Nonalcoholic fatty liver disease (NAFLD) is one of the main liver diseases today, and may progress to steatohepatitis, cirrhosis, and hepatocellular carcinoma. Some studies have shown the beneficial effects of aerobic exercise on reversing NAFLD. To verify whether chronic aerobic exercise improves the insulin resistance, liver inflammation, and steatohepatitis caused by a high fat diet (HF) and whether PPARα is involved in these actions. C57BL6 wild type (WT) and PPAR-α knockout (KO) mice were fed with a standard diet (SD) or HF during 12 weeks; the HF mice were trained on a treadmill during the last 8 weeks. Serum glucose and insulin tolerances, serum levels of aspartate aminotransferase, hepatic content of triacylglycerol, cytokines, gene expression, and protein expression were evaluated in all animals. Chronic exposure to HF diet increased triacylglycerol accumulation in the liver, leading to NAFLD, increased aminotransferase, hepatic content of triacylglycerol, cytokines, gene expression, and protein expression were evaluated in all animals. The liver lipid accumulation was not associated with inflammation; trained KO mice, however, presented a huge inflammatory response that was probably caused by a decrease in PPAR-γ expression. We conclude that exercise improved the damage caused by a HF independently of PPARα, apparently by a peripheral fatty acid oxidation in the skeletal muscle. We also found that the absence of PPARα together with exercise leads to a decrease in PPAR-γ and a huge inflammatory response.

TABLE 1. Sequence of primers forward and reverse used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer forward</th>
<th>Primer reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL-19</td>
<td>CAATGCCCACACTCCGTCGA</td>
<td>GTTTTITCCGGCAACAGA</td>
</tr>
<tr>
<td>ACC</td>
<td>CCAAGCAGTGTCGAAATC</td>
<td>ACTGGTGACTCTGCACCA</td>
</tr>
<tr>
<td>FAS</td>
<td>GATCGGTGGTATCTGCT</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>AMPK</td>
<td>AGTCTTGCAACAAGTA</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>NFRB</td>
<td>CCACTGCGAGCTATTGAC</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>PPARγ</td>
<td>ATCTTAATCCCGGACTGC</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>SCD1</td>
<td>TCCAGAAATGCTATCGATG</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>F4/80</td>
<td>GAATCCTGTGAAACTCGAGA</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>CD86</td>
<td>GTACTGTGGTCAAGGATCGCA</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
<tr>
<td>MyD88</td>
<td>TCCAGGGTCCAACAGAAG</td>
<td>GATCGGTGGTATCTGCT</td>
</tr>
</tbody>
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is considered an anti-inflammatory agent by decreasing hepatic expression of pro-inflammatory cytokines, increasing expression of anti-inflammatory cytokines, and activating the peroxisome proliferator-activated receptors (PPARs) family (Passos et al., 2015). PPAR-α and γ are the most common isoforms expressed in the liver. PPAR-γ plays a central role in inflammation response by decreasing NF-κB activity and promoting M2 macrophages polarization, whereas PPARα acts modulating the transcription of enzymes involved on β-oxidation, favoring FA oxidation (Cherkaoui-Malki et al., 2012) and preventing the transcription of pro-inflammatory proteins and chemoattractant molecules through inhibiting the translocation of NF-κB P65 subunit to the nucleus. This mechanism help controlling hepatic inflammation (Ramanan et al., 2008). Endurance exercise has been suggested as a modulator of PPARα mRNA expression in the liver (Maciejewska et al., 2011; Zhang et al., 2011).

Based on the findings, we investigated whether the beneficial effects of chronic exercise on restoring liver lipid accumulation and steatohepatitis are PPAR-α dependent, and the anti-inflammatory actions of PPAR-γ in the liver.

