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**PPAR $\alpha$ -LXR as a novel metabolostatic signaling axis in skeletal muscle which acts to optimize substrate selection in response to nutrient status**

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**Key words: SCD1, PGC-1 $\alpha$ ; PDK4; SIRT1**

## Synopsis

Liver X Receptor (LXR) and peroxisome proliferator-activated receptor (PPAR)  $\alpha$  are nuclear receptors that control expression of genes involved in glucose and lipid homeostasis. Using wild type and PPAR $\alpha$ -null mice fed an LXR agonist-supplemented diet, the study analysed the impact of pharmacological LXR activation on the expression of metabolically-important genes in skeletal muscle, testing the hypothesis that LXR activation can modulate PPAR action in skeletal muscle in a manner dependent on nutritional status. In the fed state, LXR activation promoted a gene profile favouring lipid storage and glucose oxidation, increasing *SCD1* expression and downregulating *PGC-1 $\alpha$*  and *PDK4* expression. PPAR $\alpha$  deficiency enhanced LXR stimulation of *SCD1* expression, and facilitated elevated *SREBP1* expression. However, LXR-mediated downregulation of *PGC-1 $\alpha$*  and *PDK4* was opposed and reversed by PPAR $\alpha$  deficiency. During fasting, prior LXR activation augmented PPAR $\alpha$  signaling to heighten fatty acid oxidation and decrease glucose oxidation by augmenting fasting-induced upregulation of *PGC-1 $\alpha$*  and *PDK4* expression, effects opposed by PPAR $\alpha$  deficiency. Starvation-induced downregulation of *SCD1* expression was opposed by antecedent LXR activation in wild-type mice, an effect further enhanced by PPAR $\alpha$  deficiency, which may elicit increased channeling of FA into TAG to limit lipotoxicity. Our data also identified potential regulatory links between the protein deacetylases SIRT1 and SIRT3 and PDHK4 expression in fasted muscle, with a requirement for PPAR $\alpha$ . In summary, we therefore propose that a LXR-PPAR $\alpha$  signaling axis acts as a metabolostatic regulatory mechanism to optimize substrate selection and disposition in skeletal muscle according to metabolic requirement.

**Abbreviations:** ER, endoplasmic reticulum; FA, fatty acid; LXR, Liver X Receptor; NEFA, non-esterified FA; NR, nuclear receptor; PDC, pyruvate dehydrogenase complex; PDHK, pyruvate dehydrogenase kinase; PGC-1 $\alpha$ , PPAR $\gamma$  coactivator-1 $\alpha$ ; PPAR, peroxisome proliferator-activated receptor; SCD, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein-1c; TAG, triglyceride; UPR, unfolded protein response; XBP1, X-box binding protein 1.

## Introduction

Skeletal muscle, because of its mass and total energy requirement, plays a key role in maintaining metabolic homeostasis and energy balance. The increasing recognition that subtle changes in energy balance, when considered over prolonged periods of time, represent a significant risk factor for the development of metabolic abnormalities including insulin resistance, hypertriglyceridemia and T2DM has heightened the search for specific cell signals that regulate metabolic control in skeletal muscle. Nuclear receptors (NRs) are members of a superfamily of ligand-activated transcription factors that regulate gene expression in response to nutritional and physiological stimuli. One class of NRs function as metabolic sensors, binding to substrate and end-product component molecules of metabolic pathways such as lipids and fatty acids (FA). This class of NRs includes the liver X receptors (LXR) and the peroxisome proliferator-activated receptors (PPARs).

LXRs  $\alpha$  and  $\beta$  act as sensors of cellular cholesterol, binding oxysterols, and stimulate lipogenesis by enhancing expression of genes involved in FA biosynthesis. While LXR activation is thought to be predominantly insulin sensitising [20] due to suppression of hepatic glucose output secondary to an LXR-led reduction in gluconeogenic capacity [35], it can also elicit dramatic increases in hepatic triglyceride (TAG) production [2] which, if excessive, would be predicted to be insulin desensitising through excessive ectopic lipid accumulation [7]. This has led to complications with the use of LXR agonists as therapies for type 2 diabetes mellitus (T2DM) [32]. However, it has been suggested that, although skeletal-muscle TAG is increased in insulin resistant states, storage of FA as TAG may divert them from cytotoxic pathways and oppose the development of lipotoxicity [24]. The lipogenic actions of the LXRs are therefore considered to directly oppose the action of PPAR $\alpha$ , which promotes FA oxidation.

The main target tissues of the LXRs are considered to be those known to be important in lipid biosynthesis, particularly the liver. LXR $\alpha$  expression predominates in metabolically-active tissues, including the liver, but also small intestine, kidney, macrophages, and adipose tissue. In contrast, LXR $\beta$  is more widely expressed (reviewed in [45]) and LXR target genes have been identified in other tissues that are not major sites of lipogenesis, including oxidative muscle [5,27]. LXR activation has been reported to increase the expression of *SREBP1* (which encodes the lipogenic transcription factor SREBP-1c) in skeletal muscle *in vivo* [27] and increase expression of *SCD1* (which encodes stearoyl-CoA desaturase (SCD) 1) in cultured muscle cells [5]. The SCDs are rate limiting for the biosynthesis of mono-unsaturated FA from saturated FA which are used more effectively for FA esterification to form TAG. Overexpression of SCD1 in cultured myotubes also prevents the inflammatory and endoplasmic reticulum (ER) stress responses to exposure to the saturated FA palmitate [30]. In cultured skeletal muscle, LXR activation by the synthetic LXR agonist TO-901317 increases the uptake, distribution into cellular lipids (FAs, diacylglycerol, TAG) and oxidation of palmitate but, at the same time, increases glucose uptake and oxidation [19]. Concomitant increases in rates of oxidation of both FA and glucose are unusual as there is generally reciprocal regulation of the use of these two energy substrates (the glucose/FA cycle). In the longer term, this is achieved by up-regulation of pyruvate dehydrogenase kinase (PDHK) 4, which catalyses inhibitory phosphorylation of the pyruvate dehydrogenase complex (PDC) (reviewed in [37]).

PPARs  $\alpha$  and  $\delta$  play a critical role in maintenance of lipid homeostasis (oxidation and production), regulating transcription of genes involved in FA oxidation, binding and transport and, in skeletal muscle, that encoding PDHK4. Exposure of insulin-sensitive tissues to excess non-esterified FA (NEFA) and circulating TAG induces insulin resistance [7] that can be corrected by the administration of PPAR $\alpha$  activators by actions to promote removal of intracellular lipid through tissue FA oxidation (see e.g. [42]). PPAR $\alpha$  null mice show an inability to up-regulate genes involved in FA uptake and oxidation after fasting, and as a consequence become hypoglycaemic and hypoketonemic, with accumulation of lipid in their tissues. PPAR $\gamma$ , primarily expressed in adipocytes, but also in other tissues, including skeletal muscle [26], promotes FA synthesis and storage.

The metabolic actions of PPARs are coactivated by the inducible PPAR $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) (reviewed in [36]). In skeletal muscle and liver, PGC-1 $\alpha$  is found in complex with GCN5, an acetyltransferase which acetylates PGC-1 at several lysine residues thereby inhibiting its transcriptional activity [10,22]. The balance between relative levels and activity of GCN5 and the protein deacetylase SIRT1 (the mammalian Sir2 ortholog), which can deacetylate PGC-1 $\alpha$  [10,33], provides a potential regulatory convergence point for induction of PPAR gene targets. Induction of PPAR $\alpha$  gene targets requires the interaction of PPAR $\alpha$  and PGC-1, often in complex with other enzymes and coactivators such as SIRT1 [31]. PGC-1 coactivators can also interact with other regulatory elements, including the lipogenic transcription factor sterol regulatory element-binding protein-1c (SREBP-1c), abnormalities in which have been implicated in the development of T2DM.

