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Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin resistant hamsters

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Abstract

The present study was undertaken to determine whether supplementation with polymethoxylated flavones (PMFs) could ameliorate the fructose-induced hypertriglyceridemia and other metabolic abnormalities associated with insulin resistance (IR) in hamsters. Following feeding with the fructose diet, hamsters were supplemented orally with PMF-L or PMF-H (62.5 and 125 mg/kg/day) for 4 weeks. Both PMF-treated groups showed a statistically significant ($p<0.05$) decrease in serum triglyceride (TG) and cholesterol levels compared to the fructose-fed control group. The fructose control group at the end of the study showed elevated serum insulin and impaired insulin sensitivity (glucose intolerance). On the other hand, PMF-supplemented groups showed a reversal in these metabolic defects, including a decrease in insulin level and an improvement in glucose tolerance. PMF supplementation also reduced TG contents in the liver and heart and was able to regulate adipocytokines by significantly suppressing TNF-α, INF-γ, IL-1β and IL-6 expression and increasing adiponectin in IR hamsters. The mechanism of PMF on the activation of peroxisome proliferator-activated receptors (PPAR) was also explored. PMF-H supplementation significantly increased PPARα and PPARγ protein expression in the liver. This is the first report of positive effects of PMF on adipocytokine production and on PPAR expression in IR hamsters. This study suggests that PMF can ameliorate hypertriglyceridemia and its anti-diabetic effects may occur as a consequence of adipocytokine regulation and PPARα and PPARγ activation.

Keywords: Polymethoxylated flavones; Insulin resistance; Triglyceride; Adipocytokines; Peroxisome proliferator-activated receptor

Introduction

The metabolic syndrome/IR disorder has become increasingly common in Western society; it is estimated that 20–25% of the US adult population is affected. Current non-pharmaceutical risk-reducing therapies include body weight reduction and regular physical activity. Intervention by nutraceuticals aimed at correction of hyperlipidemia and maintenance of glycemic control could be another promising option but, so far, very few natural products have been investigated for their efficacy as hypolipidemic and anti-diabetic drugs.

PMF, a group of highly methoxylated phenolic compounds belonging to a class of flavones, are found specifically in citrus. The two most common PMFs, tangeretin and nobiletin, occur largely in the peel of tangerines and oranges (Horowitz and Gentili, 1977). PMFs play an important role in a number of biological functions including anti-cancer properties (Silalahi, 2002), immunomodulatory and anti-inflammatory properties (Lin et al., 2003), antioxidant and neuroprotective properties (Datla et al., 2001). Previously, the role of citrus PMFs in modulating hypolipidemic responses in HepG2 cells in a hamster model of diet-induced hypercholesterolemia was reported to inhibit apo B secretion (Kurowska and Manthey, 2002). Further studies demonstrated that tangeretin modulated triacylglycerol metabolism via suppression of diacylglycerol acyltransferase (DGAT) activity and an inhibition of microsomal triglyceride transfer protein mediated lipidation of nascent apo B within the ER lumen

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(Kurowska et al., 2004). Studies have also shown that supplementation with 1% tangeretin or with 1% PMF mixtures containing largely tangeretin and nobiletin significantly reduced serum total, VLDL and LDL cholesterol and also decreased serum and liver TG in hamsters with hypercholesterolemia induced by feeding casein-based semi-purified diet (Kurowska and Manthey, 2004).

IR, obesity and diabetes are now recognized as inflammatory disorders (Dandona et al., 2004). An ever increasing number of molecules that are best known for their roles in immune and inflammatory cells are now considered as key modulators of energy metabolism in insulin targeted tissues. Adipose tissue serves as the site of TG storage and free fatty acid (FFA)/glycerol release in response to changing energy demands. It not only stores excess energy but also secretes a variety of proteins including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), adiponectin, leptin, resistin, and plasminogen-activator inhibitor type 1 (Hotamisligil et al., 1993; Scherer et al., 1995; Tsuchida et al., 2005). Since several of these adipocytokines influence insulin sensitivity and glucose metabolism, they might provide a molecular link between increased adiposity and impaired insulin sensitivity and help to find the molecular targets for anti-diabetic drugs and dietary supplements. TNF-α plays an important role in the immune response and in inflammatory processes; however, this cytokine has increasingly been recognized as a key modulator of glucose homeostasis and lipid metabolism in adipose tissue (Sethi and Hotamisligil, 1999). IL-6 is an important systemic signaling molecule in an inflammatory state and inhibits early hepatic insulin receptor signaling and downstream insulin action in vivo, actions which might cause IR at the adipocyte and hepatocyte levels (Kanemaki et al., 1998; Yudkin, 2003). Another factor in adipose tissue signaling is adiponectin, the most abundant circulating adipocytokine in both rodents and human subjects. Adiponectin has an important role in regulating adiposity and IR (Xydakis et al., 2004).

The activation of PPAR, a nuclear transcription factor, mediates pleiotropic effects including stimulation of lipid oxidation, modulation of lipoprotein metabolism and inhibition of vascular inflammation. PPARγ activation leads to adipocyte differentiation and improved insulin signaling of mature adipocytes. Thus, PPARs play a prominent role in several physiological processes including the control of lipid and lipoprotein metabolism, glucose homoeostasis and the inflammatory response (Fruchart et al., 1999). Recent studies have suggested that PPARα and PPARγ agonists may alleviate inflammation (Hofmann et al., 1994; Jiang et al., 1998; Tsuchida et al., 2005). It has been previously shown that tangeretin also activated PPAR, which enhances cellular fatty acid (FA) β-oxidation and therefore reduces the availability of FA for incorporation into cellular TG (Kurowska et al., 2004).

