

TITLE:

TLR2 and TLR4 activation induces p38 MAPK-dependent phosphorylation of S6 kinase 1 in C2C12 myotubes

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RUNNING TITLE

TLR2 and TLR4 activate S6K1 in muscle cells

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ABBREVIATIONS:

PA: palmitic acid, LPS: lipopolysaccharide; MAPK: mitogen activated protein kinase; NF- κ B: Nuclear factor kappa B; S6K1: ribosomal S6 kinase 1; TLR: toll-like receptor

Abstract

Toll-like receptors 2 (TLR2) and 4 (TLR4) are present in the plasma membrane of skeletal muscle cells where their functions remain incompletely resolved. They are able to bind various extra-cellular ligands, such as FSL-1, lipopolysaccharide (LPS) and/or palmitic acid (PA). The purpose of this study was to investigate the link between PA, TLR2/4, and ribosomal S6 kinase 1 (S6K1) in C2C12 myotubes. Incubation with agonists of either TLR2 or TLR4, as well as incubation with high concentration of PA, led to an increase in S6K1 phosphorylation level. Canonical upstream kinases of S6K1, protein kinase B (PKB) and mammalian target of rapamycin complex 1 (mTORC1), were regulated in the opposite way by PA, indicating that those kinases were probably not involved. By using the SB202190 inhibitor, we evidenced that p38 MAPK was a key mediator of PA-induced phosphorylation of S6K1. Down-regulation of either *tlr2* or *tlr4* gene expression by small interfering RNAs prevented the activation of both p38 MAPK and S6K1 by FSL-1, LPS or PA. In summary, our results showed that TLR2 and TLR4 agonists are able to increase the level of S6K1 phosphorylation in a p38 MAPK dependent way in C2C12 myotubes. As PA induced the same intracellular signaling, we evidenced for the first time an atypical pathway for PA that is induced at the cellular membrane level and results in a higher phosphorylation state of S6K1.

INTRODUCTION

Toll-like receptors (TLRs) play a major role in the immune response and are implicated in septic shock and in chronic diseases, amongst other. TLRs are expressed in various tissues. In white blood cells, they are known to induce the production of cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 α (IL1 α) and interleukin-6 (IL6), via nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinases (MAPK) signaling (Akira et al., 2003, West et al., 2006). More than 70% of the TLR family is expressed in C2C12 myoblasts and myotubes, but their roles are still under investigation (Frost et al., 2006). The most frequently expressed, TLR2 and TLR4, have been shown to play a role in insulin resistance and atherosclerosis induced by a high-fat diet in animal models (Caricilli et al., 2008, Madan and Amar, 2008, Radin et al., 2008).

Palmitic acid (PA), a long chain saturated fatty acid, is acutely and highly increased during endurance exercise. While it is chronically and moderately elevated in obesity and type 2 diabetes. These two conditions are associated with both insulin resistance and proinflammatory cytokines production, the first situation being transitory whereas the second one being chronic (Boden, 2003). PA is capable of activating TLR2 pathway in C2C12 myogenic cells (Senn, 2006) and TLR4 in the RAW264.7 macrophage cell line and in 3T3-L1 preadipocytes (Shi et al. 2006). Therefore, besides its role as energy substrate, extra-cellular PA is suggested to activate intra-cellular signaling pathways (Clarke, 2004).

Insulin activates the PKB (protein kinase B) pathway leading to an increased activity of mTORC1 (mammalian target of rapamycin complex 1) and S6K1 (ribosomal S6 kinase 1). The latter is a Ser/Thr protein kinase that catalyses the phosphorylation of S6 protein, a component of the eukaryotic ribosomal 40S subunit which is essential for increasing protein synthesis (Baar and Esser, 1999, Pullen and Thomas, 1997). Activity of S6K1 changes in response to growth factors and nutrients signaling, and besides its role in protein synthesis, S6K1 may play a role in various pathologies, including obesity, diabetes, ageing and cancer. For a detailed review see (Fenton and Gout, 2011).

