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# Mice fed with a high fat diet show a decrease in the expression of “toll like receptor (TLR)2 and TLR6 mRNAs in adipose and hepatic tissues

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### Abstract

**Introduction:** Pattern-recognition receptors (PRRs), which include Toll-like Receptor (TLRs) and Nacht leucine-rich repeat proteins (NLRP/NALPs), are molecules of innate immunity able to recognize a wide variety of ligands present in microorganisms and human tissues. Adipocytes (fat cells) may play an important role in the physiological regulation of their own immune responses via TLRs. During obesity, the inflammatory pathway is triggered and insulin responsiveness is altered in fat tissue as a result of TLR4 activation by dietary lipids.

**Objective:** Here, we investigate if other PRR family members could also participate in the inflammatory processes in the adipose tissue of obese mice.

**Methods:** The mRNA expression of TLRs, the NLRP3-inflammasome (NLRP3, ASC, caspase-1 and IL-1beta), IL-6, and TNF $\alpha$  in the hepatic and adipose tissues of mice fed with a high fat diet (HFD) were studied by RT-PCR.

**Results:** Adipose tissue from mice fed with a HFD had decreased expression levels of TLR2, TLR6 and TLR7 and was similar to the pattern in hepatic tissue HFD mice. IL-6 and TNF- $\alpha$  expression also were decreased in adipose tissue of mice fed with a HFD. NLRP3-inflammasome expression was not modified.

**Conclusion:** These results suggest that the low expression of TLR2, and TLR6 in the mice fed with a HFD could be regulating the inflammation induced by the diet employed in this study.

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Key words: TLR. NLRP3. Adipose. Liver. Fat.

### EL TEJIDO ADIPOSO Y EL TEJIDO HEPÁTICO DE LOS RATONES ALIMENTADOS CON UNA DIETA ALTA EN GRASA TIENEN UN DECREMENTO EN LA EXPRESIÓN DEL mRNA DEL “TOLL LIKE RECEPTOR” (TLR)2 Y DEL TLR6

### Resumen

**Introducción:** Los receptores que reconocen patrones (PRRs), que incluyen a los “Toll like receptors” (TLRs) y a las “Nacht leucine-rich repeat proteins” (NLRP/NALPs), son moléculas que participan en la inmunidad innata y éstos pueden reconocer una variedad de ligandos presentes en los microorganismos y en los tejidos del humano. La respuesta inmune conducida por los TLRs de las células de los adipocitos tiene un papel importante en la regulación fisiológica del tejido graso. Durante el desarrollo de la obesidad, la alta presencia de los lípidos activa el TLR4 encendiendo un proceso inflamatorio que conduce a una resistencia a la insulina en el tejido graso.

**Objetivo:** En este trabajo, nosotros investigamos si los otros miembros de la familia de los PRRs podrían también participar en el proceso inflamatorio del tejido adiposo de los ratones obesos.

**Métodos:** La expresión del mRNA de los TLRs, del NLRP3-inflammasoma (constituido por las moléculas NLRP3, ASC, caspasa-1 e IL-1beta), de las citocinas inflamatorias IL-6 y TNF $\alpha$  fue estudiado, por RT-PCR, en el tejido adiposo y en el tejido hepático de ratones alimentados con una dieta alta en grasa (HFD).

**Resultados:** El tejido adiposo de los ratones alimentados con una HFD tuvieron un nivel bajo de expresión de TLR2, TLR6 y TLR7 en comparación a los ratones controles y este nivel fue también presente en el tejido hepático. La expresión de la IL-6 y el TNF- $\alpha$  se decremento en el tejido adiposo de los ratones alimentados con HFD. La expresión del NLRP3-inflammasoma no fue modificado.

**Conclusión:** Estos resultados sugieren que el nivel bajo de expresión del TLR2 y del TLR6 en los ratones alimentados con HFD podrían estar regulando la inflamación inducida por la dieta empleada en este estudio.

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Palabras clave: TLR. NLRP3. Adiposo. Hígado. Grasa.