Materials and Methods

Animal procedure

Male C57BL/6j wild type (WT) and PPAR-α knockout (KO) mice (10–12 weeks of age) were obtained from the Jackson Laboratory and maintained on a 12:12-h light-dark cycle (lights on at 06:00). In the first moment mice were fed with a high-fat diet (HF, 59% of calories from fat, 15% from proteins, and 26% from carbohydrate) (Reeves et al., 1993). The fat composition was proportionally 44% of monounsaturated fatty acids of which 39% was Oleic acids, and 37% of saturated fatty acids of which 23.3% was composed of palmitic acid. Standard diet (SD, 9% of calories from fat, 15% from protein, and 76% from carbohydrate) (Reeves et al., 1993) for 12 weeks. Both genotypes of HF mice were divided in two groups: non-exercised (HF) or exercised (HFT). At this first moment, there were 10 mice on each group (SD, HF WT, HFT WT, HF KO, HFT KO).

Exercised group performed 1 week of treadmill adaptation, beginning at the 4th week, in which they walked at a 10 min/mile pace during 5 days. From week 5 until week 12, they were submitted to an aerobic training for 5 days/week during 60 min at 60% of the maximal speed (MS). The MS was derived from a maximal speed tests at the end of 4th, 8th, and 12th week. The tests consisted of a warm up (5 min at 10 m/min phase) and from the 6th min; the treadmill speed increased 3 min/mile every minute until the animals had run mechanical alterations, featuring exhaustion. After previous results, our aim, in a second moment, was to investigate whether the PPARγ inhibition in the liver was related to hepatic inflammation in trained mice and if it could be reversed by rosiglitazone treatment. In this second experimental design there were six mice on each group (HF KO, HFT KO, and HFT KO); N = 6. Therefore, the HF KO mice were divided in HF KO, HFT KO, and HFT KO treated with rosiglitazone (HFT RG KO) and this
group was submitted to the same experimental protocol of the first groups (diet and exercise) plus 15 mg/kg/day of oral rosiglitazone.

After 12-weeks experimental protocol, mice were fasted for 6 h before being euthanized for blood collection and tissue sampling. The epididymal, subcutaneous, and retroperitoneal adipose tissue were dissected and weighed, and the sum of their weights represents the adipose tissue index. Liver weight was also assessed and tissue was stored for further RNA and protein analysis.

Analytical procedures

Serum non-esterified fatty acids (NEFA) (HR Series NEFA-HR (Golay et al., 1984)—Wako Pure Chemical Industries, Ltd., Richmond, VA), total cholesterol, triacylglycerol levels, and alanine aminotransferase (ALT) activity were determined by enzymatic methods (Labtest, Lagoa Santa, MG, Brazil) (Srisawasdi et al., 2011).

Histological analyses

Small liver pieces were fixed with paraformaldehyde (4%), embedded in paraffin and serially cross-sectioned. The slices were stained with hematoxylin and eosin for steatosis analysis (Yin et al., 2012).

Assessment of triacylglycerol levels in the liver

Lipids were extracted from the liver with chloroform–methanol, as described by Folch (FOLCH et al., 1957). Tissue triacylglycerol levels in the lipid extract were determined by enzymatic assay (Labtest, Lagoa Santa, MG, Brazil).

Insulin and glucose tolerance tests

Four hours fasted mice received an intraperitoneal injection of insulin (1 U/kg body weight) or o-glucose (2 g/kg body weight). For insulin tolerance tests, blood samples (5 μl) were collected from the tail vein before and at 10, 20, 30, and 40 min after the insulin bolus injection. The constant for serum glucose disappearance (KITT) was calculated by linear regression of the glycemic levels measured between 5 and 30 min after insulin injection, as the interval in which the glucose linear phase decay occurs (Bonora et al., 1989). Similarly, for glucose tolerance tests, blood samples were collected from the tail vein before and at 15, 30, 60, 90, and
120 min after the glucose bolus injection (Bergmeyer, 1973; Chomczynski and Sacchi, 1987). The differences in glycemia before and during the glucose administration were used to calculate the areas under the curve (AUC). The levels of serum glucose were measured using the glucometer Accu-Chek® performa (ROCHE®, São Paulo, SP, Brazil).

