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ARTICLE

Soy protein isoflavones differentially regulate liver X receptor isoforms to modulate lipid metabolism and cholesterol transport in the liver and intestine in mice

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Abstract
Aims/hypothesis Liver X receptor (LXR)α regulates the genes involved in cholesterol, fatty acid and glucose metabolism. Soy protein (SP) consumption reduces the hepatic accumulation of cholesterol and triacylglycerol, and improves insulin sensitivity. However, it is not known whether these effects are mediated via LXRα. We therefore investigated whether the consumption of SP regulates metabolic changes in cholesterol metabolism and insulin sensitivity via LXRα.
Methods Wild-type (WT) and *Lxrα*$^{-/-}$ (*Lxrα*, also known as *Nr1h3*) mice were fed an SP diet with or without cholesterol for 28 days. The expression of LXRα target genes was measured in liver and intestine, as were hepatic lipid content and faecal bile acid concentration. Oral glucose and insulin tolerance tests were also performed. Hepatocytes were used to study the effect of isoflavones on LXR activity.
Results The livers of WT and *Lxrα*$^{-/-}$ mice fed an SP high-cholesterol diet showed less steatosis than those fed casein. The SP diet increased the expression of the ATP-binding cassette (ABC) sub-family genes *Abca1*, *Abcg5* and *Abcg8* in the liver and intestine, as well as increasing total faecal bile acid excretion and insulin sensitivity in WT mice compared with mice fed a casein diet. However, these effects of SP were not observed in *Lxrα*$^{-/-}$ mice. The SP isoflavone, genistein, repressed the activation of LXRα target genes by T0901317, whereas it stimulated the activation of LXRβ target genes. The AMP-activated protein kinase inhibitor, compound C, had the opposite effects to those of genistein.
Conclusions/interpretation Our results suggest that SP isoflavones stimulate the phosphorylation of LXRα or LXRβ, resulting in different biological effects for each LXR isoform.

Keywords AMPK · Cholesterol · Insulin resistance · Intestine · Isoflavones · Liver · LXRα · LXRβ · Mice · Soy protein

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Abbreviations

ABC	ATP-binding cassette
ACC	Acetyl-CoA carboxylase
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
ITT	Insulin tolerance test
LXR	Liver X receptor
LXRE	LXR response element
RCT	Reverse cholesterol transport
SP	Soy protein
WT	Wild-type

Introduction

Obesity, a major public health problem around the world [1, 2], is associated with several metabolic abnormalities, which include hypertension, hypercholesterolaemia, hypertriacylglycerolaemia and insulin resistance [3, 4], and lead to type 2 diabetes and cardiovascular disease [5–8]. Serum cholesterol levels are clinically managed through the use of statins [9]. However, a number of dietary modifications have been suggested to reduce serum cholesterol concentrations [10].

Several meta-analyses have shown that the consumption of soy protein (SP) reduces total and LDL-cholesterol [11, 12]. Studies in experimental animals have also demonstrated that SP reduces blood lipids, hepatic cholesterol and triacylglycerol [13–15], and also increases insulin sensitivity [16]. The precise mechanism by which SP reduces serum and hepatic cholesterol has not been established, although it has been suggested that these effects occur through an increase in bile acid excretion [17].

The inter-organ cholesterol flux and the synthesis of bile acids from cholesterol are in part regulated by the transcription factor, liver X receptor (LXR)α [18, 19]. LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily [20]. The LXR subfamily consists of two isoforms, LXRα and LXRβ, which form obligate heterodimers with the retinoid X receptor and regulate gene expression by binding to LXR response elements (LXREs) in the promoter regions of their target genes [21], some of which are involved in reverse cholesterol transport (RCT) [22]. LXRα is highly abundant in the liver, intestine, kidney, adipose tissue and macrophages, whereas LXRβ is ubiquitously produced [23]. However, LXRα is the dominant isoform in the liver, the activation of which increases biliary cholesterol secretion and limits cholesterol absorption [24]. Recent evidence suggests that LXRα activity can be regulated by phosphorylation [25, 26]. There is evidence that isoflavones are weak ligands for certain other nuclear receptors [27–29], but it is not known whether the consumption of SP or its isoflavones (mainly genistein and daidzein) modulates transcriptional control of LXRs or regulates the phosphorylation of these nuclear receptors.

Our aim, therefore, was to use wild-type (WT) and $Lxr\alpha^{-/-}$ (*Lxrα* also known as *Nr1h3*) mice and investigate whether metabolic changes that increase bile acid excretion after the consumption of an SP diet are mediated via LXRα, thus leading to the upregulation of genes involved in bile acid synthesis and RCT in the liver and intestine. Our study also sought to determine whether isoflavones are able to activate LXRα, directly or indirectly, and whether LXRα can mediate the transcriptional effects of SP on the regulation of genes involved in fatty acid synthesis, RCT and insulin sensitivity.

Methods

Animals, diet formulation and feeding Male $Lxr\alpha^{-/-}$ mice were obtained from Gustafsson's laboratory. These mice were backcrossed for ten generations in C57BL/6J mice [30]. C57BL/6J control mice were purchased from Taconic Europe (Lille Skensved, Denmark). Male mice at 3 to 4 months of age had free access to water and one of the experimental diets. These isocaloric diets, the composition and sources of which are shown in electronic supplementary material (ESM) Table 1, were administered in dry form. The isolated SP used in these studies had 88% purity. The $Lxr\alpha^{-/-}$ or WT mice were divided into four experimental groups as follows (n=10 each): (1) 20% casein; (2) 20% casein plus 2% cholesterol; (3) 20% SP; and (4) 20% SP plus 2% cholesterol. The animals were housed in microisolators with a 12 h light/dark cycle and received the experimental diets for 28 days. At the end of the study, the animals were fasted for 8 h, killed by carbon dioxide inhalation and decapitated. The blood was collected and serum, obtained by centrifugation at 1,000 *g*, stored at −70°C until further analysis. Liver, ileum and gall bladder were frozen in liquid nitrogen and stored at −70°C until further analysis. The animal protocol was approved by the Animal Committee of the National Institute of Medical Sciences and Nutrition, Mexico City.

Cholesterol and triacylglycerol analysis Liver lipids were extracted with chloroform-methanol (0.09 g of tissue) according to the method described by Folch [31]. Cholesterol and triacylglycerol in serum and liver were measured with an enzymatic colorimetric commercial kit (DiaSys Diagnostic Systems, Holzheim, Germany) in a chemistry analyser (RA-50; Technicon Ames, Tarrytown, NY, USA).