PA is often considered a negative regulator of insulin sensitivity through decreased activation of the PKB pathway but the molecular links between PA and PKB are not clear. As TLR are likely candidates, the initial purpose of the present study was to explore a potential relationship between PA, TLR2/4 and the PKB/mTOR/S6K1 pathway. Contrary to our hypothesis, the intermediates of this pathway were not regulated in the same way by PA: the phosphorylation state of S6K1 was increased whereas PKB and mTOR were dephosphorylated. Therefore, we decided to explore a potential regulation of PA on S6K1 which we hypothesized to be transduced by TLR and p38 MAPK.

2. MATERIALS AND METHODS

2.1. Cell culture

C2C12 cells were purchased from the ATCC (Manassas, VA) and incubated at 37°C in a humidified atmosphere of 5% CO₂. Myoblasts were grown in

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μ M non essential amino acids (NEAA), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4.5 g/l glucose until 70% confluence and differentiated for 72-96h in a medium in which FBS was replaced by 1% horse serum.

FSL-1 (Pam2Cys-GDPKHPKSF), Ultrapure lipopolysaccharide from 0111:B4 E.coli (LPS) and PA were added to culture medium at a final concentration of 0.75 μ M, 1 μ g/ml, and 0.75 mM for 30 min, 3 hours and 3 hours, respectively. SB202190 was added to culture medium 15 minutes prior addition of either TLR activator. LPS and FSL-1 were dissolved in water, SB202190 in DMSO, and PA in ethanol; control vehicle contained the same solvent.

FSL-1 and LPS were obtained from InvivoGen (Toulouse, France), palmitic acid (PA, P5585) was purchased from Sigma-Aldrich (Bornem, Belgium) and p38 MAPK inhibitor SB202190 was obtained from Alexis Biochemicals (Zandhoven, Belgium).

2.2 Protein extraction

Cells were extracted using the method described by Deldicque et al. (2007). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and homogenised in an ice-cold lysis buffer containing 20mM Tris, pH 7.0, 270mM sucrose, 5mM EGTA, 1mM EDTA, 0.1% Triton X-100, 1mM sodium orthovanadate, 50mM sodium β -glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM 1,4-dithiothreitol (DTT), and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium). Cells homogenates were centrifuged at 10,000g for 10min at 4°C. Protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Cells lysates were stored at -80°C until subsequent analyses.

2.3 Western blotting.

Protein lysates (10-20 μ g) were combined with Laemmli and separated by SDS-PAGE (10-12%). After electrophoretic separation (40mA), the proteins were transferred to PVDF membranes at 80V for 2h. Membranes were blocked for 60min in Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% milk. Then, the membranes were incubated overnight at 4°C in TB ST containing 1% bovine serum albumin and one of the following antibodies: anti-phospho-p38 Thr180/Tyr182 (1:1,000), anti-total p38 (1:1,000), anti-phospho-PKB Ser473 (1:1,000), anti-total PKB (1:1,000), anti-phospho-mTOR Ser2448 (1:1,000), anti-total mTOR (1:1,000) and anti-TLR2 (1:1,000) were obtained from Cell Signaling Technology (Leiden, The Netherlands). Anti-phospho-S6K1 Ser 389 (1:1,000), anti-total S6K1 (1:1,000) and anti-TLR4 (1:1,000) were from Santa Cruz Biotechnology. Anti-GAPDH (1:20,000) was obtained from Abcam (Cambridge, UK). Anti-actin (1: 20000) was from BD Transduction laboratories (Erembodegen, Belgium). Membranes were washed three times with TBST and were incubated with a secondary antibody at room temperature for 60min [anti-rabbit (1:10,000) or anti-mouse (1:20,000) from Sigma (Bornem, Belgium)]. After three additional washes, chemiluminescent detection was carried out

using an ECL Western blotting kit (Amersham ECL Plus, GE Healthcare, Diegem, Belgium). Bands were visualized on film, scanned and quantified by densitometry using the ImageMaster 1D image analysis software (Amersham-GE Healthcare, Diegem, Belgium).

