Rapamycin pre-treatment abrogates Tumour Necrosis Factor-α down-regulatory effects on LXR-α and PXR mRNA expression via inhibition of c-Jun N-terminal kinase 1 activation in HepG2 cells

Di-Lin Ng[1] · Shin-Wei Tie[2] · Pei-Chin Ong[1] · Wyi-Sian Lim[2] · Tengku-Sifzizul Tengku-Muhammad[3]
Quok-Cheong Choo[1] · Choy-Hoong Chew[2] ✉

1 Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, Kampar,
   Perak, Malaysia

2 Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, Kampar,
   Perak, Malaysia

3 Department of Biological Science, Faculty of Science and Technology, Universiti Malaysia Terengganu, Kuala Terengganu,
   Terengganu, Malaysia

✉ Corresponding author: chewch@utar.edu.my

Abstract The Liver X Receptor (LXR) and Pregnane X Receptor (PXR) are members of the nuclear receptor superfamily. Previously, they have been classified as important regulators of lipid homeostasis. However, recent studies have shown that they may be implicated in anti-inflammatory responses as well. This study shows that Tumour Necrosis Factor-α (TNF-α) treatment reduces both LXR-α and PXR mRNA expression. However, pre-treatment with rapamycin, an mTOR inhibitor, followed by TNF-α stimulation, significantly induces LXR-α and PXR mRNA expression to ~17- and ~2-fold, respectively. This suggests that mTORC1, a multi-molecular complex of which mTOR is a member, may act as a negative regulator that inhibits the induction of LXR-α and PXRas anti-inflammatory genes. It is also shown here that inhibition of JNK1 via the mTOR/Akt pathway coincides with the up-regulation of LXR-α and PXR mRNA, after TNF-α treatment. Together, these observations suggest that JNK1 possibly act downstream of mTORC1 as an LXR-α and PXR inhibitor. From the results gleaned in this study, rapamycin (and its analogues) may be used to reduce acute inflammation by promoting the induction of LXR-α and PXR as anti-inflammatory genes.

INTRODUCTION

Several members of the nuclear receptor family, including LXR and PXR have emerged as important regulators of inflammatory signalling (Castrillo and Tontonoz, 2004). Previously, LXRs were only classified as cholesterol sensors that regulate the expression of genes involved in lipid metabolism in response to specific oxysterol ligands (Repa and Mangelsdorf, 2000). Conversely, the canonical function of the PXR is to sense elevations in xenobiotics and endobiotics and to orchestrate a response that promotes xenobiotic/endobiotic metabolism and excretion (Kliewer, 2003). Numerous studies have established that LXRs regulate gene expression linked to cholesterol metabolism in a tissue-specific manner. For example, LXR activation in rodent liver up-regulates Cyp7a1, a member of the cytochrome P450 family that is critical for bile acid synthesis (Peet et al. 1998). In the intestine, LXR controls the reabsorption of cholesterol via the expression of ABCG5 and ABCG8 (Yu et al. 2002). In peripheral cells such as macrophages, LXRs regulates the expression of a panel of genes involved in reverse cholesterol transport. In response to macrophage cholesterol overload, LXRs induce expression of the cholesterol efflux transporters ABCA1 and ABCG1, the apolipoproteins apoE and apoCs, and the phospholipid transfer protein, PLTP (Repa and Mangelsdorf, 2000; Zelcer and Tontonoz, 2006). PXR, on the other hand, regulates the expression of several key enzymes controlling the bile acid synthesis

pathway, lipid metabolism and glucose homeostasis (Eloranta and Kullak-Ublick, 2005; Handschin and Meyer, 2005; Moreau et al. 2008). The roles of LXR and PXR as intermediates in inflammatory signalling has also been described (Castrillo et al. 2003; Joseph et al. 2003; Langmann et al. 2004; Shah et al. 2006; Zhou et al. 2006). Since LXR and PXR are involved in both metabolic and inflammatory pathways, this makes them potentially attractive targets for the modulation of inflammation responses, especially towards TNF-α.

TNF-α down-regulates specific targets which include the nuclear receptors LXR and probably PXR (Kim et al. 2007). Upon TNF-α stimulation, the c-Jun N-terminal kinases (JNK) will be activated via its upstream kinase MKK7 (Bogoyevitch and Kobe, 2006). Subsequently, the activated JNK would translocate into the nucleus to activate its cognate transcription factor, c-Jun (Bogoyevitch and Kobe, 2006). There have been reports linking the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway to JNK activation (Wajant and Scheurich, 2001). However there was no direct attempt at studying the mechanisms of the mTOR/Akt/JNK interplay. There are still doubts as to whether rapamycin will inhibit JNK directly or indirectly via its many upstream kinases. This study will attempt to demonstrate that LXR-α and PXR mRNA repression by TNF-α via the JNK cascade is abrogated by mTOR inhibition. Insights obtained from this study may enable restoration of normal lipid homeostasis in acutely inflamed human cells in the future.

MATERIALS AND METHODS

Cell line and culture

The human hepatocarcinoma cell line, HepG2 cells, were obtained from ATCC (Manassas, VA, USA) and grown in Minimum Essential Medium with Earle salts (Invitrogen; Carlsbad, CA, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 units ml^{-1}) in a 5% CO_2 incubator maintained at 37ºC.

Cell treatment

For expression studies, cells were stimulated with 20 ng/mL of TNF-α (Millipore Corporation; Billerica, MA, USA). For rapamycin inhibition studies, 10 nM of rapamycin (Sigma-Aldrich; St. Louis, MO, USA) was added 2 hrs prior to TNF-α stimulation for 2 hrs.

Real-time RT-PCR

Expressions of human *Lxr-α, Pxr, Jnk1* and *β-actin* mRNA transcripts were detected by real-time RT-PCR. Briefly, total RNA from HepG2 cells was isolated using the RNeasy Mini kit (QIAGEN; Dusseldorf, Germany) and a real-time one-step RT-PCR performed using Quantifast SYBR Green (QIAGEN; Dusseldorf, Germany) on a MyIQ iCycler (Bio-Rad; Berkeley, CA, USA) using the protocol: reverse transcription at 10 min at 50ºC, initial denaturation for 5 min at 94ºC, 34 cycles of 10 sec at 94ºC, 30 sec at 58ºC (*Lxr-α*, *Pxr* and *β-actin*) or 63ºC (*Jnk1*), and 30 sec at 72ºC, followed by a 10 min final extension step at 72ºC. Melt curve analysis was performed as follows: 1 min at 55ºC , followed by 10 sec at 55ºC with an incremental 0.5ºC for 90 cycles. Primer sequences used to amplify the human *Lxr-α* and *Pxr* were as follows: 5'-TCAGGCGGATCTGTTCTTCT-3' (*Lxr-α* reverse), 5'-CGGGCTTCCACTACAATGTT-3' (*Lxr-α* forward), resulting in a 213-bp RT-PCR product, and 5'-TCCCTGTCCGTTCACTTTTC-3' (*Pxr* reverse), 5'-CAAATCTGCCGTGTATGTGG-3' (*Pxr* forward), resulting in a 289-bp RT-PCR product. Primer sequences used to amplify the human *Jnk1* were as follows: 5'-GCCTGTCGCACGTGATTGA-3' (*Jnk1* reverse) and 5'-CGTCTTCGTTCGCACTGTTG-3' (*Jnk1* forward), resulting in a 175-bp RT-PCR product. *β-actin* primers were used as an internal control. The primer sequences for human *β-actin* were as follows: 5'-CGTACCACTGGCATCGTGAT-3' (forward), 5'-CCATCTCTTGCTCGAAGTTC-3' (reverse), which yielded a 280-bp RT-PCR product.