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## Introduction

Pattern-recognition receptors (PRRs) are molecules of innate immunity able to intrinsically recognize a wide variety of pathogen associated molecular patterns (PAMPs) present in microorganisms.<sup>1</sup> There are several PRRs such as Toll-like Receptors (TLRs), nucleotide-binding oligomerization domain proteins (NODs), Nacht leucine-rich repeat proteins (NLRPs or NALPs), C-type lectin receptors and peptidoglycan receptor proteins (PGRPs) are also able to detect invariant molecular signatures of invading pathogens.<sup>1</sup>

TLRs have three domains: the extracellular domain, which is a leucine-rich region (LRR); the transmembrane domain; and the intracellular domain, called Toll/Interleukin-1 receptor (TIR) domain.<sup>1</sup> The LRR domain recognizes a specific PAMP, e.g. TLR4 recognizes lipopolysaccharide (LPS), TLR9 recognizes bacterial unmethylated DNA, and TLR3 and TLR7 recognize double- and single-stranded RNAs, respectively.<sup>2,3</sup> TLR2 is the most promiscuous<sup>2</sup>, recognizing a variety of PAMPs such as peptidoglycan, lipoteichoic acid, lipopeptides, zymosan, etc. TLRs are expressed in sentinel cells, such as dendritic cells and macrophages, which drive the innate immune response.<sup>4</sup> Other cell types can also express TLRs, e.g. epithelial,<sup>5</sup> fibroblasts,<sup>6</sup> myoblasts,<sup>7</sup> keratinocytes<sup>8</sup> and microglia cells.<sup>9</sup> Ligand-TLR interactions drive activation of TIR domain followed by the recruitment of multiple intracellular adaptor and signaling proteins, including MyD88, TRIF, and TRAF6,<sup>10</sup> subsequently the MAP kinase pathways and the NF- $\kappa$ B and interferon response factor (IRF) transcription factors, are activated, leading to the induction of antimicrobial peptides or proinflammatory cytokines such as interleukin-6 (IL-6), IL-12, tumor necrosis factor (TNF)- $\alpha$ , and type I interferons (IFNs).<sup>4</sup>

Nevertheless, other PRRs such as NLRPs also participate in innate immunity. Fourteen NLRPs members have been identified in the human genome. The NLRPs have three structural domains, LRR domain (carboxy-terminal), an intermediary NACHT (NBS; NOD) domain and a pyrin domain (amino-terminal) (PYD).<sup>11</sup> A well-established feature of NLRPs is their ability to interact with apoptosis-associated speck-like protein (ASC) through a PYD-PYD interaction. ASC encodes a 22-kDa protein which contains a carboxy-terminal domain CARD along with the amino-terminal PYD, and serves as a conjunction molecule between PYD- and CARD-containing proteins. NLRP and ASC can interact with inactivated caspase-1 (procaspase-1) or caspase-5 (procaspase-5), and they create an intracellular complex named inflammasome.<sup>11-12</sup> An interaction between ASC and NLRPs was shown initially for NLRP1 (NALP1/DEFCAP/NAC/CARD7) and subsequently also found for NLRP2 and NLRP3 (NALP3/PYPAF1/Cryopyrin/CIAS1). The PYD of ASC interacts with the PYD of several NLRPs, whereas ASC's CARD binds to

procaspase-1 CARD for its activation.<sup>12-13</sup> The active caspase-1 is required for the production of active IL-1 $\beta$  and IL-18 in activated monocytes and macrophages.<sup>14</sup>

Obesity appears to cause chronic low-grade inflammation which contributes to a systemic metabolic dysfunction associated with obesity-linked disorders. Palmitic acid and stearic acid can activate TLR4<sup>15</sup> producing proinflammatory cytokines such as IL-6 or TNF- $\alpha$ . A high uptake of fat is conducive to a metabolic alteration in adipose tissue that increases free fatty acids in blood circulation. A result of this alteration is the generation of activated macrophages which produce proinflammatory cytokines via TLRs, resulting in a state of inflammation in adipose tissue. Recent studies suggest that adipocytes may play an important role in the physiological regulation of immune responses on fat deposits via TLR signalling cascades,<sup>16-17</sup> and that TLR4 activation via dietary lipids triggers inflammatory pathway and alters insulin responsiveness in the fat tissue during obesity.<sup>18</sup> NLRP3 is considered a sensor of damaged tissues, because it can be activated by uric acid, intracellular ATP, asbestos, and  $\alpha$ -amyloid polymers. NLRP3-*knockout* mice do not increase weight when fed a high fat diet; thus, the NLRP3-inflammasome is involved in the obesity process.<sup>19</sup> These results indicate a role for PRRs in the development of obesity.

