based on published literature mentioned earlier⁽⁷⁾. In the present study, 1.5% BMJ had no detrimental effects on liver function as measured by serum aspartate aminotransferase, alanine amino transferase and lactate dehydrogenase levels as reported in previous studies⁽³²⁾.

Although BM contains numerous chemicals, the active hypoglycaemic and hypolipidaemic compounds are unknown. Compounds isolated from the fruit and seeds of the BM plant that are believed to contribute to its hypoglycaemic activity include charantin (a steroid glycoside) and polypeptide 'p' or plant insulin (a 166 residue insulin mimetic peptide)⁽³³⁾. BM is also known to contain additional glycosides such as mormordin, vitamin C, carotenoids, flavanoids and polyphenols^(34,35). Recent studies indicate that cucurbitanoid compounds are the active principals of BM which possess hypoglycaemic properties⁽³⁶⁾.

So far, only a few, non-randomized clinical studies have investigated the anti-diabetic effects of BM in man^(37–41). It is therefore crucial to conduct adequately powered, randomized, placebo-controlled clinical trials before BM can be recommended as an effective alternative and/or complementary

therapy for diabetic dyslipidaemia. Administration of BM with other hypoglycaemic/hypolipidaemic agents must be performed under medical supervision and monitoring, due to its hypoglycaemic properties^(37,42). Some negative side-effects such as diarrhoea and hepatotoxicity in man have been noted⁽⁴⁰⁾, which could be due to excessive consumption.

The present data are the first to demonstrate that the primary mechanism of BMJ action to restore hepatic glucose and lipid metabolism is associated with increased post-IR signal transduction linked to tyrosine phosphorylation of IR β and IRS proteins thereby leading to PI3K activation. Further studies are warranted not only to characterize and identify the active ingredients of BM that regulate lipid metabolism, but also to standardize physiologically relevant dosage in man.

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