Research Article

Toll-like receptor 4 activation in skeletal muscle of diet-induced obese rats

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Abstract

Toll-like receptor 4 (TLR4) is found in the membrane of skeletal muscle cells. A variety of factors can activate TLR4. It has been shown that TLR4 expression reduce after aerobic training, but more studies considering the influences of different types of training on TLR4 expression are necessary. The purpose of this study was to evaluate the influence of 8 weeks of aerobic training on muscle TLR4 Expression in rats. Twenty Male Wistar rats (200±20 g) divided into four groups: control, training, high fat diet (HFD) and HFD+exerise. High fat diet was made by adding 10% animal oil, 2% cholesterol and 0.5% colic acid to standard rodent chow. Training group performed a swimming training protocol (1 h/day, and 5 days/week for 8 weeks). Forty eight hours after the final session of training, the rats were sacrificed and their gastrocnemius muscle was removed for determination of TLR4 expression. Training significantly decreased TLR4 messenger RNA and protein expression ($p<0.05$). Levels of TLR4 expression in the HFD group was significantly ($p<0.05$) higher tahn control ones. Our result displayed that training in rats induceed a critical suppression in the TLR4 signaling in muscle. These data give noticeable progress in our knowledge of the events that link physical training to an improvement in inflammation.

Key Words: **Aerobic training, High fat diet, Toll-like receptor 4, Inflammation, Skeletal muscle**

Introduction

Low-grade inflammation is general manifestations and could play a role in the pathogenesis of obesity (Jialal et al., 2014). A disorder of adipose tissue biology plays a latent role in the initiation of inflammatory events in obesity (Fresno et al., 2011). Currently, various studies have provided awareness in the expression and activation of innate immune receptors such as Toll-like receptors (TLRs) in different cells. TLRs seem to tend to the chronic inflammatory condition of obesity (Kawai & Akira, 2011). TLRs are related to the increased release of cytokines and the stimulation of antimicrobial activity by both the acquired and innate immune system. One of these TLRs, TLR4, upon stimulation with lipopolysaccharide (LPS), in conjunction with CD14 (LPS receptor), mediates several processes in the inflammatory cascade, such as the production of tumor necrosis factor-α (TNF-α) and other inflammatory cytokines (Kim & Sears, 2010). TLR4 activation stimulates a range of intracellular signaling pathways that coordinate the extent, form and duration of the inflammatory response. Studies have been demonstrated that the TLR4 may play a main role in the link between insulin resistance, inflammation, and obesity. TLR4 is a pivotal modulator in the cross-talk between inflammatory and metabolic pathways (Zwagerman et al., 2010). The regulation of TLRs as key factor involved in the inflammatory responses to training has received great attention. Chronic and Acute training change the function and the number of circulating cells of the immune system and also induce a cytokine response with a role that not only handles training-induced inflammation, but also regulates the accessibility of metabolites for muscle contraction (Wu et al., 2014). The anti-inflammatory effects of training could be partially related to muscle cytokine production profile induced by muscular contraction. Tumor necrosis factor a (TNF-a), a key proinflammatory cytokine with a significant role in the modulation of cellular processes, and the anti-inflammatory cytokine interleukin 10 (IL-10), is secreted by the human adipose stores (Simpson et al., 2009). The expression of these inflammatory factors is dependent on the nuclear factor kB (NF-kB) path. TLR4 may activate NF-kB, which is a transcription co-

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-ponent the inflammatory response. It is known that TLR4 expression decreases in young subjects following one session of aerobic training performed at high. Furthermore, TLR4 mRNA expression from muscle-homogenate is reduced by endurance training in humans, and resistance training induces a decreased expression of TLR4 in rat skeletal muscle (Nickel et al., 2012). Stewart et al., (2005) showed that aerobic and resistance training together decreases TLR4 expression in old and young population. Similar findings have been demonstrated by Timmerman, where resistance and aerobic training did not change total TLR4 expression, but lowered TLR4 expression in pro-inflammatory monocytes (Timmerman et al., 2008).