## Objective

The aim of this work is to know if other TLR family members are involved in the TLR-mediated inflammatory processes in the obese adipose tissue. Thus, in this work the mRNAs expression of NLRP3, ASC, caspase-1, IL-6, TNF $\alpha$ , IL-1 $\beta$ , and all TLRs in the adipose and hepatic tissues of mice fed with a high fat diet (HFD) was studied.

## Methods

### *Diets*

Two diets were used: (i) a control diet for rodents (Harlan Tekla® Madison, WI, USA) and (ii) a HFD designed by a nutritionist utilizing Mexican foods such as butter and lard that contained 32% carbohydrate, 18% protein, and 50% saturated fat.<sup>20</sup>

### *Mouse study*

Animal management was supervised by a veterinarian in accordance with the principles set forth in the NIH guide for the care and use of laboratory animals and approved by the Animal Care Committee of the Instituto de Ciencias de la Salud, UAEH. Male CD-1 mice of approximately 21-23 g were provided by the animal facility of the Instituto de Ciencias de la Salud,

UAEH. To analyze the effect of HFD on the expression of PRRs, 10 CD-1 mice were divided into two groups of 5: the control mice group fed with a normal diet for rodent (i), and the test group were fed HFD (ii).

#### RNA isolation and RT-PCR analysis

Mice were killed by cervical dislocation. Liver and fat organs were obtained from each mouse. Liver and fat organs were washed in D-PBS to eliminate blood contamination. Total RNA extraction was performed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) supplemented with RNase-free DNase I and RNA was re-extracted with TRIzol reagent. For the reverse transcriptase (RT) reaction, total RNA (3 µg) with 0.5 µg of oligo-(dT)<sup>15-18</sup> (Invitrogen) was denatured at 70°C for 10 min. Then, 1X single strand buffer, 0.5 mM DTT, 500 µM of each dNTP's and 200 U of MMLV reverse transcriptase (Invitrogen) were added. The RT reactions were performed at 42°C for 1 hour. The polymerase chain reactions (PCR) were performed with 1 µl of the cDNA, 1X buffer, 1 mM MgCl<sub>2</sub>, 200 µM of each dNTP's, 0.2 µM of each TLRs and NLRP3-inflammasome and β-actin specific primers<sup>6,21</sup> and 2 U of TaqDNA polymerase (Invitrogen). Optimal PCR conditions were 30 cycles of 30 seconds at 92°C, 30 seconds at 60°C, and 30 seconds at 72°C.

#### Semiquantitative PCR

The intensity of the amplified bands was analysed with Alpha Imager software. The band intensities were normalized to the corresponding β-actin signal (PRRs/β-actin rate). The results were analyzed by a t-student with correction of Welch.

## Results

#### Body weight of high-fat diet mice

The mice fed with a HFD showed a significant increase in body weight at 3<sup>rd</sup> and 4<sup>th</sup> month ( $p < 0.05$ ) compared to control group (fig. 1). The results demonstrate that the HFD was efficient for increasing the body weight of mice.

#### Expression of TLRs mRNAs in mice fed with a HFD

Analysis of expression levels of all TLRs mRNAs in adipose and hepatic tissues of mice fed with HFD was performed. The results presented in figure 2, showed that adipose tissue from control mice expressed all the TLRs, whereas that mice fed with HFD only TLR2, 3, 4, 5, and 6. However, adipose tissue from mice fed with HFD showed lower expression levels of TLR2, 6 and 7 than adipose tissue from control mice ( $p < 0.05$ ), moreover