The hypolipidemic and anti-inflammatory effects of tangeretin and nobiletin observed in vitro and in vivo, combined with the ability of tangeretin to act as a PPAR agonist in HepG2 cells, prompted us to hypothesize that PMF may be effective in the treatment of dyslipidemia associated with IR by regulating inflammatory cytokines and adipocytokines via PPAR activation. The fructose-fed hamster model was used in the present study to determine whether supplementation with PMF could ameliorate hypertriglyceridemia and other metabolic abnormalities associated with IR. The mechanism of PMF on the activation of PPAR was also explored.

Materials and methods

Reagents

Routine reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Induction of hypertriglyceridemia and IR

Male Golden Syrian hamsters (approx. 130–150 g, *Mesocricetus auratus*, Charles River, Wilmington, MA) were acclimatized and given free access to water and a rodent chow for 2 weeks prior to the initiation of the experimental diet. The Animal Care and Use Committee, University of Hawaii, and The University of Western Ontario Council approved animal experimental protocols. All surgical procedures were done under isoflurane (4% in pure oxygen by mask). Euthanasia was conducted under isoflurane gas and by cutting the diaphragm via a thoractomy to create negative pressure. Hamsters were housed individually and maintained at an ambient temperature of 22 °C under a photoperiod of 12 h of light and 12 h of darkness.

Hamsters were weighed and randomized into four groups of 7 hamsters each: chow; control fructose-fed; fructose+low dose PMF (PMF-L, 62.5 mg/kg body weight per day); and fructose+high dose PMF (PMF-H, 125 mg/kg body weight per day). Hamsters were fed fructose-enriched diet that contained 60% fructose and 22% casein (Dyets Inc., no. 161506, Bethlehem, PA) for two weeks to induce hypertriglyceridemia and IR as previously described (Taghibiglou et al., 2000). The chow-fed group was not fed fructose. The hamsters were assessed at the end of 2 weeks of fructose feeding for the development of their IR status by monitoring body weight, plasma TG, cholesterol and glucose.

PMF treatment

The PMF supplement, in powder form, was obtained from KGK Synergize Inc. (London, Ontario, Canada) and analyzed to contain 41% PMF (mainly tangeretin and nobiletin, 1 : 1 w/w) and 0.3% synephrine. The respective hamster groups were orally fed daily doses of either PMF-L or PMF-H for the remaining 4 weeks in addition to continued fructose feeding. To make the extract palatable, the PMF was mixed with 300 mg of Nutri-Cal (sweetener) as a carrier. The control group also received equivalent quantities of the sweetener.

Biochemical analyses

Blood samples from 16 h-fasted hamsters were collected from the retroorbital venous plexus into EDTA-coated and non-anticoagulant tubes at baseline (first day of fructose feeding) and week 3 (first day of PMF administration). At the end of the study, the blood samples were collected by cardiac puncture.

Serum was collected after centrifugation at 3000 ×*g* for 20 min at 4 °C and kept at −80 °C until use for the determination of biochemical markers. Serum TG, cholesterol, and glucose levels were determined by enzymatic colorimetric assays using commercially available kits from Randox Laboratories (Oceanside, CA) and a Shimadzu UV-1201 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Serum insulin (Linco Research, St. Charles, MO) and adiponectin (B-Bridge International, Sunnyvale, CA) protein levels were measured using specific enzyme-linked immunosorbent assays (ELISA) methods according to the manufacture's instructions.

Glucose tolerance test

An intra-peritoneal glucose tolerance test (i.p. GTT) was performed on a separate group of animals. Treatment time and feeding were identical as described above. Hamsters were fasted overnight and a 0 h blood sample was taken by retro-orbital bleed. A 30% glucose solution dissolved in 0.9% NaCl, at a dose of 3 g/kg body weight, was injected intraperitoneally. Four more blood samples were taken by retro-orbital bleed at 30, 60, 90 and 120 min after glucose administration. Total volume was less than 0.9 mL per hamster and all blood samples were collected in EDTA-coated tubes. Serum glucose concentration was measured using the enzymatic colorimetric assay kit (Randox Laboratories, Oceanside, CA) and a Shimadzu UV-1201 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

Quantitative analysis of TG mass in tissues

The tissues including heart, liver, epididymal fat pad, and skeletal muscle, were dissected and washed with saline before freezing in liquid nitrogen. TG mass was measured essentially as described previously (Scribner et al., 2000) with the exception that chloroform/methanol was substituted for hexane/isopropanol. Briefly, 100 mg of frozen tissue were minced and homogenized in 2 ml of sucrose buffer (0.3 mol/L sucrose, 25 nmol/L 2-mercaptoethanol, and 10 mmol/L EDTA, pH 7.0) and mixed with hexane/isopropanol (3:2 v/v). The organic phase was separated, dried, and the lipids resuspended in 100 μL of ethanol. TG mass was performed according to the manufacturer's procedure. TG measurements were normalized to the weight of each tissue (milligrams of TG per gram of tissue).

