Original Research

Rice protein isolate improves lipid and glucose homeostasis in rats fed high fat/high cholesterol diets

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Abstract

In order to understand the molecular mechanisms underlying effects of feeding rice protein on lipid and glucose homeostasis, weanling rats were fed AIN-93G diets made with casein or rice protein isolate (RPI) for 14 d. Peroxisome proliferator-activated receptor (PPAR)α genes and proteins involved in fatty acid degradation were upregulated by feeding RPI ($P < 0.05$), accompanied by increased promoter binding and nuclear expression of PPARα and its heterodimerization partner retinoid X receptor ($P < 0.05$). Effects of RPI feeding on hepatic PPARγ signaling were significant but less robust. Feeding RPI also increased hepatic genes involved in cholesterol metabolism and transport. However, feeding RPI had no effect on binding of liver X-receptor (LXR)α to the cytochrome P450 (CYP)7A1 promoter. The effect of RPI feeding on PPARα signaling appeared to be direct and was reversed when RPI diets were switched to casein. In another experiment, male Sprague–Dawley rats were fed casein diets from postnatal day (PND) 24 to PND64 or were fed high fat 'Western' diets containing 0.5% cholesterol made with either casein or RPI. Increased liver triglyceride content, hypercholesterolemia and insulin resistance in the 'Western' diet-fed rats were partially prevented by feeding RPI ($P < 0.05$). mRNA and protein expression of hepatic enzymes involved in fatty acid synthesis were suppressed by feeding 'Western diets' containing RPI ($P < 0.05$), despite a lack of effects on nuclear concentrations of sterol regulatory element binding protein-1c. These data suggest that attenuation of metabolic syndrome observed in RPI-fed rats after consumption of diets high in fat and cholesterol occur as a result of improved lipid and glucose homeostasis partly as a result of activation of PPARs.

Keywords: rice, metabolic syndrome, lipid, cholesterol, PPAR, insulin resistance

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Introduction

Cancers of the breast, prostate and colon and cardiovascular disease are all considered Western diseases with significantly lower incidence in certain Asian countries compared with the United States.[1] Diet is considered an important factor responsible for the increased risk of these diseases in Western countries and protection in Asian populations.[1–3] Traditional Asian diets in countries such as Japan and China contain more fish, vegetables, rice and soy and less red meat and fat than the American diet. Of these diet components, soy and soy-associated phytochemicals such as isoflavones have received considerable attention for their potential improved insulin sensitivity and lipid homeostasis and protection against cardiovascular disease and metabolic syndrome.[1,3–6] However, rice is consumed by these populations in considerably larger quantities than soy. Rice and rice products are increasingly recognized as also having health beneficial properties in relationship to cancer and cardiovascular disease.[7–14] Most of these studies have focused on phytochemicals such as tocotrienols and oryzanols found in rice bran, bran oil and colored rice.[15–18] Much less attention has been paid to the potential health effects of rice protein isolates (RPI) and phytochemicals bound to RPI, even though such isolates are in widespread use in the food industry.[7,9,19,20] RPI has been reported to have protective effects against chemically induced mammary carcinogenesis in animal models[7] and RPI-associated phytochemicals have anti-tumor activity against human tumor cell lines *in vitro*.[8] In addition, feeding RPI has been suggested to improve lipid homeostasis and protect against atherosclerosis.[9,10]

In the current report, we studied the metabolic and endocrine effects of feeding RPI to rats during early development in comparison to feeding diets where casein was the sole protein source. Growth, body composition and the growth hormone-insulin-like growth factor (GH-IGF-1) axis were

assessed. In addition, expression of hepatic genes involved in fatty acid, cholesterol and glucose homeostasis, and the expression and activation of three nuclear receptors, peroxisome proliferator-activated receptor (PPAR)α, PPARγ and liver X-receptor (LXR)α known to be involved in the regulation of these genes, were all studied. Finally, we examined the effects of RPI early in life on the physiological, endocrine and metabolic responses associated with consumption of a high fat, high cholesterol 'Western' diet.

Materials and methods

Materials

RPI was a gift from Riceland Foods Inc. (Stuttgart, AR, USA). RPI was produced from rice germ using a proprietary process similar to that previously published[7] and employed amylase hydrolysis of starch to syrup. The syrup was removed through consecutive water washes and the remaining protein dried. Although processing of bran from germ removes some phytochemicals such as oryzanols, others such as vitamin E derivatives are enriched in rice germ.[13] We have previously published the profile of phytochemicals, which remain bound to RPI after processing. This consists mainly of a wide variety of fatty acid and phospholipid metabolites.[8] All the chemicals, unless otherwise noted, were purchased from Sigma Aldrich (St Louis, MO, USA). For Western blotting, goat polyclonal antibodies against rat cytochrome P450 (CYP)7A1 (sc-14423), goat polyclonal antibodies against rat SKl-1/S1P (sc-9785), mouse monoclonal antibodies against human sterol regulatory element binding protein (SREBP)-1 (sc-13551) and rabbit polyclonal antibodies against retinoid X receptor (RXR) (sc-553) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sheep polyclonal antibodies specific for rat liver CYP4A1 were generated as previously described[21] and were a gift from the late Dr Gordon Gibson, (University of Surrey, Surrey, UK). Rabbit polyclonal antibodies against PPARα and CD36 were from Novus Biologicals (Littleton, CO, USA). Rabbit polyclonal antibodies against fatty acid synthase (FASN) and PPARγ were from Cell Signaling (Danvers, MA, USA). Mouse monoclonal antibodies against actin and rabbit polyclonal antibodies against proliferating cell nuclear antigen (PCNA) were from Sigma and Santa Cruz Biotechnology, respectively. Mouse monoclonal antibodies to rat CYP2C11 were the gift of Dr Paul Thomas (Rutgers University, Newark, NJ).[22] A rabbit polyclonal antibody was raised against a peptide coding for a unique region of rat LXRα: NH2-PRVSSPPQVLPQLSP-OH (Bio synthesis Inc, Lewisville, TX, USA).[6]

Animals and diets

The experiments received prior approval from the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (UAMS). Weanling and time-impregnated Sprague–Dawley rats were obtained from Charles River (Wilmington, MA, USA) and were housed in polycarbonate cages in a Association of Laboratory Animal Care-approved animal facility in an environmentally controlled room at 22°C with a 12 h light/dark cycle.

Experiment 1: Effects of RPI feeding on body composition, PPAR and LXR-mediated gene expression during early development

To determine if feeding RPI affected body composition and pathways regulating lipid and glucose metabolism during early development, time-impregnated female rats were given *ad libitum* access to water and AIN-93G diets formulated with casein as the sole protein source (CAS) from gestational day 4 (GED4).[6] At birth, litters were culled to five male and female female pups per litter. Dams continued to be fed CAS throughout lactation. Groups of $n = 10$ pups of each sex selected at random from CAS-fed litters were then weaned onto either the same CAS diet or onto diets where casein was replaced by RPI. Both diets were made according to the AIN-93G formulation (Table 1) and were isonitrogenous. Different essential amino acids were added to achieve National Research Council recommended concentrations, and corn oil was used in place of soy oil.[6] Pups were scarified on postnatal day (PND) 33. Serum and liver were collected and stored at -70°C until use. The GH-IGF-1 axis and expression of PPAR- and LXR-regulated genes involved in fatty acid degradation, glucose homeostasis and cholesterol metabolism and transport were assessed.

