

# Infliximab reverses steatosis and improves insulin signal transduction in liver of rats fed a high-fat diet

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## Abstract

Non-alcoholic fatty liver disease, induced by nutritional factors, is one of the leading causes of hepatic dysfunction in the modern world. The activation of proinflammatory signaling in the liver, which is induced by systemic and locally produced cytokines, and the development of hepatic insulin resistance are two important factors associated with the progression from steatosis to steatohepatitis, a pre-cirrhotic condition. The objective of the present study was to evaluate the effect of inhibition of tumour necrosis factor (TNF)- $\alpha$ , using the monoclonal antibody infliximab, on the expression of cytokines, induction of steatosis and fibrosis, and insulin signal transduction in the liver of Wistar rats fed a high-fat

diet. Ten days of treatment with infliximab significantly reduced the expression of the proinflammatory markers, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and SOCS-3, in the liver of rats fed a high-fat diet. This was accompanied by reduced fat deposition and fibrosis and by improved insulin signal transduction through insulin receptor (IR)/IR substrate/Akt/FOXO1 and JAK2/STAT3 pathways. In conclusion, short-term inhibition of TNF- $\alpha$  with infliximab reduces inflammation and steatosis/fibrosis, while improving insulin signal transduction in an animal model treated with a high-fat diet.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) comprehends a large spectrum of clinicopathological conditions of the liver, ranging from steatosis to cirrhosis, and including steatohepatitis (NASH) and fibrosis (Browning & Horton 2004). In several regions of the world, predominantly those that have adopted westernized diet standards, NAFLD has become the most common cause of abnormal liver function, leading, frequently, to severe hepatic insufficiency (Browning *et al.* 2004, Williams 2006). However, not all patients (and animal models) exposed to a high-fat diet progress from steatosis to the pre-cirrhotic conditions, NASH, and fibrosis, and the factors that predispose to this progression are not completely known (Day & James 1998, Browning & Horton 2004).

In recent years, the characterization of the frequent association between NASH and hepatic insulin resistance has opened a new venue for investigation in this field (Medina *et al.* 2004). In several tissues of the body, including the liver, insulin resistance may evolve as a consequence of proinflammatory signaling generated by cytokines produced by the enlarged adipose tissue in overnourished people (Hotamisligil 2006). These cytokines activate intracellular serine kinases capable of targeting and inhibiting key elements of the insulin signaling pathway (De Souza *et al.* 2005a, Hotamisligil 2006). In the liver, both systemic and locally produced cytokines are thought

to play a role in the progression to NASH and, also, to favor the installation of hepatic insulin resistance (Tilg & Diehl 2000, Anty *et al.* 2006, Poniachik *et al.* 2006, Tomita *et al.* 2006). Among these cytokines, tumour necrosis factor (TNF)- $\alpha$  seems to play a predominant role since it can simultaneously activate a program that coordinates the expression of other cytokines and promotes intracellular proinflammatory signaling (De Souza *et al.* 2005a, Hotamisligil 2006, Tomita *et al.* 2006).

In clinical practice, the inhibition of TNF- $\alpha$  activity is currently in use for the treatment of diseases such as rheumatoid arthritis, Crohn's disease, and psoriatic arthritis (Chang & Lichtenstein 2006, Scott & Kingsley 2006). The anti-TNF- $\alpha$  monoclonal antibody, infliximab, is one of the available drugs approved for human use and is employed by thousands of patients in the world (Scott & Kingsley 2006). Some recent studies have evaluated the effect of TNF- $\alpha$  inhibiting approaches for the treatment of different inflammatory liver diseases, but the results are still controversial (Tilg & Diehl 2000). For example, at least two studies have observed favorable responses to TNF- $\alpha$  inhibiting approaches in ethanol-induced liver injury in rodents (Iimuro *et al.* 1997, Yin *et al.* 1999). Conversely, in a group of 20 patients with alcoholic steatohepatitis, only the combined use of infliximab and prednisone resulted in some clinical improvement after 28 days of treatment (Spahr *et al.* 2002). Because consumption

of fat-rich diets seems to play an important role in the pathogenesis of NAFLD and on its progression to NASH (Lieber *et al.* 2004, Zou *et al.* 2006), in the present study, we utilize an animal model fed on a high-fat diet to evaluate the effect of infliximab upon liver cytokine expression, morphology, and insulin signal transduction. The results show that, after a short period of treatment, significant reductions in proinflammatory markers in the liver are accompanied by reduced steatosis and fibrosis, and by improved insulin signal transduction.

## Materials and Methods

### Antibodies, chemicals, and buffers

The reagents for SDS-PAGE and immunoblotting were purchased from Bio-Rad. HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, and BSA (fraction V) were from Sigma.  $^{125}\text{I}$ -protein A and nitrocellulose paper (BA85, 0.2  $\mu\text{m}$ ) were from Amersham. Amobarbital and human recombinant insulin (Humulin R) were from Lilly. The anti-TNF- $\alpha$  monoclonal antibody, infliximab, was from Centocor (Horsham, PA, USA). Anti-insulin receptor (IR; sc-711, rabbit polyclonal), anti-IR substrate 1 (IRS1; sc-560, rabbit polyclonal), anti-IR substrate 2 (IRS2; sc-8299, rabbit polyclonal), anti-JAK2 (sc-278, rabbit polyclonal), anti-Akt (sc-1618, goat polyclonal), anti-phospho [Thr $^{183}$ /Tyr $^{185}$ ]JNK (sc-6254, mouse monoclonal), anti-phosphotyrosine (pY) (sc-508, mouse monoclonal), anti-phospho Ser $^{473}$ Akt (sc-7985-R, rabbit polyclonal), anti-STAT3 (sc-7179, rabbit polyclonal), anti-phospho [Tyr $^{705}$ ]STAT3, anti-FOXO1 (sc-11350, rabbit polyclonal), anti-phospho [Ser $^{256}$ ]FOXO1 (sc-22158-R, rabbit polyclonal), anti-PGC-1 $\alpha$  (sc-5816, goat polyclonal), anti-HNF-4 $\alpha$  (sc-6556, goat polyclonal), anti-TNF- $\alpha$  (sc-1350, goat polyclonal), anti-IL-1 $\beta$  (sc-7884, rabbit polyclonal), anti-IL-6 (sc-1265, goat polyclonal), anti-IL-10 (sc-1783, goat polyclonal), anti-SOCS-3 (sc-9023, rabbit polyclonal), and anti-actin (sc-10731, rabbit polyclonal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Experimental model and treatment protocols

