Oxidative stress status and liver tissue defenses in diabetic rats during intensive subcutaneous insulin therapy

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Abstract

Long-term insulin delivery can reduce blood glucose variability in diabetic patients. In this study, its impact on oxidative stress status, inflammation, and liver injury was investigated. Diabetes was induced in Wistar rats with a single dose of streptozotocin (100 mg/kg). Untreated rats and rats administered Insuplant® (2 UI/200 g/day) through a subcutaneous osmotic pump for one or four weeks were compared with non-diabetic controls. Body weight, fructosamine level, total cholesterol, Insulin Growth Factor-1 (IGF-1) level, lipid peroxidation, and total antioxidant capacity were measured. Hepatic injury was determined through the measurement of glycogen content, reactive oxygen species (ROS) production, and macrophage infiltration. Liver oxidative stress status was evaluated through the measurement of superoxide dismutase (SOD), catalase (CAT), and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) expression, and p38 mitogen-activated protein kinase (p38MAPK) activation. Induction of diabetes led to increased plasma oxidative stress and inflammation. Moreover, ROS production and macrophage infiltration increased in addition to SOD, CAT, and NADPH oxidase expression. Intensive insulin therapy improved metabolic control in diabetic animals as seen by a restoration of hepatic glycogen, plasma IGF-1 levels, and a decrease in plasma oxidative stress. However, insulin treatment did not result in a decrease in acute inflammation in diabetic rats as seen by continued ROS production and macrophage infiltration in the liver, and a decrease of p38MAPK activation. These results suggest that the onset of diabetes induces liver oxidative stress and inflammation, and that subcutaneous insulin administration cannot completely reverse these changes. Targeting oxidative stress and/or inflammation in diabetic patients could be an interesting strategy to improve therapeutic options.

Keywords: Insulin therapy, oxidative stress, diabetes, liver

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Introduction

Diabetes is one of the most common chronic diseases worldwide affecting more than 340 million people. It is characterized by prolonged hyperglycemia in the postprandial and/or fasting state[1,2] as a result of impaired insulin-mediated glucose metabolism. Uncontrolled hyperglycemia leads to the progressive development of microvascular and macrovascular complications, causing morbidity and mortality in diabetic patients.[3–6] Indeed, hyperglycemia is the pivotal factor leading to increased oxidative stress and reactive oxygen species (ROS) production which can activate pathways including glucose autoxidation, protein glycation, and the subsequent oxidative degradation of glycated proteins.[7–9] Moreover, fluctuations in acute glucose concentration are related to oxidative stress.[10] While a certain level of ROS is necessary to maintain cell survival, high levels of ROS lead to activation of cell defenses and, if cells cannot re-establish equilibrium, to cell death.[11,12] One of the major sources of ROS in the tissues of diabetic patients, in response to high glucose, is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,[13,14] which catalyzes the generation of superoxide anions. Superoxide is well known as a mediator of inflammation and an inducer of apoptosis. When overproduced, ROS can lead to intracellular protein, lipid (lipid peroxidation), membrane, and DNA damage, and activate inflammation *via* several stress pathways such as nuclear factor-kappa B, stress-activated protein kinase/c-Jun NH(2)-terminal kinase, or p38 mitogen-activated protein kinase.[15]

ROS levels are regulated through a variety of cellular defense mechanisms consisting of enzymatic antioxidants.[16,17] Maintenance of acceptable and necessary levels of ROS, through their sequestration and transformation, is carried out by a pool of dedicated enzymes including superoxide dismutase (SOD) and catalase (CAT).[18,19] Reports in the literature regarding the effects of diabetes-induced hyperglycemia on antioxidant enzymes are contradictory. Hyperglycemia has been shown to enhance, inhibit, or maintain the level of antioxidant enzyme expression in diabetic animals, with wide variations depending on animal age and duration of diabetes,[20–22] or tissues examined.[23,24] These discrepancies may arise due to variations in enzyme activity over time; for example, compensatory increases in enzyme activity in response to increased oxidative stress or direct inhibitory effects of ROS depending on the type of tissue under examination. With diabetes some tissues, such as heart, kidney, and retina, are more susceptible to damage, while others are more resistant.[25]

The liver plays a pivotal role in glucose homeostasis through glycogen storage in the fed state and glucose production through glycogenolysis and gluconeogenesis in the postabsorptive period; under physiological conditions, hepatocytes are the main site of glucose metabolism. Insulin enhances glycogen synthesis within the liver and prevents glucose production.

