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Journal of Nutritional Biochemistry 23 (2012) 265–271

Journal of Nutritional Biochemistry

# Intake of trans fatty acids during gestation and lactation leads to hypothalamic inflammation via TLR4/NFκBp65 signaling in adult offspring☆

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Received 11 August 2010; received in revised form 30 November 2010; accepted 2 December 2010

**Abstract**

We examined whether feeding pregnant and lactating rats with hydrogenated vegetable fats rich in trans fatty acids led to an increase in serum endotoxin levels and inflammation and to impaired satiety-sensing pathways in the hypothalamus of 90-day-old offspring. Pregnant and lactating Wistar rats were fed either a standard chow (Control) or one enriched with hydrogenated vegetable fat (Trans). Upon weaning, the male offspring were divided in two groups: Control-Control (CC), mothers and offspring fed the control diet; and Trans-Control (TC), mothers fed the trans diet, and offspring fed the control diet. The offspring's food intake and body weight were quantified weekly and the offspring were killed on the 90th day of life by decapitation. The blood and hypothalamus were collected from the offspring. Food intake and body weight were higher in the TC rats than in the CC rats. TC rats had increased serum endotoxin levels and increased hypothalamic cytokines, IL-6, TNF-α and IL1-β, concentrations ($P<.05$). TLR4, NFκBp65 and MyD88 were higher ($P<.05$) in the TC rats than in the CC rats. AdipoR1 was lower in the TC rats than in the CC rats. Thus, the present study shows that the mothers' hydrogenated vegetable fat intake during pregnancy and lactation led to hypothalamic inflammation and impaired satiety-sensing, which promotes deleterious metabolic consequences such as obesity, even after the withdrawal of the causal factor. In other words, the effect remains after the consumption of the standard chow by offspring.


*Keywords:* Trans fatty acids; Hypothalamic inflammation; Cytokines; Adiponectin receptor; Fetal programming

## 1. Introduction

Recently, studies have showed that altered nutritional experiences during early periods of life and have caused the rise of obesity-associated diseases due, in part, to fetal programming [1,2]. Although the term "fetal programming" was developed to explain the effects of maternal undernourishment on offspring [3], recent studies have reported the effects of maternal obesity and/or a high-fat diet rich in saturated fatty acids on an offspring's metabolism in early and adult life [1,4–7]. Unfortunately, almost half of all babies who are born to mothers who are either overweight or obese during pregnancy will have a greater risk of developing a metabolic syndrome later in childhood [6,8]. In other words, there is a greater risk [AU1]that the exposure to abundant dietary conditions later in life will then initiate the development of obesity [1,4,6,9].

Several studies have shown that the intake of saturated fatty acids leads to hypothalamic inflammation, resistance to insulin as well as leptin and body mass gain [7,9,10–17].

Inflammatory response can also activate the mammalian target of rapamycin (mTOR) pathway, which regulates several processes of the cell, including energy metabolism. Once hyperactivated, mTOR leads to endoplasmic reticulum (ER) stress, activation of p70S6K (S6K1) and insulin resistance [18]. Furthermore, obesity is also associated with increased mTOR activity, and a deficiency of S6K1 can protect animals against diet-induced obesity and insulin resistance [19,20]. Another cellular fuel sensor is adiponectin, which acts in the hypothalamus by reducing food intake, activating an insulin/leptin-like effect and

☆ Funding sources: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).
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doi:10.1016/j.jnutbio.2010.12.003

improving the activation of insulin signaling mainly via AdipoR1 [21,22]. However, the effects of the intake of trans fatty acids on hypothalamic AdipoR1 expression have not yet been evaluated.

The relationship between the hypothalamic inflammation pathway and a high-fat diet rich in saturated fatty acids has been studied only in adult animals [10,12–14]; this is also the case for studies showing hypothalamic insulin and leptin resistance in animals fed trans or saturated fatty acids [5,7,11]. Despite this, and the fact that these findings are consistent with the knowledge that trans fatty acids are incorporated to the central nervous system [5,23], little is known about potential mechanisms by which trans fatty acids lead to hypothalamic inflammation and disrupt insulin signaling and satiety-sensitizing pathways in offspring during their adult life. Thus, the aim of the present study was to examine whether feeding pregnant and lactating rats with hydrogenated vegetable fats rich in trans fatty acids leads to an increase in serum endotoxin levels and inflammation, and impaired satiety-sensitizing pathways in the hypothalamus of 90-day-old offspring.