Training in diet-induced obesity (DIO) rats, causes a significant suppression in the TLR4 signaling path in the muscle and adipose tissue. TLR expression has consequently shown to be decreased after a blend of aerobic and resistance training, but more researches reflecting the effects of different training intensities, type and duration on TLR expression are required. Weight rise following use of a high-calori/high-fat diet is related to increase of endogenous lipopolysaccharide and glucose, which can change TLR4 expression and therefore monocyte functional capability. In addition, TLR4 has a role in the body fat build-up, since mice with a knockout of TLR4 progress the Adonis phenotype that is identified by low body fat and high bone mineral density nevertheless of dietary fat amount (Mignot et al., 2012). It has showed that inactive non-obese adults have a higher TLR4 expression than active individuals (Mignot et al., 2012). More research is necessary to compare the impact of physical inactivity and weight gain on TLR4. It would be helpful to know if the aerobic swimming training can also cause more positive immunological changes. We hypothesized that high-fat diet-induced weight gain would be associated with an increase in TLR4 expression and aerobic training would cause greater alterations in inflammation. Therefore, the aim of this study was to investigate the effects of 8 weeks of swimming training training and high fat diet on muscle TLR4 expression in rats.

Materials and Methods

Animals

Male Wistar rats with age of 8 weeks (200±20 g) were kept under observation for one week before the experimentation to be acclimatized with environmental conditions. During the experiment, all the rats were kept in standard cage in a room with standard temperature (22±2 °C) and humidity (%55±5) with a 12 h light/dark cycle and free access to water and food. All protocols were allowed by the institutional animal ethics committee of the Baqiyatallah University of Medical Sciences, which heeds the National Institutes of Health guidelines for the use and care of animals.

Experimental groups and design

Rats were haphazardly divided into four groups (n=5 per group): control healthy rats that stayed sedentary (C), trained, healthy rats that performed training for 8 weeks (E), high fat diet rats that remained inactive and fed with high fat diet (HFD), and trained high fat diet rats that performed training for 8 weeks and fed with high fat diet (HFD&E). High fat diet was built by adding 2% cholesterol powder (Merck, Germany), 0.5% colic acid powder (Merck, Germany) and 10% animal oil to standard rodent chow (Reyna et al., 2013).

Training protocol

The animals were adapted to swimming in the first week of training (rats swam for 10 min at the first day; duration of training was added daily 10 min until swimming time for 60 min) in a rubber swimming tank with dimension of 55×100×60 cm that was enough for 6 rats to swim, concurrently. Water temperature was maintained at 32±2 °C. The training protocol consisted of daily swimming sessions (1 h/day; 9:00–11:00 AM, 5 days/week, for 8 weeks) (Teerapornpuntakit et al., 2009). The control groups (C and HFD) remained sedentary in the filled swimming tank that animal's paws reached to the bottom of the tank and their head were out of water. Initially, weekly and final body weights were determined for all the rats by digital scale in a certain day of the week. The rats were killed with an overdose of anesthetic (chloral hydrate) for 48 h after the last session. Gastrocnemius muscle was removed and after washing with phosphate buffered saline and snap freezing in liquid nitrogen, kept in -80 ºC for assessment of protein and gene expression of TLR4.