Experiment 2: Direct effects of RPI diets versus metabolic imprinting

To determine if the effects of RPI diet on PPARα-regulated fatty acid degradation were direct effects of dietary components that would disappear when RPI consumption ended, or if PPARα activation would persist as the result of metabolic programming during early development, pregnant rats (GED4) were randomly assigned to either CAS- or RPI-based AIN-93G diets (Table 1) throughout gestation and lactation ($n = 6$/group). At birth litters were culled to six male and six female pups per litter. At weaning on PND21, one male pup from each litter was either weaned onto their respective diet or was switched from RPI to CAS. Rats were sacrificed on PND33 and expression of the PPARα-regulated hepatic gene CYP4A1 was measured by realtime reverse transcription-polymerase chain reaction (RT-PCR).

Experiment 3: Effects of RPI on metabolic syndrome produced as a result of feeding 'Western diets'

To determine if RPI feeding could protect against the development of obesity and metabolic sequelae resulting from consumption of a typical high fat/high cholesterol 'Western diet' in early development, male rats ($n = 10$) were purchased from Charles River on PND24 and had *ad libitum* access to water and the CAS AIN-93G diet (Table 1, Casein), from PND24 until PND64. Additional groups of rats ($n = 10$) were pair-fed a high fat/high cholesterol 'Western' diet (Harlen Teklad TD88137) containing 42% of energy from lipids, mainly from milk fat and 0.5% cholesterol made with casein ('Western casein') or with

Table 1 Diet composition

	AIN-93G		High fat/high cholesterol 'Western diet'	
Nutrition information	**CAS**	**RPI**	**CAS**	**RPI**
Energy (kcal/g)	4.34	4.34	4.50	4.50
Protein	Casein	RPI	Casein	RPI
% Energy	19	19	15	15
Amino acids (g/kg)*				
L-Cysteine	3.00	0	0	0
DL-Methionine	0	1.50	3.00	3.00
L-Lysine HCl	0	4.41		4.41
Threonine	0	0.19		0.19
Fat	Corn oil	Corn oil	Anhydrous milk fat	Anhydrous milk fat
% Energy	17	17	42	42
Carbohydrate†				
% Energy	64	64	43	43
Ratio	3.9:1.3:1	3.9:1.3:1	1:0:2.3	1:0:2.3
Cholesterol (g/kg)	0	0	5	5
Cellulose fiber (g/kg)	45.4	45.4	50.0	50.0
Vitamin mix	AIN-93-VX	AIN-93-VX	Teklad (40060)	Teklad (40060)
Choline bitartrate (g/kg)	2.5	2.5		
Mineral mix	AIN-93-MX	AIN-93-MX	AIN-76-MX	AIN-76-MX
Calcium carbonate (g/kg)	0	0	4.0	4.0
Antioxidant (g/kg)	TBHQ (0.014)	TBHQ (0.014)	Ethoxyquin (0.04)	Ethoxyquin (0.04)

CAS, casein; RPI, rice protein isolate; TBHQ, *tert*-butylhydroquinone
*Individual amino acids supplemented to meet National Research Council recommendations
†Corn starch:maltodextrin:sucrose

RPI ('Western RPI') as the sole protein source (Table 1). Oral glucose tolerance tests were performed on PND57. After the rats were sacrificed on PND64, serum and liver were collected and stored at -70°C until use for endocrine and metabolic analysis as described below.

Body composition analysis

Body composition was assessed via whole animal body composition by nuclear magnetic resonance (Echo Medical Systems, Houston, TX, USA) performed in conscious unanesthetized rats[23] and using postmortem dissected weights of retroperitoneal fat pads. Liver sections were stained for lipid droplets using a 60% Oil red O stock solution as described previously.[24]

Endocrine and biochemical analyses

Serum from Experiment 1 was assayed for IGF-1 by radioimmunoassay using a kit from Diagnostic Systems Laboratories (Webster, TX, USA). Pituitary GH content was assessed by radioimmunoassay of pituitary homogenates as described previously.[25] 16α-Hydroxylation of testosterone was measured in hepatic microsomes from Experiment 1 using [^{14}C]testosterone and the high-performance thin-layer chromatography method of Ronis *et al.*[26]

Seven days prior to sacrifice, five rats from each group in Experiment 3 were given an oral glucose tolerance test.[23] Serum insulin concentrations were measured in tail bleeds using an enzyme-linked immunosorbent assay for rat insulin (Linco Research, St Charles, MO, USA). Serum insulin and serum glucose were also measured at sacrifice. Glucose was assayed using the glucose oxidase method (Synermed, Westfield, IN, USA). Serum triglycerides and total cholesterol were measured using commercially available reagents (Synermed, Westfield, IN, USA). Lipids were extracted from liver homogenates with chloroform/methanol (2:1 v/v) and triglycerides and cholesterol concentrations assayed as described above for serum.

Western immunoblot analysis

Hepatic microsomes were prepared by differential ultracentrifugation.[26] Nuclear extracts were prepared using a Cell Lytic kit (Sigma). Microsomes were analyzed for the expression of CYP2C11, CYP4A1 and CYP7A1 apoprotein and CD36. PPARα, RXR and SREBP-1c protein expression was assessed in nuclear extracts. FASN and SKI-1/S1P proteins were immunoquantitated from whole liver homogenates. Even loading of protein onto gels was confirmed by Coomassie blue protein staining of duplicate gels. Loading variation of proteins across gels was $\pm 3\%$. Nuclear SREBP-1c was normalized to PCNA.

Realtime RT-PCR analysis of mRNA expression

Realtime RT-PCR was conducted on $n = 3$ mRNA preparations each isolated from liver tissue prepared by pooling tissue from three livers selected at random from each diet group in Experiments 1 and 3 and from $n = 6$ individual livers/group in Experiment 2 as described previously[24] using gene-specific probes with previously published primer sequences,[6,24] except for the following genes: delta 6-desaturase – 5′-CCG TGG CTT TTT CTT CTC TCA-3′ Forward, 5′-CTT TCC GCC CTT CTC TTT GA-3′ Reverse; 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase – 5′-CTT GGT TTC TGG CTC TTT CAG

AGA-3′ Forward, 5′-AAC GAG CTG CAG TCA CAT CTG T-3′ Reverse; and NPC1L1 – 5′-TGC CAG CTG TGA GGA CCT TC-3′ Forward, 5′-TGC CTC TTG CTA TCC AGG GA-3′ Reverse. Glyceraldehyde 3-phosphate dehydrogenase or cyclophilin amplicons were used to normalize the values. No differences between the groups were observed in the expression of these housekeeping genes.