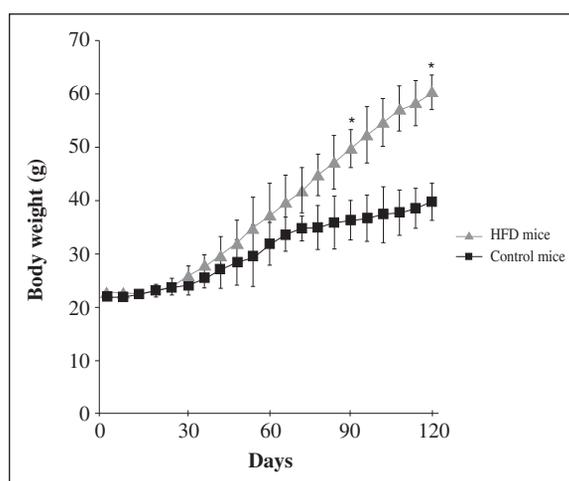


Fig. 1.—Effect of a high fat diet in mice CD-1 on body weight. Control mice were fed with diet for rodents (Harlan Tekla® Madison, WI, USA) and HFD mice were fed with a high fat diet during 4 months. \*Comparison between control mice and HFD mice in 3<sup>rd</sup> and 4<sup>th</sup> month, analyzed by a one-way ANOVA with a Tukey's test.

TLR3, 4, and 5 did no changes their expression levels to both mice groups (fig. 2). This same pattern was observed in the hepatic tissue of both mice groups (fig. 2).

#### Expression of proinflammatory cytokine mRNAs in mice fed with a HFD

As is known, the proinflammatory cytokines can be induced by TLR activation, IL-6 and TNF-α were measured in adipose and hepatic tissues of mice fed with a HFD. As shown in figure 3, IL-6 mRNA was expressed in adipose tissues from control mice group but not in adipose tissues of mice fed with HFD ( $p < 0.05$ ). The same result was obtained in hepatic tissues from both mice groups. The expression levels of TNF-α decreased only in adipose tissue of mice fed with HFD (fig. 3), while in other tissues there was no change in the expression level of this cytokine.

#### Expression of NLRP3-inflammasome mRNAs in mice fed with a HFD

NLRP3-inflammasome is constituted by NLRP3, ASC, caspase-1, and IL-1β molecules, hence it was interesting to us to measure them in the mice fed with a HFD. Overall, the mRNAs expression levels of these molecules were similar in both groups for adipose and hepatic tissues ( $p > 0.05$ ; fig. 4).

## Discussion

The expression level of TLR2, 6 and, 7 in adipose and hepatic tissues of mice fed with a HFD found in