Numerous studies have demonstrated the existence of hepatic and systemic oxidative stress in diabetic animal models and tested antioxidant molecules;[26–29] however, few of these were carried out on insulin-treated diabetic rats mimicking the human therapeutic approach. A reduction in blood glucose variability in response to long-term insulin delivery has been reported previously;[30,31] however, its impact on oxidative stress and liver metabolism has not yet been described.

In the present study, we developed an experimental diabetic animal model in which type 1 diabetes was induced by a single high dose of streptozotocin (STZ). We then administered insulin subcutaneously through an osmotic pump for either one or four weeks and assessed the levels of plasma and hepatic oxidative stress, and liver tissue defenses during insulin treatment.

Materials and methods

Chemicals

Amyloglucosidase (AMGD) was purchased from Roche Diagnostics (Meylan, France); glucose and phosphate-buffered saline from Fisher Scientific (Illkirch, France); and eosin, Harris hematoxylin, ethanol, and toluene from Labonord (Templemars, France). All other products were purchased from Sigma-Aldrich (St Quentin Fallavier, France).

Animals and ethics statement

This study was performed in accordance with 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). The laboratory in which the work was conducted was licensed by the Department of Veterinary Service (license N° B67-482-28), and the protocol of the study was approved by the Departmental Direction of Populations Protection (license N° 67-318). All efforts to minimize animal suffering and reduce the numbers of animal used were made. Male Wistar rats (180–200 g) (Depré, Saint Doulchard, France) were housed in a thermoneutral environment ($23 \pm 1^{\circ}C$) with a 12:12 h photoperiod and provided food (Safe-A04, Villemoisson-sur-Orge, France) and water *ad libitum*. All rats were weighed once a week and on the day of death.

Induction of diabetes

Diabetes mellitus was induced by a single intraperitoneal injection of STZ (100 mg/kg, n = 30). After three days, glycemia was measured in tail-vein blood samples using a glucometer (Accu-Chek Performa, Roche Diagnostic, France). Rats with a blood glucose level >250 mg/dL and c-peptide level <100 ng/mL were considered. Only 20 diabetic rats were then selected and randomized into two diabetic groups: untreated (DIAB) (n = 10) or treated with Insuplant® (Sanofi-Aventis Deutschland GmbH, Germany) (INS) (n = 10) which was administered through a subcutaneous osmotic pump. The control group (C) was composed of non-diabetic rats of the same age (n = 10).

Surgery and samples

Twenty-four hours after diagnosis of diabetes, the INS group were anesthetized (ketamine/xylazine (100 µL/100 g)) and an osmotic minipump (Alzet, model 2006, Charles River Laboratories Inc., Wilmington, MA, USA) was inserted subcutaneously. The infusion rate was set to 2 UI/200g rat/day for either one or four weeks.

One week and four weeks after starting insulin treatment, five rats per group were killed. After general anesthesia induced by a mixture of 2.7 mL of xylazine (Rompun® 2%, Bayer, Puteaux, France) and 10 mL of ketamine (Imalgène® 1000, Merial, Lyon, France) at a dose of 100 µL/100 g; the stages of sleep of animals (vibrissae inert, loss of palpebral reflex, non-responsiveness to external stimuli) were checked. Blood was sampled from the abdominal aorta and rats were killed by exsanguination. Plasma samples were taken for measurement of plasma, metabolic, and oxidative parameters. Liver tissue and vessels were embedded in Tissue-Tek® O.C.T. (Leica Microsystem SAS, Nanterre, France) and snap frozen in liquid nitrogen.

Biochemical analysis of plasma

Metabolic parameters. Tail vein blood glucose at death was measured before anesthesia using a glucometer. To determine the efficiency of subcutaneous insulin therapy, human insulin was measured by ELISA (Mercodia Insulin ELISA, Uppsala, Sweden). Plasma fructosamine was measured using a colorimetric method from the Cerba Laboratory (Cergy Pontoise, France), and Insulin Growth Factor-1 (IGF-1) was measured with a Quantikine® mouse/rat IGF-1 immunoassay (R&D systems Inc.,

Minneapolis, MN, USA) according to the manufacturer's instructions. Plasma cholesterol was measured using the Cholesterol RTU™ (Biomérieux, Lyon, France) colorimetric method and a cholesterol calibrator.

Inflammatory parameters. α2-macroglobulin was measured according to the manufacturer's instructions (Rat α2-macroglobulin ELISA, GenWay Biotech, Inc. San Diego, USA).