RNA isolation and quantitative PCR The total RNA from liver and ileum was extracted as described by Chomczynski and Sacchi [32]. Total RNA was reverse-transcribed and PCR amplification performed (Applied Biosystems, Foster

City, CA, USA) using TaqMan assays (Applied Biosystems). Assays for each gene were carried out in triplicate in 96-well optical plates with a sequence detection system (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). β-Actin was used as the invariant control for liver and intestine analyses.

Histological analysis Liver sections were obtained, fixed by immersion in 10% formaldehyde (vol/vol) dissolved in phosphate buffer and subsequently dehydrated and embedded in paraffin. Sections (3 μm width) were obtained and stained with haematoxylin and eosin.

Fatty acid analysis Total lipids from the liver were extracted as described by Folch [31] and the fatty acids then methylated as previously described [33]. The methylated fatty acids were analysed by gas chromatography (Agilent 6850; Agilent, Santa Clara, CA, USA) with flame ionisation detector, (Agilent)using an HP-1 capillary column (J&W Scientific, Albany, CA, USA).

Bile acid measurements The amounts of bile acid were determined from the gall bladder (~0.05 ml bile per animal) and faeces. For measurement of faecal bile acid excretion, stools from WT and *Lxrα*$^{-/-}$ mice were collected during the final 3 days of the study, and dried, weighed and ground. The bile acid was derivatised as described by Keller and Jahreis [34]. The trimethylsilyl bile acids were analysed by gas chromatography (Agilent 6850 with flame ionisation detector) using a capillary column (Innowax; J&W Scientific) as previously described [34].

Cell culture and co-transfections HepG2 cells were grown in DMEM, with glucose (25 mmol/l), 10% fetal bovine serum, penicillin (200 IU/ml) and streptomycin (100 mg/ml), in a humidified CO_2 incubator at 37°C. Cells were co-transfected with the empty expression vector or an expression vector containing *Lxrα* or *Lxrβ*, along with a reporter vector containing three repeats of the consensus LXRE cloned in pGL3 Basic (Promega, Madison, WI, USA). Cells were seeded in 24-well plates, co-transfected for 8 h and genistein or daidzein added at the concentrations indicated. The synthetic ligand, GW3965 (Enzo Life Sciences, Farmingdale, NY, USA), and the natural oxysterol, 22(R)-hydroxycholesterol (Sigma-Aldrich, St. Louis, MO, USA), were used as positive controls. After 16 h of incubation, the cells were collected and lysed. The luciferase activity was measured using a commercial luciferase assay kit (luciferin-ATP; BioThema, Umeå, Sweden) and a luminometer (Infinite 200; Tecan, San Jose, CA, USA).

Culture of primary mouse hepatocytes and transfection Mouse hepatocytes were isolated by the collagenase perfusion technique and separated from non-parenchymal liver cells by centrifugation at 325 *g* [35]. On day 0, primary hepatocytes were plated in a six-well plate (9.6 cm^2/well) (Corning CellBIND, Tewksbury, MA, USA). On day 1, mouse *Lxrα* or *Lxrβ* (also known as *Nr1h2*) expression vectors (400 ng) were transfected using a transfection reagent (FuGENE HD; Roche Diagnostics, Mannheim, Germany). At 4 h after transfection, genistein or daidzein (15 μmol/l), and/or 10 μmol/l T0901317 were added. Total RNA from the hepatocytes was obtained using Trizol reagent.

Protein extraction and western blotting Primary mouse hepatocytes were homogenised in lysis protein RIPA buffer containing 1 mmol/l sodium fluoride, 2 mmol/l sodium orthovanadate and complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany). Total protein (30 μg) was loaded on 8% polyacrylamide gels, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with non-fat dry milk (Bio-Rad, Hercules, CA, USA) and incubated overnight at 4°C with the following primary antibodies: anti acetyl-CoA carboxylase (ACC) and anti phospho-ACC at Ser79 (pACC) (Millipore, Temecula, CA, USA), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bands were analysed using ImageJ 1.42p digital imaging processing software (http://rsb.info.nih.gov/ij/March/27/2012).

OGTT and intraperitoneal insulin tolerance tests Mice were fasted for 6 h before the OGTT and insulin tolerance test (ITT). The OGTT was performed by administering glucose (1.0 g/kg body weight) by gavage. Insulin (0.8 unit/kg) was injected intraperitoneally. Blood samples were obtained via tail nick and glucose was measured with a glucometer (OneTouch Ultra Accu-Chek Sensor; Roche Diagnostics). The AUC values were calculated as follows: [(glc)T1+(glc)T2)*(T2−T1)]/2 [36], where glc is glucose and T is time.

Statistical analysis The results are presented as the means ± SEM. The statistical analysis was performed using one-way ANOVA followed by Fisher's protected least-square difference test to determine significant differences between the groups. Differences were considered significant at $p<0.05$. Analysis was by Statview statistical analysis program, version 4.5 (Abacus Concepts, Berkeley, CA, USA).

Results

Effect of SP on body weight and serum lipids No difference in body weight was observed between WT and *Lxrα*$^{-/-}$ mouse groups after 28 days (ESM Table 2). However, the

liver weight of $Lxr\alpha^{-/-}$ mice fed diets with cholesterol was significantly higher than that of the other groups.

Serum cholesterol levels in all groups were comparable. The addition of cholesterol to the diets significantly increased serum cholesterol levels in $Lxr\alpha^{-/-}$, but not in WT mice (Fig. 1a). The profile of serum cholesterol particles in $Lxr\alpha^{-/-}$ mice showed a significant increase in the number of atherogenic particles in comparison with WT mice. In particular, WT mice fed SP had the lowest amount of atherogenic particles and the highest concentration of HDL particles (Fig. 1b). Serum triacylglycerol did not increase in $Lxr\alpha^{-/-}$ mice on either an SP or a casein diet (Fig. 1c).

Regulation of hepatic lipids by SP Macroscopically, livers of WT mice fed casein (ESM Fig. 1a) were pale compared with those fed SP (ESM Fig. 1b), this difference being augmented in $Lxr\alpha^{-/-}$ mice (ESM Fig. 1e, f). The addition of cholesterol to the SP or casein diets increased the fatty appearance (ESM Fig. 1c, d), particularly in $Lxr\alpha^{-/-}$ mice (ESM Fig. 1g, h).