2.4 Gene silencing

Ambion Silencer® Select Pre-designed siRNAs against mouse *tlr2* and *tlr4* and Silencer® Validated siRNA against *gapdh* were purchased from Ambion International/Applied Biosystems (Huntingdon, UK). Transfection of C2C12 cells was performed with Lipofectamine 2000 (Invitrogen, Belgium), according to manufacturer's instructions. Briefly, cells were differentiated for 72 hours, then transfected with 50 nM of siRNA in the presence of 4 μ l Lipofectamine 2000. Transfection durations were 17 and 6 hours for siRNA against *tlr2* and *tlr4*, respectively. Transfection with siRNA against *gapdh* was included for both durations. Since transfection, antibiotics were omitted from the medium for a 40-hour period, and then re-added. Cell harvesting was done 72 hours after transfection.

2.5 Treatment with palmitic acid.

PA was dissolved in 100% ethanol to a final stock solution of 75mM. The final concentration used in all experiments was 0.75mM, diluted in cell DMEM media supplemented with 100 μ M nonessential amino acids. Ethanol was added in all control samples as a vehicle. After 4 days of differentiation, matures myotubes were exposed to PA for 3h.

2.6 LAL test

Limulus Amebocyte Lysate (LAL) concentration was determined using a disposal kit (PYROGENT® Ultra – LONZA). LAL test is a qualitative test from Gram-negative bacterial endotoxin. It was performed according the instructions of the manufacturer. Briefly, Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. A lysate provided (Limulus polyphemus) was used in order to determinate the labeled concentration (EU/ml) as Standard Endotoxin. LAL reagent water was used as a negative control. 100 μ l of standard, sample (DMEM with ethanol and DMEM with ethanol and palmitic acid) or water was diluted with 100 μ l of reconstituted lysate into the appropriate 10 x 75 mm tube. After being mixed, the tubes were placed in a 37°C hot water for a 60-minute incubation. After exactly 60 min, the absorbance was measured at 405nm using a microplate reader FLUOstar OPTIMA (BMG Labtech).

2.7 Statistical analysis

All results are shown as means $\pm$ SEM from 4 independent experiments. Student's unpaired t-test was used for comparing groups, with the exception of figure 2 after incubation with palmitic acid, FSL and LPS with or without p38 MAPK inhibitor, where a two-way ANOVA and Bonferroni post-hoc tests were used. Statistical threshold was set at $P < 0.05$.

3. RESULTS

3.1 Incubation of C2C12 myotubes with palmitic acid results in a PKB-independent p38 MAPK-dependent increase in the phosphorylation state of S6K1

In C2C12 myotubes, a 3 hour incubation with 0.75 mM PA resulted in a 40% decrease in PKB phosphorylation, as compared to incubation with the vehicle alone ($P < 0.05$; Fig. 1A). Likewise, phosphorylation level of mTOR, a kinase downstream of PKB, was reduced by ~50% after C2C12 myotube incubation with PA ($P < 0.05$; Fig. 1B). However, in the same conditions, incubation with PA resulted in a 1.7-fold increase in p38 MAPK and in a 1.6-fold increase in S6K1 phosphorylation levels ($P < 0.05$ in both case; Fig. 1C & 1D).

A 30 minute incubation with 0.75 μ M FSL-1 (a synthetic diacylated lipoprotein TLR2 agonist) resulted in a 2-fold increase in S6K1 phosphorylation ($P < 0.001$; Fig. 2). This effect was not observed when the cultures were pre-treated with SB202190, suggesting that p38 MAPK is controlling S6K1 phosphorylation. Likewise, TLR4 activation induced by 3 hour incubation process with 1 μ g/ml LPS (lipopolysaccharide, a TLR4 agonist) resulted in a 1.5-fold increase in S6K1 phosphorylation ($P = 0.002$), which was repressed by the inhibitor of p38 MAPK. The same effect was observed after incubation with 0.75 mM PA for 3 hours (1.7-fold increase, $P = 0.002$).