Western blot analysis

Cells were harvested, washed in phosphate-buffered saline (PBS) and centrifuged. Cytoplasmic and nuclear proteins were extracted using the Nuclear and Cytoplasmic Extraction kit (Pierce, Thermo Fisher Scientific; Waltham, MA, USA). The cell lysates were subsequently resolved using SDS-PAGE,

transferred onto Immobilon-P membranes (Millipore Corporation; Billerica, MA, USA), and immunoblotted with anti-JNK/SAPK #9252, anti-phospho-SAPK/JNK (Thr183/Tyr185) #9251, anti-c-Jun #9165, anti pc-Jun (Ser 73) #9164, anti-MKK7 #4172, anti-phospho-MKK7 (Ser271/Thr275) #4171, anti-mTOR #2972, anti-phospho-mTOR (Ser2448) #2971, anti-Akt #9272, anti-phospho-Akt (Ser473) #9271, anti-rabbit IgG HRP-linked antibody (#7074). Anti-β-actin (#4967) was utilised as an internal control. The nuclear fractions were probed against c-Jun and pc-Jun only, while the cytoplasmic fractions were probed against the other antibodies. All antibodies were purchased from Cell Signaling Technology; Beverly, MA, USA. Immunoreactive bands were visualised and their densities analysed using the Fluorchem FC2 system (Alpha Innotech; San Leandro, CA, USA).

Statistical analysis

A one-way ANOVA was used to compare mean values for independent variables. Significant differences at 95% confidence ($p < 0.05$) and 99% confidence ($p < 0.01$) are depicted with an asterisk (*) and two asterisks (**) on each graph, respectively. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Previously, it has been shown that TNF-α decreases the expression of LXR-α mRNA (Kim et al. 2007). To determine whether PXR mRNA expression will exhibit a similar pattern, HepG2 cells were treated with varying dosages of TNF-α. From Figure 1, 20 ng/mL of TNF-α significantly reduces both LXR-α and PXR mRNA expression to ~0.5- and ~0.4-fold respectively. The reduction in LXR-α mRNA expression agrees with a recent study using Hep3B cells by Kim et al. (2007). Similar observations were made in HK2 (human proximal tubular) cells, 3T3-L1 cells and rabbit adipocytes, when TNF-α treatment significantly reduced LXR-α mRNA expression (Wang et al. 2005; Lu et al. 2006; Zhao and Dong, 2007). Indeed, RXR-α, which heterodimerise with LXR-α, was reduced upon stimulation with TNF-α as well (Kim et al. 2007). As for PXR, it has been reported that stimulation with the pro-inflammatory cytokines IL-6, IL-1 and LPS activates NF-κB, a pro-inflammatory transcription factor, which in turn inhibit PXR mRNA expression (Assenat et al. 2006). Reciprocally, once the expression of NF-κB was repressed, PXR mRNA expression was increased (Zhou et al. 2006). These findings conclusively show that the lipid metabolism and inflammatory pathways would eventually converge at some point. The characteristic changes in lipid metabolism that occur during acute inflammation include hypertriglyceridemia, decreased hepatic fatty acid oxidation and ketogenesis, inhibition of bile acid synthesis, and decrease in serum HDL levels (Khovidhunkit et al. 2004). As both LXR-α and PXR mRNA expression were down-regulated by TNF-α, they could be closely related to each other physiologically. It would be logical if their expression patterns mirror each other when exposed to certain external stimuli, such as ultraviolet radiation or reactive oxygen species, and in this particular study, TNF-α.
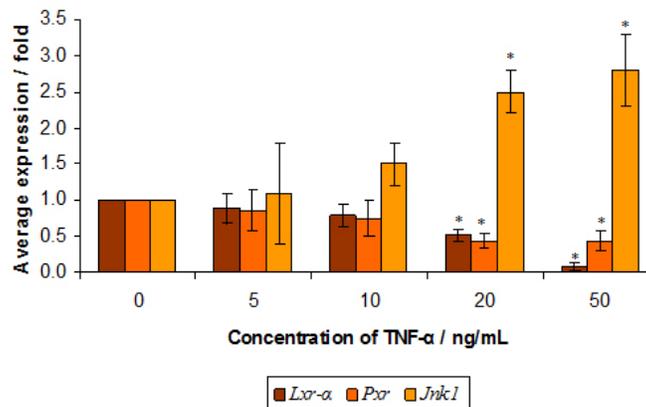

Fig. 1 Average mRNA expression of LXR-α, PXR and JNK1 as normalised to β-actin when treated with 0, 5, 10, 20 and 50 ng/mL of TNF-α for 24 hrs. * significant increase at $p < 0.05$ vs. control. Treatment of 20 ng/mL of TNF-α alone resulted in a significant decrease in mRNA expression for LXR-αand PXR mRNA expression. However, JNK1 mRNA was significantly increased.

The repression of LXR-α and PXR mRNA when exposed to TNF-α would be mediated by kinases involved in signal transduction. As TNF-α was often implicated in JNK1 signalling (Bogoyevitch and Kobe, 2006), it is possible that JNK1 would be one of the important intermediates in this signalling cascade. Based on Figure 1, JNK1 mRNA expression increased ~2.5-fold upon stimulation with 20 ng/mL of TNF-α. This increase in mRNA expression coincides with the decrease of LXR-α and PXR mRNA expression (Figure 1). Unfortunately, there is not enough substantive evidence in this study to suggest a concrete link between the two. However, knockdown of JNK1 has been shown to up-regulate PGC-1β (Yang et al. 2007), as a key activator of hepatic lipogenesis and lipoprotein secretion through activation of LXR-α (Wolfrum and Stoffel, 2006). As LXR-α auto-regulates its own transcription (Laffitte et al. 2001; Li et al. 2002, Ulven et al. 2004), it is conceivable that JNK1 may act to repress *Lxr-α* transcription through LXR-α inhibition. Kim et al. (2009) has proposed a contradictory argument, in which they hypothesised that JNK1 increases LXR-α activity, although this may be due to different cell types and experimental models. In any case, these reports suggest that there could be an interplay between JNK1 and LXR-α. Similar to our findings, Wang et al. (2010) has shown that activation of JNK1 coincides with reduced *Pxr* expression. As JNK1 is the most downstream of the mitogen activated protein kinases (MAPK) which is involved in inflammation (Wagner and Nebreda, 2009), it is probable that there may be still other upstream kinases which induction may cause a decrease in LXR-α and PXR mRNA levels. One viable option would be the mTOR. Sustained mTOR activity may contribute to steatosis by impairing lipid homeostasis (Parent et al. 2007) and also causes lipogenesis deregulation (Laplante and Sabatini, 2010).