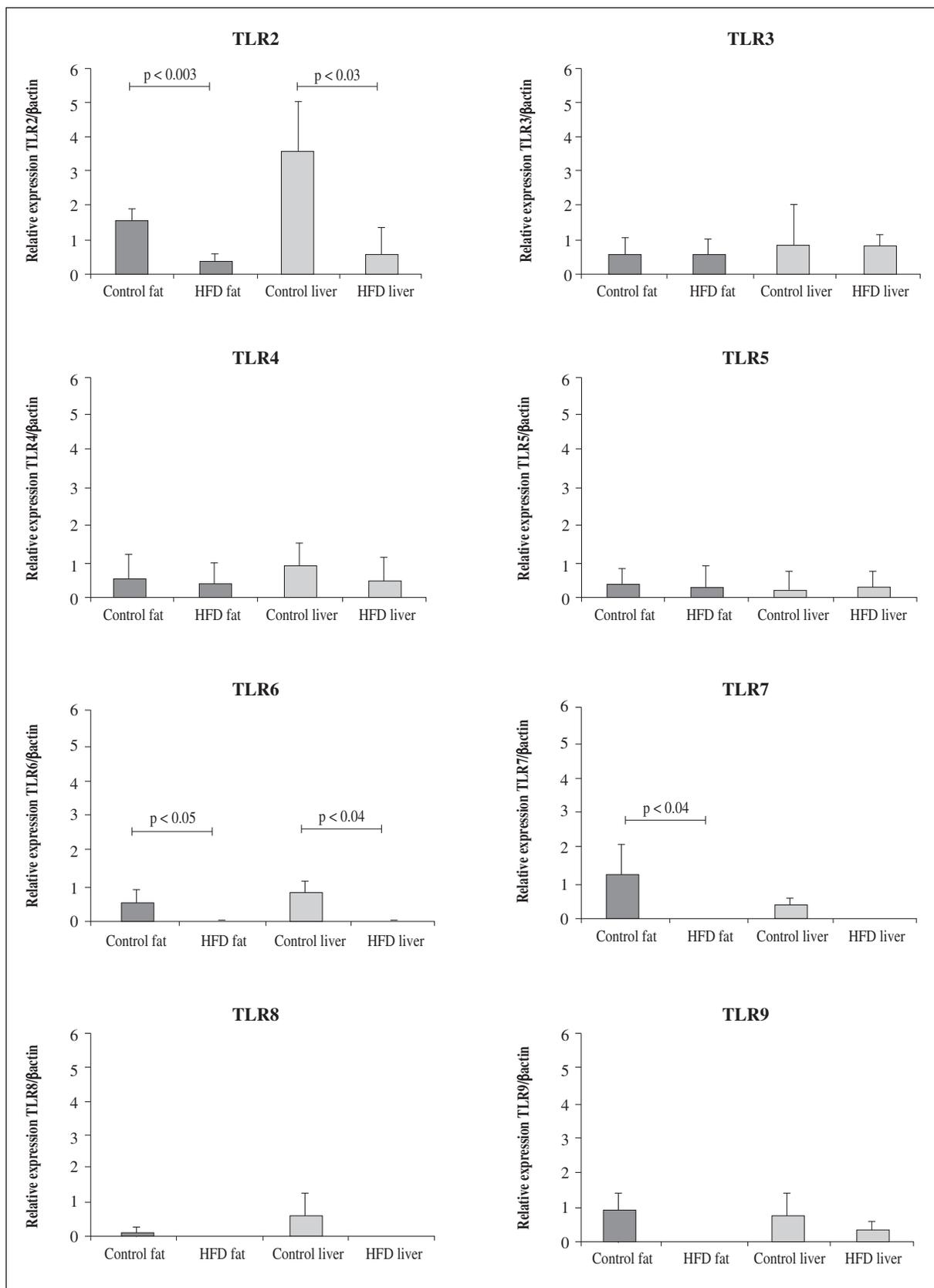


Fig. 2.—Intensity TLRs mRNAs expression of mice fed with a high fat diet. RT-PCR was performed in control mice and HFD mice of hepatic and adipose tissues. The intensity of each band was measured in an Alpha Imager system. Each bar correspond to the mean value ( $\pm$  SD) of the relation TLR/ $\beta$ -actin mRNA expression. \*Show statistical difference ( $p < 0.05$  according to t-student with correction of Welch).