In the present study, we analysed the consequences of pharmacological LXR activation by TO-901317, which activates LXR $\alpha$  and LXR $\beta$  with approximately equal potency [44], on the expression of metabolically-important genes in skeletal-muscle *in vivo*. Genes encoding proteins regulating TAG storage selected for study were *SREBP1*, which encodes SREBP-1c, and *SCD1*. Key regulators of lipid and glucose oxidation selected were PGC-1 $\alpha$  and PDK4. Since overexpression of SCD1 in cultured myotubes prevents ER stress [30], we also studied the expression of X-box binding protein 1 (XBP1), which is established as a key effector of the mammalian unfolded protein response (UPR) in skeletal muscle [3]. Since changes in PGC-1 $\alpha$  co-transcriptional activity in skeletal muscle may be modulated by post-translational modification, including reversible acetylation, we additionally investigated the impact of LXR activation on SIRT1 and GCN5 expression. Studies were undertaken in the well-fed state, where the glucose supply is ample, insulin levels are high, and incoming FA are predominantly esterified rather than oxidised. We also evaluated the impact of pharmacological LXR activation on the response of metabolically-important genes to starvation, where PPAR $\alpha$  signalling becomes dominant, FA are oxidised and glucose is conserved. To elucidate further the potentially complex interplay between LXR and PPAR $\alpha$  in regulating skeletal-muscle gene expression, we utilised PPAR $\alpha$  null mice.

## Experimental

### Animals

All procedures performed in this study were approved by the Local Ethics Review Committee of the University of Oxford and were carried out under the authority of the appropriate Home Office (UK) personal and project licences in accordance with the Home Office Animals (Scientific Procedures) Act 1986. Tissue and blood samples from male PPAR $\alpha$ -null mice, bred on to a Sv/129 genetic background, were kindly provided by Professor G.F. Gibbons (University of Oxford, U.K.). Wild-type male Sv/129 mice were used as controls. Mice were maintained on a reverse light/dark cycle (12h dark phase 03:00-15:00, 12h light phase 15:00-03:00). Wild type (WT) mice were provided with *ad libitum* access to either standard high-carbohydrate/low-fat rodent laboratory diet or standard diet additionally containing the non-sterol LXR agonist N-(2,2,2-trifluoroethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl) phenyl] sulfonamide (TO-901317) at a final concentration of 0.01% (w/w) for 5 days. PPAR $\alpha$  null mice were fed only on diet containing TO-901317 (0.01%; w/w). After 5 days mice were either killed at the mid point of the dark phase on day 5 or starved for 24 h after 5 days of maintenance on either normal or TO-901317-supplemented diet before being killed. Dietary administration of TO-901317 to WT and PPAR $\alpha$  mice was kindly undertaken by Professor G.F. Gibbons (University of Oxford, U.K.).

### Tissue and blood sampling

Mice were anaesthetised by injection of sodium pentobarbital (60 mg/ml in 0.9 % NaCl; 1 ml/kg body wt. i.p.) and, once locomotor activity had ceased, liver and mixed hind-limb skeletal muscle were rapidly excised and freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen.

Frozen tissue was stored in liquid nitrogen prior to analyses. Blood was sampled from abdominal aorta. Blood samples were centrifuged for 5 min at 12,000 g and plasma was stored at -20°C.

### Measurement of plasma insulin and blood glucose

Insulin was measured using a mouse insulin double-antibody ELISA according to manufacturer's instructions (Mercodia, Uppsala, Sweden). Glucose was determined using a commercially available kit (Roche Diagnostics, Lewes, U.K.).

### Quantitative RT-PCR

Gene expression was measured by qRT-PCR, using Taqman or Sybr green methodology. Gene expression was determined by  $\Delta\Delta C_T$  normalised against 18S control RNA (Applied Biosystems, Warrington, U.K.). Primer sequence (Eurogentec, Southampton, UK) details are shown in Supplementary Table 1.

### Immunoblotting

Solubilised protein samples (10 µg; measured and equalized in each fraction using an RC-DC BioRad system (BioRad, UK)) were separated by SDS-PAGE and transferred onto PVDF membrane (GE Healthcare, Amersham, UK). Blots were blocked in 5% (w/v) milk protein in Tris-buffered saline/0.1% Tween-20 (TBS/T) solution and then incubated in primary antibody. Antibodies used in this study were anti-SIRT1, anti-GCN5, anti-XBP1, anti-PGC1alpha (all SCBT, CA, USA); anti-SIRT3 (Abgent, CA, USA). Following primary antibody incubation, blots were incubated in either anti-mouse, anti-rabbit or anti-goat secondary antibodies (all Jackson ImmunoResearch, PA, USA). Detection of bands was achieved by using the chemiluminescence substrate SuperSignal West Pico (Pierce, Rockford, IL, USA). Reference protein measurements were made with mouse monoclonal anti-β-actin (clone AC-15; Sigma, Poole, UK) primary antibody in a 3% (w/v) milk/TNT solution, at 4°C. For each representative immunoblot presented, the results are from a single gel exposed for a uniform duration, and each lane represents a preparation from a different mouse.

### Statistical analysis

Results are presented as the mean ± standard error mean (S.E.M.), with the numbers of mice in parenthesis. Statistical analysis was performed by ANOVA followed by Fisher's post-hoc tests for individual comparisons or Student's t-test as appropriate (Statview, Abacus Concepts, inc., Berkeley, CA, USA). A *P* value of < 0.05 was considered to be statistically significant.

## Results

### Metabolic characteristics of the groups

In fed mice, LXR activation (closed bars) decreased plasma glucose concentrations in both PPARα null mice (21%; *P* < 0.01) and wild-type mice (17%; *P* < 0.05; Fig. 1A). The hypoglycemic effect of TO-901397 in the fed state is consistent with the reported effects of LXR activation to suppress hepatic glucose output [35]. Plasma glucose levels were decreased in fasted compared to fed mice (30.1%; *P* < 0.001; Fig. 1). However, antecedent LXR activation exerted a potent effect to further decrease glycemia in fasted PPARα null mice (30%; *p* < 0.001; Fig. 1B). Plasma insulin levels were decreased in fasted mice relative to fed (40 %; *P* < 0.01; Fig. 1). Plasma insulin levels were not significantly affected by LXR activation under fed and fasted conditions in WT mice or PPARα null mice, although LXR activation elicited modest increases in plasma insulin levels in PPARα null mice (1.5 fold and 1.4 fold in fed and fasted mice respectively; Fig. 1C and 1D). Insulin/glucose ratio analysis suggested that LXR activation resulted in a loss of insulin sensitivity in PPARα null mice, but not in WT mice (Fig. 1).