Six-week-old male Wistar rats (*Rattus norvegicus*) from the University of Campinas Central Animal Breeding Center were used in the experiments. The rats were allowed access to chow and water *ad libitum*. The animals were maintained on a 12 h light:12 h darkness artificial cycle and housed in individual cages. Food was withdrawn 12 h before the experiments. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23 revised 1996). Rats

were treated either with a regular rodent chow or with a fat-rich (HL) diet (Table 1). The HL diet was prepared using predominantly fat from pork source and is rich in saturated fat (Araujo de Vizcarrondo *et al.* 1998). At 0 and 35 days after introduction of diets, rats were evaluated for metabolic parameters (Table 2). In the first part of the study, some rats were randomly selected at 5, 10, 15, 20, 25, 30, or 35 days after introduction of diets, for evaluation of liver histology and expression of cytokines, cytokine-responsive protein, and proteins involved in liver metabolism. After setting 20 days of HL diet as the time when steatosis was installed and levels of cytokines in liver were increased, some rats were randomly selected for treatment with saline (100  $\mu\text{l}$ /dose, *i.p.*, twice a day) or with infliximab (100  $\mu\text{g}$  in 100  $\mu\text{l}$  saline/dose, *i.p.*, twice a day). The infliximab dose was adjusted for rat metabolic rates, as compared with human metabolic rates (in clinical practice the human dose is 5.0–10.0 mg/kg every 8 weeks). Since the half-life of immunoglobulin is about 2–3 weeks, we believe that treating the experimental animals on a daily basis leads to stable anti-TNF- $\alpha$  immunoglobulin levels at about 7 days of treatment. In this part of the study, the experiments were conducted 10 days after starting infliximab use (which corresponded to day 30 of HL diet introduction). In addition, to evaluate the effect of infliximab to inhibit TNF- $\alpha$  signaling, we performed a dose-response experiment. For that, rats were treated once, *i.p.*, with different doses of the drug (0, 10, 50, 100, 200, and 400  $\mu\text{g}$  in 100 ml saline), and after 24 h, liver was obtained for evaluation of JNK activation by immunoblot as described later.

### Determination of glucose, insulin, leptin, and TNF- $\alpha$

Glucose was determined by the glucose oxidase method, as previously described (Trinder 1969). Insulin was determined by RIA as described (Scott *et al.* 1981). Leptin was determined using a commercially available ELISA kit (Crystal Chem. Inc., Chicago, IL, USA), following the recommendations of the manufacturer. TNF- $\alpha$  was determined using a commercially available ELISA kit (Pierce Biotechnology Inc., Rockford, IL, USA), following the instructions of the manufacturer.

### Liver histology

Hydrated 5  $\mu\text{m}$  sections of paraformaldehyde-fixed, paraffin-embedded liver specimens were stained by regular

**Table 1** Macronutrient composition of the diets

	Standard chow		High-fat chow	
	g%	kJ%	g%	kJ%
Protein	19	18	19	12
Carbohydrate	77	73	45	27
Saturated fat	4	9	36	61
kJ/g	15.8		24.5	

**Table 2** Clinical, biochemical, and hormonal parameters of experimental animals

	Control		HL	
	D0	D35	D0	D35
Body mass (g)	156 ± 11	212 ± 18*	154 ± 13	276 ± 22* <sup>†</sup>
Glucose (mg/dl)	91 ± 6	95 ± 5	92 ± 7	97 ± 7
Insulin (ng/ml)	2.45 ± 0.44	2.48 ± 0.46	2.42 ± 0.31	3.35 ± 0.33* <sup>†</sup>
Leptin (ng/ml)	2.88 ± 0.23	3.01 ± 0.19	2.79 ± 0.22	3.97 ± 0.32* <sup>†</sup>

*n* = 5; \**P* < 0.05 vs D0 of the same group; <sup>†</sup>*P* < 0.05 vs D35 of control. D0, day 0 of treatment; D35, day 35 of treatment.

hematoxylin–eosin (HE) and Masson's trichrome (MT) methods for evaluation of liver histology and fibrosis (Lin *et al.* 1998).

#### Liver glycogen and lipid content

At the end of the experimental period, five rats from each experimental group were randomly selected for determination of glycogen and total lipid content in the liver. For that, fragments of liver were collected and digested in pre-warmed KOH solution (30%) for glycogen measurements as previously described (Pimenta *et al.* 1989). Total lipid was determined by the gravimetric method (Mackenzie *et al.* 1970).

#### Morphometric analysis

A point counting procedure was carried out using a microscope with a sampling stage and a semiautomatic advancer. In HE- or MT-stained sections, fat or fibrosis areas respectively were delimited using the Adobe Photoshop software, and the respective areas were quantified using an Optomax Image Analysis System (Optomax, Burlington, MA, USA). For each group, livers from five rats were prepared and stained, and six fragments from each liver were analyzed. Morphometric results are presented as area fractions (the percentage of specific counts in relation to the total number of counted points; Lin *et al.* 1998, Thirone *et al.* 2002).

#### Immunoprecipitation and immunoblotting

For evaluation of cytokine and protein content, the abdominal cavities of anesthetized rats were opened and fragments of liver (3.0 × 3.0 × 3.0 mm) were gently removed and immediately homogenized in solubilization buffer at 4 °C (1% Triton X-100, 100 mmol/l Tris–HCl (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium orthovanadate, 2.0 mmol/l PMSF, and 0.1 mg aprotinin/ml). The protein concentration of the supernatants was determined by the Bradford dye-binding method. Samples containing 0.2 mg protein extracts were separated by SDS–PAGE, transferred to nitrocellulose membranes and blotted with antibodies against TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, phospho [Thr<sup>183</sup>/Tyr<sup>185</sup>]JNK, PGC-1 $\alpha$ , HNF-4 $\alpha$ , IR, IRS1, IRS2,

Akt, FOXO1, JAK2, STAT3, and SOCS3. For evaluation of insulin signal transduction in liver, the abdominal cavities of anesthetized rats were opened and the animals were injected insulin (100  $\mu$ l, 10<sup>-6</sup> mol/l) or saline (100  $\mu$ l) through the cava vein. After different intervals (described in Results), fragments (3.0 × 3.0 × 3.0 mm) of liver were excised and immediately homogenized in solubilization buffer as mentioned previously. Samples of total protein extracts containing 2.0 mg protein were used for immunoprecipitation with antibodies against IR, IRS1, IRS2 or JAK2 at 4 °C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes, and blotting with anti-phosphotyrosine (pY) antibodies. Some samples were analyzed by direct immunoblotting using antibodies against phospho [Ser<sup>473</sup>]Akt, phospho [Ser<sup>256</sup>]FOXO1, and phospho [Tyr<sup>705</sup>]–STAT3. Specific bands were labeled with <sup>125</sup>I–protein A, and visualization was performed by exposure of the membranes to RX-films (Velloso *et al.* 1993). Protein loading in gels was evaluated by Coomassie blue staining and by reblotting membranes with anti-actin antibody.