Electrophoretic mobility shift analysis and chromatin immunoprecipitation analysis

Nuclear extracts were prepared by pooling tissue from three livers selected at random from each diet group in Experiments 1 and 3. These nuclear extracts which represent $N = 3$ pools derived from nine rats/diet group were used for electrophoretic mobility shift analysis (EMSA) of PPARα binding to the peroxisome proliferator response element (PPRE) on the acyl Co-A oxidase (ACO) gene as described previously in our laboratory.[27] Chromatin immunoprecipitation (ChIP) analysis was utilized to assess PPARγ binding to the PPRE element in the promoter of the glucokinase gene and of LXRα binding to the LXR response element in the promoter of the CYP7A1 gene as described by Ronis *et al.*[6] ChIP was carried out using a ChIP assay kit (ChIP-IT™, Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± standard error of mean. For Experiments 1 and 3, since the feeding experiments were initiated after weaning, the individual rat was used as the unit of measure ($n = 10$), except for realtime RT-PCR analysis of hepatic gene expression and for EMSA and ChIP analysis. In this case, nuclear extracts each pooled from $n = 3$ rats/diet group were used in the analysis and for statistical comparisons $n = 3$. In Experiment 2, since diet manipulations occurred during gestation and lactation, treatment groups were each derived from $n = 6$ litters and the litter was used as the unit of measure. All data were analyzed using Sigma Stat for Windows (version 3.5 Systat Software Inc, San Jose, CA, USA). For multiple group comparisons, the data in Experiment 1 were analyzed by two-way analysis of variance (ANOVA) and the data in Experiments 2 and 3 by one-way ANOVA. Where variances were unequal such as in the case of FASN expression, data were analyzed by ANOVA on ranks. Differences among means were determined by the Student–Neuman–Keuls test and were considered significant if $P < 0.05$.

Results

Experiment 1

Growth and body composition

In Experiment 1, decreased mean body weight, weight gain and slightly reduced fat pad weight was observed on PND33 after weaning male rats onto AIN-93G diets made with RPI compared with those fed CAS ($P < 0.05$) (Table 2). These effects were less pronounced in female rats. Decreased weight gain coincided with disruption of GH signaling after RPI consumption, since pituitary GH and serum IGF-1 concentrations were reduced ($P < 0.05$) in rats fed RPI compared with rats fed CAS. Furthermore, the expression and activity of male hepatic CYP2C11, which is dependent on male patterns of GH secretion[25] were reduced ($P < 0.05$) relative to the CAS group after RPI feeding (Table 2, Figure 1).

Table 2 Differences in growth and GH-axis signaling in rats fed CAS or RPI*

	CAS	RPI
Male		
Final body weight (g)	118.00 ± 4.00	103.00 ± 3.90†
Body weight gain (g/d)	3.90 ± 0.10	3.20 ± 0.12†
Peri-renal adipose tissue (g/100 g body wt.)	0.16 ± 0.01	0.13 ± 0.01†
Plasma IGF-1 (mg/L)	860.00 ± 52.00	655.20 ± 64.70†
Anterior pituitary GH (ng/mg protein)	6500.00 ± 600.00	4400.00 ± 1100.00
Liver weight (%)	4.64 ± 0.10	4.52 ± 0.18
Testosterone 16α-hydroxylase (nmol/mg/min)	2.70 ± 0.60	0.74 ± 0.20†
CYP2C11 apoprotein‡	1.00 ± 0.09	0.19 ± 6.10†
CYP2C11 mRNA¶	1.00 ± 0.19	0.24 ± 9.20†
Female		
Final body weight (g)	101.00 ± 3.00#	93.00 ± 2.00†,#
Body weight gain (g/d)	3.10 ± 0.20#	2.70 ± 0.12#
Peri-renal adipose tissue (g/100 g body wt.)	0.17 ± 0.04	0.13 ± 0.01
Plasma IGF-1 (mg/L)	1047.00 ± 68.00#	803.90 ± 71.10†
Anterior pituitary GH (ng/mg protein)	7200.00 ± 600.00	4900.00 ± 500.00†

CAS, casein; GH, growth hormone; RPI, rice protein isolate; IGF-1, insulin-like growth factor-1; CYP, cytochrome P450
*Values are means ± standard error of mean, $n = 10$ per group
†Different from corresponding value in CAS, $P < 0.05$
‡Immunoquantitation of Western blots of CYP2C11 apoprotein expression in hepatic microsomes relative to CAS, which was set at 1.0
¶Expression of CYP2C11 mRNA normalized to GADPH (housekeeping gene) expressed relative to CAS, which was set at 1.0
#Male and female different, $P < 0.05$

PPARα-regulated pathways

As shown in Table 3, the expression of hepatic genes involved in peroxisomal fatty acid ω-oxidation (CYP4A1);

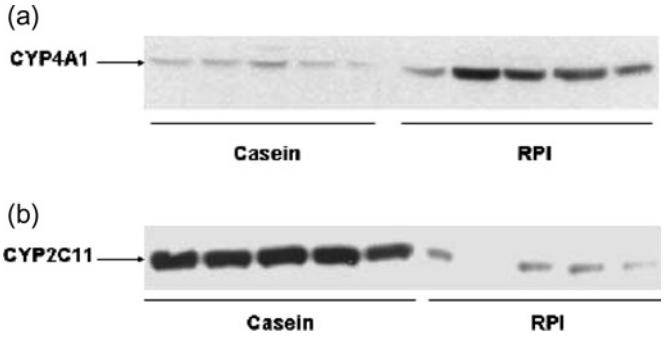

Figure 1 Effects of feeding casein or RPI for 14 d beginning at weaning on expression of hepatic microsomal CYP4A1 (a) and CYP2C11 (b) in male rats in Experiment 1. Representative Western blots. Each lane represents a single animal. CYP, cytochrome P450; RPI, rice protein isolate

Table 3 PPARα-regulated pathways in rat liver are upregulated by feeding RPI

Gene name	Function	Relative expression	
		CAS	RPI
CYP4A1 mRNA[†] ω-hydroxylation	Microsomal		
Male		1.00 ± 0.21	4.66 ± 1.05*
Female		0.85 ± 0.25	4.66 ± 1.05*
CYP4A1 apoprotein[‡]			
Male		1.00 ± 0.12	4.21 ± 0.29*
Female		0.96 ± 0.10	3.57 ± 0.55*
Acyl Co-A oxidase mRNA[†]	Peroxisomal β-oxidation		
Male		1.00 ± 0.06	1.78 ± 0.18*
Female		1.33 ± 0.13	2.05 ± 0.20*
Carnitine palmitoyltransferase 1A mRNA[†]	Fatty acid transport into mitochondria		
Male		1.00 ± 0.15	4.75 ± 0.25*
Female		1.12 ± 0.05	6.40 ± 0.19*
Mitochondrial trifunctional protein α-subunit (HADHA) mRNA[†]	Mitochondrial β-oxidation		
Male		1.00 ± 0.12	1.46 ± 0.09*
Female		1.16 ± 0.06	1.69 ± 0.10*
Delta 6-desaturase mRNA[†]	Fatty acid desaturase		
Male		1.00 ± 0.06	1.78 ± 0.09*
Female		1.24 ± 0.11	1.88 ± 0.18*
PPARα mRNA[†]			
Male		1.00 ± 0.08	2.76 ± 0.30*
Female		0.70 ± 0.05	1.85 ± 0.10*