Enzyme-linked immunosorbent assay (ELISA)
Liver tissue samples (80–100 mg) were carefully homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4) containing 10 μg/ml of protease inhibitor cocktail (Sigma–Aldrich®, St. Louis, MO). Homogenates were centrifuged and the supernatant was utilized to determine the protein concentration by Bradford assay (Bio-Rad®). Aliquots of each sample with the same concentration of total protein (25 mg) were diluted in Laemmli buffer. The samples were then subjected to electrophoresis on SDS–polyacrylamide gel (SDS–PAGE), then transferred from the gel to a nitrocellulose membrane, which was incubated first with an antibody against Phospho-AMPKα (Thr 172) (1:1000) from Cell Signaling (Cell Signaling), GAPDH (1:1000) from Santa Cruz (Santa Cruz®) or ACC (1:1000) from Cell Signaling (Cell Signaling), and then with anti-IgG antibody conjugated with peroxidase. After these process, membranes were incubated with the peroxidase substrate (ECL kit, Biorad®) and quantified by optical densitometry.

Western blotting
The livers were carefully homogenized in extraction buffer containing proteases and phosphatases inhibitors. After proper centrifugations, the proteins were determined by Bradford assay (Bio-Rad®). Aliquots of each sample with the same concentration of total protein (25 mg) were diluted in Laemmli buffer. The samples were then subjected to electrophoresis on SDS–polyacrylamide gel (SDS–PAGE), then transferred from the gel to a nitrocellulose membrane, which was incubated first with an antibody against Phospho-AMPKα (Thr 172) (1:1000) from Cell Signaling (Cell Signaling), GAPDH (1:1000) from Santa Cruz (Santa Cruz®) or ACC (1:1000) from Cell Signaling (Cell Signaling), and then with anti-IgG antibody conjugated with peroxidase. After these process, membranes were incubated with the peroxidase substrate (ECL kit, Biorad®) and quantified by optical densitometry.

120 min after the glucose bolus injection (Bergmeyer, 1973; Chomczynski and Sacchi, 1987). The differences in glycemia before and during the glucose administration were used to calculate the areas under the curve (AUC). The levels of serum glucose were measured using the glucometer Accu-Chek® performa (ROCHE®, São Paulo, SP, Brazil).

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Statistical methods

The data are presented as mean ± SEM (standard error of the mean) and analyzed by one-way analysis of variance (one-way ANOVA) followed by Bonferroni posttests. Analyses were performed using GraphPad Prism 5.0 software. Differences were considered significant when \( P < 0.05 \).

Results

KO SD mice behaved the same as shown in the literature (Supplementary Table S1).

HF feeding was able to increase both genotype mice body weight, but HF KO showed lower weight gain when compared to HF WT (Table 2). This weight gain was accompanied with an increase in white adipose tissue depots (data not shown) as well as the adiposity index, and dyslipidemia (Table 2). Exercise was efficient to reduce body weight gain, adiposity index, and dyslipidemia in both animals’ lineages (Table 2). The fasting glucose levels were higher in the HF WT and lower in the HFT WT; KO mice showed low levels when compared to WT and presented no difference when trained (Fig. 1A).

Our results showed that HF WT animals had glucose intolerance, characterized by an increase on glycemia during the GTTs (Fig. 1B and C), and a severe insulin resistance measured in the ITT (Fig. 1D and E). Both parameters were restored by exercise. KO animals showed low basal glycemia as well as lower glucose intolerance when compared to WT; however, these animals presented severe insulin resistance, which was not restored by exercise.

The HF feeding during 12 weeks caused liver damage, both animals’ lineages showed an increase in total liver weight (Table 1) and lipid contents, observed in their histological slice (Fig. 2C). HFT KO mice also showed higher hepatic TAG (Fig. 2A), but aspartate aminotransferase (AST) was reduced only in HFT WT (Fig. 2B). The CAE decreased lipid stored in hepatocytes in both genotypes (Fig. 2).

Regarding lipogenic genes, we observed that HF diet upregulated mRNA expression of SREBP and FAS in WT mice,
Fig. 4. Hepatic protein levels of phosphorylation of AMP-activated protein kinase (AMPK) in Thr172 (A) and its respective quantification (B); and phosphorylation of Acetyl-CoA carboxylase (ACC) in Ser79 (C) and its respective quantification (D) in WT and KO mice fed to high fat diet (HF) and trained (HFT). The line represents control group (SD). Data are mean ± SD. *P < 0.05. **P < 0.05 comparing to control (one-way ANOVA followed by Bonferroni). (n = 4 to all groups).