#### Statistical analysis

Specific protein bands present in the blots were quantified by digital densitometry (ScionCorp Inc., Frederick, MD, USA); areas of interest in liver histology were quantified by point counting (Optomax Image Analysis System). Means  $\pm$  S.E.M. obtained from densitometric scans, area measurements, and the values for blood insulin, leptin TNF- $\alpha$  and glucose, and body mass were compared by ANOVA (Tukey–Kramer or Dunnett, as appropriate) with *post hoc* test (Bartlett). A *P* value of < 0.05 was accepted as statistically significant. In time course experiments for determination of protein content and in morphometric analysis, the results were normalized by the values of the controls and expressed as fold variation.

## Results

#### Metabolic characteristics of the experimental animals

Table 2 shows that 35 days of treatment with the HL diet promoted a significant increase in body mass and serum insulin, and leptin levels. No modification in glucose levels was detected in HL-treated rats.

*HL diet leads to hepatic steatosis and fibrosis*

The livers of rats on HL diet were clearly steatotic (Fig. 1A-upper panels, HE). The intra-hepatocyte fat depots were predominant in the perivenular zone with extensions to the external areas of the lobule. Morphometric analysis revealed an increase of 19.3-fold ( $\pm 2.2$ -fold;  $P < 0.05$ ,  $n = 5$ ) in fat depot areas, as compared with the control. In MT-stained livers, fibrosis was observed predominantly in portal spaces with some extension toward centro-lobular zones (Fig. 1A-lower panels, MT). The morphometric analysis revealed an increase of 3.7-fold ( $\pm 0.6$ -fold;  $P < 0.05$ ,  $n = 5$ ) in fibrotic areas in the livers of HL rats.

*Time-dependent increase in cytokine expression in livers of HL rats*

HL diet consumption led to a progressive increase in the expression of the pro-inflammatory cytokines, TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , of the regulatory cytokine IL-10 and the cytokine suppressor protein, SOCS-3, in the livers of rats (Fig. 1B). These events were accompanied by the reduced expression of a transcription factor involved in the control of gluconeogenesis, HNF-4 $\alpha$ , and the increased expression of a co-activator

of transcription involved in the regulation of lipid metabolism and storage in hepatocytes, PGC-1 $\alpha$  (Fig 1C).

*Infliximab reduces pro-inflammatory cytokine expression in livers of HL rats*

The efficiency of infliximab to inhibit TNF- $\alpha$  action was tested by two different methods. First, mice on HL diet were treated with a single dose of infliximab according to a dose-response protocol described under Materials and Methods section, and the basal activation of JNK was determined in liver. As shown in Fig. 2A, a dose as low as 50  $\mu$ g infliximab was capable of significantly inhibiting JNK. Secondly, the blood concentration of TNF- $\alpha$  was determined in mice submitted to a 10-day infliximab treatment protocol. Consumption of the HL diet promoted a significant increase of TNF- $\alpha$  blood levels from  $76.3 \pm 6.9$  to  $166.0 \pm 9.7$  pg/ml, in control and HL mice respectively ( $n = 5$ ,  $P < 0.05$ ). Upon infliximab treatment, the levels of TNF- $\alpha$  in HL mice reduced 35% to  $107.4 \pm 8.1$  pg/ml ( $n = 5$ ,  $P < 0.05$  versus HL not treated with infliximab; Table 3). Infliximab did not promote significant changes in body mass, food intake, and blood levels of insulin and leptin (Table 3). To evaluate the effect of TNF- $\alpha$  inhibition upon liver cytokine