## 2. Methods

### 2.1. Animals and diets

The Experimental Research Committee of the Federal University of Sao Paulo approved all procedures for the care of the animals used in this study. Rats were kept under controlled conditions of light (12:12 h light-dark cycle with lights on at 06:00) and temperature (22±1°C). Three-month-old female Wistar rats were left overnight to mate, and copulation was verified the following morning by the presence of sperm in the vaginal smears. On the first day of gestation, rats were isolated in individual cages and randomly divided into two groups, receiving either a commercial diet (Control) or a diet enriched with hydrogenated vegetable fat (Trans), according to AIN-93G. The diets were maintained throughout pregnancy and lactation. On the day of delivery, considered as day zero of lactation, each mother was given eight male pups. On the 21st day of life the animals were weaned and the offspring were divided in two groups and were fed until the 60th day with AIN-93G and following until 90th day, with AIN-93M: Control-Control group (CC), mothers and offspring were fed the control diet; and Trans-Control (TC), mothers were fed the trans diet, and offspring were fed the control diet.

Both diets were prepared according to the recommendations of the American Institute of Nutrition (AIN-93 G and M) [24] and were similar in calories and lipid content. The source of lipids for the Control diet was soybean oil, and the principal source for the Trans diet was partially hydrogenated vegetable fat, rich in trans fatty acids. Moreover, both diets had already been used in others works of our group [4,5,25]. The centesimal composition of the diets is presented in Table 1.

The fatty acid profile of each diet was previously published by our group [4,5,25]. In the Table 2, it has been showed the 18:1 isomers trans and 18:2 trans fatty acid of each diet.

### 2.2. Serum endotoxin levels

Serum endotoxin was assayed using a chromogenic limulus amebocyte lysate (LAL) test, which is a quantitative test for gram-negative bacterial endotoxin (Cambrex). Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL. The initial rate of activation is directly determined by the concentration of endotoxin. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from the colorless substrate Ac-lle-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405–410 nm following termination of the reaction. The correlation between the absorbance and endotoxin concentration is linear.

The LAL assay represents the same limitations for quantifying endotoxin in serum, due to the fact that blood samples contain a number of substances that can interfere with the LAL test, e.g., certain proteins in the blood have the ability to neutralize endotoxins, which can be troublesome when attempting to quantify the endotoxin level of a sample. Furthermore, blood contains serine proteases that are also known to interfere with the LAL assay. For the purposes of this study, all samples were run in duplicate within the same plate; therefore, no inter-assay variability was observed.

To assess recovery of endotoxin within the assay, known concentrations of recombinant endotoxin (0.25 and 1.00 EU/mL) were added to diluted serum to determine whether the expected concentration correlated closely with the actual observed value and whether there were any variations in serum contents due to the reaction. Lyophilized endotoxin (E. coli origin) was used to generate a standard curve with the chromogenic LAL test kit from Cambrex and produced a corresponding curve in accordance with the manufacturer's instructions.

Table 1
Composition of the control diet and diet enriched with trans fatty acids according to AIN-93

| Ingredients | Diet (g/100 g) | |
|---|---|---|
| | Control | Trans |
| Casein | 20 (14) | 20 |
| L-cysteine | 0.3 (0.18) | 0.3 |
| Cornstarch | 62 (71.1) | 62 |
| Soybean oil | 8 (5) | 1 |
| Hydrogenated vegetable fat | - | 7 |
| Butylhydroquinone | 0.0014 (0.0008) | 0.0014 (0.0008) |
| Mineral mixture | 3.5 | 3.5 |
| Vitamin mixture | 1.0 | 1.0 |
| Cellulose | 5.0 | 5.0 |
| Choline bitartrate | 0.25 | 0.25 |
| Energy (kcal/g) | 4.0 (3.8) | 4.0 (3.8) |

The first number refers to the growth diet (AIN-93G) and the number in parentheses refers to the maintenance diet (AIN-93M) when its composition differs from that of the growth diet. The control diet contains soybean oil and the trans diet contains partially hydrogenated vegetable oil.