### LXR activation increases SCD1 and XBP1 expression in skeletal muscle in the absence of increased SREBP1c expression

*SREBP1*, which encodes the lipogenic transcription factor SREBP1-c, is reported as a key LXR target gene in skeletal muscle [27]. Furthermore, the *SREBP1* promoter contains two LXR response elements that are essential for insulin-dependent activation of *SREBP1* [2]. This has led to the suggestion that insulin activates the *SREBP1* promoter via increasing the activity of the LXRs, possibly through production of a LXR specific ligand [2]. Both *SREBP1* and *SCD1* gene expression are upregulated in liver under lipogenic conditions, including insulin stimulation and high-carbohydrate feeding (reviewed in [28]). In the present study, starvation (24 h) of WT mice resulted in a marked decline in both *SREBP1* (93%;  $P < 0.001$ ) and *SCD1* (71%) gene expression (Fig. 2A; see also [25]). *SCD1* expression was also significantly suppressed in skeletal muscle following starvation (85%,  $P < 0.001$ ; Fig. 2F), whereas skeletal-muscle *SREBP1* expression increased 5-fold ( $P < 0.001$ ) (Fig. 2B), despite lowered circulating insulin levels. We failed to detect altered *SREBP1* gene expression in response to pharmacological LXR activation in hind-limb skeletal muscle of fed WT mice sampled at the mid point of the dark cycle (Fig. 2C), when insulin concentrations peak, and endogenously-generated agonists of LXR would be predicted to contribute to expression of LXR target genes. Although LXR activation failed to augment *SREBP1c* expression, it elicited a modest (1.4-fold) increase in gene expression of *SCD1* in skeletal muscle of WT mice (Fig. 2G). In contrast to its limited effects in wild-type mice, LXR activation significantly increased both *SREBP1-c* and *SCD1* expression levels, by 3.3 fold ( $P < 0.001$ ) and 8.8 fold ( $P < 0.05$ ) respectively, in fed  $\text{PPAR}\alpha$  null mice and by 8.5 fold ( $P < 0.001$ ) and 4.1 fold ( $P < 0.05$ ) respectively compared to WT LXR agonist-treated mice (Figs. 2C and 2G).

In contrast to observations in fed mice, antecedent LXR activation increased expression of both *SREBP1* and *SCD1* in skeletal muscle of fasted mice (Fig. 2D and 2H).  $\text{PPAR}\alpha$  deficiency blocked LXR-mediated induction of skeletal-muscle *SREBP1* expression after starvation (Fig. 2D), whereas it further enhanced the expression of *SCD1* (Fig. 2H).

Recent studies have reported a key role for *XBPI* in induction of hepatic lipogenic genes, including *SCD1* [21]. In liver, starvation-induced decreases in lipogenic gene expression were not associated with a decline in *XBPI* mRNA or protein expression (Fig. 2I); however, increased *XBPI* expression in skeletal muscle of fasted mice (5.3-fold;  $P < 0.05$ ; Fig. 2J) closely paralleled changes in *SREBP1*, rather than *SCD1* expression. Modest LXR-mediated upregulation of *SCD1* was associated with increased expression of *XBPI* (3.4 fold) in skeletal muscle of fed WT mice, while the more dramatic LXR-mediated upregulation of *SREBP1* and *SCD1* expression in fed  $\text{PPAR}\alpha$  null mice was also paralleled by increased *XBPI* expression by 7.1 fold ( $P < 0.01$ ) compared to WT-fed mice, and by 1.8 fold ( $P < 0.05$ ) compared to WT LXR agonist-treated mice (Fig. 2C). Moreover, in WT fasted mice, skeletal-muscle *XBPI* levels were markedly enhanced following LXR activation (1.6 fold;  $P < 0.001$ ), a response that was blocked in  $\text{PPAR}\alpha$  null mice (Fig. 2I).

### Effects of TO-901317 on $\text{PGC-1}\alpha$ and $\text{PDK4}$ expression in fed WT and $\text{PPAR}\alpha$ null mice

In skeletal muscle,  $\text{PPAR}\alpha$  and  $\text{PPAR}\delta$  are key regulators of FA oxidation (reviewed in [37]). In addition, the NR coactivator  $\text{PGC-1}\alpha$  is recognized to play important roles in regulating glucose and lipid metabolism in liver and skeletal muscle by interacting with a range of NRs, including  $\text{PPAR}\alpha$  and the LXRs (see e.g. [36]). Hepatic  $\text{PGC1}\alpha$  gene was significantly increased in WT starved compared with fed mice (Fig. 3A), as is consistent with its role in coordinating the response of hepatic nutrient handling to starvation (reviewed in [36]). The upregulation of  $\text{PGC1}\alpha$  gene expression seen in response to fasting (Fig. 3B) was more robust in skeletal muscles compared to livers of WT mice (2.2 fold,  $P < 0.01$  vs. 89%,  $P < 0.05$ ). Protein expression of  $\text{PGC1}\alpha$  was enhanced by fasting to similar extents in liver and skeletal muscle (Figs. 3A and 3B).  $\text{PGC1}\alpha$  expression levels were decreased (59%,  $P < 0.05$ ) by LXR activation in skeletal muscle of fed WT mice (Fig. 3C). Since  $\text{PGC-1}\alpha$  cooperates with LXR to regulate gene expression, this could represent a feedback loop. However, the effect of LXR activation to decrease skeletal-muscle  $\text{PGC1}\alpha$  required

the participation of PPAR $\alpha$ , since *PGC1 $\alpha$*  expression was increased (by 3.4-fold;  $P < 0.001$ ) in response to pharmacological LXR activation in skeletal muscle of fed PPAR $\alpha$  null mice (Fig. 3C).

The gene expression of PDHK4, a regulatory kinase that inactivates PDC, blocking glucose oxidation and thereby forcing fat oxidation, is PPAR $\alpha/\delta$  regulated (reviewed in [37]). As expected, fasting elevated hepatic PDHK4 expression, with a greater (3.2-fold;  $P < 0.01$ ) increase in protein expression (Fig. 3E) than gene expression (see also [14]). Fasting also increased PDHK4 expression in skeletal muscle, with increases of 2.0-fold ( $P < 0.001$ ) and 2.2-fold ( $P < 0.01$ ) in gene and protein expression respectively (Fig. 3F) (see also [13]). In fed WT skeletal muscle, similar to changes in *PGC1 $\alpha$*  expression, LXR activation suppressed *PDK4* expression (61%;  $P < 0.05$ ) (Fig. 3G). In addition, *PDK4* expression, like that of *PGC1 $\alpha$* , was enhanced (2.2 fold,  $P < 0.001$ ) in response to LXR activation in skeletal muscle of fed PPAR $\alpha$  null mice (Fig. 3G).

Whilst LXR activation lowered *PGC1 $\alpha$*  expression in skeletal muscle of fed WT mice, antecedent LXR activation significantly induced *PGC1 $\alpha$*  expression in fasted WT mice (3.2-fold;  $P < 0.001$ ; Fig. 3D). The inductive effect of LXR activation on *PGC1 $\alpha$*  gene expression was abolished in PPAR $\alpha$  null mice, with expression levels of *PGC1 $\alpha$*  mRNA exhibiting an approximate 50% reduction compared to untreated WT fasted mice (Fig. 3D). The response of *PDK4* expression to starvation was exaggerated by antecedent LXR activation in WT mice (3.8 fold;  $P < 0.001$ ); however this response was opposed by PPAR $\alpha$  deficiency (Fig. 3H).