Cytokine determination by cytometric bead array (CBA) and ELISA

Hamster blood samples were allowed to clot for 30 min at room temperature before centrifuging for 20 min at 2000 ×*g*. The sera were stored at −80° C until assayed for cytokines. IL-2, IL-4, IL-5, TNF-α and IFN-γ were simultaneously detected using mouse Th1/Th2 cytokine CBA kit (BD Biosciences, Brisbane, QLD) according to the manufacturer's instructions. Briefly, 50 μl of the sample were mixed with 50 μl of the mixed capture beads and 50 μl of the mouse Th1/Th2 phycoerythrin (PE)-conjugated detection antibodies. The mixture was incubated at room temperature for 2 h in the dark followed by a wash step. The samples were then resuspended in 300 μl of wash buffer before acquisition on the FACS Calibur (BD Bioscience, Brisbane, QLD). The data were analyzed using CBA software (BD Biosciences, Brisbane, QLD). Standard curves were generated for each cytokine using the mixed bead standard provided in the kit. The concentration of cytokine in the hamster serum was determined by interpolation from the appropriate standard curve. The detection limits for IL-2, IL-4, IL-5, TNF-α and IFN-γ were 5, 5, 5, 2, and 6.3 pg/ml, respectively. IL-1β and IL-6 in sera were measured by using specific mouse ELISA kit (R&D Systems, Sydney, NSW). The detection limits were at 20 pg/mL.

PPAR assay

Liver and adipose tissue were homogenized in a PBS lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mg/mL EDTA as described previously (Rahimian et al., 2001). After homogenization, samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C. The supernatant was collected and the protein concentration determined using a protein assay kit (BioRad Laboratories, Hercules, CA). Protein (40 μg) was separated by SDS-PAGE and transferred onto PVDF membrane (BioRad Laboratories, Hercules, CA) overnight at 4 °C. After transfer, the membrane was blocked by incubation with 5% fat-free dry milk diluted in TBST (20 mM Tris–HCl, 0.9% saline, 0.1% Tween-20) for 2 h at room temperature. After blocking, the membrane was probed with a goat polyclonal antibody against PPARα (sc-1985) or PPARγ (sc-1984) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5 h at room temperature (diluted 1:2500). After being washed, the membrane was again probed with a HRP-conjugated anti-goat IgG antibody for 1 h at room temperature (diluted 1:8000). The peroxidase complex was visualized using the Lightning Chemiluminescent Detection kit (Perkin-Elmer, Boston MA). Protein bands were densitometrically evaluated using the BioRad Gel Doc 2000 system (BioRad Laboratories, Hercules, CA).

Statistical analysis

All data were presented as mean±SD. Statistical analysis was performed using SPSS 11.5. ANOVA was used to

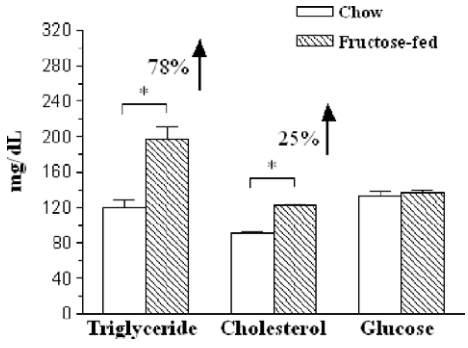

Fig. 1. Hypertriglyceridemic and IR hamster model. The concentrations of serum TG, cholesterol and glucose were determined by enzymatic colorimetric assays in hamsters after feeding high fructose diet for 2 weeks. Values are represented as mean±SD from 7 animals of each group. *$p<0.05$.

Table 1
Effect of PMF supplementation on metabolic variables

	Fructose-fed (n=7)	PMF-L (n=7)	PMF-H (n=7)
Before PMF supplementation			
Body weight (g)	148±4	153±11	151±7*
TG (mg/dL)	198±22	188±17*	214±28*
Cholesterol (mg/dL)	120±6	112±8	118±7*
Glucose (mg/dL)	116±9	137±8	133±9
After PMF supplementation			
Body weight (g)	157±4	143±7**	143±9**
TG (mg/dL)	213±13	112±7**	98±7**
Cholesterol (mg/dL)	120±6	104±3	91±6**
Glucose (mg/dL)	116±9	121±9	112±6
LDH (U/L)	594±72	627±70	687±99
Liver (g)	6.2±0.7	5.4±0.7**	5.2±0.5**
Epididymal fat (g)	3.9±0.4	3.3±0.8	3.0±0.4**
Heart (g)	0.6±0.1	0.6±0.1	0.6±0.1
Muscle (g)	7.0±0.9	7.5±0.3	7.9±0.9

Hamsters were fructose-fed for 2 weeks followed by PMF supplementation at two dosages: PMF-L and PMF-H for another 4 weeks in addition to continued fructose feeding. The body weights were monitored weekly to adjust the administration of PMF. Values represent the mean±SD of 7 animals per group. *p<0.05 vs. before and after PMF supplementation. **p<0.05 vs. control fructose-fed hamster at end of the study after PMF supplementation.

examine the effect of treatment over time on serum TG, cholesterol, glucose concentrations and body weight. The differences between the experimental groups were analysed by two tailed unpaired Student's t-test. Spearman correlation coefficient r was determined to assess the relationship between different parameters. Statistical significance was established at p<0.05.