To see if mTOR inhibition affects the induction of LXR-α and PXR mRNA, the HepG2 cells were treated with rapamycin, a mTOR inhibitor, before the individual experiments were analysed. In Figure 2, TNF-α treatment significantly reduced the expression of LXR-α and PXR mRNA to ~0.5-fold. This is in accordance with earlier experiments in this study (Figure 1). Rapamycin treatment alone causes a reduction in expression in LXR-α and PXR mRNA to ~0.5- and ~0.25-fold respectively (Figure 2). This shows that mTOR inhibition would not drive the expression of LXR-α and PXR mRNA by itself. However, pre-treatment with rapamycin, followed by TNF-α stimulation, resulted in a ~17- and ~2.0-fold increase in LXR-α and PXR mRNA expression respectively (Figure 2). From these observations, it could be deduced that mTOR acts as an inhibitor in TNF-α-mediated anti-inflammatory responses involving LXR-α and PXR. When the cells were stimulated with TNF-α alone, mTOR –possibly via JNK1– inhibits the anti-inflammatory action of LXR-α and PXR, as shown in the decrease in the mRNA expression of both genes and the corresponding increase in JNK1 mRNA expression (Figure 1). Removing the inhibitor –mTOR in this instance– will lead to up-regulation of LXR-α and PXR mRNA expression only when challenged with TNF-α. The observations here suggest that rapamycin augments the anti-inflammatory response only when stimulated with TNF-α by inducing the transcription of LXR-α and PXR mRNA. This hypothesis is backed by considerable evidence that has emerged indicating that, in addition to inducing genes involved in reverse cholesterol transport, LXRs reciprocally repress a set of inflammatory genes after bacterial, LPS, TNF-α, or IL-1β stimulation (Joseph et al. 2003). Examples of such genes include those involved in generation of bioactive molecules such as iNOS and COX2, IL-6 and IL-1β, the chemokines monocyte chemoattractant protein-1 (MCP-1) and MCP-3, and MMP9 (Castrillo et al. 2003; Joseph et al. 2003). PXR has been shown to have a significant effect on ablating the inflammatory response mediated by exogenous toxins and to have an important role in modulating inflammatory diseases of the bowel (Langmann et al. 2004; Shah et al. 2006; Zhou et al. 2006).

Subsequently, the role of JNK1 in regulating LXR-α and PXR mRNA expression was further examined. Recently, LXR-α, mTOR and JNK1 have been implicated in hepatic steatosis (Parent et al. 2007; Sabio et al. 2009; Kim et al. 2010). This provides a solid basis to further investigate their interplay. JNK1 has also been further identified as important regulators in hepatic steatosis and insulin resistance (Schattenberg et al. 2006; Sabio et al. 2009; Singh et al. 2009). Based on Figure 3a, JNK1 was constitutively expressed. JNK1 was activated after TNF-α stimulation (up to ~1.77-fold) in rapamycin-untreated cells. However, rapamycin treatment abrogated JNK1 activation in HepG2 cells (Figure 3b). Subsequent stimulation with TNF-α failed to re-establish JNK1 activation (Figure 3b). Apparently, it would seem here that rapamycin pre-treatment (and subsequent TNF-α stimulation) would up-regulate LXR-α and PXR mRNA expression (Figure 2), but abrogates JNK1 activation (Figure 3b).

To ensure that the abrogation of JNK expression is valid, the expression and activation of c-Jun, the canonical substrate of JNK, was studied. Figure 3c shows that total c-Jun expression remained constant regardless of any stimulation/treatment. From Figure 3d, TNF-α stimulation alone is shown to activate c-Jun in rapamycin-untreated samples, up to ~2.29-fold. However, rapamycin treatment alone

was also sufficient to induce c-Jun activation, as seen in rapamycin-treated samples (Figure 3d). Here, it would seem that c-Jun activation does not require prior JNK1 activation, as activated JNK1 was not detected under rapamycin treatment (Figure 3b). This suggests that there may be other kinases which may replace JNK1's role in activating c-Jun. Indeed, ERK8 has been shown to phosphorylate c-Jun directly in HCT15 colorectal cells (Xu et al. 2010). In neurons, the inhibition of JNK activity could not block either the c-Jun activation or up-regulation or their subsequent death (Dunn et al. 2002). In COP9 signalosome-directed c-Jun activation/stabilisation (Neumann et al. 1999) and calcium-regulated activation of c-Jun (Zanger et al. 2001), c-Jun activation was increased without corresponding JNK activation. The reduction in c-Jun activation (~0.62-fold) after rapamycin pre-treatment and subsequent TNF-α stimulation (Figure 3d) correlates with the increase in LXR-α and PXR mRNA expression (Figure 2). It is postulated here that rapamycin removes the molecular inhibitor that will prohibit LXR-α and PXR to counteract inflammation in cells (as induced here with TNF-α).