### Effects of LXR activation, PPAR $\alpha$ deficiency and fasting on SIRT1 and GCN5 expression

In skeletal muscle, PGC-1 $\alpha$  target genes for FA oxidation are induced by the deacetylase SIRT1 [8], whilst GNC5 has been reported to acetylate and suppress PGC-1 $\alpha$  activity in skeletal muscle and in liver [10,22]. Moreover, SIRT1 can activate hepatic LXR [23]. *SIRT1* gene expression was markedly upregulated in response to fasting in livers of WT mice, with a concomitant 81% increase in SIRT1 protein expression ( $P < 0.01$ ) (Fig. 4A; see also [9]). In contrast, hepatic GCN5 gene expression was unaffected by fasting, although there was a 2.6-fold increase ( $P < 0.05$ ) in GCN5 protein expression (Fig. 4E), suggestive of post-transcriptional control. The responses to fasting of SIRT1 and GCN5 in skeletal muscle differed to those observed in liver. SIRT1 gene and protein expression were unaffected by starvation (Fig. 4B), whereas *GCN5* gene expression in skeletal muscle was suppressed by 46% ( $P < 0.01$ ) in response to starvation (Fig. 4F). The decline in skeletal-muscle *GCN5* gene expression seen in response to fasting was not, however, paralleled by a decline in GCN5 protein expression (Fig. 4F).

We observed that LXR activation decreased gene expression of SIRT1 (by 29%;  $P < 0.05$ ) in skeletal muscle of fed WT mice, an effect which was opposed by PPAR $\alpha$  deficiency (Fig. 4C). LXR activation also decreased GCN5 mRNA expression in skeletal muscle of fed WT; however, this response was not opposed by PPAR $\alpha$  deficiency (Fig. 4G). Thus PPAR $\alpha$  deficiency selectively affects LXR-mediated suppression of SIRT1 in the fed state.

In contrast to the fed-state, pharmacological LXR activation prior to starvation elicited a marked increase in both *SIRT1* expression (93%;  $P < 0.01$ ; Fig. 4D) and *GCN5* expression (3.8 fold;  $P < 0.001$ ; Fig. 4H) in fasted WT skeletal muscle. Upregulation of both *SIRT1* and *GCN5* expression in fasted mice by antecedent LXR activation was abolished by PPAR $\alpha$  deficiency (Fig. 4D and 4H).

### SIRT1 and SIRT3 are differentially regulated by LXR activation in the fed, but not fasted, state

Recent findings show that acetylation appears to target most enzymes in most metabolic pathways including those occurring within mitochondria, such as the Krebs cycle and mitochondrial  $\beta$ -oxidation [40,46]. SIRT3 is localized in the mitochondrial matrix, where it regulates the acetylation levels of metabolic enzymes, and its expression is up-regulated in liver and brown adipose tissue in response to starvation [34], leading to promotion of FA oxidation [12]. In the present study, SIRT3



gene expression was suppressed in both liver (by 40%;  $P < 0.05$ ; Fig. 4I) and skeletal muscle (by 69%;  $P < 0.001$ ; Fig. 4J) of WT mice. However, unlike SIRT1, changes in SIRT3 gene expression were not associated with corresponding changes in SIRT3 protein expression. Thus, starvation elicited a significant (2.2-fold;  $P < 0.01$ ) increase in SIRT3 protein expression in liver (Fig. 4I), together with a modest and non-significant increase (32%) in skeletal muscle (Fig. 4J). The response of *SIRT3* gene expression to LXR activation also differed to that of SIRT1. Thus LXR activation increased gene expression of SIRT3 in skeletal muscles of WT mice (by 73%;  $P < 0.01$ ), a response that, unlike that of SIRT1, was unaffected by PPAR $\alpha$  deficiency (Fig. 4K), demonstrating that LXR regulation of the mitochondrial SIRT3 gene expression is PPAR $\alpha$  independent. Antecedent LXR activation opposed the fasting-induced decline in SIRT3 gene expression such that SIRT3 mRNA levels were increased by 7.5 fold ( $P < 0.05$ ) compared to untreated WT fasted mice. The action of LXR to abolish the effect of fasting on SIRT3 was however completely blocked by PPAR $\alpha$  deficiency (Fig. 4L).

## Discussion

Previous studies in human muscle cells in vitro demonstrated that LXR activation by TO-901317 directly affects gene expression of key enzymes regulating nutrient handling, and modifies glucose and fatty acid metabolism. For example, LXR activation by TO-901317 in human muscle cells increases oleate and palmitate uptake and oxidation, as well as glucose uptake and oxidation [11,19]. Treatment with TO901317 also increases lipogenesis and lipid accumulation in myotubes [18], together with the number of lipid droplets [11]. LXR activation by TO901317 in cultured muscle cells also increases the expression of lipogenic enzymes, including SREBP-1c, FAS and SCD-1 [5]. The present study investigated potential interactions between LXR and PPAR $\alpha$  in modulating the expression of genes encoding proteins regulating FA storage as TAG in skeletal muscle in vivo. We also investigated the expression of metabolically-important genes encoding key regulators of lipid and glucose oxidation in skeletal muscle in vivo. Fasting was employed as a metabolic challenge associated with an increased demand for and/or supply of FA for oxidation. To assess the impact of impaired lipo-oxidative capacity on the response to LXR activation, we also administered TO-901317 to PPAR $\alpha$  null mice with for 5 days. We demonstrated that in the fed state, LXR activation elicited modest increases in *SCD1* paralleled by downregulation of expression of *PGC-1 $\alpha$*  and *PDK4* in skeletal muscle. PPAR $\alpha$  deficiency significantly enhanced LXR stimulation of *SCD1* expression, which was associated with significantly elevated *SREBP1* expression. PPAR $\alpha$  can inhibit SREBP-1c promoter activity induced by LXR [43]. Our studies therefore indicate that adequate PPAR $\alpha$  signaling exists in skeletal muscle of fed WT mice to oppose LXR-induced upregulation of *SREBP1* expression (as might be predicted since lipid synthesis and oxidation are generally reciprocally regulated). Downregulation of *PGC-1 $\alpha$*  and *PDK4* in skeletal muscle elicited by LXR activation in WT mice was opposed and reversed by PPAR $\alpha$  deficiency. Given that PPAR $\alpha$  deficiency also selectively allows an effect of LXR activation to increase *SREBP1* expression in the fed state, these data could imply that a lipid or cholesterol metabolite generated secondary to LXR activation, whose accumulation in skeletal muscle in the fed state is opposed by PPAR $\alpha$  signalling, acts to increase *PDK4* and *PGC1 $\alpha$*  expression. In WT mice, *SREBP1* was upregulated in skeletal muscle in response to starvation, whereas it was down-regulated in liver. *SREBP1* upregulation in skeletal muscle in response to fasting was augmented by antecedent LXR activation. This was linked to PPAR $\alpha$  signaling as the responses were blunted in PPAR $\alpha$  null mice. In contrast, downregulation of *SCD1* expression was elicited by starvation in both liver and skeletal muscle. The response of *SCD1* in skeletal muscle to starvation was opposed by antecedent LXR activation in WT mice, an effect further enhanced by the absence of PPAR $\alpha$  signaling. *PGC-1 $\alpha$*  and *PDK4* gene and protein expression were upregulated in both liver and skeletal muscle in response to fasting. Responses of *PGC-1 $\alpha$*  and *PDK4* expression to fasting after prior LXR activation were opposite to those observed in the fed state, namely augmentation of their fasting-induced upregulation. Unlike the fed state, LXR-

mediated effects in fasting were opposed by PPAR $\alpha$  deficiency, supporting the critical role of PPAR $\alpha$  in the response to fasting.