Oxidative parameters. Lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) using the OxiSelect™ TBARS Assay Kit-MDA (Malondialdehyde) Quantitation (Cell Biolabs Inc., San Diego, USA) according to the manufacturer's instructions.

Total antioxidant capacity (TAOC) was measured using a Trolox ((+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity method described by Re *et al.*[32] Briefly, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (7 mmol/L) was added to potassium persulfate (2.5 mmol/L) to generate $ABTS^{\bullet+}$ radical cations. The $ABTS^{\bullet+}$ (pH 7.4) was used at an absorbance of 0.7 ± 0.02 at 734 nm as measured using a microplate reader (iMarkTM, Biorad, Marnes-la-Coquette, France). Exactly 4 min after the addition of 200 μL diluted $ABTS^{\bullet+}$ solution to 2 μL plasma or Trolox (0.4 μmol/L) standards, absorbance was read at 734 nm. Results are expressed as μM equivalent of Trolox.

Histological studies

Hepatic glycogen quantification. A piece of fresh liver (100 mg) was placed in a tube with 450 μL sodium acetate buffer (0.2 mol/L, pH 4.5) and mixed briefly with an ULTRA-TURRAX (Polytron PT-MR2100, Kinematica AG, Luzern, Switzerland). An aliquot of homogenate was mixed with AMGD and incubated at 55°C for 90 min to degrade glycogen into glucose residuals. Samples (10 μL) or reference standards of glucose (0–1 g/L) were incubated with 250 μL of extemporary glucose reagent (250 μL of ortho-dianisidine 10 g/L in distilled water + 50 mL glucose oxidase 0.25 g/L/peroxidase 0.04 g/L in phosphate buffer) at room temperature for 10 min, and absorbance was read at 450 nm. Samples were analyzed in duplicate, and the results expressed as microgram glycogen per microgram liver (adapted from Gustavsson et al.[33]).

Characterization of hepatic oxidative stress. The oxidative fluorescent dye dihydroethidine (2.5 μmol/L, 30 min, 37°C) was used to evaluate *in situ* formation of ROS on unfixed 10 μm-thick sections of liver using a method described by Dal-Ros *et al.*[34] The level of ROS was determined using a microscope and the whole fluorescence of tissue samples was quantified with the imaging software (NIS-Elements BR, Nikon, France) at a 20 × magnification. Levels of ROS were expressed as the percentage of red pixels per five random fields per animal in comparison with control values (100%).

Hepatic inflammation. *In situ* macrophages were measured on frozen 10 μm sections of liver. After fixation with 4% paraformaldehyde, sections were incubated with rabbit anti-ionized calcium binding adapter molecule 1 (Iba 1) (1:1000, rabbit, Wako Chemicals GmbH, Germany), then incubated with an anti-rabbit IgG biotinylated secondary antibody (1:200, goat, Vector Laboratories Inc., Burlingame, USA) followed by conjugated horseradish peroxidase (HRP) streptavidin (Vector Laboratories), and finally by incubation with 3,3′-diaminobenzidine (Vector Laboratories) and counterstaining with diluted Harris hematoxylin. Macrophage density was expressed as the percentage of brown pixels per five random fields per animal in comparison with control values (100%).

Western blotting assays

Preparation of liver tissue homogenates. Liver tissue (5 mg) from experimental rats was homogenized using NP-40 buffer (NaCl 150 mmol/L, 1.0% Triton X-100, Tris 50 mmol/L, pH 8) with a protease/phosphatase inhibitor cocktail (Roche Diagnostics, Meylan, France) using an ULTRA-TURRAX. Supernatants were collected and protein contents measured by the Bradford method.[35]

Western blotting. Total protein (20–40 μg) was separated on a 4–12% Bis-Tris Criterion™ XT Precast Gel (Bio-Rad, Marne-La-Coquette, France) and transferred to an Immobilon PVDF membrane (Millipore, Molsheim, France). Anti-GAPDH (1:500, rabbit, Cell Signaling, Danvers, MA, USA), -CAT (1:1000, mouse, Sigma-Aldrich), -MnSOD (manganese superoxide dismutase, 1:500, mouse, Sigma-Aldrich), -phosphorylated-p38 (1:500, rabbit, Cell Signaling, Danvers, MA, USA), and -p67 phox subunits of NADPH oxidase (1:100, mouse, Santa Cruz, CA, USA) antibodies were incubated with membranes overnight at 4°C. Membranes were incubated for 1 h at room temperature with a HRP-conjugated secondary antibody (1:2000–1:4000) and developed using the Luminata™ Forte Western HRP substrate (Millipore, Molsheim, France) with Chemidoc XRS (Bio-Rad, Marne-La-Coquette, France). The relative quantity of the protein of interest compared with the reference protein GAPDH was measured with Image J software (NIH, USA).