The livers of WT mice fed high-cholesterol diets showed increased accumulation of cholesterol and triacylglycerol compared with WT mice on diets without cholesterol, although those fed SP had lower lipid levels (ESM Fig. 2i, j). The livers of $Lxr\alpha^{-/-}$ mice fed either SP or casein contained significantly higher concentrations of both lipids than the livers of corresponding WT mice. Dietary cholesterol dramatically increased the hepatic concentration of cholesterol and triacylglycerol in $Lxr\alpha^{-/-}$ mice (ESM Fig. 2i, j). $Lxr\alpha^{-/-}$ mice fed a casein diet exhibited 10.2- and 3.3-fold increased levels of hepatic cholesterol and triacylglycerol, respectively, compared with those fed a diet without cholesterol. In $Lxr\alpha^{-/-}$ mice fed an SP diet with cholesterol, the corresponding increases were 7.3- and 0.8-fold, respectively, compared with those on diets without cholesterol.

Effect of SP on hepatic histological abnormalities WT mice fed casein had some hepatocytes with small- and medium-sized cytoplasmic lipid vesicles (ESM Fig. 2a), with addition of cholesterol increasing the number of large lipid vesicles (ESM Fig. 2c). These histological changes were clearly smaller in WT mice fed the SP diet (ESM Fig. 2b, d). In contrast, hepatocytes of $Lxr\alpha^{-/-}$ mice fed casein only showed large lipid vesicles (ESM Fig. 2e); the addition of cholesterol to the diet produced a greater increase in the number of large lipid vesicles, resulting in severe hepatic steatosis, and abundant chronic inflammatory infiltrate and necrosis (ESM Fig. 2g). These abnormalities were associated with a dramatic increase (5.2-fold) in the serum levels of alanine transaminase (ALT) compared with $Lxr\alpha^{-/-}$ mice fed the casein diet without cholesterol (ESM Fig. 2k). Although these histological changes were clearly attenuated in $Lxr\alpha^{-/-}$ mice fed SP or SP with cholesterol (ESM Fig. 2f, h), the addition of cholesterol increased serum ALT levels by 4.9-fold (ESM Fig. 2k).

Regulation by SP of hepatic genes involved in bile acid synthesis and RCT On measuring the expression of genes that are responsible for the synthesis of bile acids and dependent on LXR, we observed significant differences in the *Cyp7a1* mRNA concentration between $Lxr\alpha^{-/-}$ mice and WT mice fed either the casein or SP diets (Fig. 2a). Whereas only the expression of *Cyp27a1* was increased in $Lxr\alpha^{-/-}$ mice fed diets without cholesterol, the addition of cholesterol repressed *Cyp27a1* expression in WT and $Lxr\alpha^{-/-}$ mice (Fig. 2b).

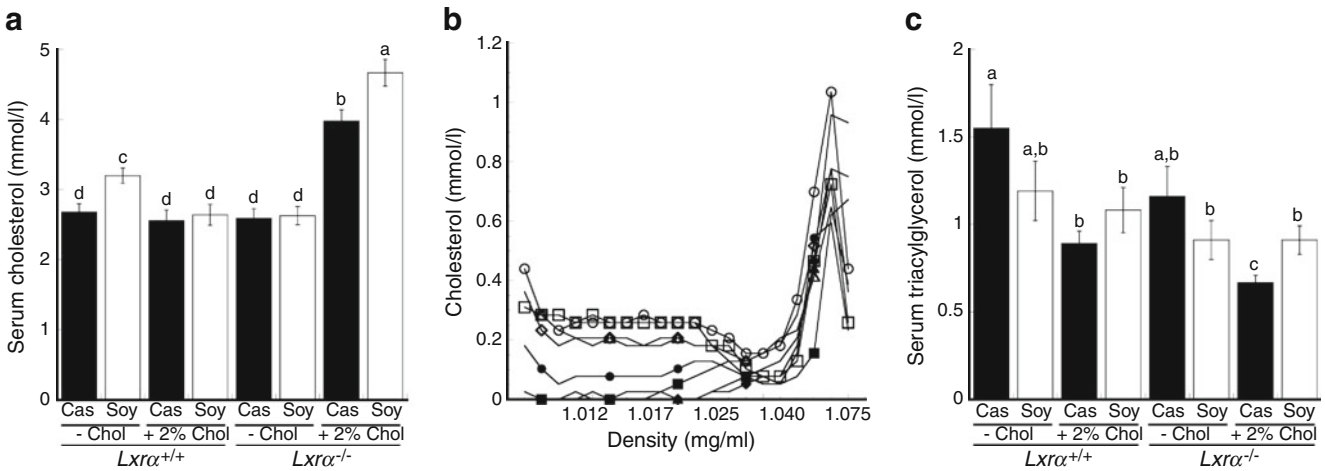

Fig. 1 Serum lipid concentrations in WT and $Lxr\alpha^{-/-}$ mice fed soy or casein (Cas) diets with or without cholesterol (Chol). (**a**) Serum cholesterol concentrations and (**b**) the lipoprotein profile of cholesterol particles; black diamonds, Soy $Lxr\alpha^{+/+}$; black triangles, Cas $Lxr\alpha^{+/+}$; black circles, Soy+Chol $Lxr\alpha^{+/+}$; black squares, Cas+Chol $Lxr\alpha^{+/+}$; white diamonds, Soy $Lxr\alpha^{-/-}$; white triangles, Cas $Lxr\alpha^{-/-}$; white circles, Soy+Chol $Lxr\alpha^{-/-}$; white squares, Cas+Chol $Lxr\alpha^{-/-}$. (**c**) Serum triacylglycerol concentrations. The mice were fed the experimental diets for 1 month and blood was obtained after overnight fasting. The results are expressed as means ± SEM; n=10 mice per group; mean values with different superscript letters within a row are significantly different (p<0.05)