HADHA, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase; CAS, casein; PPARα, peroxisome proliferator-activated receptor casein α; RPI, rice protein isolate; IGF-1, insulin-like growth factor-1; CYP, cytochrome P450

*Different from corresponding value in CAS, $P < 0.05$

[†]Gene expression data are expressed as (gene expression/expression of the housekeeping gene GAPDH) relative to mean expression in CAS male liver = 1. Data are mean ± standard error of mean, $n = 3$ mRNA pools each derived from the livers of three rats fed CAS or RPI

[‡]Immunoquantification of Western blots using hepatic microsomes relative to mean expression in CAS male liver = 1. Data are mean ± standard error of mean for $N = 10$/group

peroxisomal and mitochondrial fatty acid β-oxidation (ACO and the mitochondrial trifunctional protein α-subunit [HADHA]); fatty acid transport into the mitochondria (carnitine palmitoyl transferase 1A); and fatty acid desaturation (delta 6-desaturase) were all elevated in RPI-fed male and female rats relative to rats fed CAS in Experiment 1 ($P < 0.05$). All these genes are known to be positively regulated by the transcription factor PPARα.[6,24,28,29] Elevation of the microsomal CYP4A1 protein after feeding RPI ($P < 0.05$) was confirmed in male liver by Western immunoblot (Figure 1, Table 2). EMSA analysis of protein binding to the PPRE element in the ACO promoter demonstrated that PPARα binding was increased two- to three-fold in both male and female rats fed RPI in this experiment ($P < 0.05$) (Figure 2a). Feeding RPI also increased the expression of hepatic PPARα mRNA in both sexes ($P < 0.05$) (Table 3). PPARα protein and protein expression of its heterodimerization partner RXR[28,29] in hepatic nuclear extracts were higher in CAS-fed females than males and were elevated in both sexes after RPI feeding ($P < 0.05$) (Figure 2b and c).

PPARγ-regulated pathways

Glucokinase, a key enzyme involved in the regulation of glucose metabolism and CD36, a scavenger receptor involved in hepatic fatty acid uptake, are two hepatic genes known to be regulated by PPARγ.[30,31] Both genes were expressed more highly in female than male liver ($P < 0.05$) (Table 4). Consumption of RPI in Experiment 1 increased expression of both genes in males relative to rats fed casein ($P < 0.05$). In females, glucokinase mRNA was lower, but CD36 mRNA was higher after RPI feeding. Increased mean expression of two proteins of similar molecular weight recognized by antibodies to CD36 was observed in liver microsomes from RPI-fed male rats by Western immunoblot (Figure 3a). ChIP analysis showed that feeding RPI in males modestly increased PPARγ binding to its response element on the glucokinase promoter relative to male rats fed casein and increased expression of hepatic PPARγ mRNA ($P < 0.05$) (Figure 3b, Table 4).

Cholesterol homeostasis

The expression of genes involved in the maintenance of cholesterol homeostasis was also examined *in* Experiment 1. These included HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis;[32] the CYP450 enzyme CYP7A1, which catalyzes cholesterol 7α-hydroxylation, the rate-limiting step in the formation of bile acids;[33] the sterol transporters ABCA1, ABCG5 and ABCG8;[34] and the newly characterized intestinal cholesterol transporter NPC1L1.[35] The data are presented in Table 5. Hepatic HMG-CoA mRNA expression was increased ($P < 0.05$) only in RPI-fed males. The expression of CYP7A1 and ABCG8 mRNA was increased by feeding RPI in both sexes ($P < 0.05$). In females, RPI feeding also increased expression of hepatic ABCA1 and ABCA5 mRNA ($P < 0.05$). No effects of RPI were observed on the expression of NPC1L1 mRNA in the jejunum of either sex. Induction of CYP7A1 by feeding RPI was confirmed at the apoprotein level by Western immunoblot (Figure 4a). Since CYP7A1 and the ABC transporters can all be regulated via the transcription factor LXRα, ChIP analysis of LXRα binding to a response element identified in the CYP7A1 promoter was conducted. RPI did not significantly affect LXRα binding (Figure 4b).

Experiment 2

To determine if exposure to RPI diets during gestation and lactation were capable of imprinting PPARα signaling, we measured the expression of CYP4A1 mRNA in the liver of male rats on PND33 from Experiment 2 in which animals were fed CAS or RPI throughout development or in rat pups whose dams were fed RPI diet during gestation and lactation and who were switched to CAS at weaning. Only rats fed RPI throughout development had significantly induced CYP4A1 ($P < 0.05$) (Figure 5).

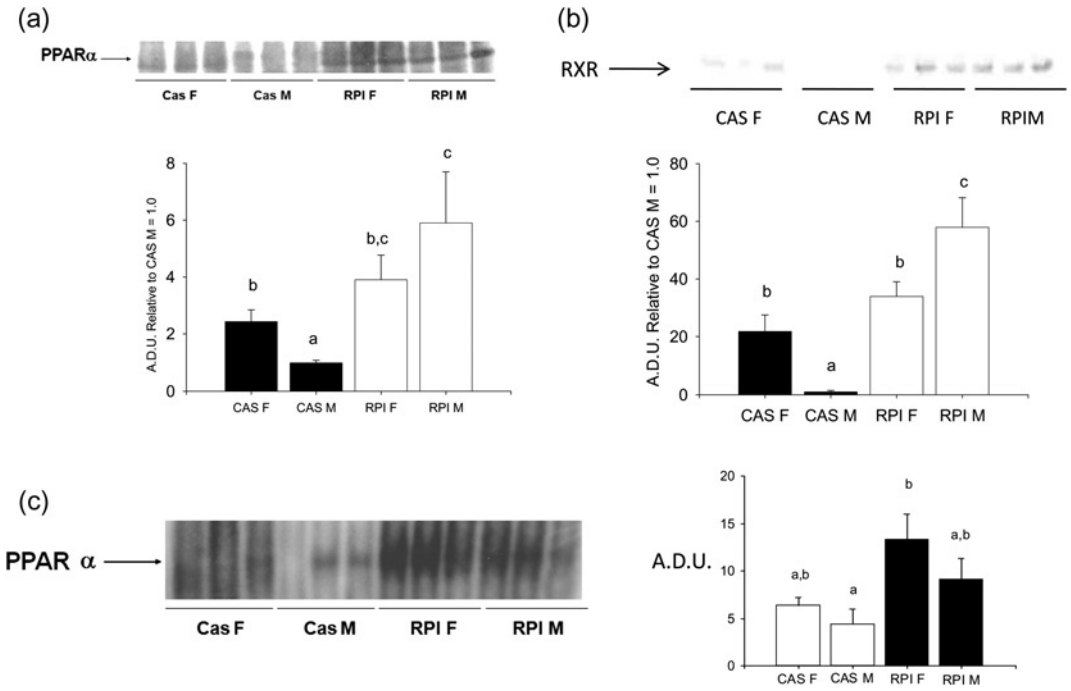

Figure 2 Effects of feeding CAS or RPI for 14 d beginning at weaning on activation of hepatic PPARα signaling in male (M) and female (F) rats in Experiment 1. (a) Expression of PPARα protein in Western blots of nuclear extracts; (b) expression of RXR protein in Western blots of nuclear extracts; and (c) electrophoretic mobility shift assay of PPARα binding to the PPRE on the ACO promoter. Each lane represents samples pooled from three rats/group. Means without a common letter differ significantly, a < b < c using two-way ANOVA followed by Student–Neuman–Keuls *post hoc* analysis. CAS, casein; RPI, rice protein isolate; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; ACO, acyl Co-A oxidase