Table 2
Trans fatty acid determination in diets

| Fatty acid | Diet (% of total fatty acids) | | | |
|---|---|---|---|---|
| | Growth | | Maintenance | |
| | Control | Trans | Control | Trans |
| Total TFA of C18:1 | ND | 11.62±0.11 | ND | 13.68±0.15 |
| C18: 1 6–8t | ND | 0.98±0.04 | ND | 1.10±0.10 |
| C18:1 n-9t | ND | 8.43±0.07 | ND | 9.98±0.08 |
| C18: 1 10t | ND | 0.56±0.03 | ND | 0.74±0.02 |
| C18: 1 11t | ND | 0.78±0.04 | ND | 0.87±0.09 |
| C18: 1 12–14t | ND | 0.87±0.01 | ND | 0.99±0.10 |
| C18:2 n-6 trans | ND | 0.43±0.17 | ND | 0.22±0.01 |
| Total TFA | ND | 12.05±0.14 | ND | 13.9±0.08 |

TFA, trans fatty acid; ND, not detected.

### 2.3. Hypothalamic TNF-α, IL-6, IL-1β and IL-10 protein level determined by ELISA

Following decapitation, brains were removed, and the hypothalamus was dissected, homogenized and centrifuged at 12,000 *g* for 30 min at 4°C; the supernatant was saved, and the protein concentration was determined using the BCA assay (Bio-Rad, Hercules, California) with bovine serum albumin (BSA) as a reference. Quantitative assessment of TNF-α, IL-6, IL-10 and IL-1β proteins was carried out by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN) following the recommendations of the manufacturer. All samples were run as duplicates and the mean value was reported.

### 2.4. Protein analysis by Western Blotting

After euthanasia, the hypothalami were dissected and homogenized in 1.0 mL of solubilization buffer at 4°C [1% Triton X-100, 100 mm Tris-HCl (pH 7.4), 100 mm

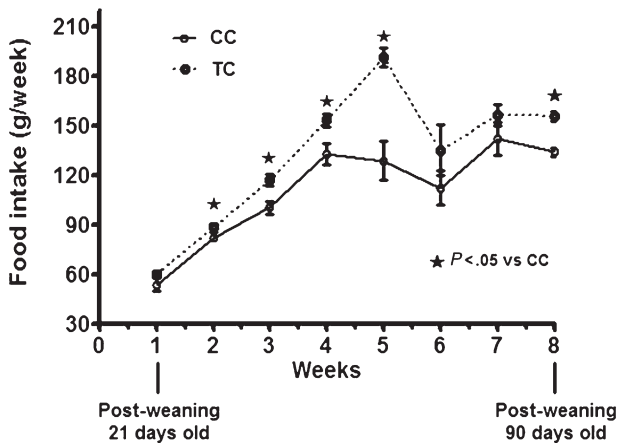


Fig. 1. Offspring food intake (in grams per week). CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. Data are expressed in ME±SEM of 12 rats per group.

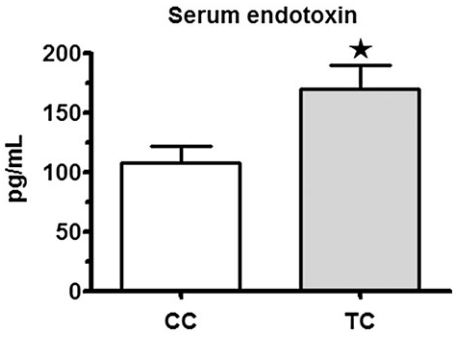


Fig. 2. Serum endotoxin levels. CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of animals studied per group was 5. Data are expressed in ME±SEM. *$P$<.05 vs. CC.

sodium pyrophosphate, 100 mm sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 2.0 mm phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg aprotinin/mL] with a Polytron (model 713T; Fisatom Equipamentos Científicos, São Paulo, SP/Brazil). Insoluble material was removed by centrifugation for 30 min at 9,000×g in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4°C. The protein concentration of the supernatants was performed by the BCA assay (Bio-Rad, Hercules, CA, USA). Proteins were denatured by boiling (5 min) in a Laemmli sample buffer [26] containing 100 mM DTT, run on 10% SDS-PAGE in a Bio-Rad miniature slab gel apparatus.