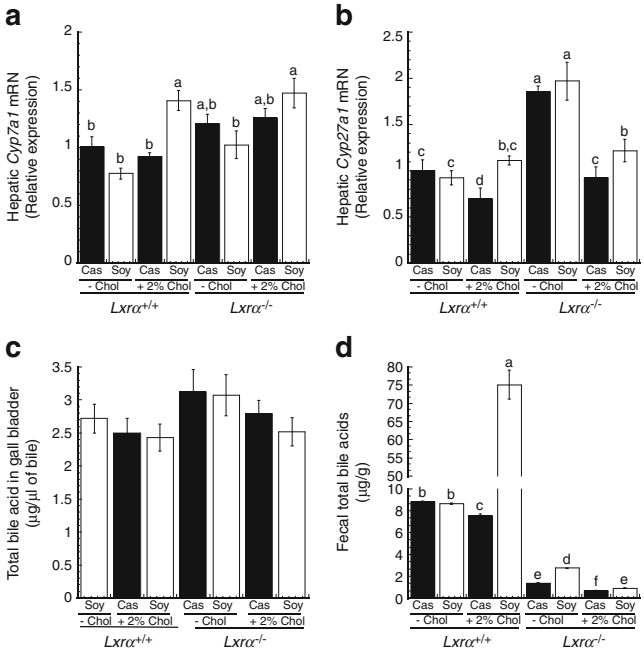

Fig. 2 Hepatic expression of genes encoding the enzymes of bile acid metabolism, and bile acid concentration in WT and $Lxr\alpha^{-/-}$ mice fed casein or SP diets with or without cholesterol (Chol). (**a**) *Cyp7a1* and (**b**) *Cyp27a1* mRNA expression. (**c**) Total bile acid concentration in bile and (**d**) total faecal bile acids. The results are expressed as means ± SEM; $n=10$ mice per group; mean values with different superscript letters within a row are significantly different ($p<0.05$)

We observed significant changes in the expression of cholesterol transporters. The addition of cholesterol to the diet in WT and $Lxr\alpha^{-/-}$ mice significantly increased the expression of the ATP-binding cassette (ABC) sub-family genes *Abca1*, *Abcg5* and *Abcg8*, with groups fed SP having the highest respective mRNA levels in the liver (ESM Fig. 3a–c). The expression of these transporters in $Lxr\alpha^{-/-}$ mice is possibly mediated by LXRβ. Analysis of the relative expression of these genes, based on the abundance of hepatic cholesterol and calculated as the ratio of the relative mRNA abundance and the hepatic cholesterol concentration, showed that $Lxr\alpha^{-/-}$ mice fed SP or casein had dramatically decreased expression upon the addition of dietary cholesterol in comparison with WT mice, indicating in the former an inability to remove cholesterol (ESM Fig. 3d–f).

There were no differences in the total amount of bile acids in the bile (Fig. 2c). However, faecal total bile acids excreted from WT mice on an SP diet with cholesterol were ninefold higher than in the control groups or in mice fed casein with cholesterol (Fig. 2d). These differences were not observed in the $Lxr\alpha^{-/-}$ groups.

SP regulates bile acid excretion and intestinal expression of RCT genes To understand this change in bile acid excretion, we measured the abundance of cholesterol and bile acid transporters in the ileum. The expression of *Fxr* (also known as *Nr1h4*), *Ibabp* (also known as *Fabp6*) and *Ibat* (also known as *Slc10a2*) decreased in WT mice fed high-cholesterol diets (ESM Fig. 4a–c), whereas $Lxr\alpha^{-/-}$ mice fed diets with or without cholesterol did not express these genes at levels above control levels. Interestingly, WT mice fed SP without cholesterol showed increased *Abcg5*, *Abcg8* and *Abca1* mRNA levels compared with those fed casein (ESM Fig. 4d–f). Moreover, the addition of cholesterol increased the expression of these genes by 111%, 23% and 40%, respectively, compared with expression in mice fed casein. These differences were abolished when $Lxr\alpha^{-/-}$ mice were fed the experimental diets with or without cholesterol.

Regulation of LXR isoform activity by SP isoflavones To explore whether soy isoflavones were responsible for activating production of the ABC transporters via LXRα or LXRβ, we conducted functional assays in HepG2 cells to analyse the effect of the soy isoflavones (genistein and daidzein) on the LXREs. The addition of daidzein or genistein did not increase luciferase activity compared with the respective controls, whereas the synthetic ligand, GW3965, and the natural oxysterol, 22(R)-hydroxycholesterol, significantly increased luciferase activity (ESM Fig. 5a, b). These data strongly suggest that the observed increase in the expression of genes encoding the ABC transporters was not mediated via a direct LXR agonist effect. Studies with LXRβ showed similar results (data not shown).

Next, we studied whether isoflavones could regulate LXRα indirectly. We showed that the incubation of hepatocytes with T0901317 increased *Srebp1* (also known as *Srebf1*) mRNA abundance by 18.4-fold. Interestingly, the hepatocytes transfected with the *Lxrα* vector incubated with daidzein or genistein significantly reduced the stimulatory effect of T0901317 by 40% and 51%, respectively (Fig. 3a). However, the repressive effect of isoflavones on *Srebp1* expression was not observed in hepatocytes transfected with the *Lxrβ* vector (Fig. 3c). Similar effects were observed for *Abca1*, another LXR target gene (Fig. 3b–d). Surprisingly, the response of the *Abcg5* and *Abcg8* genes was the opposite of that observed for *Srebp1* and *Abca1*. The incubation of stimulated transfected hepatocytes with the *Lxrβ* vector in the presence of isoflavones further stimulated the mRNA expression of *Abcg5* and *Abcg8*, particularly upon the addition of genistein (2.4- to 2.8-fold) (Fig. 4a–d). These data suggest that isoflavones may differentially regulate the expression of LXR target genes.

There is evidence that the activity of LXRα can be downregulated by phosphorylation of AMP-activated protein kinase (AMPK) [25]. We therefore used the AMPK inhibitor, compound C, to explore whether isoflavones could regulate the activity of LXRα via AMPK. As observed in Fig. 5a, the ability of T0901317 to increase *Srebp1*