Results

Metabolic effects of PMF in fructose-fed hamsters

Hamsters were fed fructose-enriched diet over a 2-week period and developed hypertriglyceridemia associated with IR. A significant increase in serum TG levels by approximately 78% (p<0.001 vs. chow-fed) and cholesterol by approximately 25% (p<0.01 vs. chow-fed) was observed (Fig. 1). Glucose levels remained essentially unchanged in the chow- and

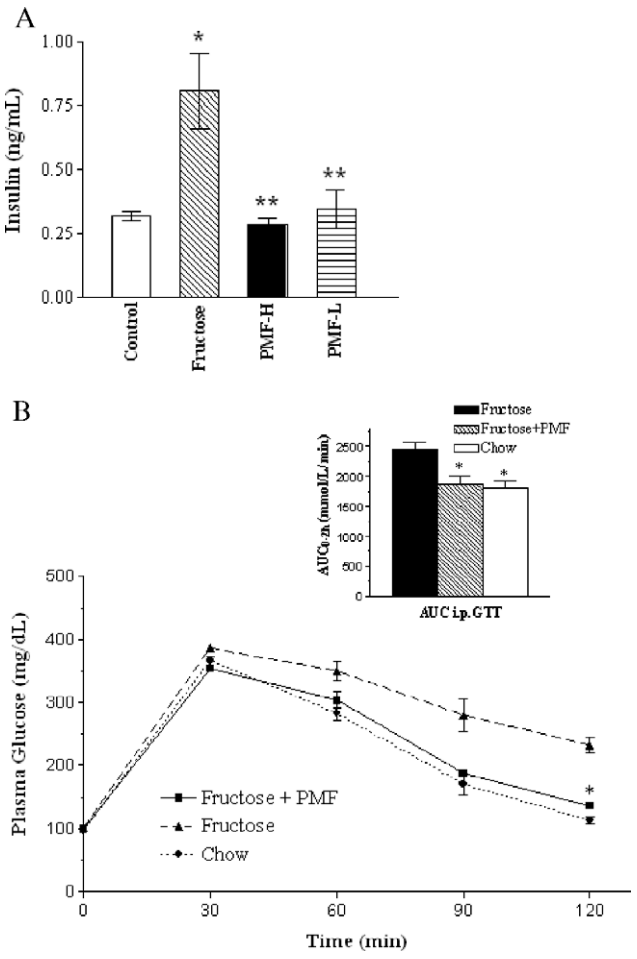

Fig. 3. (A) Serum insulin levels are increased in fructose-fed hamsters. ELISA was done to determine serum insulin in chow-fed, fructose-fed and PMF-L and PMF-H hamsters. Values represent the mean±SD of seven animals per group. *p<0.05 vs. baseline value; **p<0.05 vs. control fructose-fed hamster at week 7. (B) Glucose tolerance test (GTT) profile of hamsters. Blood was collected at 0, 30, 60, 90 and 120 min after glucose administration from the chow-fed, fructose-fed and PMF-H groups. Glycemic responses were determined as changes in serum glucose concentrations over 2 h (at 30 min intervals) following the i.p. injection of glucose, 3 g/kg body weight. Values are represented as mean±SD for 4 animals per group. *Significant difference between PMF-H group and fructose-fed group (p<0.05). Insert shows graphic representation of the total AUC.

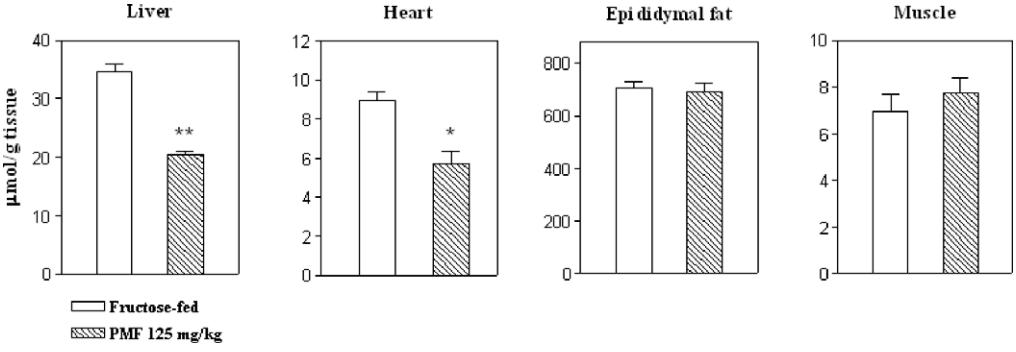

Fig. 2. PMF increases TG mass of liver and heart. Hamsters were fructose-fed for 2 weeks followed by PMF supplementation at two dosages: PMF-L and PMF-H for another 4 weeks in addition to continued fructose feeding. TG mass was measured in liver, heart, epididymal fat and skeletal muscle at the end of the study from the fructose-fed and PMF-H groups. Values are represented as mean±SD of seven animals per group. *p<0.05 vs. fructose-fed; **p<0.001 vs. fructose-fed.