Fig. 5. Hepatic mRNA expression of NLR pyrin domain containing 3 (NLRP3) (A), Caspase 1 (Casp-1) (B), Toll-like receptor 4 (TLR-4) (C), nuclear factor Kappa B (NF-κB) (D) in WT and KO mice fed to high fat diet (HF) and trained (HFT). The line represents control group (SD). Data are mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. ###P < 0.001 comparing to control (one-way ANOVA followed by Bonferroni). (n = 8 to all groups). a.u, arbitrary units.
however, exercise was not able to decrease them (Fig. 3E and D). Interestingly, HFT WT showed an increase on ACC mRNA expression, while HFT KO mice had all these parameters reduced (Fig. 3C). The protein levels of total ACC in the liver decreased in HF animals, however, exercise has shown a positive effect on ACC Ser79 phosphorylation recovery in WT mice (Fig. 4).

Exercised groups exhibited a decrease in lipid oxidative enzyme-related genes such as AMPK and CPT-1 (Fig. 3A and B), but we observed a tendency of AMPK phosphorylation Thr172 recovery in WT mice (Fig. 4).

HF diet overregulated hepatic gene expression of NLRP-3, TLR-4, and caspase-1 in WT and NF-κB in KO when compared to SD WT. CAE reduced NLRPL-3 and TLR-4 in both mice genotype and NF-κB in HFT KO (Fig. 5). PPAR-α KO mice, when exercised, showed a huge inflammatory response marked by an increase in TNF-α, IL-12, IL-6, and IL-1β (Fig. 6A–E), otherwise these animals also showed an increase in anti-inflammatory cytokine IL-4 (Fig. 6H). HFT WT showed a decrease in monocyte chemotactic protein 1 (MCP1) (Fig. 6F), additionally, IL-10 was elevated for both genotypes when exercised compared to SD mice.

Fig. 6. Hepatic levels of tumor necrosis factor-a (TNFa) (A) interleukin-8 (IL-8) (B), IL-12 (C), IL-6 (D), IL-1β (E), monocyte chemotactic protein 1 (MCP1) (F), IL-10 (G), and IL-4 (H) in WT and KO mice fed to high fat diet (HF) and trained (HFT). The line represents control group (SD). Data are mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, ****P < 0.001 comparing to control (one-way ANOVA followed by Bonferroni). (n = 8 to all groups).
We also observed decrease in gene and protein expressions of PPAR-γ in the liver of HFT KO mice (Fig. 7). To investigate if the inflammation in response to exercise was caused by a PPAR-γ reduction, we treated these animals with rosiglitazone during the exercise protocol.

Rosioglitazone administration was efficient in reversing the insulin resistance present in this genotype (Supplementary Table S1).

The protein expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and MCP-1 was decreased by rosiglitazone treatment (Fig. 8). HFT RG KO mice also showed a decrease in NF-κB and IL-1β gene expression and an increase in TLR-4 and MyD88 mRNA (Fig. 9). We did not observe an increase in PPAR-γ gene expression in HFT RG KO; however, there was an increase in its target genes PGC-1α and FAS to this group when compared to HFT, and a tendency to recover SCD-1 and SREBP-1 to HF levels. CPT-1 gene expression was even smaller in HFT RG KO mice. F4/80 expression was reduced in both exercise groups, and CD86 gene expression had a decrease only in the HFT RG KO group (Fig. 10).

Discussion

In the present study, CAE has been shown efficient in decreasing the lipid storage in hepatocytes, body weight gain, and adiposity index in an experimental model of NAFLD, characterized by the loss of the nuclear receptor PPAR-α (PPAR-α knockout mice), as well as in wild-type animals fed with a HF diet during 12 weeks. Taken together, these results suggest that CAE exerts beneficial effects on reducing NAFLD, body weight gain, and adiposity index by mechanisms that do not require PPAR-α. In our model of HF, we did not find the association between hepatic inflammation and NAFLD. However, interestingly, exercise training increased the hepatic inflammation in PPAR-α KO by reduction of PPAR-γ inhibition.