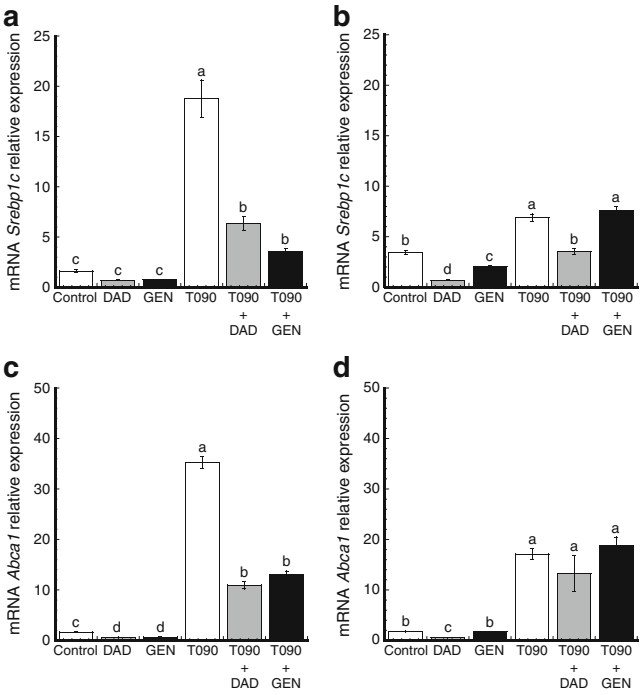

Fig. 3 The effect of soy isoflavones on the expression of *Srebp1* and *Abca1* in mouse hepatocytes overexpressing *Lxrα* or *Lxrβ*. The cells were transfected with the expression vector for (**a**, **c**) *Lxrα* and (**b**, **d**) or *Lxrβ*, and the hepatocytes were incubated with daidzein (DAD) or genistein (GEN), with or without the synthetic LXR ligand T0901317 (T090). The experiments were performed in quadruplicate; mean values with different superscript letters within a row are significantly different ($p<0.05$)

expression by activating LXRα was highly stimulated by the addition of compound C. Nonetheless, genistein was able to repress the synergistic effect of T0901317, even in the presence of compound C. Conversely, in hepatocytes overproducing LXRβ, genistein significantly increased the expression of *Abcg5* in the presence of T0901317 with compound C (Fig. 5b). We showed that genistein was able to activate AMPK, since this isoflavone increased the phosphorylation of ACC, a well-known target protein of AMPK. The addition of T0901317 did not alter ACC phosphorylation, while compound C, an inhibitor of AMPK, prevented the stimulatory effect of genistein on ACC phosphorylation (ESM Fig. 6a, b).

Regulation by SP of expression of genes involved in cholesterol and fatty acid synthesis We measured the mRNA levels of several genes involved in hepatic cholesterol and fatty acid metabolism in WT and *Lxrα*$^{-/-}$ mice. Our data show that the expression of *Srebp2* (also known as *Srebf2*) and HMG-CoA reductase in the livers of WT mice was significantly reduced in animals fed cholesterol diets (ESM Fig. 7d, e); however, mRNA levels for the LDL receptor were not significantly different among the WT groups (ESM Fig. 7f). Interestingly, *Lxrα*$^{-/-}$ mice fed casein or SP diets without cholesterol had significantly higher expression of *Srebp2* mRNA and its target genes, which encode HMG-CoA reductase and the LDL receptor, than the corresponding WT mice (ESM Fig. 7d, e). However, levels of *Srebp1* mRNA changed only slightly among WT groups (ESM Fig. 7a). Again, the expression of *Srebp1* and its target gene *Fasn* were significantly increased in the liver of *Lxrα*$^{-/-}$ mice fed diets without cholesterol, an effect that was repressed by the addition of cholesterol (ESM Fig. 7b).

Hepatic *Scd1* showed a different pattern of expression in WT and *Lxrα*$^{-/-}$ mice (ESM Fig. 7c). Nonetheless, there was no significant correlation between *Scd1* mRNA and the saturated:monounsaturated fatty acid ratio in the liver. This ratio was dependent upon the addition of cholesterol to the diets (Table 1). WT or *Lxrα*$^{-/-}$ mice fed diets with cholesterol had lower ratios than the corresponding groups fed diets without cholesterol. The ratios were determined mostly by the high concentration of oleate in the livers of mice fed high-cholesterol diets and were independent of the type of dietary protein (Table 1).

SP regulates insulin sensitivity There is strong evidence that the accumulation of lipids in muscle and liver is associated

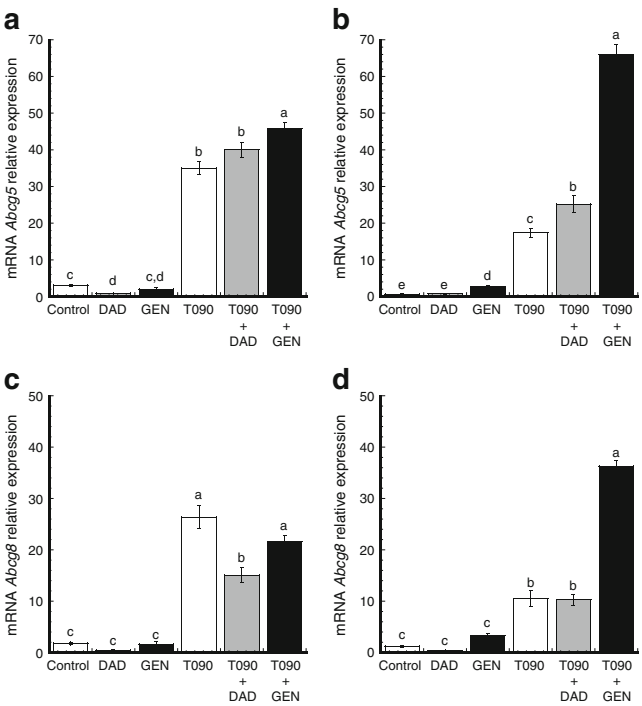

Fig. 4 The effect of soy isoflavones on the expression of *Abcg5* and *Abcg8* in mouse hepatocytes overexpressing *Lxrα* or *Lxrβ*. Cells were transfected with the expression vector for (**a**, **c**) *Lxrα* or (**b**, **d**) *Lxrβ*, and hepatocytes were incubated with daidzein (DAD) or genistein (GEN), with or without the synthetic LXR ligand T0901317 (T090). The experiments were performed in quadruplicate; mean values with different superscript letters within a row are significantly different ($p<0.05$)