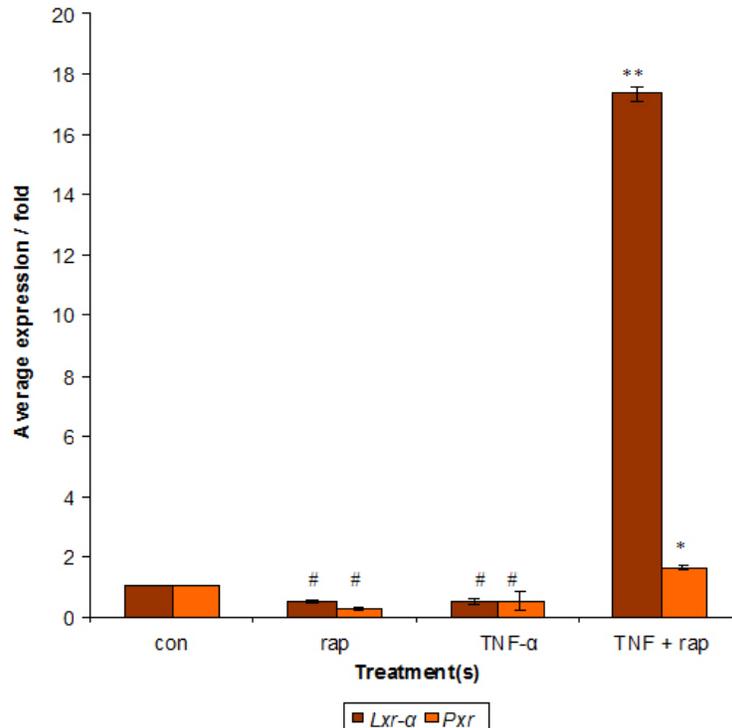**Fig. 2 Average mRNA expression of LXR-α and PXR as normalised to β-actin; untreated control (denoted by con bars), when treated with 100 ng/mL rapamycin only for 2 hrs (denoted by TNF-α bars) when stimulated with 20 ng/mL TNF-α only for 2 hrs (denoted by TNF-α bars) and when pre-treated with 100 ng/mL rapamycin for 2 hrs, subsequently followed by 20 ng/mL TNF-α for 2 hrs (denoted by TNF + rap bars).** # significant decrease at $p < 0.05$ vs. control. * significant increase at $p < 0.05$ vs. control. ** significant increase at $p < 0.01$ vs. control. Treatment of TNF-α alone resulted in a significant decrease for LXR-α and PXRmRNA expression. Rapamycin treatment by itself causes a significant reduction in both *Lxr-α* and *Pxr*. However, pre-treatment with rapamycin followed by TNF-α stimulation caused a significant increase in induction for both LXR-α and PXR mRNA.

Next, the interaction between JNK1 and mTOR/Akt was studied. Previous experiments have shown that rapamycin, an mTOR inhibitor, successfully abrogates JNK1 activation as well as reduces LXR-α and PXR mRNA expression (Figure 2 and Figure 3b). Therefore, Western blots were performed using antibodies of components in the JNK1 cascade and the mTOR/Akt pathway, to look for any direct link that may herald cross-signalling between the two pathways. Based on Figure 4a, total MKK7, the specific JNK activator (Bogoyevitch and Kobe, 2006; Haeusgen et al. 2010), was detected in all samples. However, its activated form was only detected in rapamycin-treated samples (Figure 4b). As with the case of mTOR and Akt as discussed in the following sections, JNK1 and MKK7 could both be involved in a scaffolding complex, which was first described in yeast by Whitmarsh et al. (1998) and

further elucidated by Whitmarsh (2006). Scaffold proteins for the JNKs have been identified. These include the JNK interacting protein (JIP) group of putative scaffolds, which includes the JIP1, JIP2 and JIP3 proteins. The JIP1 and JIP2 proteins are closely related proteins that bind to JNK, MKK7, and mixed-lineage protein kinases (Whitmarsh et al. 1998; Yasuda et al. 1999). JIP1 and JIP2 binds to JNK, MKK7, and members of the mixed-lineage group of MAPKKK (Kelkar et al. 2000; Weston and Davis, 2002). Indeed, JIP1-deficient mice (Jaeschke and Davis, 2007) exhibit defects in JNK activation and insulin resistance. This observation was also expressed by Song and Yong (2005), who hypothesised that JIP1 assembles Akt, MKK7 and JNK1. As JNK1 and MKK7 would be complexed together in a signalling scaffold in JIP1, MKK7 would not need to be phosphorylated to exert its kinase activity (Nihalani et al. 2003). As shown in Figure 4b, consistent with the scaffolding hypothesis, MKK7 was not observed to be phosphorylated in rapamycin-untreated samples. An inactive scaffold may cause MKK7 to be independently activated, and this was shown in Figure 4b where rapamycin pre-treatment activated MKK7. In fact, MKK7 was shown to preferentially activated by TNF-α and cellular stresses (Moriguchi et al. 1997). Based on Figure 3d, this correlates with the results obtained in this experiment, where c-Jun was activated with corresponding MKK7 activation. p-JNK1 would be detected as it would be freed from the complex to translocate into the nucleus to act on transcription factors (Cavigelli et al. 1995).
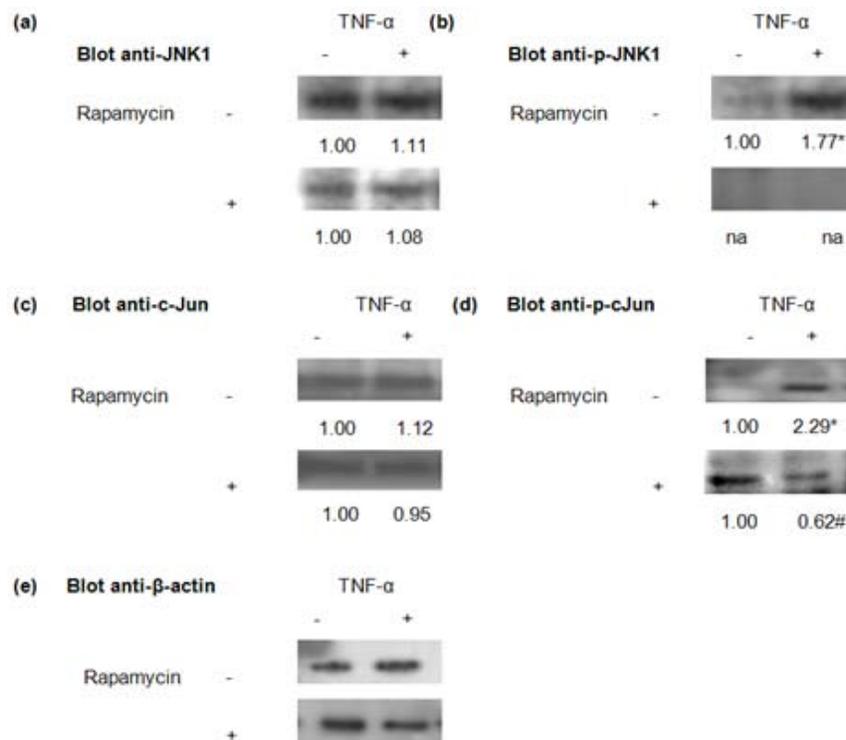

Fig. 3 Western blots of (a) JNK1, (b) p-JNK1, (c) c-Jun, (d) pc-Jun, (e) β-actin. This is a representation of three separate experiments. Rapamycin '-' indicates no pre-treatment and '+' indicates 2 hrs of rapamycin pre-treatment. TNF-α '-' signifies unstimulated samples and '+' signifies 2 hrs of TNF-α stimulation. * significant increase at $p < 0.05$ vs control. # significant decrease at $p < 0.05$ vs control. Treatment with rapamycin abrogated JNK1 activation. Rapamycin and TNF-α both activate c-Jun singly, but treatment with TNF-α reduces c-Jun activation after rapamycin treatment. β-actin was used as loading control. Densities for bands were recorded beneath each blot and were normalised to their respective loading controls.