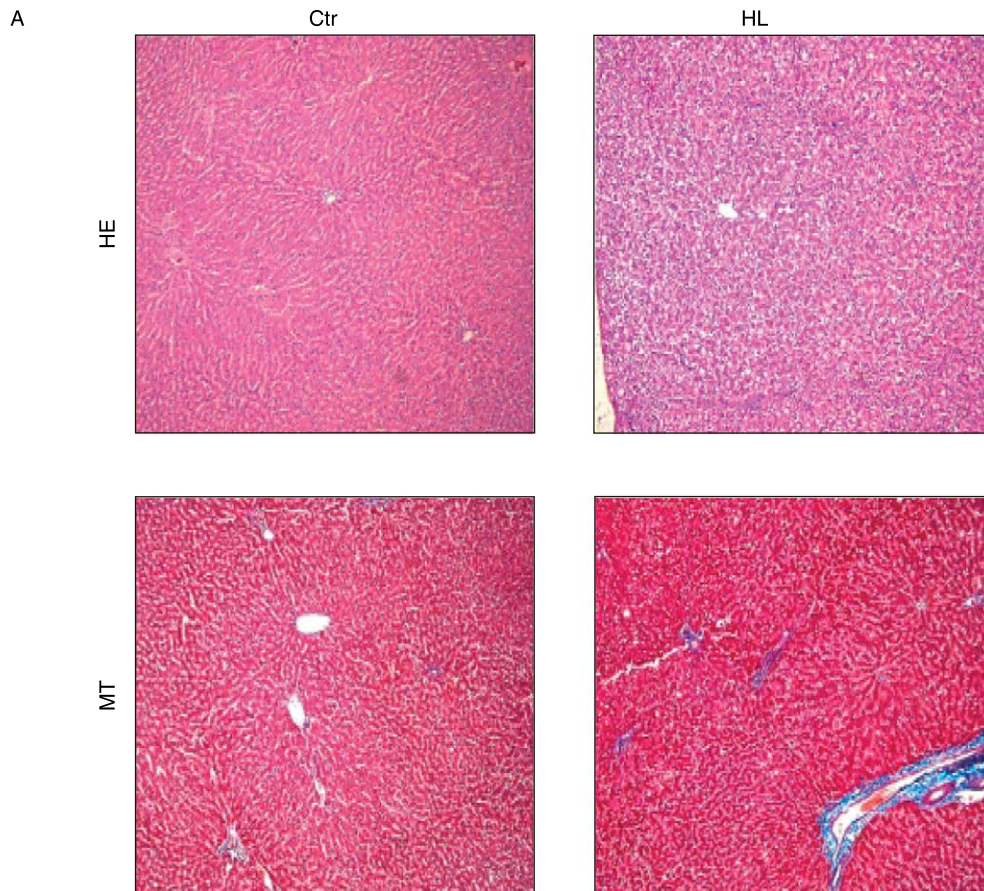
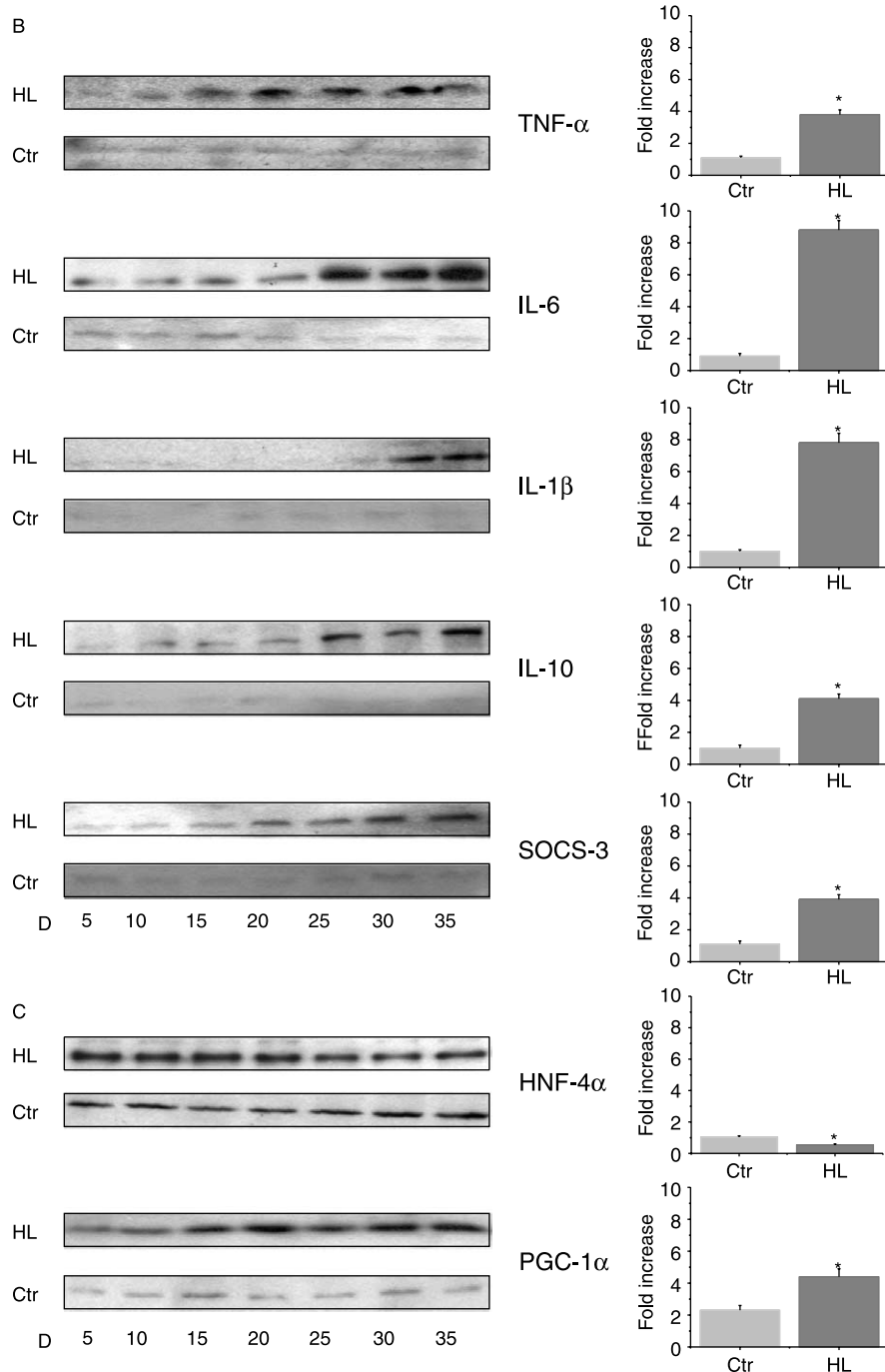
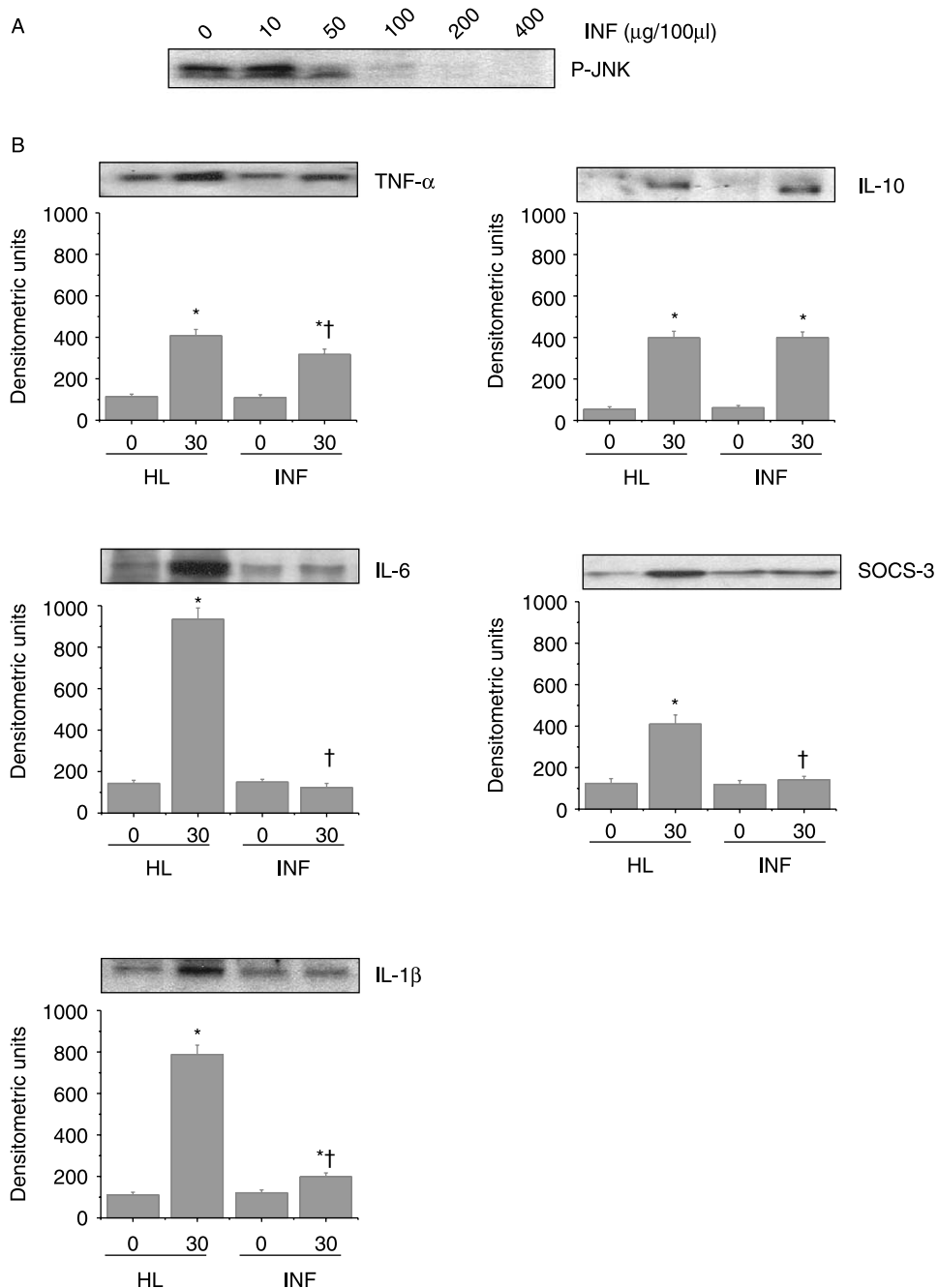