Experiment 3

Insulin sensitivity and lipid homeostasis after feeding 'Western diets'

As we have reported previously,[6] feeding a 'Western casein' diet to weanling male rats from PND24 to PND64 (Experiment 3) did increase weight gain, % body fat and % liver weight relative to the Casein-fed AIN-93G control group ($P < 0.05$) (Table 6). Feeding a 'Western RPI' diet provided no protection against the effects of fat and cholesterol on weight gain or body composition. Analysis of oral glucose tolerance test after 33 d of 'Western casein' diet feeding demonstrated increased serum insulin at 60, 90 and 150 min postglucose challenge compared with the Casein control or 'Western RPI' diet groups ($P < 0.05$) (Figure 6). Area under the insulin/time curve was 92.3 $\pm$ 43 ng/mL (150 min) (Casein control) versus 171 $\pm$ 54 ng/mL (150 min) ('Western casein') versus 62 $\pm$ 8.6 ng/mL (150 min ('Western RPI). Insulin sensitivity was greater in the 'Western RPI' diet group. This was accompanied by partial reversal of the increased plasma glucose observed in the 'Western casein' group relative to the Casein control group (Table 6). Feeding 'Western casein' diet increased serum and hepatic triglyceride and cholesterol concentrations and liver weight, relative to Casein-fed controls ($P < 0.05$) (Table 6). Feeding 'Western RPI' diet significantly reduced serum cholesterol and hepatic triglyceride content and liver weight relative to feeding the 'Western casein' diet, but increased serum triglycerides ($P < 0.05$) (Table 5, Figure 7a–c).

Table 4 Effects of feeding CAS or RPI on PPARγ-regulated pathways in rat liver

Gene name	Function	Relative expression* CAS	RPI
Glucokinase	Glucose sensor, regulator of hepatic glucose		
Male		1.00 $\pm$ 0.24	18.50 $\pm$ 1.50†
Female		5.43 $\pm$ 0.38‡	2.10 $\pm$ 0.20†
CD36	Scavenger receptor, fatty acid transporter		
Male		1.00 $\pm$ 0.06	4.50 $\pm$ 0.25†
Female		3.53 $\pm$ 0.28‡	5.36 $\pm$ 0.15†
PPARγ			
Male		1.00 $\pm$ 0.08	2.25 $\pm$ 0.30†
Female		1.20 $\pm$ 0.10	1.30 $\pm$ 0.25

CAS, casein; PPARα, peroxisome proliferator-activated receptor casein α; RPI, rice protein isolate

*Gene expression data are expressed as (gene expression/expression of the housekeeping gene GAPDH) relative to mean expression in CAS male liver = 1. Data are mean $\pm$ standard error of mean, $n = 3$ mRNA pools each derived from the livers of three rats for CAS or RPI

†Different from corresponding value in CAS, $P < 0.05$

‡Male and female different, $P < 0.05$

Fatty acid synthesis and SREBP-1c activation

We also examined the effects of feeding the 'Western RPI' diet in comparison to feeding the 'Western casein' in Experiment 3 on activation of SREBP-1c, a transcription factor that regulates the expression of rate-limiting genes in fatty acid synthesis and desaturation such as FASN,

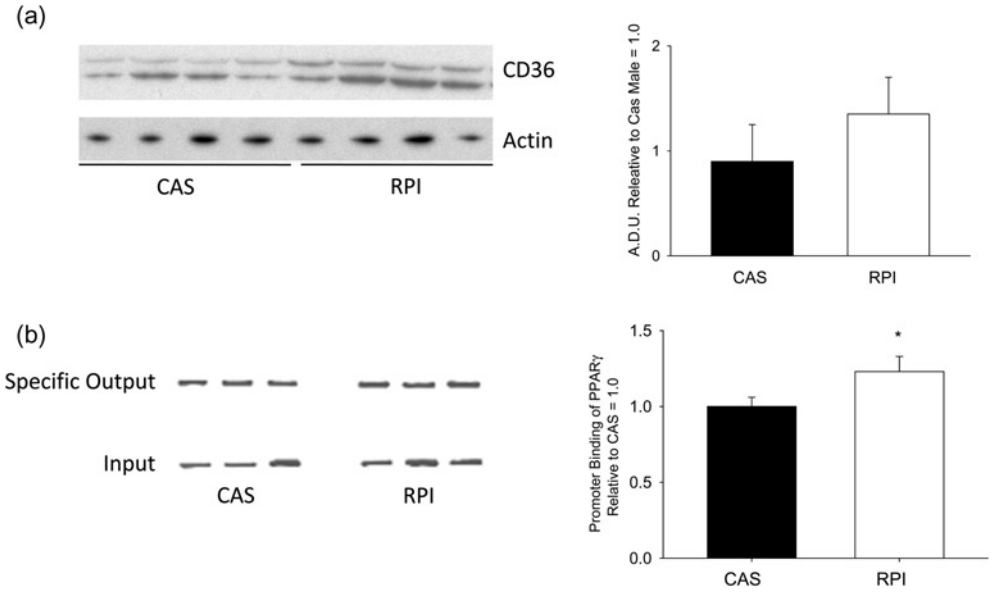

Figure 3 Effects of feeding CAS or RPI for 14 d beginning at weaning on activation of hepatic PPARγ signaling in male rats in Experiment 1. (a) Western immunoblot analysis of CD36 protein expression in hepatic microsomal fractions. Each lane represents a single animal. Immunoquantitation data are mean $\pm$ SEM relative to CAS = 1.0. (b) Chromatin immunoprecipitation analysis of PPARγ binding to the PPRE on the glucokinase promoter. Each lane represents nuclear extracts pooled from $N = 3$ rats/group. Data are mean $\pm$ SEM specific output/input relative to CAS = 1.0. *Statistically significant, $P < 0.05$. CAS, casein; RPI, rice protein isolate; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; ACO, acyl Co-A oxidase; SEM, standard error of mean

acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase-1 (SCD1).[36] The expression of mRNAs for ACC1 and SCD1 were reduced in the liver of the 'Western RPI' group compared with the 'Western casein' group ($P < 0.05$) (Table 7). Changes in hepatic FASN mRNA expression were smaller, but a 50% reduction in hepatic FASN protein was observed in the 'Western RPI' group in Western immunoblots compared with both casein groups (Figure 7d). Realtime RT-PCR analysis and Western immunoblot analysis demonstrated a small increase in hepatic expression of the serine protease SKI-1/S1P, which is responsible for processing of SREBP-1c from an inactive form tethered to the cytoskeleton to a smaller molecular weight 'activated' form which migrates to the nucleus, in the 'Western casein' group relative to Casein controls ($P < 0.05$) (Table 7, Figure 7e). However, no effects of either Western diet or RPI feeding were observed on nuclear expression of processed SREBP-1c (Table 7, Figure 7f).