3.2 Downregulation of TLR2 or TLR4 protein expression prevents palmitic acid-induced phosphorylation of p38 MAPK and S6K1 in C2C12 myotubes

To further explore the link between PA, p38 MAPK and S6K1 in muscle cells, we transfected C2C12 myotubes by either an anti-*tlr2* or an anti-*tlr4* siRNA.

First we made sure that downregulation of TLR expression using small interfering oligonucleotides was significant and specific. As compared to transfection with an anti-*gapdh* siRNA, the levels of TLR2 and TLR4 proteins were decreased by 46% ($P < 0.05$) and 35% ($P < 0.001$), respectively (Figs 3A & 3B). No significant off-target effect was observed, since neither TLR2 protein content was change after anti-*tlr4* transfection (Fig. 3A), nor TLR4 protein content was modified after anti-*tlr2* transfection (Fig. 3B). As compared to non-transfected myotubes, transfection of anti-*gapdh* siRNA resulted in a reduction of GAPDH protein expression of 58% ($P < 0.001$) and 61% ($P < 0.001$), using the protocol of anti-*tlr2* or anti-*tlr4* siRNAs, respectively (see Materials and Methods section). Furthermore, transfection with anti-*gapdh* siRNA had no effect on TLR2 and TLR4 protein expression, with actin protein being used as gel loading control (not shown).

In C2C12 cells transfected with anti-*tlr2* siRNA, no increase in the phosphorylation levels of p38 MAPK and S6K1 was observed upon incubation with PA, while it was the case when C2C12 myotubes were transfected with anti-*gapdh* siRNA (+100% for p38 MAPK, $P < 0.05$, Fig. 3C; +78% for S6K1, $P < 0.05$, Fig. 3E). Similar results were observed when comparing the effect of PA on C2C12 myotubes after either anti-*gapdh* or anti-*tlr4*: a significant increase in the phosphorylation levels of p38 MAPK (+100%, $P = 0.002$) and S6K1 (+89%, $P = 0.009$) was seen only in control anti-*gapdh* transfected cells (Figs. 3D & 3F).

3.3 In C2C12 myotubes, S6K1 phosphorylation is increased by stimulation of either TLR2 or TLR4 receptors

To ascertain that TLR stimulation results in an increase in S6K1 phosphorylation as seen after PA incubation, C2C12 myotubes were submitted to either a 30-min 0.75 μ M FSL-1 or a 3-h 1 μ g/ml LPS incubation.

In control myotubes transfected with anti-*gapdh* siRNA, FSL-1 and LPS induced a 1.8-fold ($P < 0.05$, Fig. 4A) and a 1.7-fold increase in S6K1 phosphorylation ($P < 0.05$, Fig. 4B), respectively. As expected, FSL-1 did not produce any change in S6K1 in C2C12 myotubes transfected with anti-*tlr2* siRNA (Fig. 4A). Similarly, transfection with anti-*tlr4* siRNA prevented the LPS-induced change in S6K1 phosphorylation (Fig. 4B).

To further investigate the activation of S6K1 induced by TLR2 and TLR4 agonists in muscle cells, we first measured the levels of phosphorylation of p38 MAPK. Both FSL-1 and LPS induced significant increases in control myotubes transfected with anti-*gapdh* siRNA [FSL-1: 2.2-fold ($P < 0.05$) and LPS: 1.9-fold ($P < 0.05$), Fig. 4C & 4D]. When transfected with the appropriate siRNA, this effect was not detected (Fig. 4C & 4D). Additionally, as previously mentioned, pre-incubation with p38 MAPK inhibitor SB202190 prevented both FSL1- and LPS-induced phosphorylation of S6K1 (Fig. 2) suggesting that p38 MAPK is essential to induce the TLR related S6K1 phosphorylation in cultured myotubes.