The electrotransfer of proteins from gels to nitrocellulose membranes was performed for ~1.30 h/4gels at 15 V (constant) in a Bio-Rad semi-dry transfer apparatus. Nonspecific protein binding to the nitrocellulose was reduced by preincubation for 2 h at 22°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4°C with antibodies against IR, IL-6R1, TNFα-R1, TLR2, TLR4, MyD88, TRAF6, NFκBp50, NFκBp65, AdipoR1, p70S6K, and alpha-tubulin obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Akt and mTOR obtained from Cell (Cell Signaling Technology, Inc., MA, USA) diluted in 1:1000 with blocking buffer supplemented with 1% BSA and then washed for 30 min in blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h at 22°C. For evaluation of protein loading, membranes were stripped and reblotted with an anti-alpha-tubulin antibody as appropriate. Specific bands were detected by chemiluminescence and visualization/capture was performed by exposure of the membranes to RX films. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software-Scion Corporation, Frederick, Md., USA).

### 2.5. Statistical analysis

The statistical analysis was performed using the GraphPad Prism statistics software package version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The data are expressed as the means±SEM. Implementation of the Kolmogorov-Smirnov test revealed that the results of experiments were distributed normally. The data were analyzed using Student's t-test for comparison between two groups. A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Food intake

Fig. 1 shows significant differences in the absolute food intake (g/week) among groups. The TC group shows higher absolute intake than the CC group, but the significant difference was found between the second and fifth week, as well as in the last week of treatment.

### 3.2. Serum endotoxin levels

Endotoxin concentrations in the blood were increased in the TC group (+55%) compared to the CC group (Fig. 2).

### 3.3. Hypothalamic cytokines profile

The TC group had increased hypothalamic IL-6 (+36.5%), TNF-α (+22.5%) and IL1-β (+124%) concentrations when compared to the CC group (Fig. 3A, B and C, respectively), while hypothalamic IL-10 levels were similar among the groups (Fig. 3D).

### 3.4. Insulin receptor and Akt/PKB expression in the hypothalamus

Insulin signaling was evaluated by quantification of IR and Akt/PKB expression. However, IR (Fig. 4A) and Akt/PKB (Fig. 4B) were not altered between the studied groups.