The PPARs and LXRs both bind to target genes as heterodimers with the RXR (retinoid X receptor). In the absence of ligand, PPAR-RXR and LXR-RXR can bind to target genes to repress transcription. In the presence of activating ligands, corepressor complexes are replaced by coactivator complexes, enhancing gene transcription. Cross talk between PPAR $\alpha$  and LXR is proposed to ensure that antagonistic pathways are not simultaneously activated (reviewed in [1]), either through direct interaction between PPAR and LXR or via competition for a limiting amount of shared RXR [17]. Our data add a further dimension to this scenario. On the basis of the changes in pattern of expression of key metabolic genes in skeletal muscle observed in response to LXR activation, we propose that LXR activation acts to optimise substrate selection and disposition in skeletal muscle through augmenting PPAR signalling possibly through LXR-mediated generation of an, as yet unidentified, PPAR ligand. This action is not intrinsically PPAR isotype selective, but depends on additional changes in PPAR ligand availability and/or PPAR signalling that LXR activation is able to augment. Consequently, we propose that in the fed state LXR activation promotes glucose oxidation (through downregulation of PDHK4) and enhances lipid storage both directly (via upregulation of SCD1, and possibly SREBP-1c), as has been previously demonstrated [5,27] and possibly by enhancing PPAR $\gamma$  signalling. We further propose that prior LXR activation augments PPAR $\alpha$  signalling in starvation to enhance the capacity for FA oxidation (by increasing the expression of the PPAR $\alpha$  coactivator PGC-1 $\alpha$ ) and suppress glucose oxidation (through upregulation of PDK4 expression). The increase in *PDK4* expression may reflect upregulation by PGC-1 $\alpha$  (reviewed in [6]). In support of our hypothesis the expression of a cohort of genes (including PGC-1 $\alpha$  and PDHK4) is augmented in skeletal muscle following LXR activation in WT starved mice, but not in PPAR $\alpha$  null starved mice, indicating that the effect of LXR activation is PPAR $\alpha$  dependent. Increases in PGC1 $\alpha$  and PDHK4 would favour enhanced lipid oxidation and restricted glucose oxidation and glucose sparing in the starved state. PGC-1 $\alpha$  promotes the expression of FA oxidation genes (reviewed in [6]), whilst PDHK4 is the main PDHK isoform responsible for suppression of skeletal-muscle PDC activity in starvation [38] and insulin resistance [15], which by decreasing glucose oxidation forces fat oxidation (see [15,38]).

In the fed state, in contrast, LXR activation suppresses the expression of *PGC-1 $\alpha$*  and *PDK4*, a response reversed by PPAR $\alpha$  deficiency, with augmented gene expression, together with upregulation of *SREBP1* expression. Chronic ligand activation of LXR increases PPAR $\gamma$  expression in human skeletal muscle cells [19] and PPAR $\gamma$  agonist treatment decreases *PDK4* expression in skeletal muscle [41], mimicking the response of skeletal-muscle *PDK4* expression to LXR activation in fed WT mice in the present study. Thus LXR activation of fed WT mice may increase PPAR $\gamma$  signalling in skeletal muscle, possibly via the generation of an endogenous PPAR $\gamma$  agonist. Intact PPAR $\alpha$  signalling may be required to maintain a muscle metabolic profile appropriate to the fed state. LXR activation unrestrained by PPAR $\alpha$  signalling may switch the muscle metabolic profile to one resembling that seen in fasting so as to promote clearance of excessive lipid.

*PGC-1 $\alpha$*  co-transcriptional activity in skeletal muscle is modulated by post-translational modification, including reversible acetylation, mediated by the deacetylase SIRT1 and the acetyl transferase GCN5 [10] (reviewed in [6]). Thus, increased nutrient availability would render PGC-1 $\alpha$  transcriptionally inactive through hyperacetylation by GCN5, whereas decreased nutrient availability (e.g. in fasting) would increase deacetylation of PGC-1 $\alpha$  by SIRT1 leading to enhanced PGC-1 $\alpha$  binding to target gene promoters and transcriptional activation by remodeling of the local chromatin environment [6]. In support of this, we observed that fasting upregulated of SIRT1 and PGC-1 $\alpha$  gene and protein expression in liver favouring increased responsiveness of PGC-1 $\alpha$  to the need for upregulation of FA oxidation. In contrast, in skeletal muscle upregulation of PGC-1 $\alpha$  gene and protein expression occurred in the absence of changes in SIRT1 gene or protein expression, suggesting that the mechanism of regulation of PGC-1 $\alpha$  may be tissue specific. The role of GCN5

in the regulation of transcriptional activity in response to fasting in both liver and skeletal muscle is less clear as fasting selectively downregulated *GCN5* gene expression in skeletal muscle; however, protein expression of *GCN5* was increased in both liver and skeletal muscle in response to fasting. In the present study LXR activation in the fed state suppressed *PGC-1 $\alpha$* , *GCN5* and *SIRT1* expression which may limit the influence of *PGC-1 $\alpha$*  on the skeletal-muscle metabolic profile. However, absence of *PPAR $\alpha$*  signaling in the fed state enhanced *PGC-1 $\alpha$*  and *SIRT1* expression, which may compensate for the loss of *PPAR $\alpha$*  lipo-oxidative effects. Pharmacological activation of *SIRT1* stimulates FA oxidation in skeletal muscle of high-fat fed, insulin resistant mice through upregulation of FA oxidation genes including *PPAR $\alpha$*  and *PDK4* [8]. Our data support the concept that strong regulatory links exist between *SIRT1* and *PDK4* expression in muscle. In contrast, *GCN5* deactivates *PGC-1 $\alpha$*  in skeletal muscle leading to decreased FA oxidation, mitochondrial biogenesis and energy expenditure [4,10]. Our data suggest that *GCN5* expression may also be regulated by LXR and *PPAR $\alpha$* . In fasting, expression of *PGC-1 $\alpha$* , *SIRT1* and *GCN5* are increased in skeletal muscle after prior activation of LXR favouring increased responsiveness of *PGC-1 $\alpha$*  to the need for upregulation of FA oxidation. Loss of these effects in fasted *PPAR $\alpha$*  null mice may reflect an impaired ability to upregulate FA oxidation.

The complete oxidation of acetyl-CoA derived from glucose (via PDC) or FA (via  $\beta$ -oxidation) requires the participation of the Krebs cycle, a wrapped linear pathway where the end product (oxaloacetate, derived from glucose) is essential for the first step of the pathway. Consequently, nutrient input is slowed or stopped if insufficient end product is available. In starvation, an adequate supply of oxaloacetate is likely achieved by enhanced expression of *PDHK4* which, by suppressing the use of pyruvate for acetyl-CoA formation via PDC, conserves pyruvate for oxaloacetate formation via pyruvate carboxylase. Our data raise suggest that acetylation-deacetylation metabolic control mechanisms can be up-regulated by LXR activation to allow selective amplification or acceleration of metabolic responses to transitions between nutritional states. These mechanisms are likely co-ordinated by extrinsic factors such as the lipid-sensing *PPARs* according to the prevailing dominant *PPAR* isotype and/or endogenous ligand generation or accumulation.