Akt has been shown to inhibit JNK1 activity by binding to JIP1 (Kim et al. 2002). JIP1 assembles the Mixed Lineage Kinase 3 (MLK3), MKK7 and JNK1 in a complex which can be disrupted by Akt binding. In this study, Akt expression in rapamycin-untreated samples is stable (Figure 4c), and pre-treatment with rapamycin activates Akt (Figure 4d). Akt phosphorylation was increased at serine 473 ~1.48-fold after TNF-α stimulation (Figure 4d). The increase of serine 473 phosphorylation is due to the action of TNF-α (Tsou et al. 2010). Conversely, JNK1 is also deactivated (Figure 3b). In contrast, there was no

detection of p-Akt in rapamycin-untreated samples (Figure 4d), strengthening the case that only phosphorylated Akt inhibit JNK1 signalling by binding to JIP1 and disrupting the formation of the MKK7-JNK1 scaffold. This contradicts observations from Kim et al. (2002), in which co-expression of wild-type or kinase-dead Akt reduced formation of a JIP1-mediated ternary complex between MLK3 and JNK1. However, the same authors Kim et al. (2002) conceded that JIP1 phosphorylates Akt at serine 473. Other researchers have also established indirect links to Akt-JIP1 binding (Jaeschke et al. 2004; Hao et al. 2008; Morel et al. 2010). Taken together, these experiments demonstrate that p-Akt binding to JIP1 inhibits JIP1's ability to assemble active JNK signalling complexes. Presumably, rapamycin treatment will positively affect the binding affinity of Akt towards JIP1 by activating it at its serine 473 residue.
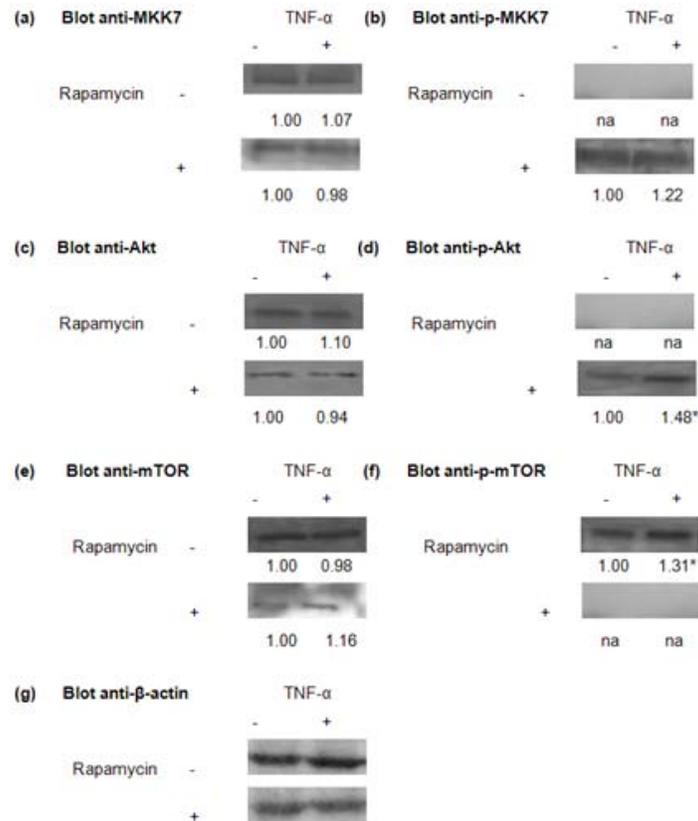

Fig. 4 Western blots of (a) MKK7, (b p-MKK7, (c) Akt, (d) p-Akt (S473), (e) mTOR, (f) p-mTOR (S2448) and (g) β-actin. Rapamycin '-' indicates no pre-treatment and '+' indicates 2 hrs of rapamycin pre-treatment. TNF-α '-' signifies unstimulated samples and '+' signifies 2 hrs of TNF-α stimulation. This is a representation of three separate experiments. * significant increase at $p < 0.05$ vs control. Pre-treatment with rapamycin activated MKK7 and Akt after TNF-α stimulation. However, rapamycin pre-treatment prevented mTOR phosphorylation at serine 2448. β-actin was used as loading control. Densities for bands were recorded beneath each blot and were normalised to their respective loading controls.

Rapamycin, and likely its analogs (CCI779, RAD001, AP23573), are cell-type-dependent inhibitors of mTORC2 function as well as universal inhibitors of the mTORC1 pathway (Sarbassov et al. 2006). Based on Figure 4d, phosphorylation of Akt at the serine 473 residue is considered a marker of mTORC2 activity (Hresko and Mueckler, 2005; Sarbassov et al. 2005; Jacinto et al. 2006). This suggests that mTOR complexes into an active form (mTORC2), and is in agreement with the hypothesis that mTOR complexes into mTORC1/2 as a more active signaling molecule (Sarbassov et al. 2005). In Figure 4e, total mTOR was detected in all samples but its activated form was only detected in rapamycin-untreated samples (Figure 4f). Stimulation with TNF-α increases its phosphorylation ~1.31-fold, which correlates with a report by Tsou et al. (2010). In rapamycin-untreated cells, mTOR may form the mTORC1, which is supported by the observation of mTOR

phosphorylation at serine 2448 (Figure 4f). The mTORC1 would be assembled by the p70 S6 kinase (Chiang and Abraham, 2005; Holz and Blenis, 2005). Therefore, phosphorylation of mTOR at serine 2448 could be considered a marker for p70 S6 kinase activity (Chiang and Abraham, 2005; Holz and Blenis, 2005). Once the cells were pre-treated with rapamycin, the assembly of mTORC1 may be disrupted by rapamycin. This could encourage mTOR to form another active complex (mTORC2), as suggested by the phosphorylation of Akt at its serine 473 residue (Figure 4f). Furthermore, mTORC2 was observed to be resistant to low dosages of rapamycin treatment, while mTORC1 was not (Sarbassov et al. 2006; Foster and Toschi, 2009). It is postulated here that TNF-α signals to c-Jun via p70-mTORC1-JNK1 in rapamycin-untreated HepG2 cells. Consistent with this hypothesis, p70 S6 was already shown to signal to JNK in bone cells (Takai et al. 2007). Conversely, with rapamycin treatment, it is postulated that TNF-α signals to c-Jun via mTORC2-Akt.