Figure 1 (continued)



**Figure 1** Effects of high-fat feeding on liver histology and protein content. (A) Hematoxylin–eosin (HE) and Masson’s trichrome (MT) staining of 5.0  $\mu\text{m}$  sections of livers of Wistar rats fed on a standard diet (Ctr) or a fat-rich diet (HL) for 35 days. (B) The protein amounts of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, and SOCS-3 were determined by immunoblotting in samples from livers of Wistar rats fed on a standard diet (Ctr) or a fat-rich diet (HL) after 5–35 days (D) of feeding with respective diets. The graphs on the right-hand side depict the respective fold variation in protein content from day 5 to day 35 in each experimental condition. (C) The protein amounts of HNF-4 $\alpha$  and PGC-1 $\alpha$  were determined by immunoblotting in samples from livers of Wistar rats fed on a standard diet (Ctr) or a fat-rich diet (HL) after 5–35 days. The graphs on the right-hand side depict the respective fold variation of protein content from day 5 to day 35 in each experimental condition. In A, the figures are representative of five different experiments. In B and C,  $n=5$ ,  $*P<0.05$  versus Ctr. Results are presented as means  $\pm$  s.e.m.



**Figure 2** Effects of infliximab on TNF- $\alpha$  signaling and cytokine expression. (A) Rats were treated intraperitoneally with a single dose of infliximab (as depicted in figure), and after 24 h, liver was obtained for immunoblot using anti-pJNK antibody. (B) The protein amounts of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, and SOCS-3 were determined by immunoblotting in samples from livers of Wistar rats fed on a fat-rich diet and treated with saline (HL) or infliximab (INF) for 10 days. Samples were collected at day 0 (before specific diet introduction) or at day 30 (30 days after specific diet introduction and 10 days after beginning daily treatment with saline or infliximab). In all experiments,  $n=5$ , \* $P<0.05$  versus time 0 in the same condition; † $P<0.05$  versus time 35 in HL. Results are presented as means  $\pm$  s.e.m.

expression and pro-inflammatory activation, rats were treated for 10 days (beginning on day 20 of HL diet) with the TNF- $\alpha$  blocking monoclonal antibody infliximab, and at the end of the experimental period, fragments of liver were obtained for immunoblotting experiments. As shown in Fig. 2B, infliximab promoted a significant reduction in IL-6 expression, which returned to levels similar to those of the control. Infliximab also promoted a significant reduction in IL-1 $\beta$  and TNF- $\alpha$  expression, but in these cases, the reductions were only partial. The levels of the regulatory cytokine, IL-10, were not affected by infliximab; however, the levels of the suppressor of cytokine signaling, SOCS-3, were completely restored to basal levels.

#### *Infliximab reduces liver steatosis and fibrosis in HL rats*

The treatment of HL rats with infliximab significantly reduced fat liver depots, as evaluated by HE staining (Fig. 3A, upper panels, HE) and determination of liver lipid content. In morphometric analyses, the magnitude of the fat area reduction was  $14.3 \pm 2.0$ -fold ( $P < 0.05$ ,  $n = 5$ ), while liver lipid content reduced from  $28.3 \pm 3.1$  g/100 g to  $6.8 \pm 2.0$  g/100 g ( $P < 0.05$ ,  $n = 5$ ). This was accompanied by a significant reduction in the fibrosis area ( $2.3 \pm 0.4$ -fold;  $P < 0.05$ ,  $n = 5$ ; Fig. 3A, lower panels, MT). In addition, treatment with infliximab resulted in stabilization of HNF-4 $\alpha$  levels, and reduction of PGC-1 $\alpha$  expression, which returned to basal levels.

#### *Infliximab improves insulin signal transduction in livers of HL rats*

Since liver insulin resistance is one of the hallmarks of diet-induced steatohepatitis, we evaluated the effect of infliximab on insulin signaling through the IR/IRS1/IRS2/Akt/FOXO1 and JAK2/STAT3 pathways. For this, rats fed on a standard (not shown) or HL diet, treated or not with infliximab, were used in immunoprecipitation and immunoblotting experiments. As compared with rats fed on a standard diet, HL feeding promoted a significant reduction in insulin-induced activation of IR, IRS1, IRS2, Akt, and FOXO1 (not shown; previously studied (De Souza *et al.* 2005b)). As shown in Fig. 4A, infliximab treatment significantly improved insulin-induced IR, IRS1, and IRS2 tyrosine phosphorylation, and Akt and FOXO1 serine phosphorylation. These

effects were not accompanied by changes in respective protein content (not shown). In addition, infliximab treatment improved insulin-induced tyrosine phosphorylation of JAK2 and STAT3 (Fig. 4B); these effects were not accompanied by changes in respective protein content (results not shown). Finally, as a consequence of improved insulin action in liver, the glycogen content in this organ increased from  $54.3 \pm 4.2$  mg/100 g to  $91.0 \pm 4.8$  mg/100 g ( $P < 0.05$ ,  $n = 5$ ).