Evaluation of TLR4 messenger RNA expression by Real-Time PCR

Total RNA was extracted using STAT- 60 reagent (Tel-Test Co.) according to the manufacturer's recommendations. To exterminate genomic pollution, RNA was treated with DNase me using a kit (EN0521; Fermentas). The cDNAs were synthesized from 500 ng DNase-treated RNA samples with a RevertAid™ First Strand cDNA Synthesis kit (K1622; Fermentas, Germany) using oligo (dT) primers. For PCR reactions, primers were adjusted from other primers, and synthesized by Cinnagen. PCRs were performed using Master Mix and SYBR Green I in an Applied Biosystems, StepOne™ thermal cycler (Applied Biosystems, USA). The PCR program began with an initial melting cycle for 5 minutes at 95°C to activate the polymerase, came after by 40 cycles of melting (30 seconds at 95°C), annealing (30 seconds at 58°C) and extension (30 seconds at 72°C). The quality of the PCR reactions was demonstrated by melting curve analyses [32]. Efficiency was determined for each gene applying a standard curve (logarithmic dilution series of cD-

-NA from the testes). For each sample, the reference gene (β2M) and target gene were amplified in the same run. Reference genes were approximately equal. The target genes were normalized to a reference gene and represented relative to a calibrator (fourth passage MSCs). Tests and CCE were used as positive controls. The TLR4 specific primers were used: forward: 5'-ATC ATC CAG GAA GGC TTC CA-3' and reverse: primer 5'-GCT GCC TCA GCA AGG ACT TCT-3'. As an internal polymerase chain reaction control, the rat ribosomal protein L32 (rpL32) (forward: 5'-TGTCCTCTAAGAACCGAAAAGCC-3' and reverse: 5'-GTTGGGATTGGTGACTCTGA- 3') was used for each sample.

Evaluation of protein expression of TLR4 by Immunohistochemistry

Protein concentrations measured using the protein assay (Bio-Rad) as stated in the instruction provided by the manufacturer. Equal amounts of protein (30 mg/well) from gastrocnemius tissue separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Bio-Rad), fixed at 4% neutral buffered paraformaldehyde and sectioned for immunohistochemical analysis. Membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline Tween-20 buffer and then incubated with the primary goat anti TLR4 antibody (Santa Cruz, 1: 300) for overnight at 4°C. After incubation with the secondary antibodies for 1 h at room temperature, the detection of immunoreactive bands was performed with the ECL system (General Electric Healthcare). Images were captured using a Zeiss LSM 5 fluorescent microscope. The intensity of TLR4 expression from different groups was quantified, and the relative protein levels were compared. Relative fold change in gene expression = $2^{-\Delta\Delta CT}$

Statistical analysis

The results were stated as the Means±STD. All statistical comparisons were done by using one-way analysis of variance (ANOVA) and Tukey test as Post hoc. All states P<0.05 was accounted as significant difference.

Results

Body weight

The weight gain of sedentary and trained rats during the experiment is shown in table 1. There was a gradual increase of body weight in all the groups during study with no significant difference.

Serum concentrations of triglyceride, cholesterol, LDL-C and HDL-C

Serum concentration of triglyceride, cholesterol, LDL and HDL are shown in Table 2 in four experimental groups. High fat diet significantly increased the serum levels of cholesterol and LDL-C in HFD group compared with the control group.

TLR4 content

It was found that in comparison to baseline levels, TLR4 mRNA and protein content significantly raised in the high fat diet rats (P<0.05) (Figure 1). In training groups (E, HFD&E), protein and mRNA expression of TLR4 were lower than those observed in non-training groups (C, HFD) (P<0.05) (Figure 1). This change indicates that training leads to an attenuated TLR4 expression after consumption of high fat diet regime.

Discussion

The purpose of the current study was to investigate the effect of 8 weeks of aerobic training on muscle TLR4 expression in rats. In addition, as a secondary purpose, this study examined the influence of high fat diet in combination with training on TLR4 expression in rats. The results indicated that forty sessions of moderate aerobic training effectively decreased TLR4 gene and protein expression in rats subjected to high fat diet. This observation is in agreement with those observed by Rodriguez-Miguelez et al., (2014) who showed that physically active subjects have considerably lower inflammatory cytokine production and TLR4 expression than inactive subjects.