4. DISCUSSION

In this study, we clearly established a link between TLR2/4, p38 MAPK and S6K1 in C2C12 myotubes. Incubation with either FSL-1, LPS or PA led to rapid increase of S6K1 phosphorylation level, in a p38 MAPK dependent way, beyond a known inhibition of the PKB-mTORC1 pathway.

4.1. Palmitic acid as a signaling molecule for myotubes

In several studies in 3T3-L1 adipocytes and C2C12 myotubes, incubating the cells with 0.75 mM free PA diminished the phosphorylation levels of PKB (Chavez and Summers, 2003, Schmitz-Peiffer et al., 1999). While this decrease was suspected to lower downstream S6K1 activation, we found, to the contrary, that PA application for 3 hours resulted in increased S6K1 phosphorylation. This observation indicates that another kinase is able to activate S6K1, reversing the effect of PKB inhibition on S6K1. Only two studies reported an increase in S6K1 phosphorylation after PA incubation in muscle (Ragheb et al., 2009, Wang et al., 2010). Both authors found concomitant decrease in the phosphorylation state of PKB.

The relationship between activation of TLR2/4 and insulin resistance was demonstrated in cell (Senn 2006), animal (Shi et al. 2006) and human (Lambert et al. 2008) models. Our results confirm that a decreased activity of the IRS/PKB pathway is implicated in this phenomenon. However, the clear relationship between the activation of TLR2/4 by various ligands amongst which PA and the dephosphorylation of PKB have still to be elucidated. Considering the results reported in the present paper, the most direct hypothesis is the negative feedback mechanism exerted by S6K1 on PKB (Tremblay and Marette, 2001). However another mechanism implicating the activation of the IKK/NF- κ B pathway by TLR2/4 might not be ruled out. Indeed, TLR2/4 activate the pro-inflammatory program of the cell via the IKK/NF- κ B pathway. Infusion of PA in WT mice resulted in an increased expression of IL6 and TNF- α whereas

the same response was not observed in TLR4^{-/-} mice (Shi et al. 2006). Therefore, the dephosphorylation of PKB reported in the present paper may potentially be related to an inhibition of IRS initiated by a pro-inflammatory process induced by TLR2/4 activation.

4.2. Crosstalk between S6K1 and p38 MAPK

We explored p38 MAPK as an upstream activator of S6K1 because we and others previously reported such crosstalk in C2C12 (Cuenda and Cohen, 1999, Deldicque et al., 2008) and because it has been showed to be phosphorylated in a TLR2 dependent manner in the same cell line (Senn, 2006). We confirmed that PA induced an increase in p38 MAPK phosphorylation in our conditions. When cells were pre-treated with SB202190, a widely used p38 MAPK inhibitor, PA-induced S6K1 phosphorylation was totally repressed, indicating the presence of a crosstalk between p38 MAPK and S6K1 during PA incubation. Since pre-treatment with SB202190 decreased the basal levels of S6K1 phosphorylation by ~60%, it is probable that the crosstalk also exists at a basal state without PA stimulation. Although the role of S6K1 in protein synthesis is well known, the role of the crosstalk between p38 MAPK and S6K1 is still unresolved (Cuenda and Cohen, 1999, Deldicque et al., 2007).

Noteworthy, long-term administration of PA to L6 muscle cells was showed to reduce the phosphorylation state of S6K1 observed after insulin administration (Dimopoulos et al., 2006). Hence, brief elevation of extracellular PA concentration, as what was reproduced in our *in vitro* conditions, could activate S6K1 via a MAPK-dependent PKB/mTORC1-independent pathway, while longer stimulation could induce PKB/mTORC1/S6K1 inhibition. This may explain how muscle adapts differentially to a steep elevation of plasma free fatty acid, as observed during exercise (van Loon et al., 2005), or to a prolonged mild elevation of PA, as observed in diabetes and obesity. S6K1 phosphorylation was correlated with increased skeletal muscle mass in both animals (Baar and Esser, 1999, Nader et al., 2005) and humans (Koopman et al., 2006, Terzis et al., 2008). However, we had no opportunity for measuring incorporation of a labelled amino acid. Therefore, the correlation between S6K1 and protein synthesis relies on previous findings in the same systems. Further confirmation that short-lasting elevation of PA is able to activate protein synthesis will bring valuable information on the *in vivo* relevance of our findings *in vitro*.