The excessive consumption of saturated fat, physical inactivity, and the increases in obesity lead to the development of NAFLD (Neuschwander-Tetri, 2001; Dietrich and Hellerbrand, 2014), which is a pathology characterized by lipid accumulation in the liver associated with insulin resistance and inflammation. Here, we observed this condition in both mouse genotypes, and 8 weeks of CAE intervention was able to reverse the lipid storage on hepatocytes, as well as decrease liver weight and liver damage. In accordance with our findings, Gonçalves et al. (2014) showed that regular aerobic training was effective in restoring mitochondrial functions and reduce lipid storage in the liver in a NAFLD mouse model.

In our study, we observed that PPAR-α KO mice developed an intense liver fat accumulation and when they were trained, they showed improvement in NAFLD parameters. During moderate aerobic exercise, the skeletal muscle requires fatty acids as an energy source (Pearsall and Palmer, 1985; Ogborn et al., 2015), which leads to a reduction of FFA and a decrease of lipid storage on hepatocytes (Oh et al., 2013; Jordy and Kiens,
Moreover, a recent study found that PPAR-α inhibits the expression of myosin heavy chain-1 (MHC-1) present in oxidative fiber muscle type 1, therefore PPAR-α knockout showed an increase in type 1 fibers allowing an increase in fatty acid oxidation during exercise (Gan et al., 2013).

In accordance with this hypothesis, we showed a decreased in gene expression of oxidative enzymes in the liver, such as AMPK in both animal genotype and CPT-1 in HFT KO mice. Besides inhibiting de novo lipogenesis and glucose hepatic production by stimulating mitochondrial genes transcription and preventing lipogenic genes expression (Foretz and Viollet, 2011), AMPK is a key regulatory enzyme that stimulates FA oxidation by increasing CPT-1. Mice were euthanized 72 h after the last session of CAE, and we observed a decrease in phosphorylation of AMPK in HFT KO and a tendency to restore these levels in HFT WT. Wherefore we did not see a significance difference in trained groups. In corroboration, Piguet et al. (2015) found a significant increase on the phosphorylation of AMPK in animals sacrificed immediately after a single bout of exercise and did not find any difference when they were sacrificed 72 h after the moderate training.

Lipogenesis in the liver is under control of many factors including the transcription factor Sterol Regulatory Element-binding Protein1 (SREBP1), PPAR-Y, fatty acid synthase (FAS), and acetyl-CoA carboxylase enzymes (ACC). Among these, ACC and FAS are key enzymes responsible for de novo lipogenesis in the liver (Nakamuta et al., 2005). Here, we showed a decrease in these parameters in HFT KO mice, suggesting a decrease in de novo lipogenesis and a reduction of TAG accumulation. We also observed a decrease on ACC total protein expression in both mice genotype when fed with a HF diet and a tendency to revert it when trained. However, we observed an increase in ACC phosphorylation Ser79 in HFT WT mice. Once Ser79 is phosphorylated by AMPK, ACC is inactivated.

Studies have shown a close relation between inflammation and NAFLD progression to NASH development (Hussein et al., 2002; Tzeng et al., 2015). Here, we showed that 12 weeks of HFD increased gene expression of NLRP-3, caspase-1, and TLR-4 in WT, and NF-κB in KO mice; important proteins involved in inflammasome pathways. However, the increase in the inflammasome pathway did not reflect on cytokine protein expression. The lack of PPAR-α was a potent inductor of the increase in immune cell recruitment by the liver and p38MAPk activity in the liver of PPAR-α KO mice fed with a HF (71% fat) (Abdelmegeed et al., 2011). Otherwise, KO mice exposed to CAE showed an increased inflammatory response.
marked by an increase in TNF-α, IL-6, and IL-1β, proteins that act in the acute phases of inflammation (Hammerich and Tacke, 2014), and IL-12 which is an important lymphocyte chemoattractant (Kawakami et al., 1999) involved in the differentiation of naive T cells into Th1 cells (O’Garra and Arai, 2000). This result is surprising, since moderate chronic exercise is known for its anti-inflammatory actions (Flynn et al., 2007). However, we observed a decrease in PPAR-γ gene and protein expression in HFT KO mice. This PPAR family isoform has been associated with anti-inflammatory actions in monocytes and macrophages to induce macrophage M2 polarization (Feng et al., 2014) and in the liver by reducing NF-κB activity (Chen et al., 2014). This decrease in PPAR-γ expression may be an important immunometabolic discussion point, since it could provoke the inflammation in HFT KO mice.