One important stimulant for decreased TLR4 signaling is lipolysis diminish, which reduces free fatty acids in circulating, since fatty acids provoke an increase in NF-kB nuclear activation by the TLR4 cascade (Rodriguez-Miguelez et al., 2014). It has been shown that TLR4 is an important role in LPS-mediated responses activating NF-kB via this cascade (Zbinden-Foncea et al., 2012).

Table 1. Weight gain (WG) of sedentary and trainingd rats during experiment

Group	WG week1	WG week2	WG week3	WG week4	WG week5	WG week6	WG week7	WG week8
C	11 ± 1	39 ± 5	56 ± 7	76 ± 7	99 ± 13	112 ± 13	119 ± 14	136 ± 17
E	13 ± 7	45 ± 5	61 ± 6	93 ±11	103 ± 9	101 ± 15	130 ± 8	140 ± 9
HFD	28 ± 7	53 ±12	72 ± 12	88 ±15	120 ± 20	139 ± 17	150 ± 19	162 ±18
HFD&E	38 ± 3	63 ± 4	76 ± 5	92 ± 7	108 ± 8	119 ± 10	126 ± 9	136 ± 8

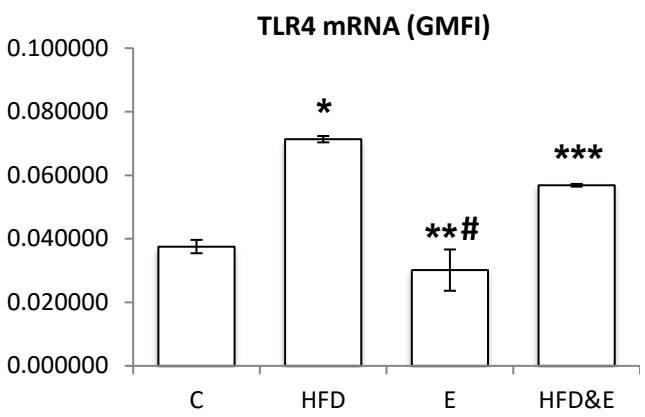


Figure 1. Changes in expression of TLR4 (GMFI; geometric mean fluorescent intensity) in response to 8 weeks swimming exercise in rats. Plot shows the levels of TLR4 mRNA expression in healthy rats that stayed sedentary €, trained healthy rats that performed training for 8 weeks €, rats that remained inactive and fed with high fat diet for 8 weeks (HFD), and rats that raining for 8 weeks and fed with high fat diet (HFD&E). Data are presented as means ± SEM of five rats per group. (*): P≤0. 05 between HFD and C. (**): P≤0. 05 between E and C. (***): P≤0. 05 between HFD&E and HFD. (#): P≤0. 05 between E and HFD.

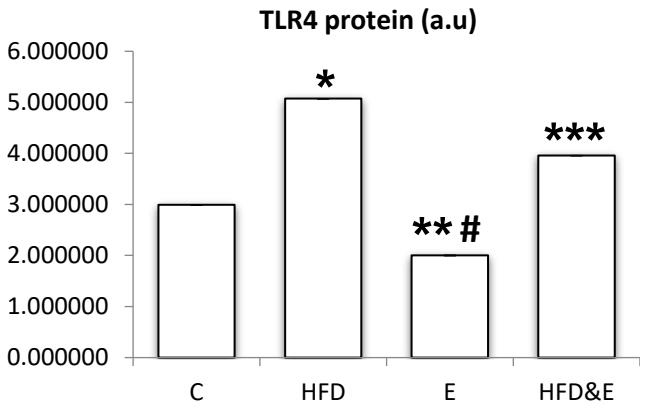


Figure 2. Effects of training on TLR4 protein in rats. Blot shows the levels of TLR4 protein in four groups: control €, training for 8 weeks €, inactive rats that fed with high fat diet for 8 weeks (HFD), and rats that raining for 8 weeks and fed with high fat diet (HFD&E). Values are arbitrary units (a.u) and presented as means ± SEM of five rats per group. (*): P≤0. 05 between HFD and C. (**): P≤0. 05 between E and C. (***): P≤0. 05 between HFD&E and HFD. (#): P≤0. 05 between E and HFD.