As conclusion, LXR-α and PXR mRNA are up-regulated upon TNF-α stimulation, only if rapamycin was administered beforehand. Although there was a lack of concrete evidence that JNK1 down-regulates LXR-α and PXR mRNA specifically, it was shown here in this study that LXR-α and PXR mRNA up-regulation (Figure 2) coincides with abrogation of JNK1 activation (Figure 3b), after TNF-α administration. Pre-treatment with rapamycin prior to TNF-α stimulation may release any inhibitory control that represses LXR-α and PXR from countering the effects of acute inflammation. Also, the formation of mTORC2 may also play a role in galvanising the response of LXR-α and PXR mRNA transcription. Based on results from this study, rapamycin (and its analogues) may be used to reduce acute inflammation by promoting the anti-inflammatory properties of LXR-α and PXR.

ACKNOWLEDGMENTS

REFERENCES

ASSENAT, E.; GERBAL-CHALOIN, S.; MAUREL, P.; VILAREM, M.J. and PASCUSSI, J.M. (2006). Is nuclear factor kappa-B the missing link between inflammation, cancer and alteration in hepatic drug metabolism in patients with cancer? *European Journal of Cancer*, vol. 42, no. 6, p. 785-792. **[CrossRef]**

BOGOYEVITCH, M.A. and KOBE, B. (2006). Uses for JNK: The many and varied substrates of the c-Jun N-Terminal kinases. *Microbiology and Molecular Biology Reviews*, vol. 70, no. 4, p. 1061-1095. **[CrossRef]**

CASTRILLO, A.; JOSEPH, S.B.; MARATHE, C.; MANGELSDORF, D.J. and TONTONOZ, P. (2003). Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *The Journal of Biological Chemistry*, vol. 278, no. 12, p. 10443-10449. **[CrossRef]**

CASTRILLO, A. and TONTONOZ, P. (2004). Nuclear receptors in macrophage biology: At the crossroads of lipid metabolism and inflammation. *Annual Review of Cell and Developmental Biology,* vol. 20, p. 455-480. **[CrossRef]**

CAVIGELLI, M.; DOLFI, F.; CLARET, F.X. and KARIN, M. (1995). Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *The EMBO Journal*, vol. 14, no. 23, p. 5957-5964.

CHIANG, G. and ABRAHAM, R. (2005). Phosphorylation of mammalian target of rapamycin (mTOR) at ser-2448 is mediated by p70S6 kinase. *The Journal of Biological Chemistry,* vol. 280, no. 27, p. 25485-25490. **[CrossRef]**

DUNN, C.; WILTSHIRE, C.; MACLAREN, A. and GILLESPIE, D.A. (2002). Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cellular Signalling*, vol. 14, no. 7, p. 585-593. **[CrossRef]**

ELORANTA, J.J. and KULLAK-UBLICK, G.A. (2005). Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism. *Archives of Biochemistry and Biophysics*, vol. 433, no. 2, p. 397-412. **[CrossRef]**

FOSTER, D. and TOSCHI, A. (2009). Targeting mTOR with rapamycin: One dose does not fit all. *Cell Cycle*, vol. 8, no. 7, p. 1026-1029. **[CrossRef]**

HAEUSGEN, W.; HERDEGEN, T. and WAETZIG, V. (2010). Specific regulation of JNK signalling by the novel rat MKK7γ1 isoform. *Cellular Signalling*, vol. 22, no. 11, p. 1761-1772. **[CrossRef]**

HANDSCHIN, C. and MEYER, U.A. (2005). Regulatory network of lipid-sensing nuclear receptors: Roles for CAR, PXR, LXR, and FXR. *Archives of Biochemistry and Biophysics*, vol. 433, no. 2, p. 387-396. **[CrossRef]**

HAO, Y.; WONG, R. and FEIG, L.A. (2008). RalGDS couples growth factor signaling to Akt activation. *Molecular and Cellular Biology,* vol. 28, no. 9, p. 2851-2859. **[CrossRef]**

HOLZ, M.K. and BLENIS, J. (2005). Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *The Journal of Biological Chemistry*, vol. 280, no. 28, p. 26089-26093. **[CrossRef]**

HRESKO, R.C. and MUECKLER, M. (2005). mTOR·RICTOR is the Ser^{473} kinase for Akt/protein kinase B in $_3T_3$-L1 adipocytes. *The Journal of Biological Chemistry*, vol. 280, no. 49, p. 40406-40416. **[CrossRef]**

JACINTO, E.; FACCHINETTI, V.; LIU, D.; SOTO, N.; WEI, S.; JUNG, S.Y.; HUANG, Q.; QIN, J. and SU, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*, vol. 127, no. 1, p. 125-137. **[CrossRef]**

JAESCHKE, A.; CZECH, M.P. and DAVIS, R.J. (2004). An essential role of the JIP1 scaffold protein for JNK activation in adipose tissue. *Genes and Development*, vol. 18, no. 16, p. 1976-1980. **[CrossRef]**

JAESCHKE, A. and DAVIS, R.J. (2007). Metabolic stress signaling mediated by mixed-lineage kinases. *Molecular Cell*, vol. 27, no. 3, p. 498-508. **[CrossRef]**

JOSEPH, S.B.; CASTRILLO, A.; LAFFITTE, B.A.; MANGELSDORF, D.J. and TONTONOZ, P. (2003). Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature Medicine,* vol. 9, no. 2, p. 213-219. **[CrossRef]**

KELKAR, N.; GUPTA, S.; DICKENS, M. and DAVIS, R.J. (2000). Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Molecular and Cellular Biology*, vol. 20, no. 3, p. 1030-1043. **[CrossRef]**

KHOVIDHUNKIT, W.; KIM, M.S.; MEMON, R.A.; SHIGENAGA, J.K.; MOSER, A.H.; FEINGOLD, K.R. and GRUNFELD, C. (2004). Thematic review series: The pathogenesis of atherosclerosis. Effects of infection and inflammation on lipid and lipoprotein metabolism: Mechanisms and consequences to the host. *The Journal of Lipid Research*, vol. 45, no. 7, p. 1169-1196. **[CrossRef]**

KIM, A.H.; YANO, H.; CHO, H.; MEYER, D.; MONKS, B.; MARGOLIS, B.; BIRNBAUM, M.J. and CHAO, M.V. (2002). Akt1 regulates a JNK scaffold during excitotoxic apoptosis. *Neuron*, vol. 35, no. 4, p. 697-709. **[CrossRef]**

KIM, M.S.; SWEENEY, T.R.; SHIGENAGA, J.K.; CHUI, L.S.; MOSER, A.; GRUNFELD, C. and FEINGOLD, K.R. (2007). Tumor necrosis factor and interleukin 1 decrease RXRα, PPARα, PPARγ, LXRα, and the coactivators SRC-1, PGC-1α, and PGC-1β in liver cells. *Metabolism*, vol. 56, no. 2, p. 267-279. **[CrossRef]**