## Discussion

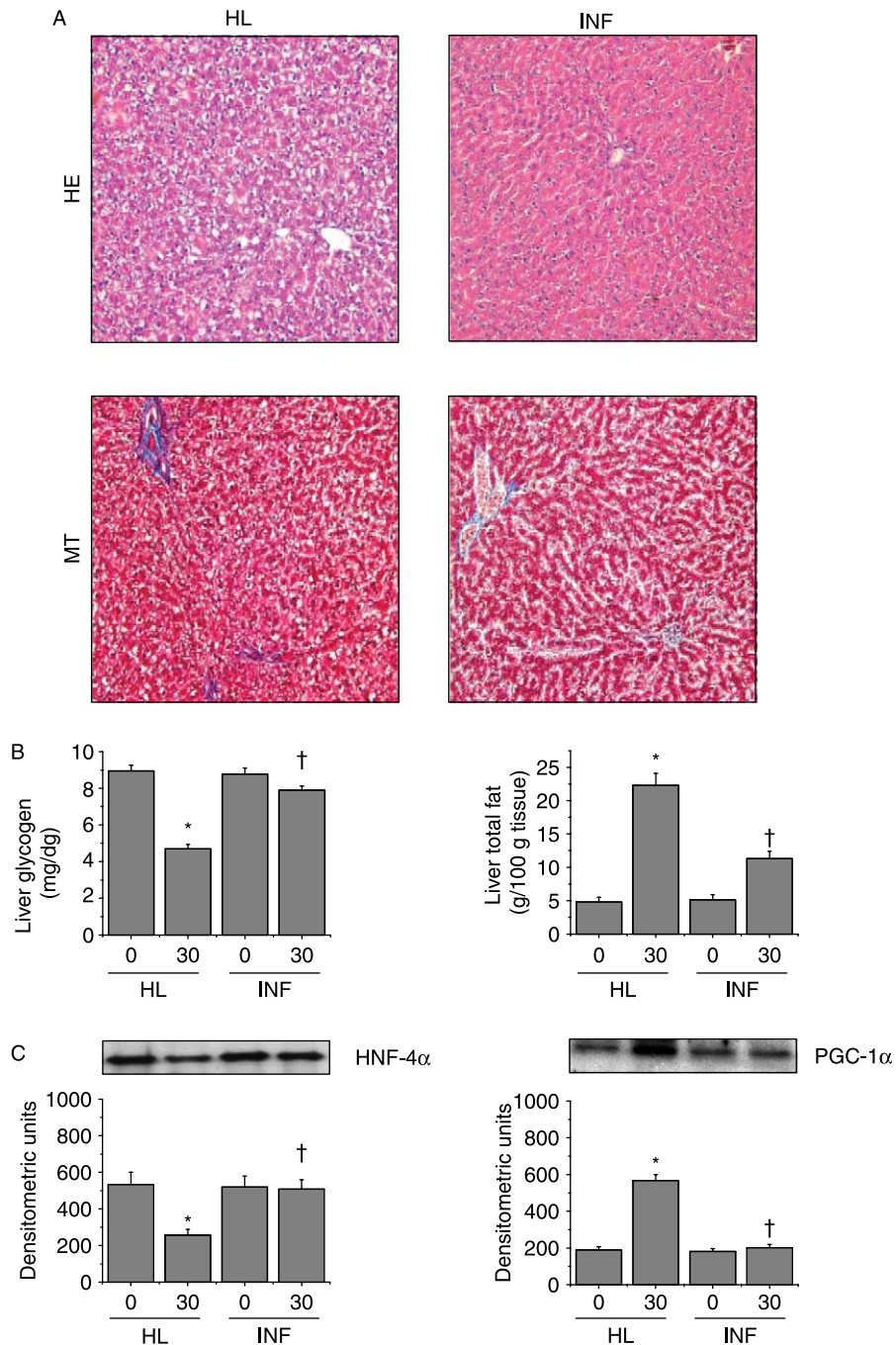
Currently, one of the most important challenges regarding NAFLD research is to understand why some patients with steatosis evolve to pre-cirrhotic conditions, such as NASH and fibrosis, while others remain stable with the rather benign state of steatosis (Day & James 1998, Tilg & Diehl 2000). Three events seem to play an important role in this progression, the consumption of fat-rich diets, the activation of proinflammatory signaling, and the development of liver insulin resistance (Day & James 1998, Tilg & Diehl 2000).

With regard to the activation of inflammatory signaling, it has become clear that systemic and locally produced cytokines are required to drive the progression from steatosis to NASH, fibrosis and cirrhosis (Day & James 1998, Tilg & Diehl 2000, Browning & Horton 2004, Larter & Farrell 2006). However, it is important to mention that no study, so far, has provided conclusive evidence of the role played by cytokines and other inflammatory factors in the initiation of steatosis (Tilg & Diehl 2000, Angulo 2002). Among the various cytokines potentially involved in this process, TNF- $\alpha$  has emerged as a key inducer of nutrient- and obesity-associated NASH (Tilg & Diehl 2000). TNF- $\alpha$  is capable of inducing the activation of stellate cells, matrix gene expression, and matrix remodeling, all of these being events that take place during the installation of NASH (Tomita *et al.* 2006). Interestingly, in addition to acting as an activator of liver inflammation and lesion, TNF- $\alpha$  can also promote insulin resistance by activating intracellular serine kinases, particularly JNK and I $\kappa$ BK, which can target and inhibit important substrates of the insulin signaling pathway (Cai *et al.* 2005, Diehl *et al.* 2005). Thus, it seems that TNF- $\alpha$  plays a central role in steatohepatitis, potentially integrating purely inflammatory signals with metabolic ones. As such, therapeutic

**Table 3** Effects of infliximab treatment on metabolic parameters and tumour necrosis factor (TNF)- $\alpha$  blood levels