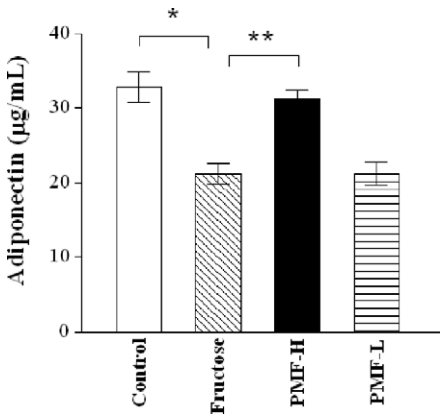

Fig. 4. Serum adiponectin levels are increased by PMF. ELISA was done to determine serum adiponectin in chow-fed, fructose-fed and PMF-L and PMF-H hamsters. Values represent the mean ± SD of seven animals per group. *$p<0.05$ vs. baseline value; **$p<0.05$ vs. control fructose-fed hamster at week 7.

fructose-fed groups. To determine whether dietary PMF supplementation can reverse the stimulatory effect of fructose on TG metabolism, IR hamsters were orally fed for another 4-week a daily dose of either the fructose diet alone, or the fructose diet with PMF-L or PMF-H.

Various serum metabolic variables, body weights and tissue weights were examined to assess the extent of the hyperlipidemia and IR upon PMF supplementation (Table 1). Both PMF-treated groups showed a statistically significant ($p<0.05$) decrease in serum TG levels compared to the fructose-fed control group after PMF supplementation. Statistically significant decreases in serum TG levels were also observed within the PMF-L and PMF-H groups before and after dietary supplementation of PMF ($p<0.05$). Serum cholesterol levels showed a significant reduction only in the PMF-H group upon PMF supplementation ($p<0.01$ vs. no PMF supplementation), while a slight, but non-significant decrease in the PMF-L group ($p=0.39$ vs. no PMF supplementation), was observed. Glucose levels over the course of the study remained essentially unchanged in the control fructose-fed, PMF-L- and PMF-H-supplemented groups ($p>0.05$ vs. baseline).

Weight gain was similar in all groups over the 2-week fructose-feeding period ($p>0.05$). Body weights at the end of the study were slightly lower in both the PMF-supplemented groups (−8.9%, $p<0.05$) as compared with the body weights prior to PMF supplementation, but not in the fructose-fed control group. The assessment of hepatotoxicity of PMF did not indicate cell membrane damage, as demonstrated by the lack of statistically significant differences ($p=0.446$ PMF-L vs. fructose-fed group and $p=0.737$ PMF-H vs. fructose-fed group) in LDH between the groups. Tissue weights were measured at the end of the study in the fructose-fed and PMF-supplemented groups. The weight of liver and epididymal adipose tissue decreased significantly in both treated groups (−13% and −20% in PMF-L group, and −16% and −30% in PMF-H group, respectively, $p<0.05$ vs. fructose-fed group). No significant changes were observed with respect to the weight of the heart and muscle ($p>0.05$ vs. fructose-fed group).

Effects of PMF on TG mass in insulin-sensitive tissues

Toxic levels of TG within insulin-sensitive tissues are thought to lead to IR. To address the possibility that PMF may reverse the accumulation of TG in insulin sensitive tissues, the TG content in liver, heart, epididymal fat pad, and skeletal muscle were examined. As shown in Fig. 2, supplementation with PMF-H significantly reduced TG accumulation in liver (−42%, $p<0.001$) and heart (−33%, $p<0.05$) compared to the fructose-fed group. PMF did not alter TG content in epididymal fat pad and skeletal muscle ($p>0.05$ vs. fructose-fed group). Liver TG mass was not determined in the chow-fed group in this study as an increase in TG mass in the liver of fructose-fed groups relative to the chow-fed groups has been shown previously (Casaschi et al., 2005).

Effects of PMF on serum insulin and changes in serum glucose in a GTT

Serum insulin levels in the chow-, the fructose-fed, and the PMF-L- and PMF-H-supplemented groups were measured by ELISA as described in Materials and methods. A significant increase in the insulin level was observed in the fructose-fed group at the end of the study as compared with baseline in the same group ($p=0.007$), Fig. 3A. This increase was reversed by

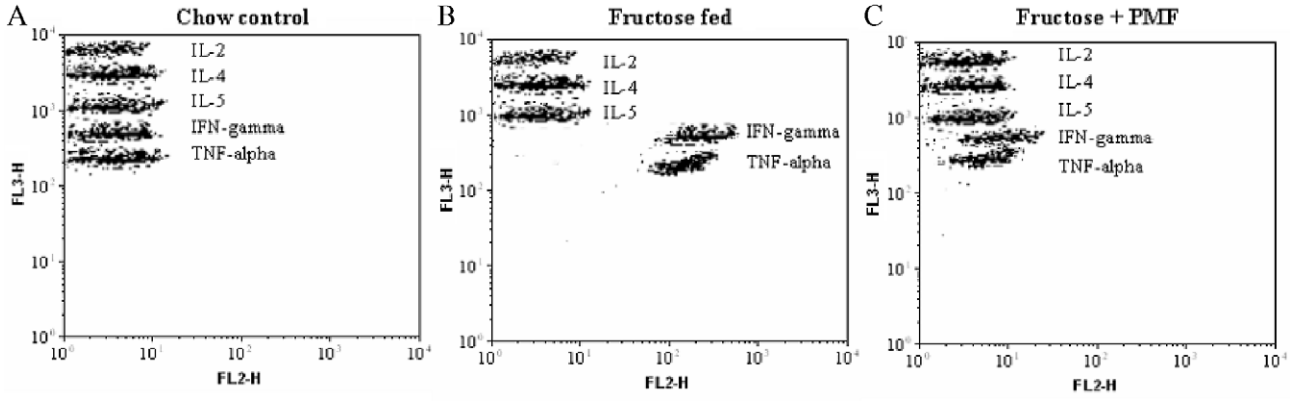

Fig. 5. PMF suppresses TNF-α and IFN-γ secretion. The representative dot plots indicate cytokine levels in (A) chow-fed (B) fructose-fed (C) PMF-H-supplemented hamsters by FACS analysis. The data presented are from duplicates.