4.3. Pharmacological and genetic controls

PA can induce cellular signaling via either intra- or extracellular action (Aas et al., 2005). We investigated the second way of action, since PA was reported to activate TLR in different cell types (Caricilli, Nascimento, 2008, Reyna et al., 2008, Senn, 2006, Shi et al., 2006, Varma et al., 2009). We further investigated TLR2 and TLR4 pathways using two techniques, namely agonist assay and gene silencing. Either FSL-1 or LPS induced an activation of S6K1 mediated by p38 MAPK, thus indicating that both TLR2 and TLR4 receptors are functional in the myotube model that we used. While p38 MAPK is one of the canonical targets of TLR2 and TLR4, further increased phosphorylation of S6K1 after incubation with TLR agonist was only reported once in macrophages (Esser et al., 2011).

Small interfering RNA against *tlr2* was efficient in reducing TLR2 protein content by 46%. Although the direct repression of TLR2 was not mentioned in his paper, Senn reported a similar ~50% decrease of interleukin 6 expression, a downstream product of TLR activation (Senn, 2006). Interestingly, a ~50% decrease in TLR2 expression was sufficient to prevent further signaling induced by FSL-1. This was also true for TLR4: LPS did not produce any downstream phosphorylation of p38 MAPK or S6K1 when the receptor was knocked down by ~50%. Therefore, we hypothesize that a TLR activation threshold (depending on the number of receptors) exists below which no activation of p38 MAPK would be possible. Furthermore, when knocking down either *tlr2* or *tlr4* gene expression, we observed a dramatic decrease in the activation of p38 MAPK and S6K1 phosphorylation by PA, thus strongly suggesting that these effects were related to the extracellular binding of PA to TLR2 or TLR4. This also suggests some kind of synergy between TLR2 and TLR4 to mediate PA-induced signaling. Although no direct interaction has been found between the receptors, cooperation between TLR2 and TLR4 has been reported in brain microglia (Laflamme et al., 2003).

4.4. Potential contaminants

To avoid any effects due to endotoxin contamination of commercially available preparations of bovine serum albumin (BSA), we added PA to culture medium, without the adjunction of BSA. Contrary to us, in the same BSA-free condition, Erridge and Samani (2009) did not report any change in p38 MAPK in C2C12 cells exposed to 0.1mM PA. The different results between both studies could be explained by the different PA concentration used and the differentiation state of the cells. To further discard a role of endotoxins, we performed a LAL test on PA-containing media, as well as media containing PA vehicle, i.e. ethanol (data not shown).

5. CONCLUSION

In conclusion, we showed that TLR2 and TLR4 agonists are able to increase the level of S6K1 phosphorylation in a p38 MAPK dependent way in C2C12 myotubes. Furthermore PA, an endogenous ligand of TLR, induced the same intracellular signaling. Our results evidenced for the first time an atypical pathway for PA that is induced at the cellular membrane level and results in a higher phosphorylation state of S6K1 via activation of TLR and phosphorylation of p38 MAPK.

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The authors have no conflict of interest

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Figure legends.

Figure 1. Effect of a 3 hour 0.75mM palmitic acid incubation on the phosphorylation state of PKB (A), mTORC1 (B), p38 MAPK (C) and S6K1 (D) in C2C12 cells. Results were normalized to the untreated (control) groups. n=4 / group. *: $P < 0.05$ versus control. Representative western blots are shown.