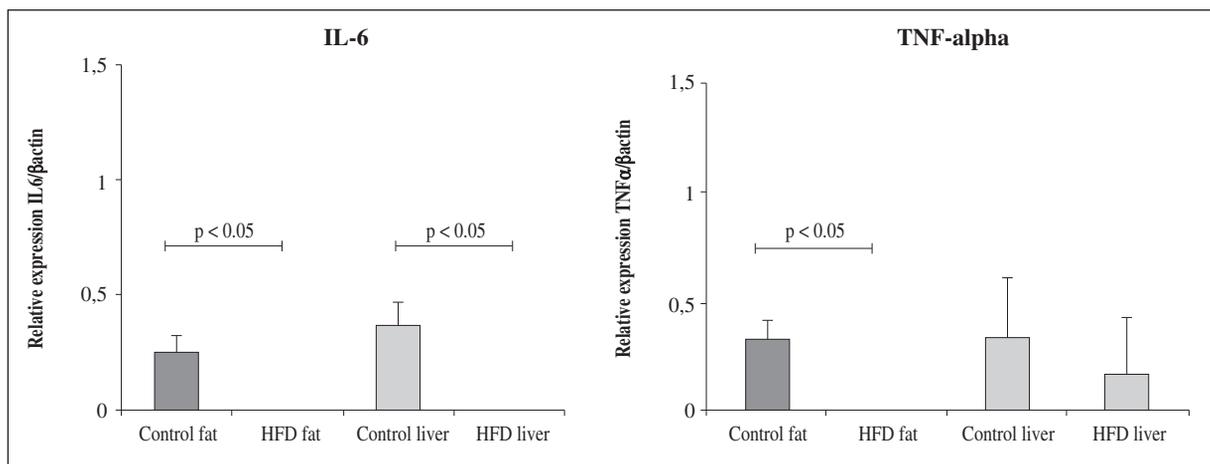


Fig. 3.—Intensity of *TNF- $\alpha$*  and *IL-6* mRNAs expression of mice fed with a high fat diet. RT-PCR was performed in control mice and HFD mice of hepatic and adipose tissues. The intensity of each band was measured in an Alpha Imager system. Each bar correspond to the mean value ( $\pm$  SD) of the relation cytokine/ $\beta$ -actin mRNA expression. \*Show statistical difference ( $p < 0.05$  according to *t*-student with correction of Welch).

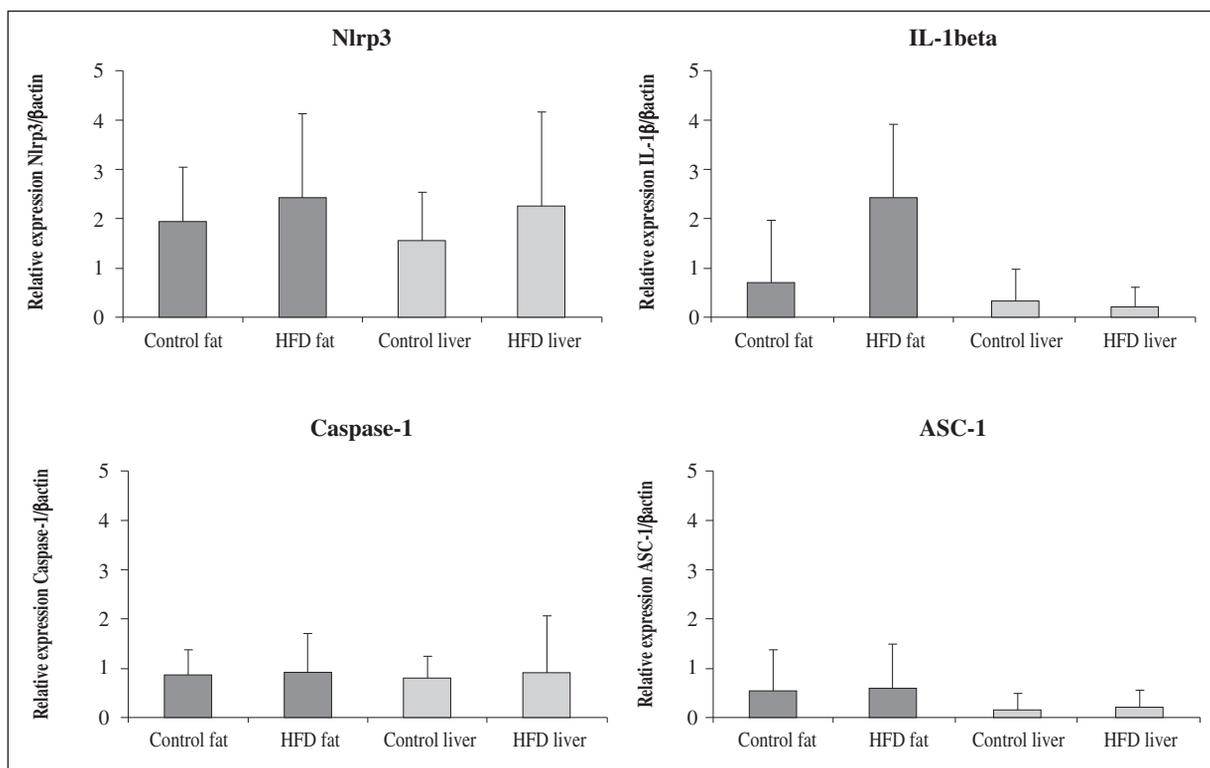


Fig. 4.—Intensity of *NLRP3*-inflammasome mRNA expression of mice fed with a high fat diet. RT-PCR was performed in control mice and HFD mice of hepatic and adipose tissues. The intensity of each band was measured in an Alpha Imager system. Each bar correspond to the mean value ( $\pm$  SD) of the relation *NLRP3* or *ASC* or *Caspase-1* or *IL-1 $\beta$* / $\beta$ -actin mRNA expression. statistical difference ( $p < 0.05$  according to *t*-student with correction of Welch).