PMF supplementation at both doses for the last 4 weeks of the study. The reductions were statistically significant ($p=0.008$ and $=0.025$, respectively) compared to the fructose-fed group

To further assess if PMF ameliorates IR, i.p. GTT was studied on a new set of hamsters ($n=4$ per group). The hamsters were subjected to the same diet program as mentioned above. After 4 weeks of PMF-H supplementation, the i.p. GTT showed significant differences between the PMF-H-supplemented group and the fructose-fed group (Fig. 3B). At the 60-, 90-, and 120-min intervals, serum glucose levels were significantly lower in the PMF-H-supplemented group than in the fructose-fed group ($p<0.05$) and essentially declined to the levels seen in the chow-fed animals. The total area under the curve (AUC) between 0 and 120 min were 2273±453 mg/L/min for the fructose-fed group and 1827±285 mg/L/min for the PMF-H group (Fig. 3B insert).

PMF increases circulating adiponectin

The insulin-sensitizing effect of PMF was further demonstrated by measuring the circulating adiponectin protein level as described in the Materials and methods section. Fructose feeding induced a marked decrease in the serum adiponectin level in IR hamsters when compared with the chow group, (Fig. 4, $p=0.003$ vs. chow group). In contrast, PMF-H restored the adiponectin level close to the level observed in the chow group with a 28% increase ($p=0.002$ vs. fructose-fed at the end of the study). There was no increase observed in the PMF-L group. A correlation analysis (Spearman) of IR markers in all hamsters showed that the decreased expression of adiponectin correlated inversely with serum TG ($r=-0.25$, $p=0.03$) and insulin ($r=-0.39$, $p=0.005$), but not with glucose and cholesterol.

Effect of PMF on cytokine secretion

To determine if PMF treatment results in the alteration of cytokines, sera from chow-fed, fructose-fed, PMF-H and PMF-L groups were measured for TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5 and IL-6 levels. The representative dot plots of cytokine levels of IL-2, IL-4, IL-5, TNF-α and IFN-γ in PMF-supplemented hamsters versus chow- and fructose-fed hamsters simultaneously detected using mouse Th1/Th2 cytokine CBA kit are shown in Fig. 5A–C. FACS analysis showed that TNF-α and IFN-γ was considerably higher in the fructose-fed hamsters compared to both the chow group and the PMF-supplemented group. No significant difference was observed in the levels of IL-2, IL-4, and IL-5 in all three groups.

Cytokine ELISA data showed that TNF-α was considerably higher in the fructose-fed hamsters when compared with the chow group (399.4 and 9.6 pg/mL, respectively, $p<0.001$), Fig. 6. Administration of PMF-H significantly decreased TNF-α concentration to the basal level (15.1 pg/mL, $p<0.001$). A reduction of TNF-α was also observed in the PMF-L group (−65%), indicating the effect of PMF was dose dependent. A similar pattern was observed with IFN-γ; both PMF-dose groups showed considerable suppression of IFN-γ expression with the reduction of 88% and 34% ($p=0.0002$ and 0.03,