KIM, Y.M.; KIM, T.H.; KIM, Y.W.; YANG, Y.M.; RYU, D.H.; HWANG, S.J.; LEE J.R.; KIM, S.C. and KIM, S.G. (2009). Inhibition of liver X receptor-α-dependent hepatic steatosis by isoliquiritigenin, a licorice antioxidant flavonoid, as mediated by JNK1 inhibition. *Free Radical Biology and Medicine*, vol. 49, no. 11, p. 1722-1734. **[CrossRef]**

KIM, K.H.; LEE, G.Y.; KIM, J.I.; HAM, M.; LEE, J.W. and KIM, J.B. (2010). Inhibitory effect of LXR activation on cell proliferation and cell cycle progression through lipogenic activity. *The Journal of Lipid Research,* vol. 51, no. 12, p. 3425-3433. **[CrossRef]**

KLIEWER, S.A. (2003). The nuclear pregnane X receptor regulates xenobiotic detoxification. *The Journal of Nutrition*, vol. 133, no. 7, p. 2444S-2447S.

LAFFITTE, B.A.; JOSEPH, S.B.; WALCZAK, R.; PEI, L.; WILPITZ, D.C.; COLLINS, J.L. and TONTONOZ, P. (2001). Autoregulation of the human liver X receptor α promoter. *Molecular and Cell Biology*, vol. 21, no. 22, p. 7558-7568. **[CrossRef]**

LANGMANN, T.; MOEHLE, C.; MAUERER, R.; SCHARL, M.; LIEBISCH, G.; ZAHN, A.; STREMMEL, W. and SCHMITZ, G. (2004). Loss of detoxification in inflammatory bowel disease: Dysregulation of pregnane X receptor target genes. *Gastroenterology*, vol. 127, no. 1, p. 26-40. **[CrossRef]**

LAPLANTE, M. and SABATINI, D.M. (2010). mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 107, no. 8, p. 3281-3282. **[CrossRef]**

LI, Y.; BOLTEN, C.; BHAT, B.G.; WOODRING-DIETZ, J.; LI, S.; PRAYAGA, S.K.; XIA, C. and LALA, D.S. (2002). Induction of human liver X receptor α gene expression via an autoregulatory loop mechanism. *Molecular Endocrinology*, vol. 16, no. 3, p. 506-514. **[CrossRef]**

LU, B.; MOSER, A.H.; SHIGENAGA, J.K.; FEINGOLD, K.R. and GRUNFELD, C. (2006). Type II nuclear hormone receptors, coactivator, and target gene repression in adipose tissue in the acute-phase response. *The Journal of Lipid Research,* vol. 47, no. 10, p. 2179-2190. **[CrossRef]**

MOREAU, A.; VILAREM, M.J.; MAUREL, P. and PASCUSSI, J.M. (2008). Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Molecular Pharmaceutics,* vol. 5, no. 1, p. 35-41. **[CrossRef]**

MOREL, C.; STANDEN, C.L.; JUNG, D.Y.; GRAY, S.; ONG, H.; FLAVELL, R.A.; KIM, J.K. and DAVIS, R.J. (2010). Requirement of JIP1-mediated c-Jun N-Terminal Kinase activation for obesity-induced insulin resistance. *Molecular and Cellular Biology*, vol. 30, no. 19, p. 4616-4625. **[CrossRef]**

MORIGUCHI, T.; TOYOSHIMA, F.; MASUYAMA, N.; HANAFUSA, H.; GOTOH, Y. and NISHIDA, E. (1997). A novel SAPK/JNK kinase, MKK7, stimulated by TNF α and cellular stresses. *The EMBO Journal*, vol. 16, no. 23, p. 7045-7053. **[CrossRef]**

NEUMANN, M.; BECH-OTSCHIR, D.; HUANG, X.; FERRELL, K. and DUBIEL, W. (1999). COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. *The Journal of Biological Chemistry,* vol. 274, no. 50, p. 35297-35300. **[CrossRef]**

NIHALANI, D.; WONG, H.N. and HOLZMAN, L.B. (2003) Recruitment of JNK to JIP1 and JNK-dependent JIP1 phosphorylation regulates JNK module dynamics and activation. *The Journal of Biological Chemistry*, vol. 278, no. 31, p. 28694-28702. **[CrossRef]**

PARENT, R.; KOLIPPAKKAM, D.; BOOTH, G. and BERETTA, L. (2007). Mammalian target of rapamycin activation impairs hepatocytic differentiation and targets genes moderating lipid homeostasis and hepatocellular growth. *Cancer Research,* vol. 67, no. 9, p. 4337. **[CrossRef]**

PEET, D.J.; TURLEY, S.D.; MA, W.; JANOWSKI, B.A.; LOBACCARO, J.M.; HAMMER, R.E. and MANGELSDORF, D.J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR*α*. *Cell*, vol. 93, no. 5, p. 693-704. **[CrossRef]**

REPA, J.J. and MANGELSDORF, D.J. (2000). The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annual Review of Cell and Developmental Biology*, vol. 16, p. 459-481. **[CrossRef]**

SABIO, G.; CAVANAGH-KYROS, J.; KO, H.J.; JUNG, D.J.; GRAY, S.; JUN, J.Y.; BARRETT, T.; MORA, A.; KIM, J.K. and DAVIS, R.J. (2009). Prevention of steatosis by hepatic JNK1. *Cell Metabolism*, vol. 10, no. 6, p. 491-498. **[CrossRef]**

SARBASSOV, D.D.; ALI, S.M. and SABATINI, D.M. (2005). Growing roles for the mTOR pathway. *Current Opinion in Cell Biology*, vol. 17, no. 6, p. 596-603. **[CrossRef]**

SARBASSOV, D.D.; ALI, S.M.; SENGUPTA, S.; SHEEN, J.H.; HSU, P.P.; BAGLEY, A.F.; MARKHARD, A. and SABATINI, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular Cell*, vol. 22, no. 2, p. 159-168. **[CrossRef]**

SCHATTENBERG, J.M.; SINGH, R.; WANG, Y.; LEFKOWITCH, J.H.; RIGOLI, R.M.; SCHERER, P.E. and CZAJA, M.J. (2006). JNK1 but not JNK2 promotes the development of steatohepatitis in mice. *Hepatology*, vol. 43, no. 1, p. 163-172. **[CrossRef]**

SHAH, Y.M.; MA, X.; MORIMURA, K.; KIM, I. and GONZALEZ, F.J. (2006). Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-κB target gene expression. *American Journal of Physiology Gastrointestinal and Liver Physiology*, vol. 292, no. 4, p. G1114-G1122. **[CrossRef]**

SINGH, R.; WANG, Y.; XIANG, Y.; TANAKA, K.E.; GAARDE, W.A. and CZAJA, M.J. (2009). Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis and insulin resistance. *Hepatology,* vol. 49, no. 1, p. 87-96. **[CrossRef]**