Discussion

Cardiovascular disease risk is lower in populations consuming traditional Asian diets rich in vegetable proteins from beans such as soy and grains such as rice.[1] Moreover, it has been suggested that low carbohydrate plant-based diets have lipid-lowering advantages and improve heart disease risk factors compared with conventional low fat diets with animal products.[37,38] Much attention has focused on improvements in body composition, lipid homeostasis and insulin sensitization produced by soy protein isolates (SPIs) and soy-associated phytochemicals such as isoflavones.[3–6] In contrast, less attention has been paid to similar effects

Table 5 Effects of feeding CAS or RPI on cholesterol metabolism and transport

Gene name	Function	Relative expression* CAS	RPI
Liver			
HMG-CoA reductase	Cholesterol synthesis		
Male	1.00 ± 0.11	$1.40 \pm 0.15^{\dagger}$	
Female	1.15 ± 0.15	1.38 ± 0.10	
CYP7A1	Cholesterol 7α-hydroxylase, bile acid synthesis		
Male	1.00 ± 0.18	$6.40 \pm 1.60^{\dagger}$	
Female	1.45 ± 0.28	$5.30 \pm 1.45^{\dagger}$	
ABCA1	Cholesterol efflux pump		
Male	1.00 ± 0.15	1.20 ± 0.13	
Female	0.72 ± 0.10	$1.87 \pm 0.08^{\dagger}$	
ABCG5	Cholesterol transport into bile		
Male	1.00 ± 0.19	1.25 ± 0.12	
Female	$0.40 \pm 0.04^{\ddagger}$	$1.90 \pm 0.15^{\dagger\ddagger}$	
ABCG8	Cholesterol transport into bile		
Male	1.00 ± 0.15	$5.71 \pm 0.20^{\dagger}$	
Female	1.65 ± 0.25	$5.00 \pm 0.40^{\dagger}$	
Jejunum			
NPC1L1	Intestinal cholesterol transport		
Male	1.00 ± 0.17	0.85 ± 0.04	
Female	1.19 ± 0.19	0.86 ± 0.10	

CAS, casein; PPARα, peroxisome proliferator-activated receptor casein α; RPI, rice protein isolate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA
*Gene expression data are expressed as (gene expression/expression of the housekeeping gene GAPDH) relative to mean expression in CAS male liver = 1. Data are mean $\pm$ standard error of mean, $n = 3$ mRNA pools each derived from the livers of three rats for CAS or RPI
$^{\dagger}P < 0.05$. Diet groups as follows: CAS and RPI
‡Male and female different, $P < 0.05$

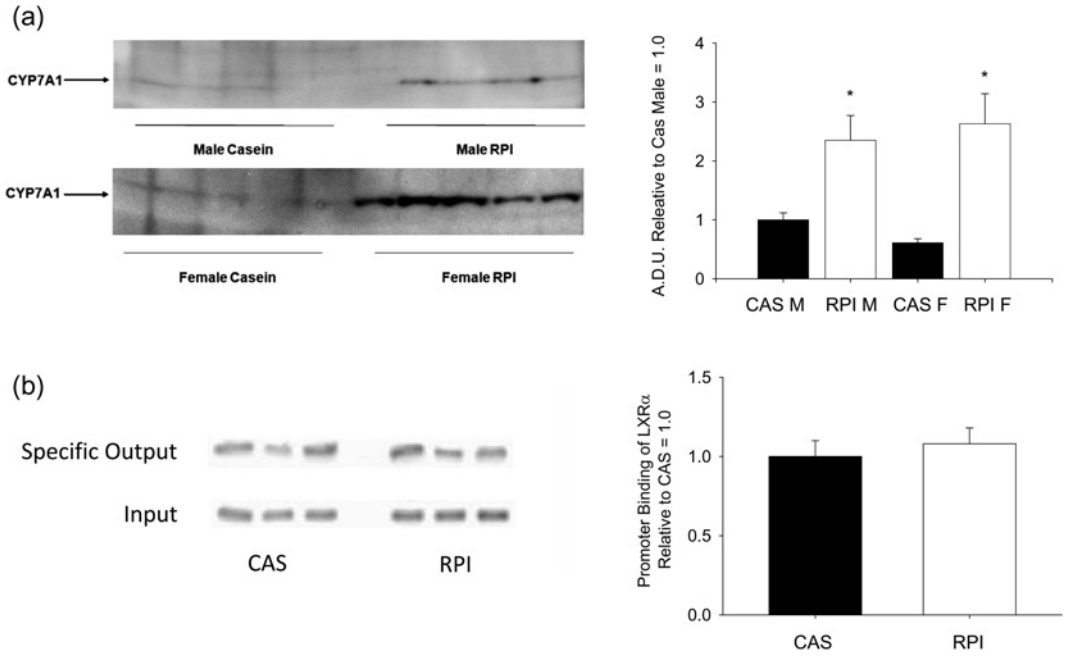

Figure 4 Effects of feeding CAS or RPI for 14 d beginning at weaning on cholesterol metabolism and LXRα activation in Experiment 1. (a) Western immunoblot analysis of CYP7A1 apoprotein expression in hepatic microsomes. Each lane represents a single animal. Immunoquantitation data are mean $\pm$ SEM relative to CAS = 1.0. *Statistically significant, $P < 0.05$. (b) Chromatin immunoprecipitation analysis of LXRα binding its response element on the CYP7A1 promoter. Each lane represents nuclear extracts pooled from $N = 3$ rats/group. Data are mean $\pm$ SEM specific output/input relative to CAS = 1.0. CAS, casein; RPI, rice protein isolate; liver X-receptor; CYP, cytochrome P450; SEM, standard error of mean

produced by rice; rice-based food products such as RPI; rice bran; bran oils; and rice-associated phytochemicals despite the significant consumption of rice products among low-cardiovascular risk populations.[9–12,14,16–18] In the current study, we assessed the potential for feeding RPI, which has previously been suggested to have anti-carcinogenic, anti-atherogenic and cholesterol-lowering properties[7,8,11,12,19] to affect body composition, lipid and glucose homeostasis in growing rats compared with those fed standard AIN-93G diets made with the animal protein casein and in rats developing metabolic syndrome in response to consumption of high fat/high cholesterol 'Western' diets.