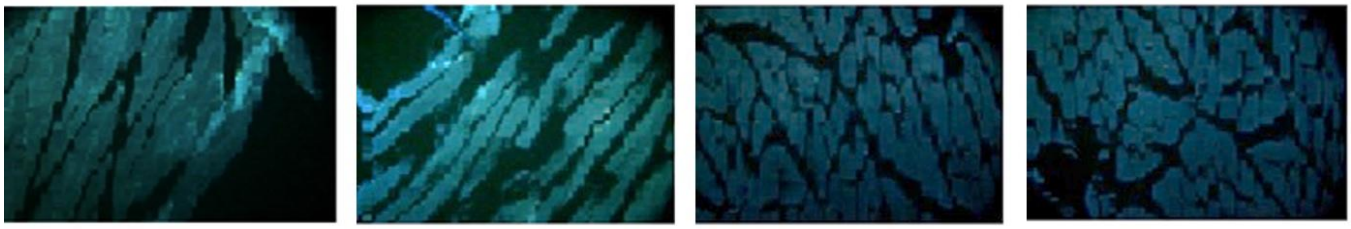
Actually, TLRs lead to cytokine expression through NF-KB pathway, which is implicated in the regulation of TLR expression (Zbinden-Foncea et al., 2012). Earlier studies have revealed that TLR4 plays an important role as a connection between inflammatory cytokine production and a physically active lifestyle. Hence, the trained rats had considerably lower inflammation and TLR4 levels than inactive animals. These information give further support for training induced downregulation of TLR4 level (Lira et al., 2010). Gleeson et al. have offered two different mechanisms whereby TLR4 expression is lowered by training, albeit the precise mechanism has yet to be clarified. First, although TLR4 induced cytokine release and therefore activation of the inflammatory cascade, expression of TLR4 appears to be negatively regulated by cytokines. The release of cytokines as a result of training decrease TLR4 expression linked with inflammatory response as an effort of the body to sustain a homeostatic balance. Indeed, previous studies have reported that training raise expression of TNF-α (cytokine that down-regulate TLR4 and lower inflammation). Second, stress hormones such as glucocorticoids that released during training, change immune function and reduce the capability of the immune system to response to diseases. TLR4 expression, depending on the ligand, causes different responses, which will affect the amount, type and duration of the inflammatory response. For example, the TLR4/MyD88 signaling pathway is used to induce the expression of proinflammatory cytokines (Rodriguez-Miguelez et al., 2014). TLR4 meditate the inflammatory response by adaptor MyD88 protein. MyD88-dependent pathway plays an important role in the inflammatory response induced by training. In support, data from the study indicate that decreased TLR4 expression induced by training, was accompanied by lower in MyD88 protein concentration (data are not shown). Trainings with different intensities and volumes may influence the MyD88 pathway differently, explaining the differences between studies (Gleeson

Table 2. Representative changes of blood triglyceride, cholesterol, LDL and HDL (mg/dl) in groups (C, E, HFD, HFD& E) after swimming training.

Group	Triglyceride (mg/dl)	Cholesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
C	104± 14	97± 11	53 ±6	8±1
E (CE)	99± 8	74±9	47±8	10±1
HFD (H)	136±15	197±29*	181±38*	9±2
HFD&E (HE)	135±24	164±20*	123±24*	13±2

All values are presented as mean±SEM. * Significant difference from normal group (P<0.05).

Figure 3 Effects of training on TLR4 protein in four groups: control (C), training (E), high fat diet (HFD) and training and high fat diet (HFD&E).



et al., 2011).