this work was lower than control group. These results contrast with several reports where the TLR2 and TLR4 of obese mice are overexpressed.<sup>17,22</sup>

It is known that TLR2 can recognize many PAMPs of microorganisms and it happens in association with TLR1 or TLR6. The TLR1-TLR2 or TLR6-TLR2 heterodimers mainly recognize a large number of lipid-

containing molecules and transducers inflammatory signalling in a variety of cell types, including insulin-responsive cells.<sup>23</sup> In our work, a low expression of TLR2 and 6 in mice fed with a HFD was found; this result suggests that these TLRs may be important in the regulation of diet-induced overweight. This assumption is supported by the following evidences: I). The

TLR2 and 4 have been involved in the developing of obesity because an increasing of their expression has been observed in genetically obese (ob/ob) mice or induced by a HFD.<sup>17,22-25</sup> II). The resveratrol inhibits visceral adipogenesis by suppressing the galanin-mediated adipogenesis signalling cascade. It may also attenuate the cytokine production in the adipose tissue by repressing the TLR2, 4-mediated pro-inflammatory signalling cascades in HFD-fed mice.<sup>26</sup> III) Stearidonic acid suppresses TLR2 but not TLR4 protein expression in the adipose stem cells.<sup>27</sup> Furthermore, stearidonic acid significantly decreases the activation and translocation of NF- $\kappa$ B, a TLR2 downstream signalling.<sup>27</sup> IV). It has been demonstrated that TLR2-deficient mice (TLR2-/- *knock-out*) fed with a HFD do not develop obesity, diet-induced adiposity, glucose tolerance, insulin resistance, hypercholesterolemia, and hepatic steatosis.<sup>23</sup> Moreover, adipose tissue from Tlr2-/- mice shows attenuation of adipocyte hypertrophy, a diminished macrophage infiltration and inflammatory cytokine expression;<sup>22-25,28</sup> these data indicate that TLR2 may be directly involved in HFD-induced inflammation and may also regulate basal and insulin-stimulated glucose uptake in adipocytes. We think the low expression of TLR2 and 6 in mice fed with a HFD could be a temporary resistance mechanism to obesity, since the low expression of TLR2 will be kept in an anti-inflammatory state decreasing the adipocyte hypertrophy.

Regarding to the others TLRs, it has been observed that the expression in adipose tissue of TLR1-9 and TLR11-13 in obesity-induced mice by a HFD or leptin deficiency is up-regulated, and the levels in the TLR1, TLR4, TLR5, TLR8, TLR9,<sup>25</sup> and TLR12 of the visceral adipose tissue were greater in diet-induced obese mice than in the ob/ob mice.<sup>16</sup> Other study reports a higher level of TLR4 expression in human adipose deposits<sup>17</sup> compared to other members of the TLR family (TLR1, 2, 7, and 8), furthermore in human adipocyte culture, the TLR2/TLR4 mRNAs expression and protein increased significantly followed by a Pam3CSK4 and LPS stimulation (ligands of TLR2 and TLR4 respectively) and also was associated with NF- $\kappa$ B p65 nuclear translocation and proinflammatory cytokine production.<sup>17</sup> In contrast, Kopp et al. 2009, reported the expression of all TLRs excepting TLR5 and TLR7 in mature adipocytes from different fat storages.<sup>29</sup> We did not find expression of TLR7 in adipose and hepatic tissues of mice fed with a HFD. However, it has been reported that mice genetically deficient in TLR5 shows hyperphagia, hyperlipidemia, hypertension, insulin resistance, and increased adiposity.<sup>30</sup> These results suggest that, besides TLR2 and TLR4, all TLRs family members are involved in the obesity and exists a functional TLR pathway in adipocytes which connects to innate immunity with the adipocyte function. Nevertheless, in our work we did not find alteration in the expression of TLRs 3 and 5.