To investigate whether the hepatic inflammation was really induced by PPAR-γ inhibition, we treated KO mice with 15 mg/kg/day of rosiglitazone during the same 8 weeks of exercise training program.

Here, we observed a decrease in serum FFA and TAG in HFT RG KO mice. There was no difference in fasting glycaemia between the groups, probably because PPAR-α knockout mice have a low-fasting glycaemia phenotype due to a reduction on hepatic gluconeogenesis (Knauf et al., 2006). However, rosiglitazone administration was efficient in reverting the insulin resistance and glucose tolerance in these mice. Our model, HFT RG KO showed an increase in subcutaneous, epididymal, and brown fat and a decrease in retroperitoneal adipose depot with no difference in the adiposity index.

Besides well-known anti-diabetic actions, studies have shown an important anti-inflammatory role of rosiglitazone. Mohanty et al. (2004) showed that obese diabetic and non-diabetic patients treated with rosiglitazone during 12 weeks presented decrease in hepatic NF-κB activity. Other studies have indicated that the activation of PPAR-γ by rosiglitazone induces macrophage M2 polarization, thus, decreasing the inflammation state (Lefevre et al., 2010; Ballesteros et al., 2014). Our results showed that rosiglitazone administration was effective in reducing the exercise-mediated inflammation in KO mice. HFT RG KO mice showed a decrease in acute pro-inflammatory proteins IL-6 and TNF-α (Hammerich and Tacke, 2014), and MCP-1; a key chemokine in the migration and infiltration of macrophages in the liver (Deshmane et al., 2009).
Interestingly, these animals increased TLR-4 and MyD88 mRNA. When activated, TRL4/MyD88 induces NF-κB translocation to the nucleus by certain signaling pathways, increasing its activity, and IL-1β production (Takeda and Akira, 2004). However, we observed a decrease in protein and gene expression of IL-1β, as well as decrease in NF-κB mRNA in HFT RG KO. Wherefore we believe that the increase in gene expression of TLR4/MyD88 served to balance the decrease of this complex activity.

In addition, we observed an increase in FAS mRNA and a tendency to increase SREBP1 and SCD1 in HFT RG KO mice. The activation of SCD1 in macrophages by rosiglitazone is an important pathway to reduce endoplasmic reticulum stress, thus decreasing cellular apoptosis, and depletory events (Ikeda et al., 2015). Furthermore, we showed an increase in PGC-1α gene expression, a PPAR co-activator which regulates genes involved in energetic metabolism and mitochondrial biogenesis (Puigserver and Spiegelman, 2003). F4/80 and CD86 are important M1 macrophages markers. A recent study reported that mice treated with rosiglitazone and other PPAR-γ agonists showed a decrease in M1 F4/80+ macrophage number (Hasegawa-Moriyama et al., 2013) and expression of CD86 (Feng et al., 2014). Here, we observed decrease in gene expression of F4/80 and CD86 in the liver, indicating a reduction of M1 macrophages.

In conclusion, these findings suggest that chronic exposure to HF increased TAG accumulation in the liver, leading to NAFLD, and that CAE can reduce this parameter, independently of PPAR-α. The development of NAFLD was not associated with liver inflammation in any mouse genotype. However, we found that the absence of PPAR-α together with chronic aerobic exercise leads to a decrease in PPAR-γ and a huge inflammatory response, which was reversed by rosiglitazone administration.

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