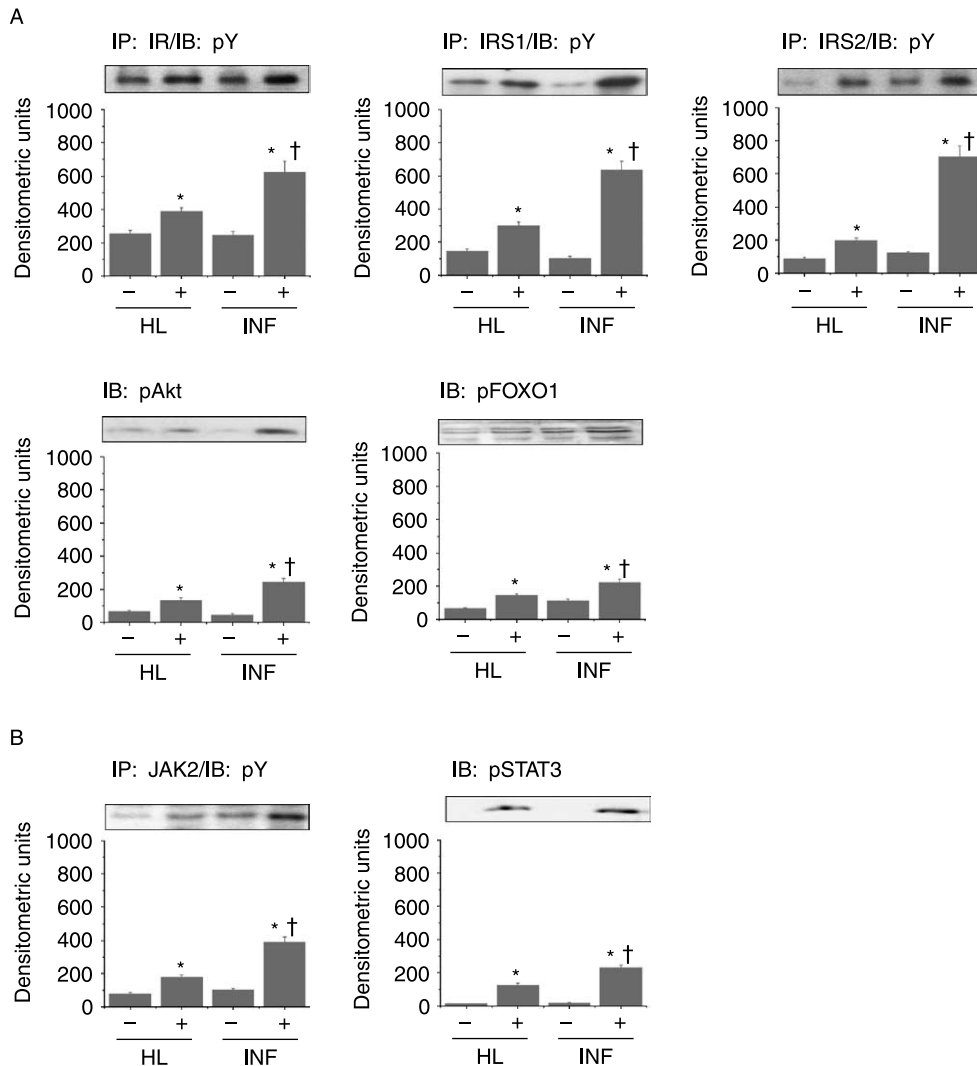
	Control		HL	
	SAL	INF	SAL	INF
Body mass (g)	214 $\pm$ 21	208 $\pm$ 16	282 $\pm$ 18	270 $\pm$ 23
Food intake (g/day)	29.2 $\pm$ 3.1	28.4 $\pm$ 1.9	26.3 $\pm$ 2.2	24.8 $\pm$ 2.3
Insulin (ng/ml)	2.49 $\pm$ 0.40	2.41 $\pm$ 0.57	3.39 $\pm$ 0.37	3.12 $\pm$ 0.30
Leptin (ng/ml)	2.99 $\pm$ 0.45	2.87 $\pm$ 0.25	3.91 $\pm$ 0.24	3.78 $\pm$ 0.31
TNF- $\alpha$ (pg/ml)	76.3 $\pm$ 6.9	NT	166.0 $\pm$ 9.7	107.4 $\pm$ 8.1*

N=5; \* $P < 0.05$  versus HL (SAL). SAL, saline treated; INF, infliximab treated.



**Figure 3** Effects of infliximab on liver histology and protein content. (A) Hematoxylin–eosin (HE) and Masson's trichrome (MT) staining of 5.0  $\mu$ m sections of livers of Wistar rats fed on a fat-rich diet for 30 days and treated for 10 days with saline (HL) or infliximab (INF). (B) Glycogen and total fat content in liver of Wistar rats fed on a fat-rich diet for 30 days and treated for 10 days with saline (HL) or infliximab (INF). (C) The protein amounts of HNF-4 $\alpha$  and PGC-1 $\alpha$  were determined by immunoblotting in samples from livers of Wistar rats fed on a fat-rich diet and treated with saline (HL) or infliximab (INF) for 10 days. Samples were collected on day 0 (before specific diet introduction) or on day 30 (30 days after specific diet introduction and 10 days after beginning daily treatment with saline or infliximab). In A, the figures are representative of five different experiments. In B,  $n=5$ , \* $P<0.05$  versus time 0 in the same condition; † $P<0.05$  versus time 35 in HL. Results are presented as means  $\pm$  S.E.M.





**Figure 4** Effects of infliximab on insulin signal transduction in the liver of rats fed on a high-fat diet. Wistar rats fed on a high-fat diet for 0 or 30 days and treated with saline (HL) or infliximab (INF) during the last 10 days (from day 20 to day 30) were anesthetized and acutely treated through the cava vein with saline (100  $\mu$ l) (–) or insulin (100  $\mu$ l,  $10^{-6}$  mol/l) (+). After 5 (IR, IRS1, and IRS2 in A; JAK2 in B), 10 (Akt in A), or 20 (FOXO1 in A; STAT3 in B) min, fragments were obtained from the liver and used in immunoprecipitation and immunoblotting experiments. In immunoprecipitation experiments, samples containing 2.0 mg total protein extracts were incubated with respective antibodies (IP), and immunocomplexes recovered with protein a sepharose were separated, under denaturing conditions, by SDS-PAGE. Nitrocellulose transfers were blotted (IB) with anti-phosphotyrosine antibodies (pY). In direct immunoblotting experiments, samples containing 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with respective antibodies. The bar graphs represent means  $\pm$  S.E.M. of densitometric values obtained for the bands. In all experiments,  $n=5$ ; \* $P<0.05$  vs (–) in the same condition; † $P<0.05$  vs (+) in HL.

strategies aiming at inhibiting TNF- $\alpha$  activity in the liver have emerged as potentially effective approaches to treat NAFLD (Tilg & Diehl 2000).