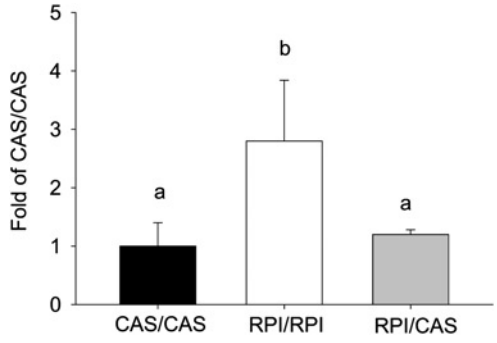

Figure 5 Effects of feeding casein (CAS/CAS) or rice protein isolate (RPI/RPI) from gestational day 4 or switching from an RPI diet to a CAS diet at weaning (RPI/CAS) on expression of hepatic CYP4A1 mRNA in male rat liver on PND33 in Experiment 2. Data represent realtime RT-PCR values for CYP4A1 mRNA/GAPDH mRNA relative to the CAS/CAS value = 1.0. Means without a common letter differ significantly, a < b using one-way ANOVA followed by Student–Neuman–Keuls *post hoc* analysis. CYP, cytochrome P450; PND, postnatal day; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance

Table 6 Effects of feeding CAS or RPI on physiological and metabolic responses of male rats to Western diets*

Diet group	Casein	Western casein	Western RPI
Final body weight (g)	388.00 ± 4.10^a	439.00 ± 4.80^b	439.00 ± 12.30^b
Body weight gain (g/d)	8.10 ± 0.10^a	9.20 ± 0.1^b	9.30 ± 0.10^b
Lean body mass (%)	78.40 ± 0.9^b	72.00 ± 0.30^a	72.60 ± 0.33^a
Body fat mass (%)	14.80 ± 0.8^a	19.00 ± 0.30^b	19.10 ± 0.60^b
Peri-renal adipose tissue (g/100 g body wt.)	0.91 ± 0.08^a	1.60 ± 0.14^b	1.20 ± 0.18^b
Liver			
Relative weight (g/100 g body wt.)	4.10 ± 0.12^a	4.70 ± 0.17^b	4.28 ± 0.21^a
Triglycerides (μmol/g wet tissue)	44.70 ± 12.70^a	100.60 ± 14.40^b	43.70 ± 7.0^a
Total cholesterol (μmol/g wet tissue)	51.00 ± 10.00	80.00 ± 22.00	50.00 ± 11.00
Serum			
Triglyceride (mmol/L)	3.40 ± 0.20^a	4.80 ± 0.24^a	6.23 ± 0.32^b
Total cholesterol (mmol/L)	2.10 ± 0.33^a	4.40 ± 1.00^b	2.30 ± 0.55^a
Glucose (mmol/L)	4.50 ± 0.13^a	5.10 ± 0.17^b	$4.80 \pm 0.33^{a,b}$
Insulin (mmol/L)	1.80 ± 0.40	1.70 ± 0.20	1.40 ± 0.50

CAS, casein; PPARα, peroxisome proliferator-activated receptor casein α; RPI, rice protein isolate

*Effects of Western diet in CAS-fed rats as reported previously.[6] Values are mean $\pm$ standard error of mean, $n = 7–10$/group. Means in a row with superscripts without a common letter differ, $P < 0.05$

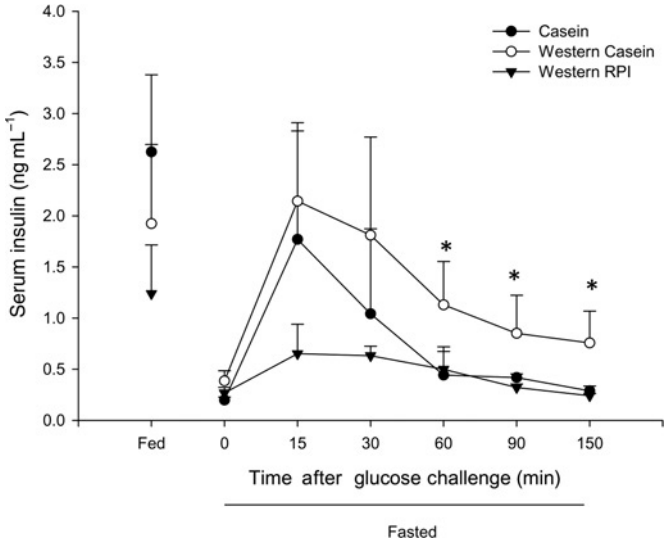

Figure 6 Insulin-sensitizing effects of RPI when fed as the sole protein source in high fat/high cholesterol 'Western' diet from weaning until PND64 in Experiment 3. Serum insulin concentrations after OGTT in male rats fed casein or casein 'Western' diets pair-fed to rats fed RPI 'Western' diets. Data are mean ± SEM for $N = 10$ rats killed at 8:00 or following an oral glucose challenge with 2.5 g/kg. *Differs significantly from casein and RPI 'Western' groups, $P < 0.05$. RPI, rice protein isolate; SEM, standard error of mean; PND, postnatal day; OGTT, oral glucose tolerance test

Table 7 SREBP-1c-regulated fatty acid synthesis in the liver of male rats fed Western diets with CAS or RPI as the protein source*

Diet group	Casein	Western casein	Western RPI
	Fold of casein		
SREBP processing			
SKI/S1P mRNA†	1.00 ± 0.20^{a}	1.90 ± 0.35^{b}	1.78 ± 0.19^{b}
SKI/S1P protein‡	1.00 ± 0.11^{a}	1.39 ± 0.10^{b}	1.30 ± 0.30^{b}
SREBP-1c protein‡	1.00 ± 0.24	1.19 ± 0.23	1.02 ± 0.27
Fatty acid synthesis			
FASN mRNA†	1.00 ± 0.16	0.93 ± 0.21	0.68 ± 0.12
FASN protein‡	1.00 ± 0.08^{b}	1.05 ± 0.24^{b}	0.54 ± 0.17^{a}
ACC1 mRNA‡	1.00 ± 0.13^{b}	1.22 ± 0.16^{b}	0.50 ± 0.07^{a}
SCD1 mRNA‡	1.00 ± 0.25^{b}	2.68 ± 0.49^{c}	0.32 ± 0.04^{a}

SREBP, sterol regulatory element binding protein; CAS, casein; RPI, rice protein isolate; FASN, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD1, stearoyl-CoA desaturase-1

*Values are means ± standard error of mean, $n = 7–10$. Means in a row with superscripts without a common letter differ, $P < 0.05$, a < b < c

†mRNA normalized to cyclophilin (housekeeping gene) and expressed relative to CAS, which was set at 1.0

‡Immunoquantitation of Western blots by densitometric scanning using PCNA to normalize expression of SREBP-1c in nuclear extracts and actin to normalize expression of FASN and SKI/S1P in whole homogenates expressed relative to casein, which was set at 1.0

Consumption of RPI produced small decreases in weight gain and adiposity when rats were weaned onto this diet in comparison to CAS in Experiment 1. This was accompanied by evidence of reduced GH-IGF-1 signaling in early development. In contrast, longer periods of RPI feeding beginning at weaning in the context of high fat/high cholesterol diets in Experiment 3 had no effect on body composition despite improved lipid profiles and evidence of insulin sensitization. This is in contrast to improvements in body composition observed in a similar study using SPI as the sole protein source in 'Western' diets.[6] The difference