Figure 2. Phosphorylation of S6K1 is induced by FSL-1, LPS and palmitic acid in C2C12 myotubes. SB202190 is a p38 MAPK antagonist. Results were normalized to the untreated (control) groups. **: $P < 0.01$ and ***: $P < 0.001$ versus controls (n = 4-5 / group).

Figure 3. TLR-mediated activation of p38 MAPK and S6K1 by palmitic acid. TLR2 and TLR4 protein expression are specifically downregulated after transfection with anti-*tlr2* (A) or anti-*tlr4* (B), respectively. The effect of 0.75mM palmitic acid on p38 MAPK and S6K1 phosphorylation was assessed in C2C12 cells transfected with anti-*gapdh* (control) anti-*tlr2* (C & E) or anti-*tlr4* (D & F) siRNA. n=4 / group. Values are reported to cells transfected with anti-*gapdh* and exposed with a vehicle. *: $P < 0.05$ and **: $P < 0.01$, versus control (n=4 / group). Representative western blots are shown.

Figure 4. Activation of p38 MAPK and S6K1 by agonist of TLR2 and TLR4 receptor. Phosphorylation levels of S6K1 and p38 MAPK protein induced by incubation with FSL-1 or LPS were measured in myotubes transfected with anti-*gapdh* siRNA (control) anti-*tlr2* (A & C) or anti-*tlr4* (B & D). S6K1 phosphorylation was analyzed without (-) and with (+) FSL-1 (A) or LPS (B) after transfection with anti-*tlr2* or anti-*tlr4*, respectively. Under the same conditions, representative western-blot reflecting p38 MAPK are shown to demonstrate a similar increase in levels of S6K1 and p38 MAPK after incubation with both FSL-1 (C) and LPS (D). *: $P < 0.05$ versus control (n=4 / group).

Figure 1.

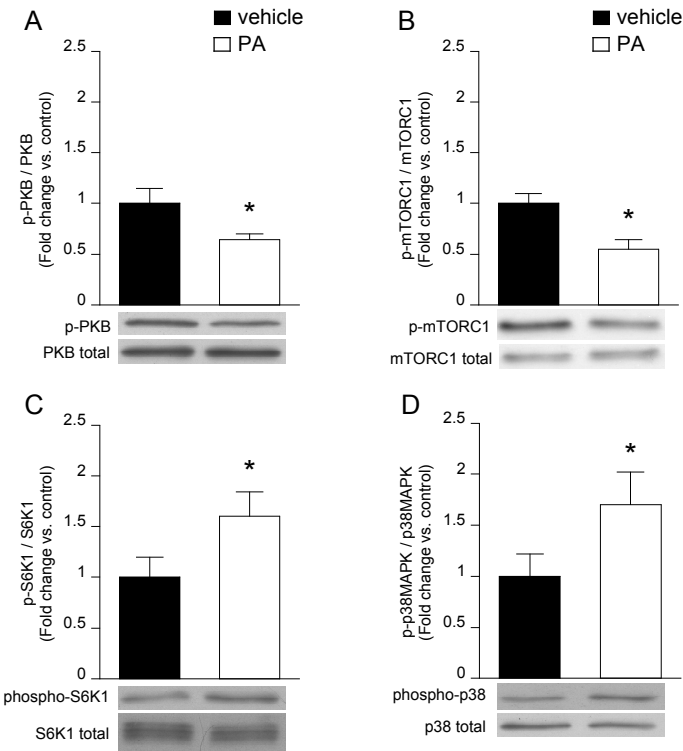


Figure 2.