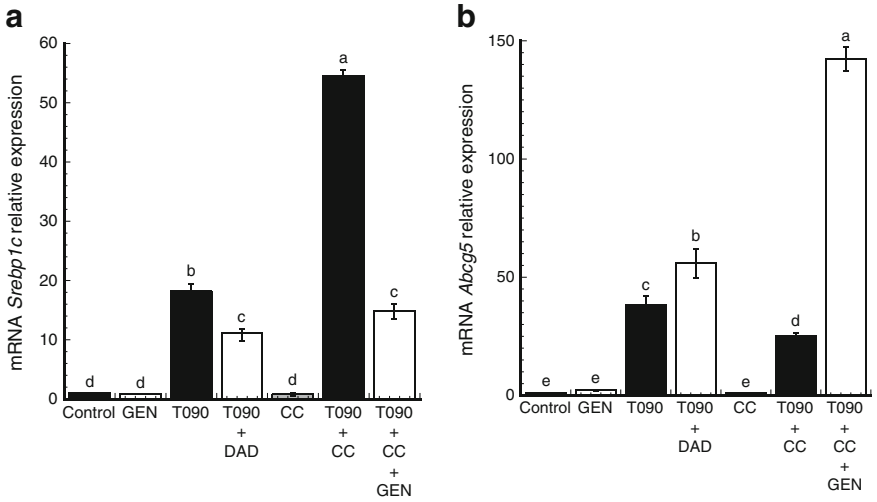

Fig. 5 (**a**) The effect of genistein on *Srebp1* expression in mouse hepatocytes overexpressing *Lxrα* and incubated with the synthetic LXR ligand, T0901317 (T090), and/or with compound C (CC), an inhibitor of AMPK. (**b**) The effect of genistein on the expression of *Abcg5* in mouse hepatocytes overexpressing *Lxrβ*; incubation as above (**a**). The experiments were performed in quadruplicate; mean values with different superscript letters within a row are significantly different ($p<0.05$)

with the development of insulin resistance [37]. Considering the elevated amounts of lipids in the livers of $Lxr\alpha^{-/-}$ mice fed casein or SP high-cholesterol diets, we investigated whether the type of dietary protein could differentially modify insulin sensitivity as measured by the OGTT and ITT. Our results showed that WT mice fed an SP high-cholesterol diet had lower fasting blood glucose levels than mice fed a casein high-cholesterol diet (Fig. 6a). Serum glucose disappearance following an oral glucose load in WT mice fed SP was significantly improved compared with mice fed casein (Fig. 6a, b). In addition, the ITT showed that glucose disappearance from the blood was improved by SP-fed mice compared with casein-fed mice (Fig. 6c) These data show that WT mice fed SP had better insulin sensitivity than the corresponding mice on a casein diet (Fig. 6d). However, in $Lxr\alpha^{-/-}$ mice fed casein or SP high-cholesterol diets, no significant differences were observed during the OGTT or ITT (Fig. 6a, c). $Lxr\alpha^{-/-}$ mice had a lower basal serum glucose concentration and improved insulin response compared with WT mice fed a casein high-cholesterol diet, suggesting that the presence of LXRα may partially affect insulin sensitivity in mice fed casein (Fig. 6d).

Discussion

The present study demonstrates that the consumption of an SP diet with cholesterol reduces the accumulation of hepatic lipids even in $Lxr\alpha^{-/-}$ mice. Macroscopic examination of the livers of WT mice fed SP cholesterol diets showed that

Table 1 Concentration of saturated and monounsaturated fatty acids in the liver of WT and $Lxr\alpha^{-/-}$ mice fed casein or SP diets with or without cholesterol for 28 days

	Wild-type				$Lxr\alpha^{-/-}$			
Variable	Cas	Soy	Cas+Chol	Soy+Chol	Cas	Soy	Cas+Chol	Soy+Chol
Palmitate (μg/g tissue)	$179\pm31^{b,c}$	$240\pm43^{a,b,c}$	$249\pm33^{a,b}$	$239\pm36^{a,b}$	108 ± 9^{d}	$135\pm21^{c,d}$	315 ± 20^{a}	296 ± 26^{a}
Palmitoleate (μg/g tissue)	$27\pm6^{b,c}$	92 ± 25^{b}	$81\pm11^{b,c}$	88 ± 21^{b}	15 ± 4^{c}	16 ± 4^{c}	239 ± 15^{a}	206 ± 20^{a}
Stearate (μg/g tissue)	$48\pm6^{a,b}$	63 ± 2^{a}	$50\pm8^{a,b}$	40 ± 3^{b}	$50\pm3^{a,b}$	$51\pm4^{a,b}$	$58\pm4^{a,b}$	63 ± 5^{a}
Oleate (μg/g tissue)	$238\pm42^{c,d}$	$355\pm80^{b,c}$	487 ± 68^{b}	$598\pm102^{a,b}$	112 ± 17^{d}	157 ± 35^{d}	982 ± 78^{a}	985 ± 79^{a}
C16/C16:1	7.0 ± 0.4^{b}	4.5 ± 1.0^{c}	$3.1\pm0.2^{c,e}$	$3.1\pm0.2^{c,e}$	8.8 ± 1.1^{a}	9.5 ± 0.6^{a}	1.3 ± 0.04^{d}	$1.4\pm0.05^{d,e}$
C18/C18:1	0.2 ± 0.01^{c}	0.2 ± 0.03^{c}	$0.1\pm0.01^{d,e}$	0.07 ± 0.01^{e}	0.5 ± 0.08^{a}	0.4 ± 0.06^{b}	0.06 ± 0.005^{e}	0.06 ± 0.002^{e}
S:M	0.9 ± 0.01^{b}	0.7 ± 0.05^{b}	0.5 ± 0.03^{c}	$0.4\pm0.02^{c,d}$	1.4 ± 0.1^{a}	1.2 ± 0.1^{a}	0.3 ± 0.01^{d}	0.3 ± 0.005^{d}

Values are means ± SEM. Mean values with different superscript letters within a row are significantly different ($p<0.05$)