*SIRT3* is located in the mitochondrial matrix and, like *SIRT1*, is an  $\text{NAD}^+$ -dependent protein deacetylase. In mice, *SIRT3* protein levels are up-regulated during fasting in brown adipose tissue and liver [12]. In liver, *SIRT3* regulates FA and ATP production during fasting by deacetylating and activating long-chain acyl-CoA dehydrogenase, that catalyses the first step of long-chain FA  $\beta$ -oxidation. In the present study, hepatic *SIRT3* gene expression was suppressed by fasting however, as reported in Ref. [12], hepatic *SIRT3* protein expression increased in response to fasting. *SIRT3* expression was also decreased in skeletal muscle in response to fasting; however, as in liver, this was associated with an increase in *SIRT3* protein levels (see also [29]). Prior LXR activation increased *SIRT3* expression in fed and fasted WT mice, but this response was abolished in fasted *PPAR $\alpha$*  null mice, suggesting that the response of skeletal-muscle *SIRT3* expression to starvation is dependent both on the preceding level of LXR activation and the degree of activation of *PPAR $\alpha$*  in starvation.

In excess, saturated FA exert lipotoxic effects leading to endoplasmic reticulum (ER) stress, inflammation, and insulin resistance. Overexpression of *SCD1* in cultured myotubes prevents the inflammatory and ER stress response to exposure to the saturated FA palmitate [30]. *SCD1* is highly expressed in skeletal muscle from obese humans [16] and obese insulin-resistant Zucker diabetic fatty rats [39]. LXR activation increases *SCD1* expression in cultured muscle cells [5]. We observed increases in skeletal muscle *SCD1* expression after LXR activation *in vivo* in the fed state and in the starved state after antecedent LXR activation. In both states, *PPAR $\alpha$*  deficiency exaggerated this effect of LXR activation, suggesting competition between LXR and *PPAR $\alpha$* , such that *PPAR $\alpha$*  limits LXR's ability to increase *SCD1*, and *PPAR $\alpha$* 's action to promote FA oxidation is dominant to the actions of LXR. Given that *SCD1* converts saturated FA into mono-unsaturated FA that can be esterified to neutral lipid (TAG), *SCD1* may exert a protective action against

lipotoxicity, by increasing disposition of lipid towards TAG synthesis when the capacity to oxidise lipid is compromised, for example by PPAR $\alpha$  deficiency.

XBP1 is a key effector of the mammalian unfolded protein response (UPR) in skeletal muscle [3]. In the present study, fasting upregulated XBP1 gene and protein expression in both liver and skeletal muscle. LXR activation in the fed state in the absence of PPAR $\alpha$  signaling also led to *XBPI* induction, which may reflect a restraint on FA oxidation. In fasting, antecedent LXR activation augments *XBPI* expression in skeletal muscle of WT mice, a response lost in PPAR $\alpha$  null mice. The increase in *XBPI* expression in WT fasted mice previously treated with the LXR agonist is also associated with induction of SCD1 expression, which may reflect an attempt to limit the lipotoxic effects by channeling FA into TAG. Furthermore, in the absence of PPAR $\alpha$  signaling, and therefore an inability to upregulate FA oxidation, SCD1 expression is induced even more and is associated with reversal of starvation-induced increases in XBP1 expression.

In conclusion, we propose a novel signalling axis comprising PPAR $\alpha$ -LXR that, via altered expression of key target genes, maximises ATP production and minimises the accumulation of toxic metabolites. Our results argue for a novel role of PPAR $\alpha$ , whereby it acts in conjunction with LXR as a metabolic regulator to maintain metabolic homeostasis ('metabolostat') under both fed and fasted conditions (via a PPAR $\alpha$ -LXR axis) forcing the skeletal muscle to a set metabolic point. This allows optimization of substrate selection and disposition in skeletal muscle through augmented PPAR signalling.

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## Figure legends

### Figure 1

Wild type (WT) mice were fed either standard rodent diet or standard diet supplemented with TO-901317 at a final concentration of 0.01% (w/w) for 5 days. Mice were either killed at the mid point of the dark phase on day 5 (fed) or starved for 24 h after 5 days of maintenance on standard diet (fed, open bars; starved, crosshatched bars) or standard diet supplemented with TO-901317 (filled bars) before being killed. PPAR $\alpha$  null (KO) mice were fed only on diet containing TO-901317 and sampled either in the fed or fasted state (filled bars). Blood samples were withdrawn for measurement of plasma glucose (panels A and B), plasma insulin (panels C and D), and insulin-glucose (I/G) ratios (panels E and F). Data are expressed as mean  $\pm$  SEM for 6-8 mice in each group. Statistically-significant effects of fasting are indicated by:  $\ddagger\ddagger$  P<0.01;  $\ddagger\ddagger\ddagger$  P<0.001. Statistically-significant effects of TO-901317 are indicated by: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. Statistically-significant effects of PPAR $\alpha$  deficiency are indicated by:  $\dagger\dagger\dagger$  P<0.001.

### Figure 2

Wild type (WT) mice were fed either standard rodent diet or standard diet supplemented with TO-901317 at a final concentration of 0.01% (w/w) for 5 days. Mice were either killed at the mid point of the dark phase on day 5 (fed) or starved for 24 h after 5 days of maintenance on standard diet (fed, open bars; starved, crosshatched bars) or standard diet supplemented with TO-901317 (filled bars) before being killed. PPAR $\alpha$  null (KO) mice were fed only on diet containing TO-901317 and sampled either in the fed or fasted state (filled bars). Liver and mixed hind-limb skeletal muscle were rapidly excised and freeze-clamped. Gene expression for *SREBP1* (panels A-D), *SCD1* (panels E-H) and *XBPI* (panels I-L) were measured in liver and hind-limb mixed muscle by qRT-PCR. Representative Western blots for XBP-1 protein expression in liver (panel I) and hind-limb skeletal muscle (panel J) of WT mice sampled in the fed or 24 h-fasted state are also shown. Data are expressed as mean  $\pm$  SEM. Statistically-significant effects of fasting are indicated by:  $\ddagger\ddagger\ddagger$  P<0.001. Statistically-significant effects of TO-901317 are indicated by: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. Statistically-significant effects of PPAR $\alpha$  deficiency are indicated by:  $\dagger$  P<0.05;  $\dagger\dagger\dagger$  P<0.001.

### Figure 3

Wild type (WT) mice were fed either standard rodent diet or standard diet supplemented with TO-901317 at a final concentration of 0.01% (w/w) for 5 days. Mice were either killed at the mid point of the dark phase on day 5 (fed) or starved for 24 h after 5 days of maintenance on standard diet (fed, open bars; starved, crosshatched bars) or standard diet supplemented with TO-901317 (filled bars) before being killed. PPAR $\alpha$  null (KO) mice were fed only on diet containing TO-901317 and sampled either in the fed or fasted state (filled bars). Liver and mixed hind-limb skeletal muscle were rapidly excised and freeze-clamped. Gene expression for *PGC-1 $\alpha$*  (panels A-D) and *PDHK4* (panels E-H) were measured in liver and hind-limb mixed muscle by qRT-PCR. Representative Western blots for PGC-1 $\alpha$  (panels A and B) and PDHK4 (panels E and F) protein expression in liver and hind-limb skeletal muscle of WT mice sampled in the fed or 24 h-fasted state are also shown. Data are expressed as mean  $\pm$  SEM. Statistically-significant effects of fasting are indicated by:  $\ddagger$  P<0.05;  $\ddagger\ddagger$  P<0.01;  $\ddagger\ddagger\ddagger$  P<0.001. Statistically-significant effects of TO-901317 are indicated by: \* P<0.05; \*\*\* P<0.001. Statistically-significant effects of PPAR $\alpha$  deficiency are indicated by:  $\dagger\dagger\dagger$  P<0.001.