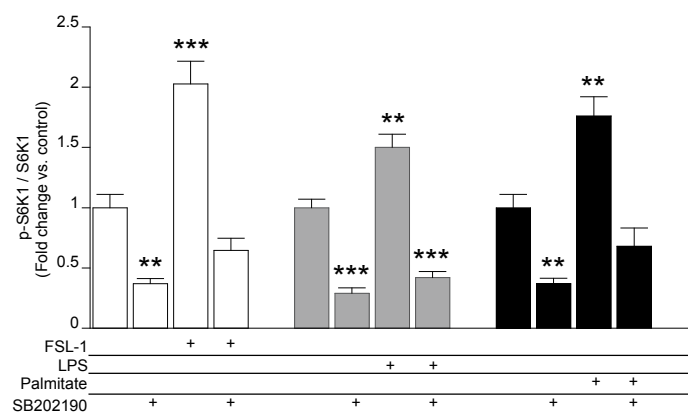


Figure 3.

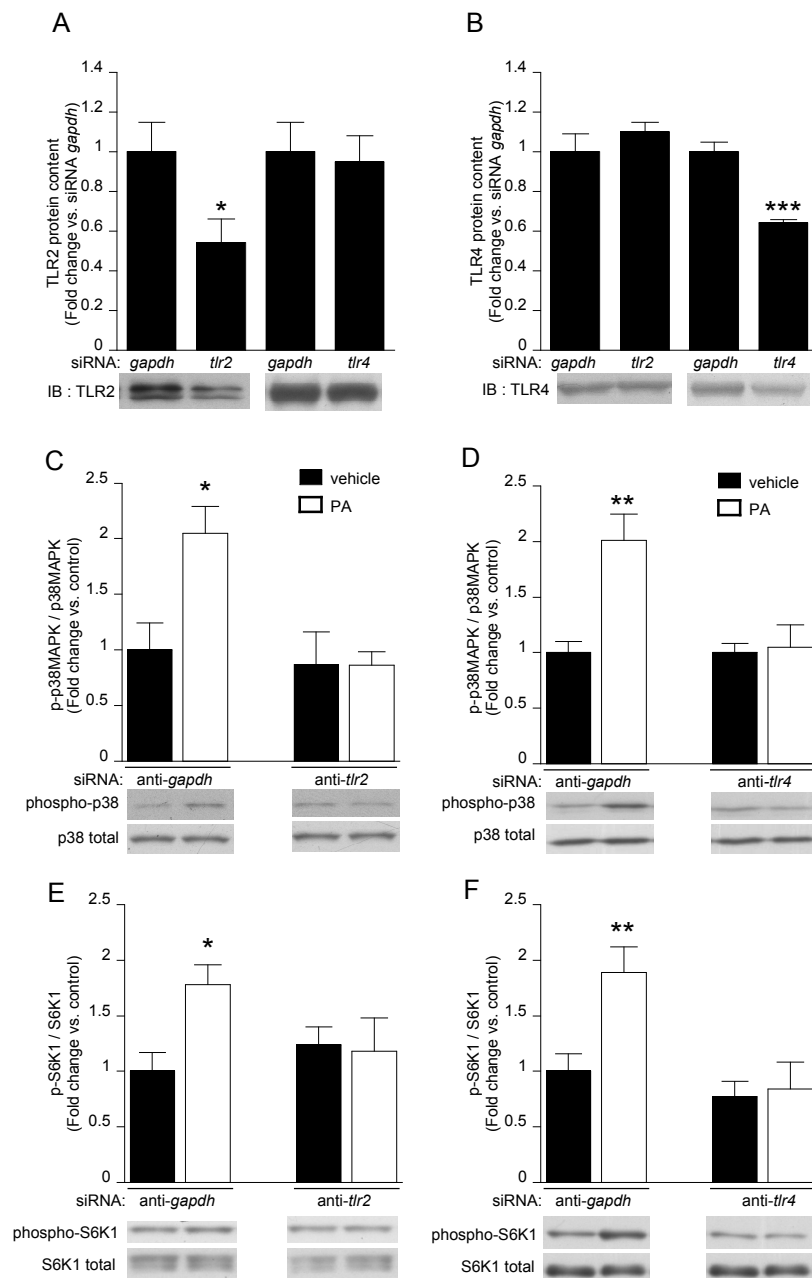


Figure 4.