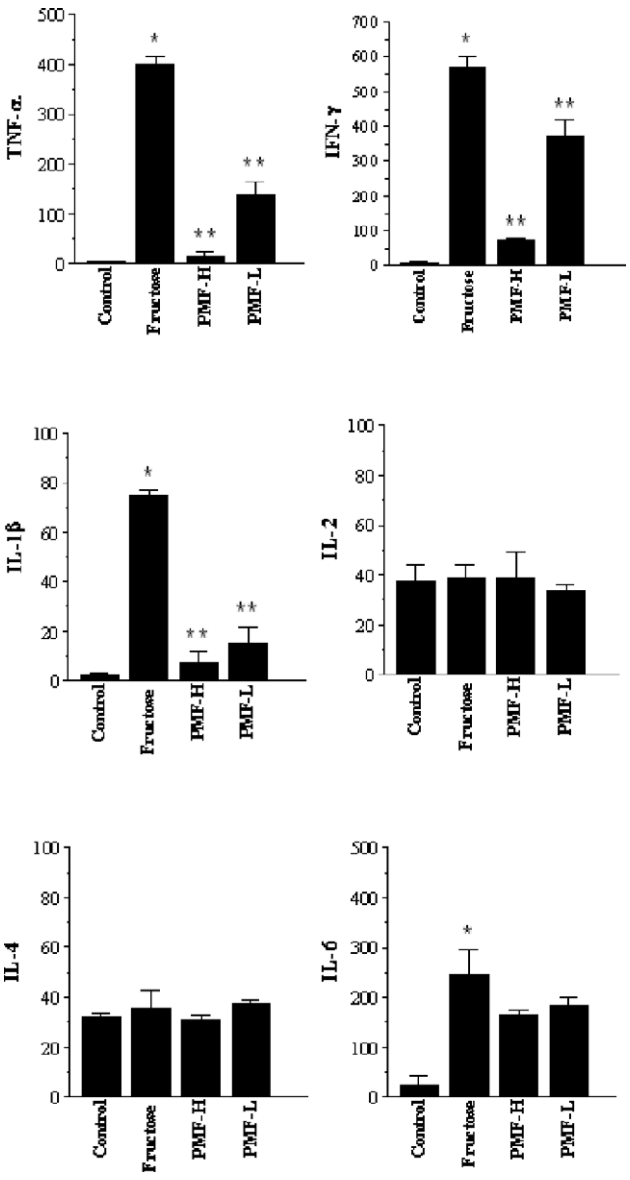

Fig. 6. PMF downregulates pro-inflammatory cytokines in IR hamsters. Hamsters were bled and serum was obtained at the end of the study from all of the 4 groups for the measurement of cytokines. TNF-α, IFN-γ, IL-2, IL-4 and IL-5 were detected using mouse Th1/Th2 cytokine CBA method. IL-1β and IL-6 were detected using specific cytokine ELISA. Values are expressed in picogram per milliliter and the mean±SD were obtained from two replicates. *Significant differences between chow control and fructose-fed, $p<0.05$; **Significant differences between fructose-fed and PMF-supplemented groups, $p<0.05$.

respectively). Similarly, the PMF groups showed suppression of IL-1β expression with the reduction of 90% and 81% ($p=0.0002$ and 0.001, respectively). IL-6 expression in the fructose-fed hamsters was significantly elevated when compared to the chow group (244.6 and 24.6 pg/mL, respectively, $p<0.01$). However, a slight decrease was observed in the PMF-H and PMF-L groups in the IL-6 level. No differences were observed in IL-2 and IL-4 between the PMF-supplemented and control groups. PMF did not modulate production of the IL-5 cytokine in this study (data not shown). There was a good correlation between the cytokines (TNF-α and IL-6) and

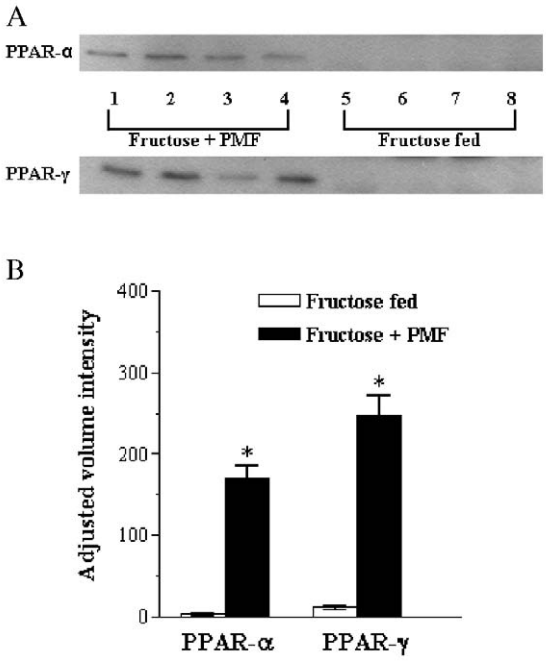

Fig. 7. PPAR protein expression in liver is up regulated by PMF. Liver tissue was collected at the end of the study from 4 animals per group, homogenized, and analyzed for PPARα and PPARγ protein expression by immunoblotting. (A) Western blots showing PPAR expression. (B) PPAR bands were quantitated by densitometry and the band intensity adjusted for band volume. Values represent the mean±SD of 4 animals per group. *$p<0.05$ vs. fructose-fed group.

adiponectin levels. Increased concentrations of TNF-α and IL-6 in the fructose group were inversely related to the decreased serum adiponectin level ($r=-0.78$, $p=0.02$).

PMF upregulates hepatic PPAR protein expression

The involvement of the lipogenic transcription factors, PPARs, which play a role in the regulation of pathways of lipid metabolism, glucose utilization and inflammatory responses in different cell types, was studied. As shown in Fig. 7A, PPARα and PPARγ protein expression in the liver was essentially undetected in the high fructose-fed hamster group. Interestingly, upon PMF-H supplementation, liver PPARα increased by 166%, while PPARγ increased by 251% in the PMF-H supplemented group ($p<0.05$ vs. fructose-fed, $n=4$ per group) (Fig. 7B).

Discussion

Previous studies regarding the effects of PMFs in inflammation, IR and dyslipidemia have shown that these compounds could downregulate the inflammatory response, prevent atherosclerosis and improve the IR both in vitro and in vivo (Kurowska and Manthey, 2004; Manthey et al., 1999; Whitman et al., 2005). In this study, the anti-diabetic effect of a PMF supplement was explored using the pre-diabetic hamster model of fructose-induced hyperlipidemia and IR (Taghibiglou et al., 2000). The hamster model of IR was established by feeding a high fructose diet for 2 weeks. Upon establishing the model system, the effects of PMF were tested on the increase in TG and cholesterol levels. After PMF supplementation for 4 weeks, significant reductions in TG and cholesterol were observed in a dose-dependent manner related to lipids, cholesterol and inflammation, indicating that the response was specific to PMFs. These data extend a previous study (from our group) demonstrating that the dietary PMF induced a hypolipidemic response in hamsters with hypercholesterolemia (Kurowska and Manthey, 2004). Furthermore, it was demonstrated that PMF could regulate adipocytokines expression in IR hamsters. This is the first report of a positive effect of PMF on adipocytokine production in IR hamsters. Lastly, it was demonstrated that PMF increases protein expression of PPAR-α and PPAR-γ in the liver. These findings support the hypothesis that the insulin-sensitizing effects of PMF may occur as a consequence of adipocytokine regulation and PPAR activation.