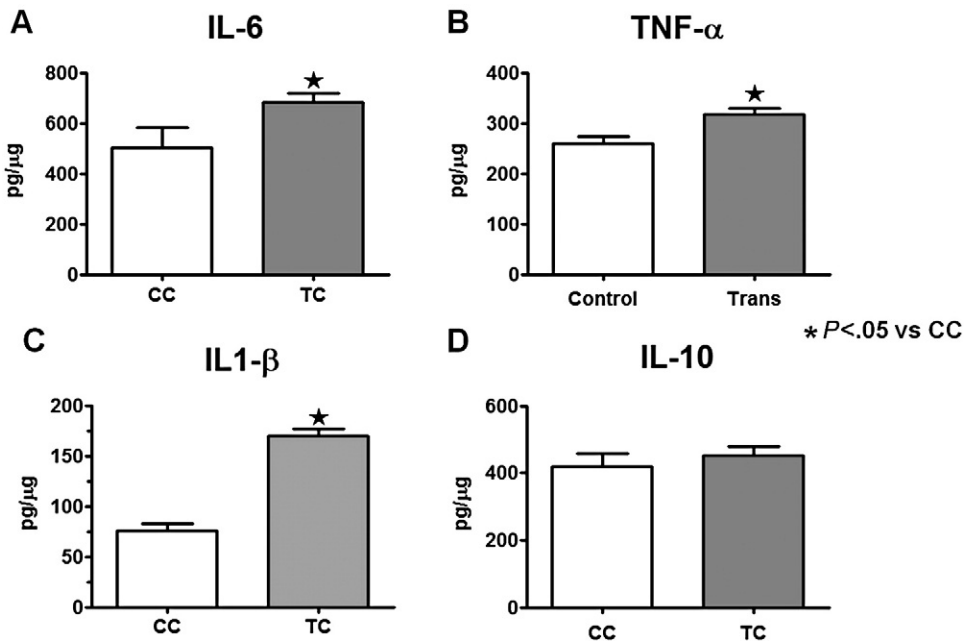


Fig. 3. Determining of hypothalamic concentrations of IL-6, TNF-α, IL-1β and IL-10 by ELISA. CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of studied animals per group was 8. Data are expressed in ME±SEM.

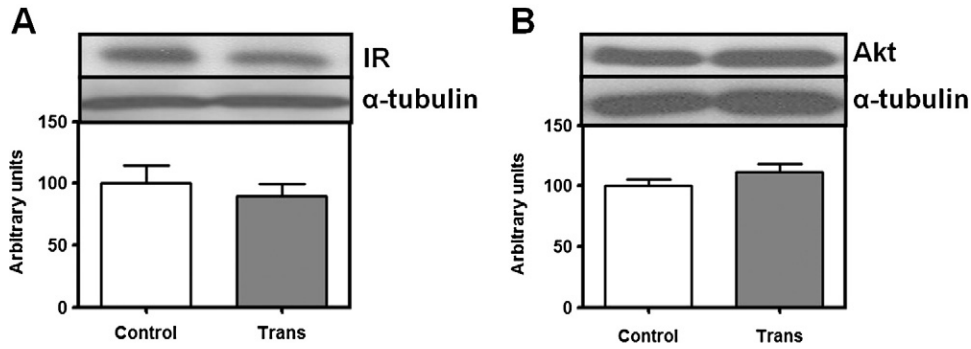


Fig. 4. Quantification of insulin receptor (A) and Akt/PKB (B). CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of animals studied per group was 7. Data are expressed in ME±SEM. The results are expressed in arbitrary units, stipulating 100 as the control value.

### 3.5. Inflammation signaling in the hypothalamus

Inflammation signaling was evaluated through cytokine receptors and TLR2/TLR4 and NFκBp50/NFκBp65 pathways in the hypothalamus.

Protein levels of the IL-6R1, TNF-α and NFκBp50 were similar among the groups (Fig. 5A, B and C, respectively). However, NFκBp65 expression in the TC group was significantly higher (+36.5%) when compared to the CC group (Fig. 5D).

Although, quantification of TLR2 and TRAF6 was similar between the studied groups (Fig. 6A and D, respectively), TLR4 (+67%) and MyD88 (68%) expression was higher in the TC group than in the CC group (Fig. 6B and C, respectively).

### 3.6. Satiety-sensing pathways in the hypothalamus

Hypothalamic mTOR and p70S6K levels were similar among the groups (Fig. 7A and B, respectively). However, the TC group showed lower (-32.5%) AdipoR1 expression than the CC group (Fig. 7C).

## 4. Discussion

In the present study, we observed that dams fed with trans fatty acids caused increased serum endotoxin levels, and hypothalamic IL-6, IL-1β, TNF-α, TLR4, MYD88, NFκBp65 and reduced adipoR1 protein expression after 90 days in male offspring fed with the control diet.

As demonstrated in our previous study [5] and in the present one, the TC group showed a higher food intake than the CC group, although a significant difference was not observed at weeks 6 and 7. The decrease in the intake during the last weeks is most likely due to a compensatory mechanism of the central nervous system that adjusts food intake to that of a habitual consumption of rat because the diet is not as much of a novelty as it is in the first weeks. Moreover, this result suggests that the hedonic reward provided by the diet rich in trans fatty acids remained activated during the weeks of treatment. In this way, several studies shown that maternal fat intake during pregnancy has a stronger influence on child fat consumption than does the maternal postnatal or prenatal fat intake [4,5,27,28]. Thus, these results could reflect uterus