Cas, casein; Chol, cholesterol; S:M, saturated:monounsaturated fatty acid ratio

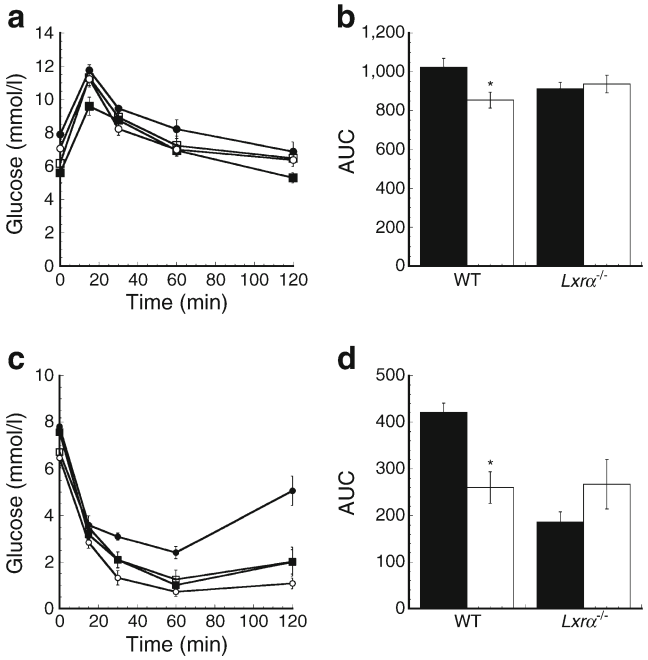

Fig. 6 OGTT and ITT results in WT and $Lxr\alpha^{-/-}$ mice. (**a**) The time curve of glucose concentrations in blood during an OGTT in WT (black circles) and $Lxr\alpha^{-/-}$ (white circles) mice fed casein, or in WT (black squares) and $Lxr\alpha^{-/-}$ (white squares) mice fed SP high-cholesterol diets for 1 month. (**b**) The AUC from OGTT detailed in (**a**). Black bars, casein diet; white bars, SP diet. (**c**) Time curve of glucose concentrations in blood during an ITT in WT and $Lxr\alpha^{-/-}$ mice as in (**a**) above. (**d**) The AUC from the ITT. Black bars, casein diet; white bars, SP diet. The results are expressed as the means ± SEM; n= 5–7 mice per group; *p<0.05 for the difference between the soy and casein groups

they were less fatty and had a better consistency than those of mice fed a casein diet with cholesterol. Moreover, the effect of SP was also observed in the livers of $Lxr\alpha^{-/-}$ mice, which normally have a fatty appearance and a very soft texture, as previously reported [18, 30]. In fact, livers of $Lxr\alpha^{-/-}$ mice fed an SP diet showed a decrease in large lipid deposits and a reduced inflammatory response. These data suggest that SP has an anti-steatotic effect, as observed in previous studies [13–15].

Previous studies and the present work have shown that the elimination of excess dietary cholesterol to prevent its hepatic accumulation occurs via increased faecal bile acid excretion [18]. We did not observe an increase in *Cyp7a1* and *Cyp27a1* expression in mice fed long-term high-cholesterol diets, nor did we see significant differences in the concentration of total bile acids in the bile. However, WT mice fed an SP diet with cholesterol had increased liver and intestinal expression of *Abcg5*, *Abcg8* and *Abca1*, an effect not observed in $Lxr\alpha^{-/-}$ mice. The highest levels of these transporters in WT mice were associated with the highest concentration of total faecal bile acids.

Although the expression of genes involved in fatty acid synthesis, bile acid synthesis and RCT is regulated by LXR [19], our data show that consumption of SP upregulated some of the genes involved in these processes and down-regulated the expression of some others. SP isoflavones were able to mediate the effects of LXR, since they are ligands for other nuclear receptors, such as the peroxisome proliferator-activated receptors and estrogen receptors [27–29, 38]. However, our data indicate that isoflavones are unable to work as direct LXR agonists and thereby stimulate transcription via a classical LXRE, a finding that is in agreement with previous results [39].

Our results in hepatocytes suggest that isoflavones are able to regulate LXR activity indirectly by promoting the phosphorylation, possibly mediated via AMPK, of LXRα or LXRβ, leading to opposing effects on the expression of certain genes. Isoflavones reduced the expression of *Srebp1* and *Abca1* via LXRα, but at the same time increased the expression of *Abcg5* and *Abcg8* via LXRβ. Our data on the use of compound C, an inhibitor of AMPK, support the hypothesis that isoflavones are able to activate AMPK, leading to the repression of *Srebp1* by the phosphorylation of LXRα and the overexpression of *Abcg5* by the phosphorylation of LXRβ. Our group and others have also demonstrated that isoflavones are able to increase the phosphorylation state of AMPK [40, 41]. More studies are needed to understand the full mechanism of this activation.

The accumulation of hepatic lipids has recently been described as one of the main causes for the development of the metabolic abnormalities obesity, primarily insulin resistance and dyslipidaemia [42]. Our results clearly show that feeding an SP high-cholesterol diet to WT mice improves insulin sensitivity compared with WT mice on a casein high-cholesterol diet. These results are in agreement with a previous study indicating that SP improves insulin sensitivity [16]. However, this beneficial effect was abolished following the deletion of $Lxr\alpha$, indicating that the effect of SP on glucose metabolism involves LXRα.

Interestingly, $Lxr\alpha^{-/-}$ mice had better insulin sensitivity independently of the type of diet, suggesting that the absence of LXRα in vivo has a beneficial rather than a negative effect on insulin sensitivity. This is in agreement with previous data from our group, which showed that $Lxr\alpha^{-/-}$ mice are more insulin-sensitive than WT mice, even after a high-fat diet leading to liver steatosis [36]. The beneficial effects of a lack of LXRα occur via LXRβ, since this isoform acts in the opposite direction to LXRα in glucose metabolism and insulin sensitivity [36]; it is also the main isoform in muscle tissue [43] and is present in adipocytes, leading to an improvement of whole-body insulin sensitivity. This suggests that the improvement of insulin sensitivity by an SP diet could be mediated by LXRα. In support of this finding, $Lxr\beta^{-/-}$ mice fed the SP diet showed improved insulin sensitivity (data not shown).

In conclusion, the differential expression of genes regulated by LXR after the consumption of SP is in part due to the capacity of isoflavones, particularly genistein, to regulate the activity of the LXR isoforms, LXRα and LXRβ, in opposing directions, possibly mediated via AMPK. The different effects of isoflavones on these nuclear receptors are likely to explain the beneficial results observed in various studies of experimental animals and in humans, where the consumption of an SP diet was shown to reduce serum cholesterol and prevent the excessive accumulation of hepatic lipids, as well as improving insulin sensitivity [13–15, 44, 45]. Further studies are needed to understand the molecular mechanisms of the regulation of LXR isoforms by isoflavones.

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Contribution statement MGG, KRS, OG, NT, MKA, JAG and ART conceived and designed the study. MGG, KRS, OG and MKA performed the experiments. VO, CAS, TJ, ADV, ALV and RHP contributed to the design, standardisation of different methods and techniques, as well as to the analysis of data. MGG, KRS, OG, NT, MKA, JAG and ART analysed and interpreted the data. MGG, KRS, OG, MKA, JAG and ART drafted the manuscript, which all authors revised for intellectual content. All authors approved the final version.