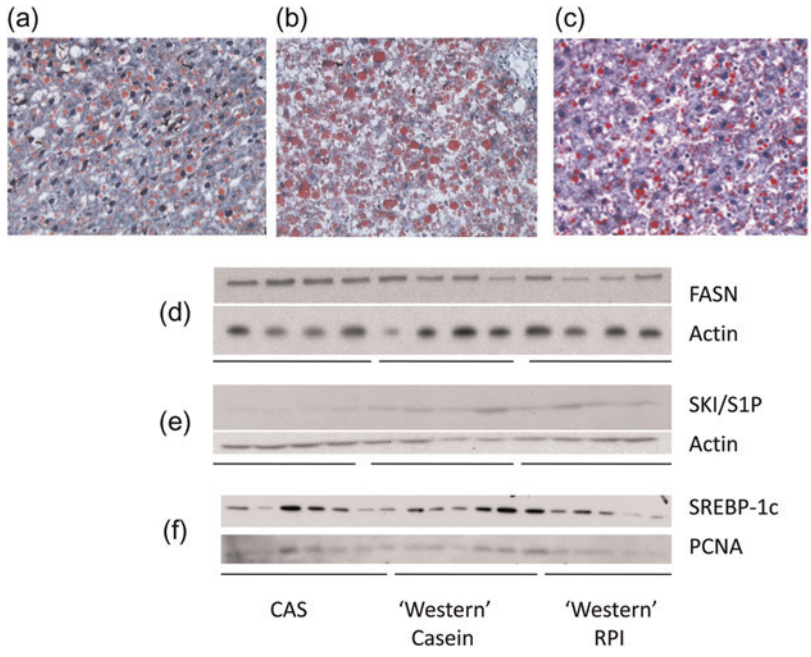

Figure 7 Effects of feeding 'Western' diets containing casein or RPI on hepatic steatosis and SREBP-regulated fatty acid synthesis in Experiment 3. Oil red O staining of fat droplets in representative 10× liver sections from PND64 male rats fed casein (a); 'Western casein' diet (b) or 'Western RPI' diet (c). (d) Representative Western blots of FASN protein expression in liver homogenates from Experiment 3. (e) Representative Western blots of SKI/S1P protein expression in liver homogenates from Experiment 3. (f) Representative Western blots of mature SREBP-1 protein expression in nuclear extracts from livers in Experiment 3. Each lane in (d)–(f) represents samples from one rat/treatment. RPI, rice protein isolate; SREBP, sterol regulatory element binding protein; PND, postnatal day; FASN, fatty acid synthase

between the two plant protein sources may be related to the unique phytochemical profile of SPI compared with RPI.[9,39] Soy-associated isoflavones such as genistein have been shown to reduce adult obesity as a result of estrogenic actions on genes regulating lipogenesis, lipolysis and adipogenesis and effects on AMP-activated protein kinase signaling in muscle and adipose tissue.[40–42] In contrast, RPI contains no isoflavones. RPI is prepared from rice germ and although this processing of the bran component reduces the concentrations of some phytochemicals such as oryzanols previously suggested to have anti-atherosclerotic and hypocholesterolemic properties,[9,10,16] other phytochemicals such as vitamin E derivatives are found at higher concentrations in the germ.[13] We have previously published analytical data demonstrating that the major phytochemicals associated with RPI are fatty acids, lysoglycero-phospholipids and fatty acid esters.[9]

Despite lack of effects on body composition, RPI consumption significantly improved both lipid and glucose homeostasis in rats developing obesity and metabolic syndrome after feeding 'Western' high fat/high cholesterol diets during development compared with rats fed similar diets made with CAS. RPI consumption reversed increases in steatosis and hepatic content of triglycerides. Moreover, a partial reversal of increases in serum glucose was accompanied by evidence of increased insulin sensitivity in oral glucose tolerance tests. Decreases in hepatic triglyceride content are consistent with our data suggesting that RPI activates hepatic PPARα-mediated fatty acid degradation and interferes with hepatic fatty acid synthesis. All aspects of PPARα signaling, downstream gene targets, binding to PPRE promoter elements and expression of both the transcription factor itself and its heterodimerization partner RXR were elevated in the livers of RPI-fed rats of both sexes. This appears to be a direct effect associated with some component of RPI rather than an imprinted metabolic effect in early development, since in Experiment 2, combined gestational and neonatal exposure to RPI via the dam resulted in no effects on hepatic expression of the PPARα target gene CYP4A1 in the male offspring on PND33 when RPI was switched to CAS diet at weaning. It is unclear if the effects of RPI on hepatic PPARα signaling are mediated via protein/peptide components or phytochemicals associated with RPI. However, PPARs are known to be activated by fatty acids and fatty acid metabolites,[43] and such compounds are known to be the major phytochemicals bound to RPI.[9] Despite consistent data suggesting that RPI feeding produces insulin sensitization, the molecular mechanisms underlying this effect are not so clear. Evidence for activation of PPARγ signaling by RPI, which would be a potential mechanism for anti-diabetic effects, was less compelling than for activation of PPARα. Induction of hepatic PPARγ target genes was not consistent and was complicated by sexual dimorphism. Moreover, ChIP analysis demonstrated only a modest $\sim$20% increase in PPARγ binding to the PPRE on the glucokinase promoter in male rats fed RPI compared with those fed CAS. It is possible that PPARγ signaling in muscle and adipose tissue is affected by RPI feeding. This remains to be investigated.

Consistent with a previous report,[12] RPI consumption reduced serum and hepatic cholesterol content. This is consistent with increased conversion of cholesterol to bile acids by CYP7A1 and reverse transport via the ABC transporters expression, all of which were increased in RPI-fed rats. However, in contrast to similar effects on these LXRα-regulated genes observed in our laboratory in 'Western' diet fed rats where SPI was the sole protein source,[6] in the current study, there was no evidence for increased activation of hepatic LXRα signaling. ChIP analysis demonstrated no increase in LXR binding to its response element on the CYP7A1 promoter after RPI feeding. It is possible that RPI consumption affected binding of co-activator or co-repressor proteins to enhance expression of LXR-regulated genes without affecting LXR binding itself. Alternatively, RPI may act via other transcription factors such as FXR, which also regulate cholesterol homeostasis.[44] This also requires further studies.

Interestingly, feeding RPI inhibited expression of hepatic SREBP-1c target genes involved in fatty acid synthesis and desaturation despite a lack of effect on nuclear concentrations of the transcription factor. It is possible that post-translational modifications of the transcription factor such as acetylation, sumoylation or phosphorylation might be affected by RPI feeding, which could alter DNA binding.[45,46] Alternatively, RPI feeding might alter the activity or expression of co-activators or co-repressors such as PPARγ co-activator-1 to alter SREBP-1c signaling independent of the transcription factor itself.[47]

In conclusion, we have demonstrated that feeding RPI-containing diets to prepubertal rats results in increased expression of hepatic genes regulated by the nuclear receptor PPARα and decreased fatty acid synthesis. These effects may partially explain the improved hepatic lipid profile in rats fed 'Western' diets where RPI replaced CAS as the sole protein source. In addition, RPI consumption improved glucose and cholesterol homeostasis in these rats, although by mechanisms which as yet remain unclear. Thus, consumption of rice protein products may improve metabolic and endocrine risk factors associated with cardiovascular disease and fatty liver disease in obese populations eating typical Western diets high in fat and cholesterol.

Author contributions: MJR supervised all aspects of the project and wrote the manuscript. JB and YC conducted all the laboratory work including establishment of several new assays and helped edit the manuscript. TMB is Director of the ACNC. He initiated research at the center using RPI, was responsible for advising Riceland Inc on RPI manufacturing procedures, was responsible for diet formulation and edited the manuscript.

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