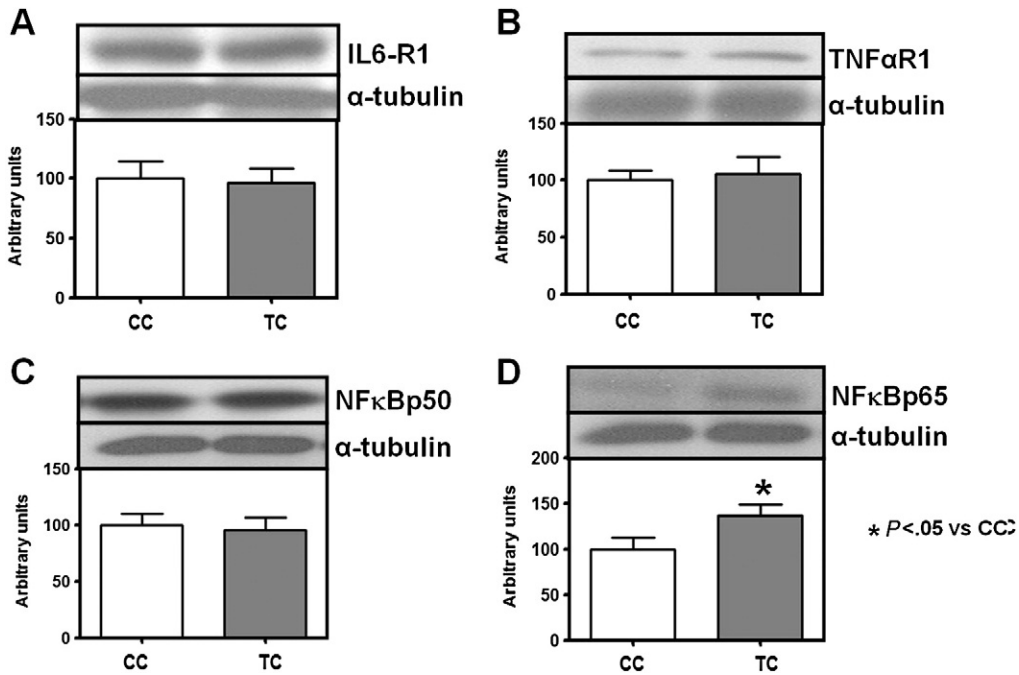


Fig. 5. Quantification of IL-6 receptor (A), TNF-α receptor (B), NFκBp50 (C) and NFκBp65 (D). CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of animals studied per group was 7. Data are expressed in ME±SEM. The results are expressed in arbitrary units, stipulating 100 as the control value.