### Figure 4

Wild type (WT) mice were fed either standard rodent diet or standard diet supplemented with TO-901317 at a final concentration of 0.01% (w/w) for 5 days. Mice were either killed at the mid point of the dark phase on day 5 (fed) or starved for 24 h after 5 days of maintenance on standard diet (fed, open bars; starved, crosshatched bars) or standard diet supplemented with TO-901317

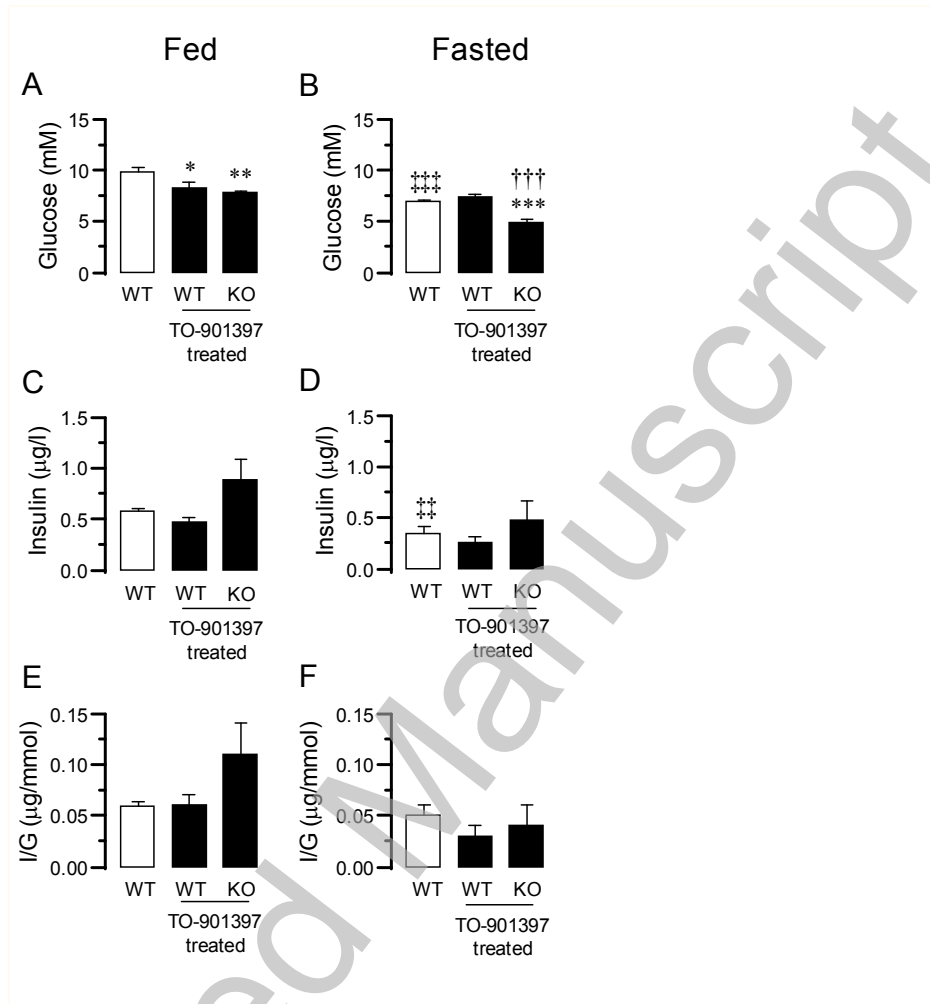


(filled bars) before being killed. PPAR $\alpha$  null (KO) mice were fed only on diet containing TO-901317 and sampled either in the fed or fasted state (filled bars). Liver and mixed hind-limb skeletal muscle were rapidly excised and freeze-clamped. Gene expression for *SIRT1* (panels A-D), *GCN5* (panels E-H) and *SIRT3* (panels I-L) were measured in liver and hind-limb mixed muscle by qRT-PCR. Representative Western blots for SIRT1 (panels A and B), GCN5 (panels E and F) and SIRT3 (panels I and J) protein expression in liver and hind-limb skeletal muscle of WT mice sampled in the fed or 24 h-fasted state are also shown. Data are expressed as mean  $\pm$  SEM. Statistically-significant effects of fasting are indicated by: ‡ P<0.05; ‡‡ P<0.01; ‡‡‡ P<0.001. Statistically-significant effects of TO-901317 are indicated by: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. Statistically-significant effects of PPAR $\alpha$  deficiency are indicated by: † P<0.05; ††† P<0.001.

**Table 1**

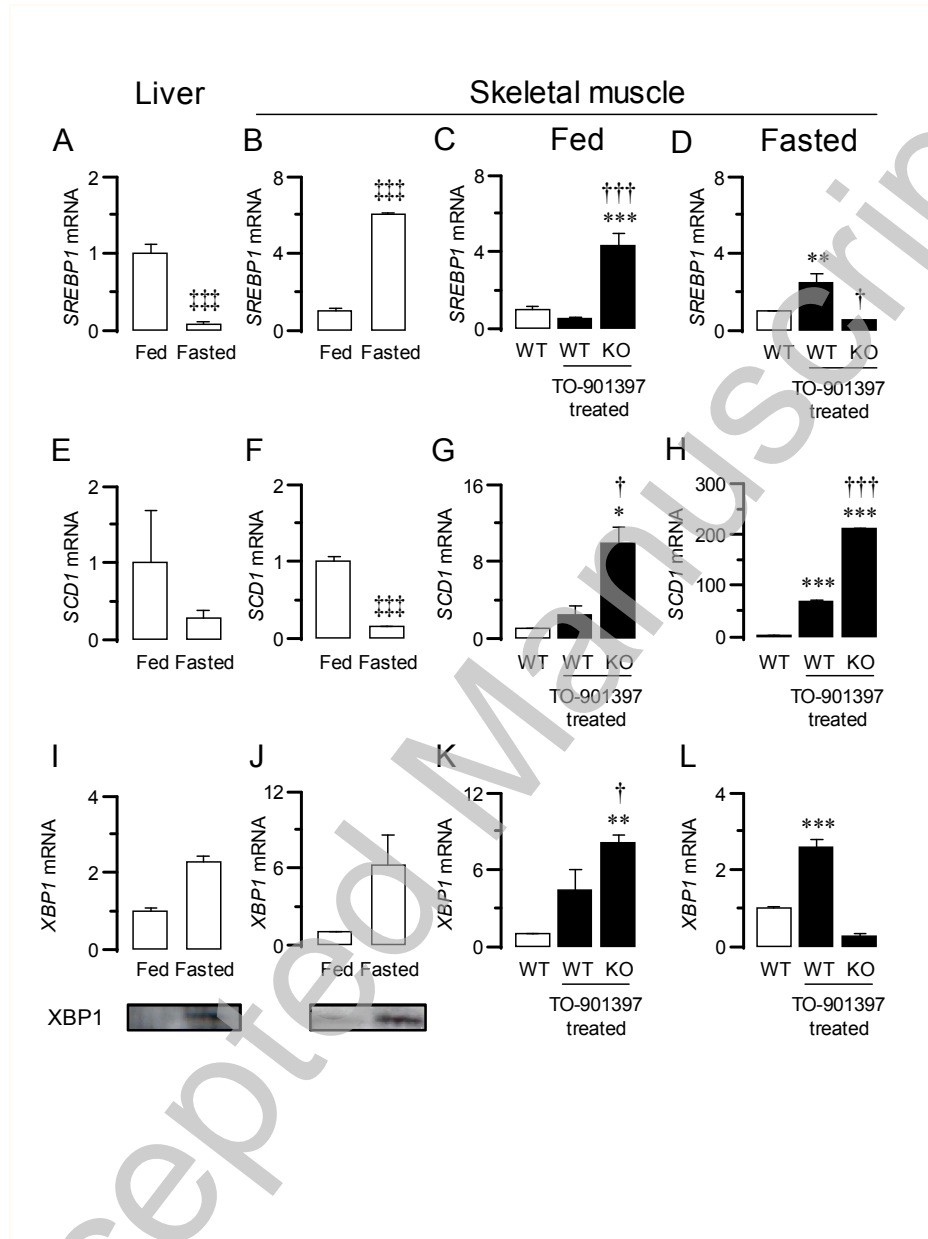
Summary of changes in hind-limb skeletal muscle gene expression in WT and PPAR $\alpha$  null fed and fasted mice in response to LXR activation.