In the present study, we evaluated the effect of the anti-TNF- $\alpha$  monoclonal antibody, infliximab, upon liver steatosis, fibrosis, and insulin signal transduction in an animal model fed on a high-fat diet (De Souza *et al.* 2005a; Pitombo *et al.* 2006). Infliximab has been widely tested in humans and animal

models with different inflammatory diseases, and it is known to produce a sustained anti-inflammatory effect (Siegel *et al.* 1995; Arend 2002). Initially, we determined the time-dependent ability of the diet to promote liver expression of proinflammatory markers and induce steatosis and fibrosis. After 20 days on the HL diet, higher expressions of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, and SOCS-3 were detected. This was accompanied by an increased expression of a co-activator of

gene transcription involved in hepatic lipid oxidation, PGC-1 $\alpha$ , and by a reduction in the expression of a transcription factor, involved in gluconeogenesis, HNF-4 $\alpha$ . In addition, the HL diet led to a significant increase in liver fat deposition and also to a significant increase in liver fibrosis. With regard to the induction of liver steatosis and fibrosis and the modulation of expression of PGC-1 $\alpha$  and HNF-4 $\alpha$ , these are well-known outcomes of nutrient-induced liver dysfunction and serve to validate the model (De Souza *et al.* 2005b). However, the determination of a time course of expression of inflammatory markers is original and shows that, in this particular model, a rather short period of treatment with a fat-rich diet is capable of inducing an inflammatory response.

The treatment with infliximab led to a significant reduction of the basal activation status of the pro-inflammatory signal-transducing protein JNK and a reduction of blood TNF- $\alpha$  levels. In addition, the treatment led to a reduction of 40% in TNF- $\alpha$  expression in liver. This is in pace with reductions observed in other studies. For example, in an animal model of colitis, the treatment with a dose of infliximab, which is similar to the one used in our study, promoted a 45% decrease in local (colonic) levels of TNF- $\alpha$  and a 50% reduction in blood levels of the cytokine (Woodruff *et al.* 2003). Furthermore, infliximab treatment effectively inhibited the expression of the pro-inflammatory cytokines, IL-6, and IL-1 $\beta$ , while the anti-inflammatory cytokine IL-10 was not affected by the treatment. The maintenance of higher levels of IL-10 may have a positive impact on the overall result of the treatment, as suggested by recent studies that show a protective effect of this cytokine in NASH development (den Boer *et al.* 2006, Elinav *et al.* 2006). Another outcome of the treatment with infliximab was the reduction of SOCS-3 expression. SOCS-3 is an intracellular modulator of proinflammatory signaling that is induced by a number of cytokines and hormones that employ the JAK/STAT signaling system (Kubo *et al.* 2003). SOCS-3 can either physically interact with the JAK/STAT complex or drive signaling intermediaries to proteasome degradation resulting, in both cases, in the negative regulation of signal transduction (Kubo *et al.* 2003, Balasubramanyam *et al.* 2005). The reduction of its expression after infliximab treatment indicates that proinflammatory signaling was considerably reduced by this approach.

In parallel with the reduction in the expression of inflammatory markers in the liver, infliximab significantly reduced steatosis and fibrosis, which was accompanied by a reduction in PGC-1 $\alpha$  and an increase in HNF-4 $\alpha$  expression. The effect of infliximab on steatosis was so remarkable that even upon macroscopic evaluation of the liver it could be noticed. Since insulin action favors fat deposition, one could argue that the improvement of insulin activity in the liver promoted by infliximab should increase and not reduce steatosis. However, it must be emphasized that liver insulin resistance affects differently two of the main physiological phenomena controlled by insulin in this tissue, i.e. gluconeogenesis and fat  $\beta$ -oxidation/deposition. While gluconeogenesis and  $\beta$ -oxidation become impaired quite early during the installation of insulin resistance, triglyceride

synthesis and deposition are affected only in late stage insulin resistance (Silverman *et al.* 1990, Baig *et al.* 2001); this implies on greater deposition of triglyceride, which is reversed as soon as insulin resistance is controlled.

Finally, to determine the effect of TNF- $\alpha$  inhibition upon liver insulin action, we evaluated insulin signal transduction through the IR/IRS1/IRS2/Akt/FOXO1 and JAK2/STAT3 signaling pathways. The first is the classical pathway activated by insulin in all insulin-responsive tissues (Saltiel & Kahn 2001). In liver, this pathway is responsible for the connection of the insulin signal with the control of gluconeogenesis (Saltiel & Kahn 2001). The outstanding improvement in the molecular activation of this pathway, achieved with infliximab, may be the result of an alleviation of the functional activities of serine kinases, such as JNK and I $\kappa$ BK, which are targets of TNF- $\alpha$  and can affect insulin signaling by promoting the inhibitory serine phosphorylation of IR, IRS1, and IRS-2 (Paz *et al.* 1997, Hirosumi *et al.* 2002, Araujo *et al.* 2005). The JAK2/STAT3 is a non-classical target of insulin signaling (Saad *et al.* 1996) that participates in the crosstalk of the insulin signaling with several other pathways (Calegari *et al.* 2005, Velloso *et al.* 2006), including inflammatory pathways activated by cytokines. In addition, the JAK2/STAT3 pathway is a primary target for leptin signaling, a hormone known to exert anti-steatotic effects (Lee *et al.* 2001) and to be modulated by a crosstalk with insulin (Carvalho *et al.* 2003). In liver, STAT-3 is known to inhibit SREBP-1c and, thus, to impose a negative signal in hepatic lipogenesis (Ueki *et al.* 2004). The inhibition of insulin-induced activation of STAT-3 in the liver of HL rats suggests that lipogenesis could be stimulated through this pathway. The reduction in SOCS-3 expression, accompanied by improved insulin-induced activation of JAK2/STAT-3 signaling after infliximab treatment may have played a positive role in the reduction of fat liver deposition and of fibrosis. Although we have not evaluated the clinical outcomes of the modification of insulin action in liver, as for example, determining liver glucose production, the comprehensive study of the signaling pathways herein performed, strongly suggests that insulin action was indeed improved by infliximab treatment.

In conclusion, the present study provides further evidence for a role for TNF- $\alpha$  as an attractive target for the therapeutics of NAFLD. Inhibition of TNF- $\alpha$  by the commercially available monoclonal antibody, infliximab, resulted not only in reversal of steatosis and fibrosis, but also in the improvement of insulin signal transduction.

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