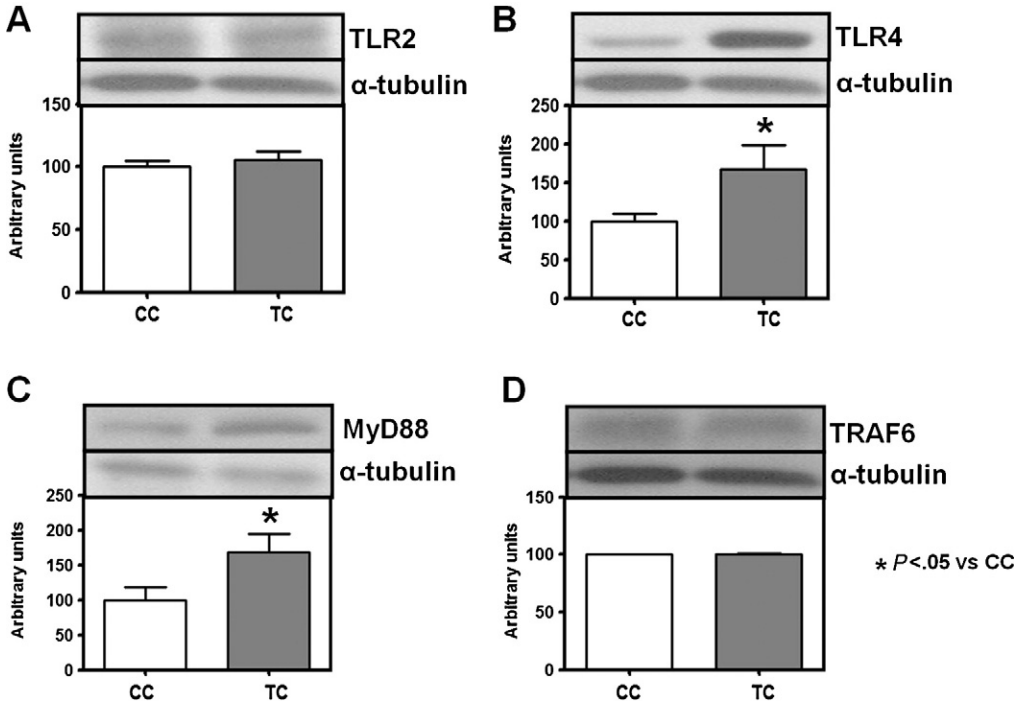


Fig. 6. Quantification of TLR2 (A), TLR4 (B), MyD88 (C) and TRAF6 (D). CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of animals studied per group was 7. Data are expressed in ME±SEM. The results are expressed in arbitrary units, stipulating 100 as the control value.

programming that occurs during the fetal stages of the offspring due to the maternal appetite and nutritional profile during pregnancy. Another factor that could justify the hyperphagia of animals fed with trans fatty acids in the present study, is stimulated hypothalamic expression of orexigenic peptides, such as neuropeptide Y (NPY), galanin and orexin in adult offspring that were fed the high-fat diet post-weaning [27,29].

We also found that the TC group had high endotoxin levels. Endotoxin, also referred to as lipopolysaccharide (LPS) has been implicated as a potent inducer of inflammation, first, due to the fact that endotoxins are ligands to TLR4, and second, because it leads to high TNF-α, IL-1β, IL-6 and reduced adiponectin concentrations [29,30]. Moreover, Laugerette et al. [31] indicated that during the digestion of lipids, several changes can occur such as alterations of the intestinal microbiota in metabolic diseases and the absorption of endogenous endotoxins.

Numerous studies [9,10,12–17,32] indicate that the activation of hypothalamic inflammation by endotoxin levels promoted by adopting a diet rich in saturated fatty acids, is a key factor causing the central nervous system to have a disrupted pathway of insulin and an inflammatory status in obesity. Thus, we clarify that rats fed another type of fatty acids, such as trans fatty acids, during pregnancy and lactation leads to an inflammatory response in the hypothalamus. To explain previous evidence that hypothalamic inflammation is not found only in animals fed a high-fat diet or in models of obesity [10,12–14,16], we evaluated pro-inflammatory cytokines receptors, TLR2/TLR4 and NFκBp50/NFκBp65 pathways in rats fed trans fatty acids. Elevated TLR4, MyD88 and NFκBp65 expression were found in the TC group.

The highly elevated hypothalamic protein expression of the inflammatory pathway (TLR4, MyD88 and NFκBp65) leads to an increase of pro-inflammatory cytokines in the hypothalamus. Recently, it was shown that pro-inflammatory cytokines, in particular TNF-α, have a dual effect in the hypothalamus: at very high doses, e.g., in infectious diseases and cancer, it is anorexigenic, whereas in smaller doses than the former, e.g., in obesity, it appears to have a potent inflammatory action leading to hypothalamic insulin and leptin resistance [9,33-35].

In the present study, reduced AdipoR1 expression was found in the hypothalamus of rats fed trans fatty acids. Acting as cellular fuel