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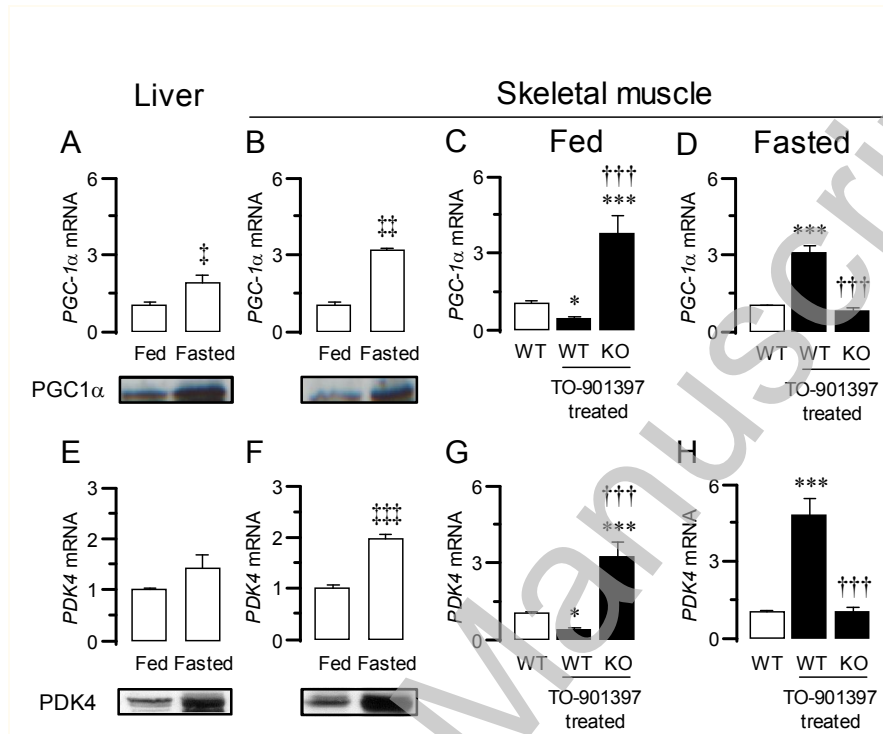
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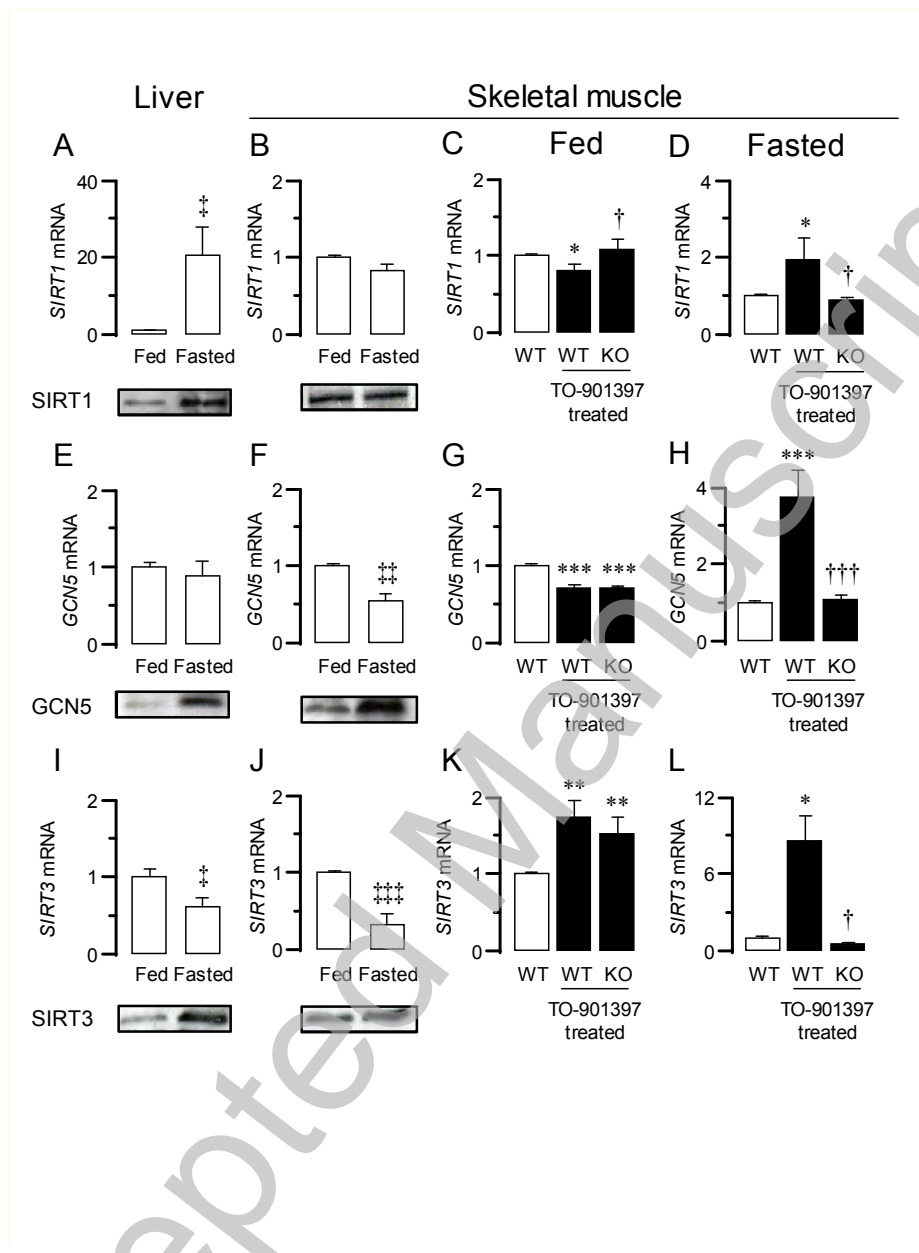
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Gene	FED		FASTED	
	Effect of LXR	Role of PPAR $\alpha$	Effect of LXR	Role of PPAR $\alpha$
<i>SREBP1</i>	↑	Required	↑	Required
<i>SCD1</i>	↑	Required	↑	Required
<i>XBP1</i>	↑	Required	↑	Required
<i>PDK4</i>	↓	Required	↑	Required
<i>PGC-1<math>\alpha</math></i>	↓	Required	↑	Required
<i>SIRT1</i>	↓	Required	↑	Required
<i>GCN5</i>	↓	Not required	↑	Required
<i>SIRT3</i>	↑	Not required	↑	Required