References

1. Wiklund P, Toss F, Weinehall L et al (2008) Abdominal and gynoid fat mass are associated with cardiovascular risk factors in men and women. J Clin Endocrinol Metab 93:4360–4366
2. Houston DK, Nicklas BJ, Zizza CA (2009) Weighty concerns: the growing prevalence of obesity among older adults. J Am Diet Assoc 109:1886–1895
3. Despres JP, Lemieux I (2006) Abdominal obesity and metabolic syndrome. Nature 444:881–887
4. Cornier MA, Dabelea D, Hernandez TL et al (2008) The metabolic syndrome. Endocr Rev 29:777–822
5. Doria A, Patti ME, Kahn CR (2008) The emerging genetic architecture of type 2 diabetes. Cell Metab 8:186–200
6. Grundy SM, Cleeman JI, Merz CN et al (2004) Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Circulation 110:227–239
7. Mathieu P, Poirier P, Pibarot P, Lemieux I, Despres JP (2009) Visceral obesity: the link among inflammation, hypertension, and cardiovascular disease. Hypertension 53:577–584
8. Olaiz-Fernandez G, Rivera-Dommarco J, Shamah-Levy T et al (2006) Encuesta Nacional de Salud y Nutricion. Instituo Nacional de Salud Publica, Cuernavaca
9. Ginsberg HN (2006) REVIEW: Efficacy and mechanisms of action of statins in the treatment of diabetic dyslipidemia. J Clin Endocrinol Metab 91:383–392
10. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (2001) Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 285:2486–2497
11. Anderson JW, Johnstone BM, Cook-Newell ME (1995) Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 333:276–282
12. Zhan S, Ho SC (2005) Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile. Am J Clin Nutr 81:397–408
13. Tovar AR, Murguia F, Cruz C et al (2002) A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome. J Nutr 132:2562–2569
14. Tovar AR, Torre-Villalvazo I, Ochoa M et al (2005) Soy protein reduces hepatic lipotoxicity in hyperinsulinemic obese Zucker fa/fa rats. J Lipid Res 46:1823–1832
15. Torre-Villalvazo I, Tovar AR, Ramos-Barragan VE, Cerbon-Cervantes MA, Torres N (2008) Soy protein ameliorates metabolic abnormalities in liver and adipose tissue of rats fed a high fat diet. J Nutr 138:462–468
16. Noriega-Lopez L, Tovar AR, Gonzalez-Granillo M et al (2007) Pancreatic insulin secretion in rats fed a soy protein high fat diet depends on the interaction between the amino acid pattern and isoflavones. J Biol Chem 282:20657–20666
17. Potter SM (1995) Overview of proposed mechanisms for the hypocholesterolemic effect of soy. J Nutr 125:606S–611S
18. Peet DJ, Turley SD, Ma W et al (1998) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell 93:693–704
19. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ (2002) Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. J Biol Chem 277:18793–18800
20. Schroepfer GJ Jr (2000) Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 80:361–554
21. Gabbi C, Warner M, Gustafsson JA (2009) Minireview: liver X receptor beta: emerging roles in physiology and diseases. Mol Endocrinol 23:129–136
22. Lewis GF, Rader DJ (2005) New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ Res 96:1221–1232
23. Repa JJ, Mangelsdorf DJ (2000) The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. Annu Rev Cell Dev Biol 16:459–481
24. Zelcer N, Tontonoz P (2006) Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest 116:607–614
25. Hwahng SH, Ki SH, Bae EJ, Kim HE, Kim SG (2009) Role of adenosine monophosphate-activated protein kinase-p70 ribosomal S6 kinase-1 pathway in repression of liver X receptor-alpha-dependent lipogenic gene induction and hepatic steatosis by a novel class of dithiolethiones. Hepatology 49:1913–1925
26. Torra IP, Ismaili N, Feig JE et al (2008) Phosphorylation of liver X receptor alpha selectively regulates target gene expression in macrophages. Mol Cell Biol 28:2626–2636
27. Shen P, Liu MH, Ng TY, Chan YH, Yong EL (2006) Differential effects of isoflavones, from *Astragalus membranaceus* and *Pueraria thomsonii*, on the activation of PPARalpha, PPARgamma, and adipocyte differentiation in vitro. J Nutr 136:899–905

28. Kuiper GG, Lemmen JG, Carlsson B et al (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263
29. Shay NF, Banz WJ (2005) Regulation of gene transcription by botanicals: novel regulatory mechanisms. Annu Rev Nutr 25:297–315
30. Alberti S, Schuster G, Parini P et al (2001) Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. J Clin Invest 107:565–573
31. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509
32. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
33. Barakat HA, Dohm GL, Loesche P, Tapscott EB, Smith C (1976) Lipid content and fatty acid composition of heart and muscle of the BIO 82.62 cardiomyopathic hamster. Lipids 11:747–751
34. Keller S, Jahreis G (2004) Determination of underivatised sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography-mass spectrometry-single ion monitoring in faeces. J Chromatogr B Analyt Technol Biomed Life Sci 813:199–207
35. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43:506–520
36. Korach-Andre M, Parini P, Larsson L, Arner A, Steffensen KR, Gustafsson JA (2010) Separate and overlapping metabolic functions of LXRalpha and LXRbeta in C57Bl/6 female mice. Am J Physiol Endocrinol Metab 298:E167–E178
37. Samuel VT, Petersen KF, Shulman GI (2010) Lipid-induced insulin resistance: unravelling the mechanism. Lancet 375:2267–2277
38. Ronis MJ, Chen Y, Badeaux J, Badger TM (2009) Dietary soy protein isolate attenuates metabolic syndrome in rats via effects on PPAR, LXR, and SREBP signaling. J Nutr 139:1431–1438
39. Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF (2005) Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. J Nutr Biochem 16:321–330
40. Hwang JT, Park IJ, Shin JI et al (2005) Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. Biochem Biophys Res Commun 338:694–699
41. Palacios-Gonzalez B, Torres N, Tovar AR (2011) Genistein stimulates AMPK-phosphorylation and expression of genes involved in fatty acid oxidation in skeletal muscle. FASEB J 25:772.777
42. Fabbrini E, Magkos F, Mohammed BS et al (2009) Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. Proc Natl Acad Sci U S A 106:15430–15435
43. Hessvik NP, Boekschoten MV, Baltzersen MA et al (2010) LXR {beta} is the dominant LXR subtype in skeletal muscle regulating lipogenesis and cholesterol efflux. Am J Physiol Endocrinol Metab 298:E602–E613
44. Anderson JW, Bush HM (2011) Soy protein effects on serum lipoproteins: a quality assessment and meta-analysis of randomized, controlled studies. J Am Coll Nutr 30:79–91
45. Jenkins DJ, Mirrahimi A, Srichaikul K et al (2010) Soy protein reduces serum cholesterol by both intrinsic and food displacement mechanisms. J Nutr 140:2302S–2311S