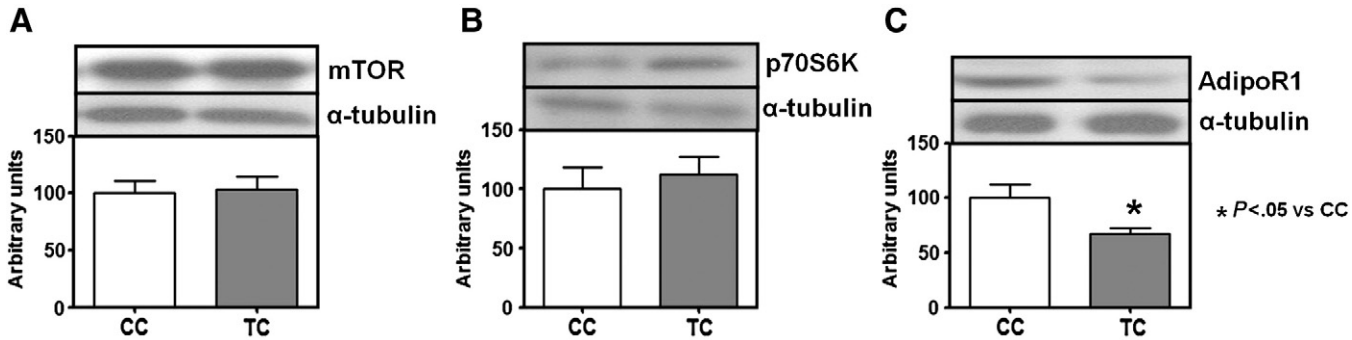


Fig. 7. Quantification of mTOR (A), p70S6K (B) and AdipoR1 (C). CC: mothers and offspring fed control diet; TC: mothers fed trans diet and offspring fed control diet. The number of animals studied per group was 7. Data are expressed in ME±SEM. The results are expressed in arbitrary units, stipulating 100 as the control value.

sensor in the hypothalamus, the adiponectin had the role of reducing food intake, activating an insulin/leptin-like effect and improving activation of insulin signaling mainly via AdipoR1 [21,22]. This result could partially explain the increase of food intake observed in the TC group. Thus, the low hypothalamic AdipoR1 expression found in animals fed trans fatty acids could initiate the phosphorylation of the serines of proteins involved in insulin signaling. On the other hand, adiponectin increases phosphorylation levels in the tyrosine of insulin receptor substrate (IRS) 1 and 2, Akt/PKB in serine and forkhead transcription factor 1 (FOXO 1), JAK2 and signal transducer as well as activator of transcription 3, indicating the existence of cross-talk between adiponectin-insulin and adiponectin-leptin pathways in the hypothalamus [21]. In accordance to Coope et al. [21], these actions were mediated by AdipoR1, the adiponectin receptor type predominantly in the arcuate and lateral hypothalamic nuclei. Moreover, in obese animals, the binding affinity of adiponectin to its receptor is also reduced [36].

Hypercaloric and hyperlipidic feeding are implicated as one of the most important environmental factors leading to the disruption of the insulin pathway in obesity. Recent studies have shown that both high-fat diets rich in saturated fatty acids and obesity models lead to hypothalamic resistance to insulin [12,13,16,35,37]. In the present study, a trans fatty acid diet did not alter hypothalamic insulin signaling protein expression, like IR, Akt/PKB, mTOR and p70S6K. It is important that the diet offered in this study is eucaloric, as the activation/inhibition of insulin signaling only is possible to evaluate through phosphorylated proteins.

In the present study, we showed an increase in hypothalamic TLR4, MyD88 and NFκBp65 expression in the TC group. These results could probably explain the reduction of AdipoR1 expression and the possible increase in food intake found in this group.

According to the fatty acid component of the diets, the hypothalamic inflammation was caused by the fatty acid intake due to the trans diet. Moreover, the present study did not consider the influence of hormones because we only evaluated male offspring. Male rats suffer a minor hormonal change and do not undergo changes in estrous cycles. On the other hand, Bangasser et al. [38] showed that female rats had brain hormone receptors that were less adaptive than those of male rats.

In summary, we showed that the partially hydrogenated vegetable oil (trans diet) intake by the mothers during pregnancy and lactation leads to hypothalamic inflammation and impaired satiety-sensing in offspring, promoting deleterious consequences such as obesity, even after the withdrawal of the causal factor. In other words, the effect remained even after the consumption of a control diet post-weaning. Furthermore, our data suggest that both nutrition during pregnancy/lactation and post-weaning should be studied in order to prevent and control hypothalamic inflammation and obesity-associated diseases.

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