Adipose tissue as a key endocrine organ may release multiple bioactive substances known as adipose-derived

secreted factors or adipokines, with pro-inflammatory or anti-inflammatory activities. Production or secretion deregulated of these adipokines might be due to adipose tissue dysfunction contributing to the pathogenesis of obesity-linked complications. Adipose tissue is mainly comprised of adipocytes, although other types of cells contribute to its growth and function, including pre-adipocytes, lymphocytes, macrophages, fibroblasts and vascular cells. Adipose tissues in obese individuals and in obese animal models are infiltrated by a large number of macrophages; this is linked to systemic inflammation and insulin resistance.<sup>31-32</sup> Moreover, the accumulation of adipose tissue macrophages is proportional to adiposity in both humans and mice,<sup>31,33</sup> and the sustained weight loss, results in a reduction in the number of adipose tissue macrophages that is accompanied by a decrease in the pro-inflammatory profiles of obese individuals.<sup>34</sup> It is well documented the relation between the elevated expression of TLR2 and inflammatory cytokines in white adipose tissue and liver of ob/ob mice.<sup>23</sup> Stearidonic acid inhibits LPS-induced IL-6 secretion and IL-6 mRNA expression in the adipose stem cells by decreasing TLR2-mediated signalling pathways.<sup>27</sup> Adipocytes from obese patients have significantly TNF- $\alpha$  level higher after stimulation of TLR2 compared with adipocytes from non-obese patients.<sup>35</sup> Mouse deficient in TLR2 attenuates local inflammatory cytokine expression specifically in the liver, indicating that TLR2 is a key mediator of hepatic inflammation.<sup>23</sup> It was observed that TNF- $\alpha$  is involved in the development of insulin resistance and that TLR2 and 4 polymorphisms may influence TNF- $\alpha$  secretion from adipocytes.<sup>36</sup> In addition, data show that the inhibition of TLR2 expression prevents the activation of MAPK8, and serine phosphorylation of IRS1 in diet-induced mice, suggesting that TLR2 is a key modulator of the crosstalk between inflammatory and metabolic pathways.<sup>22</sup> The expression level of IL-6 and TNF- $\alpha$  in our mice fed with HFD correlated with the low expression of TLR2.

The NLRP3-inflammasome is implicated in recognizing certain danger signals no microbiological leading to caspase-1 activation and subsequent IL-1 $\beta$  and IL-18 secretion. A report showed that the caloric restriction and exercise-mediated weight loss in obese individuals and with type 2 diabetes is associated with a reduction in adipose tissue expression of NLRP3 as well as with decreased inflammation and improved insulin sensitivity. Mice deficient in Nlrp3 prevent obesity-induced inflammasome activation in fat deposits and liver as well as enhance insulin signalling.<sup>19</sup> These data establish that the Nlrp3 inflammasome participates in obesity-associated danger signals and contributes to obesity-induced inflammation and insulin resistance. We did not find change in the expression levels of NLRP3, ASC, Caspase-1 and IL-1 $\beta$  (components of NLRP3-inflammasome) in mice fed with a HFD, suggesting the no participation of this inflammasome in our model.

Currently, there is a classification of obese individuals based on degree of metabolic dysfunction: those with fully metabolic dysfunction and those with mildly metabolic dysfunction. Obese individuals with the latter intermediate metabolic phenotype have lower levels of inflammatory expression marker and reduced cardiovascular risk than obese individuals with fully metabolic dysfunction.<sup>37</sup> However, in obese animal models, does not exist such. Therefore, we think that our mice developed a degree intermediate metabolic dysfunction of obesity and that the low expression of innate immunity molecules studied in adipose and hepatic tissues is due to a state between inflammation regulation and metabolic alteration induced by diet. It is possible that an extended feeding (> of 4 months) with HFD, the mice could have developed a chronic obesity (fully metabolic dysfunction) in which these molecules might have increased in both tissues.

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