It also was found that high-fat fed rats led to more TLR4 expression than low-fat fed rats. Our result was in line with those reported by Oliveira, Katie and Kawanishi who reported that high-fat fed resulted in high TLR4 expression (Oliveira et al., 2013). It is plausible that HF rats gained more weight due to less activity and increased feeding high fat content efficiency. Takahashi applied a cross-sectional model and observed that ad libitum access to a 33% fat diet was correlated with a 50% increase monocyte concentration and found that body weight was negatively associated with TLR4 expression (Takahashi et al., 2003). Because TLR4 has a role in body fat cumulating and distribution, it is logical to consider that decreased TLR4 expression may display body attempt to inhibit further weight gain and overmuch inflammation. Additional weight gain in spite of these signals may ultimately lead to the increasing TLR4 expression saw in established inflammatory situations (Oliveira et al., 2011). It has shown that high-fat feeding led to increased endogenous LPS mass and lengthened in vitro stimulation of monocytes with LPS results in lower TLR4 expression. Therefore, it is reasonable that alterations in endogenous LPS may describe dissimilarities in TLR4 expression because high-fat feeding has been linked with metabolic endotoxemia in rats (Gleeson et al., 2011). It is believed in our investigation that reduced TLR4 expression was linked with a decrease in weight gain. Nevertheless, the reason of TLR4 expression reduction after training is not completely understood. Scientists have recommended a link between TLR expression and endogenous ligands such as cytokines, necrotic cell products and HSPs, reveal that endogenous and microbial factors might use the similar pathway to activate the adaptive immune system. The alterations that befall in TLR expression after training may be associated to the production and secretion of interleukins such as IL-6 and IL-10, too. As TLR activation led to the producing of IL-6 via the NF-kB pathway, and because production and release of IL-6 into the circulation are elevated during prolonged training, it is hypothesized that high levels of IL-6 after training decrease the expression of TLRs. The HSPs (proteins that exhibited in all the cells and are elevated during physiological stress) is another key endogenous ligand that may affect TLR expression (Rodriguez-Miguelez et al., 2014). It has been proposed that HSPs, such as HSP60 and HSP72, in a same behavior to LPS, act as activators of TLR4. Training led to physiological stress because of elevated body temperature and muscle injuries, therefore it is hypothesized that HSPs in the extracellular environment are increased after training and could decrease TLR expression (Rodriguez-Miguelez et al., 2014). More investigation is needed to confirm the direct effects of decreased TLR4 expression on weight gain in rats using various models. In spite of the possible good effects of decreased TLR4 expression on weight gain, lowered TLR4 expression adjusts the ability of the immune system to respond to pathogens. Such functional declines in immunity through disruption of TLR4 result in premature mortality. Future research is needed to establish if changes in endogenous LPS are responsible for reduced TLR4 expression with diet-induced weight gain.

Conclusion

Small sample size was one of the restrictions of this study. Training-induced alterations in TLR4 expression might be correlated to soluble serum elements that change in response to training. Investigations of the future should try to evaluate the effects of various durations, forms and intensities of training on different muscle expressions of TLR4 and other cell surface receptors and their relationships between them.

What is already known on this subject?

It has been shown that TLR4 expression reduce after aerobic training, but more studies considering the influences of different types of training on TLR4 expression are necessary.

What this study adds?

Training-induced alterations in TLR4 expression might be correlated to soluble serum elements that change in response to training.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental protocols were approved by the Ethics Committee of the Baqiyatallah University of Medical Science.

Informed consent All authors consent to this manuscript submission.

Author contributions

Conceptualization: M.S., F.R.; Methodology: S.Sh., F.R.; Software: M.S., F.R.; Validation: S.Sh.; Formal analysis: M.S.; Investigation: M.S., F.R.; Resources: S.Sh.; Data curation: M.S.; Writing - original draft: S.Sh., M.S.; Writing - review & editing: M.S.; Visualization: S.Sh.; Supervision: F.R.; Project administration: M.S.; Funding acquisition: S.R.

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