SONG, J.J. and LEE, Y.J. (2005) Cross-talk between JIP3 and JIP1 during glucose deprivation. *The Journal of Biological Chemistry*, vol. 280, no. 29, p. 26845-26855. **[CrossRef]**

TAKAI, S.; TOKUDA, H.; HANAI. Y.; HARADA, A.; YASUDA, E.; MATSUSHIMA-NISHIWAKI, R.; KATO, H.; OGURA, S.; OHTA, T. and KOZAWA, O. (2007). Negative regulation by p70 S6 kinase of FGF-2–stimulated VEGF release through stress-activated protein kinase/c-*Jun* N-terminal kinase in osteoblasts. *Journal of Bone and Mineral Research*, vol. 22, no. 3, p. 337-346. **[CrossRef]**

TSOU, H.K.; SU, C.M.; CHEN, H.T.; HSIEH, M.H.; LIN, C.J.; LU, D.Y.; TANG, C.H. and CHEN, Y.H. (2010). Integrin-linked kinase is involved in TNF-α-induced inducible nitric-oxide synthase expression in myoblasts. *Journal of Celular Biochemistry*, vol. 109, no. 6, p. 1244-1253. **[CrossRef]**

ULVEN, S.M.; DALEN, K.T.; GUSTAFSSON, J. and NEBB, H.I. (2004). Tissue-specific autoregulation of the LXRα gene facilitates induction of apoE in mouse adipose tissue. *The Journal of Lipid Research*, vol. 45, no. 11, p. 2052-2062. **[CrossRef]**

WAGNER, E.F. and NEBREDA, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature Reviews Cancer*, vol. 9, no. 8, p. 537-549. **[CrossRef]**

WAJANT, H. and SCHEURICH, P. (2001). Tumor necrosis factor-associated factor (TRAF) 2 and its role in TNF signaling, *The International Journal of Biochemistry & Cell Biology*, vol. 33, no. 1, p. 19-32. **[CrossRef]**

WANG, Y.A.; MOSER, A.H.; SHIGENAGA, J.K.; GRUNFELD, C. and FEINGOLD, K.R. (2005). Downregulation of liver X receptor-α in mouse kidney and HK-2 proximal tubular cells by LPS and cytokines. *The Journal of Lipid Research*, vol. 46, no. 11, p. 2377-2387. **[CrossRef]**

WANG, K.; DAMJANOV, I. and WAN, Y.Y. (2010). The protective role of pregnane X receptor in lipopolysaccharide/D-galactosamine-induced acute liver injury. *Laboratory Investigation*, vol. 90, no. 2, p. 257-265. **[CrossRef]**

WESTON, C.R. and DAVIS, R.J. (2002). The JNK signal transduction pathway. *Current Opinion in Genetics & Development*, vol. 12, no. 1, p. 14-21. **[CrossRef]**

WHITMARSH, A.J.; CAVANAGH, J.; TOURNIER, C.; YASUDA, J. and DAVIS, R.J. (1998). A mammalian scaffold complex that selectively mediates MAP kinase activation, *Science*, vol. 281, no. 5383, p. 1671-1674. **[CrossRef]**

WHITMARSH, A.J. (2006). The JIP family of MAPK scaffold proteins. *Biochemical Society Transactions*, vol. 34, p. 828-832. **[CrossRef]**

WOLFRUM, C. and STOFFEL, M. (2006). Coactivation of Foxa2 through Pgc-1β promotes liver fatty acid oxidation and triglyceride/VLDL secretion, *Cell Metabolism*, vol.3, no. 2, p. 99-110. **[CrossRef]**

XU, Y.; ZHU, F.; CHO, Y.; CARPER, A.; PENG, C.; ZHENG, D.; YAO, K.; LAU, A.T.Y.; ZYKOVA, T.; KIM, H.; BODE, A.M. and DONG, Z. (2010). Extracellular Signal-Regulated Kinase 8-mediated c-jun phosphorylation increases tumorigenesis of human colon cancer. *Cancer Research*, vol. 70, no. 8, p. 3218-3227. **[CrossRef]**

YANG, R.; WILCOX, D.M.; HAASCH, D.L.; JUNG, P.M.; NGUYEN, P.T.; VOORBACH, M.J.; DOKTOR, S.; BRODJIAN, S.; BUSH, E.N.; LIN, E.; JACOBSON, P.B.; COLLINS, C.A.; LANDSCHULZ, K.T.; TREVILLYAN, J.M.; RONDINONE, C.M. and SUROWY, T.K. (2007). Liver-specific knockdown of JNK1 up-regulates proliferator-activated receptor γ coactivator 1β and increases plasma triglyceride despite reduced glucose and insulin levels in diet-induced obese mice. *Journal of Biological Chemistry*, vol. 282, no. 31, p. 22765-22774. **[CrossRef]**

YASUDA, J.; WHITMARSH, A.J.; CAVANAGH, J.; SHARMA, M. and DAVIS, R.J. (1999). The JIP group of mitogen-activated protein kinase scaffold proteins. *Molecular and Cellular Biology*, vol. 19, no. 10, p. 7245-7254.

YU, L.; HAMMER, R.E.; LI-HAWKINS, J.; VON BERGMANN, K.; LUTJOHANN, D.; COHEN, J.C. and HOBBS, H.H. (2002). Disruption of *Abcg5* and *Abcg8* in mice reveals their crucial role in biliary cholesterol secretion. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, p. 16237-16242. **[CrossRef]**

ZANGER, K.; RADOVICK, S. and WONDISFORD, F.E. (2001). CREB binding protein recruitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box. *Molecular Cell*, vol. 7, no. 3, p. 551-558. **[CrossRef]**

ZELCER, N. and TONTONOZ, P. (2006). Liver X receptors as integrators of metabolic and inflammatory signaling. *The Journal of Clinical Investigation,* vol. 116, no. 3, p. 607-614. **[CrossRef]**

ZHAO, S.P. and DONG, S.Z. (2007). Effect of tumor necrosis factor α in cholesterol efflux in adipocytes. *Clinica Chimica Acta*, vol. 389, no. 1-2, p. 67-71. **[CrossRef]**

ZHOU, C.; TABB, M.M.; NELSON, E.L.; GRÜN, F.; VERMA, S.; SADATRAFIEI, A.; LIN, M.; MALLICK, S.; FORMAN, B.M.; THUMMEL, K.E. and BLUMBERG, B. (2006). Mutual repression between steroid and xenobiotic receptor and NF-κB signaling pathways links xenobiotic metabolism and inflammation. *The Journal of Clinical Investigation,* vol. 116, no. 8, p. 2280-2289. **[CrossRef]**