As IR develops, adipocytes hypertrophy and become resistant to the ability of insulin to prevent the breakdown of lipids. PMF supplementation improved IR, an improvement that was also associated with decreased TG contents in the liver and heart and prevention of adipocyte hypertrophy. PMF-fed hamsters showed increased insulin sensitivity and were protected from hypertriglyceridemia induced by the high-fructose diet. Furthermore, PMF supplementation also reduced the insulin level to basal level, indicating an anti-diabetic effect.

The mechanism of PMF in improving insulin sensitivity in this study can be explained, at least in part, by its regulatory effects on adipocytokines. Adipose tissue generation of cytokines has been shown both in vitro and in vivo. Also a number of novel cytokine-like molecules, collectively known as adipocytokines, have been identified as adipocyte products. While several of these cytokines, such as TNF-α, may act predominantly in autocrine or paracrine fashion, others are released into the systemic circulation, acting as signaling molecules to remote tissues, including liver, skeletal muscle and endothelium. These signaling molecules secreted by adipose tissue play a major role in IR (Hotamisligil, 2003) since their interference with insulin signaling leads to hyperglycemia and proinflammatory changes. TNF-α has also been shown to directly lead to IR by inducing serine phosphorylation of the insulin receptor thus inhibiting insulin signaling (Hotamisligil et al., 1994). The serum concentrations of TNF-α, IFN-γ and IL-1β were found to be distinguishable between chow-fed and IR hamsters. PMF supplementation decreased these cytokine concentrations in IR hamsters. Taken together, the present study shows that TNF-α, IL-1β, and IFN-γ were elevated in IR hamsters and PMF may play a role in restoring insulin sensitivity at least in part by regulating these adipocytokines and inflammatory cytokines. IL-6 influences insulin sensitivity via directly impairing insulin signaling in primary mouse hepatocytes and 3T3-L1 adipocytes with decreased activation (Fasshauer et al., 2004). However, the present data did not show a potent alteration of IL-6 in hamsters receiving PMF supplement, indicating that the drug metabolism is considerably more complex in vivo than in vitro.

Adiponectin, which is exclusively expressed in adipose tissues and is the most abundant circulating adipocytokine in

both rodent and humans (Beltowski, 2003), has been found to be increased in obesity, diabetes and coronary heart diseases (Hotta et al., 2000; Kumada et al., 2003). Adiponectin directly affects the inflammatory response by regulating both the production and the activity of cytokines. Our results show that serum adiponectin concentration has an inverse relationship with TG concentration, IR and circulating adipocytokine concentrations in IR hamsters. This observation is consistent with the previous report that in humans the adiponectin levels are negatively correlated with insulin concentrations and positively correlated with insulin sensitivity (Weyer et al., 2001).

As more evidence emerges, there is a strong case for targeting adipose tissue in the treatment of type 2 diabetes. PPARγ agonists, for example, the thiazolidinediones, redistribute fat within the body and have been shown to enhance adipocyte insulin sensitivity, inhibit lipolysis, reduce serum FFA levels and favorably influence the production of adipocytokines. Several mechanisms are suggested by which PMF may have an impact on adipocytokines via activation of PPARα and PPARγ. It was initially speculated that these effects are at least partially mediated via a direct effect of PMF on adipose tissue since PMFs are highly hydrophobic due to the presence of multiple methoxy groups. The chemical nature of PMF might lead to its accumulation in adipose tissue; however, this hypothesis was not supported by the data of PPAR-γ expression from epididymal fat pad (data not shown). Interestingly, like tangeretin, a major constituent of PMF, which dose-dependently activated PPAR in our earlier experiments in vitro (Kurowska et al., 2004), likewise PMF appears to be a high affinity ligand of PPARα and PPARγ in the liver in this study. Thus, it may be speculated that most of the metabolic and anti-inflammatory actions of these compounds or their secondary metabolites are mediated through ligation of these transcription receptors in the liver. These observations, therefore, indicate that PMF may exert both anti-inflammatory and anti-diabetic effects as a result of its function as a PPAR agonist. Although the expression of adiponectin in adipose tissue was not investigated in this study, it would be plausible to speculate that the mechanism of increased serum adiponectin by PMF supplementation may also be secondary to the increased expression of the transcription factor PPARα and PPARγ. However, a clearer understanding of the mechanism of PMF-regulating adipose tissue signaling and its contribution to control the state of inflammation in IR will require further molecular studies.

Conclusion

This study provides novel evidence that PMF reverses hypertriglyceridimia and restores insulin sensitivity, possibly through regulating the expression of the adipocytokines by stimulating the protein expression of PPARα and PPARγ in the liver. These data provide a basis for further investigation of the role of PMF in improving IR through regulating adipocytokines and activating PPARs in human subjects and for testing dietary or therapeutic reagents for the prevention or treatment of IR.

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