Statistical analysis

All data followed a normal distribution. Values are expressed as mean ± SEM, with n indicating the number of rats. Statistical analysis was performed using the Student's *t*-test for unpaired data, or two-way ANOVA followed by Fischer's protected least-significant difference test where appropriate (Sigmastat 3.10, Systat Software, Point Richmond, United States). P value of < 0.05 was considered statistically significant.

Results

Subcutaneous insulin administration improves metabolic control in diabetic rats

After four weeks, insulin-treated diabetic rats were heavier than non-treated diabetic rats ($p < 0.01$) but lighter than the control group (Figure 1). Moreover, frequent hypoglycemia was observed in insulin-treated rats when fasting, preventing measurement of fasting glycemia. Indeed, plasma levels of human insulin in treated rats were maintained for the duration of treatment (83.75 ± 4.48 mU/L at one week, and 93.34 ± 36.67 mU/L at four weeks). Glycemia in untreated diabetic rats remained very high throughout the study (>550 g/L) (Table 1). In accordance with this data, plasma fructosamine, an indicator of glycemic control in rats, was significantly higher in untreated diabetic rats after four weeks than that in insulin-treated diabetic rats ($p < 0.001$), even if these levels remained higher than those of control rats (Table 1). In the same way, the level of plasma cholesterol was significantly higher in diabetic rats after four weeks ($p < 0.001$), although it was comparable to the control group after four weeks for insulin-treated diabetic rats (Table 1).

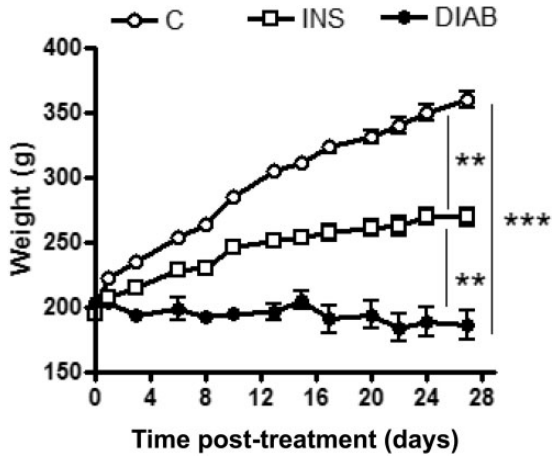

Figure 1 Weight of rats throughout the study. Results are given as mean ± SEM for control rats (C), non-treated diabetic rats (DIAB), and insulin-treated rats (INS) throughout the four weeks of study, n = 5; mean ± SEM. **$p < 0.01$, ***$p < 0.001$

Subcutaneous insulin therapy restores hepatic metabolism

Glycogen content of rat livers was comparable between insulin-treated diabetic rats and the control group throughout the study. In contrast, the level of hepatic glycogen was significantly lower in untreated diabetic rats after one ($p < 0.001$) and four weeks ($p < 0.05$) (Figure 2(a)).

As IGF-1 production is mediated by insulin, we compared the level of plasma IGF-1 between insulin-treated and untreated diabetic rats, and observed that IGF-1 production was higher in insulin-treated diabetic rats after one ($p < 0.001$) and four weeks of treatment ($p < 0.01$) (Figure 2(a)).

Subcutaneous insulin therapy does not reduce liver oxidative stress

Insulin therapy also led to a reduction in the level of plasma lipid peroxidation from one week of treatment in comparison with untreated diabetic rats ($p < 0.05$). Moreover, this reduction was maintained after four weeks attesting to the effectiveness of subcutaneous insulin administration ($p < 0.01$, Figure 3(a)). However, the TAOC of insulin-treated diabetic rats was significantly lower than that of the control rats ($p < 0.05$), and comparable to that of untreated diabetic rats, after four weeks of treatment (Figure 3(b)).

Excess ROS production in the liver of diabetic rats was only reduced after one week of insulin treatment ($p < 0.001$ compared with diabetic rats). After four weeks, the level of ROS was similar between treated and untreated diabetic rats, and was significantly higher than that of the control group ($p < 0.05$, Figure 3(c) and (d)).

Finally, a study of oxidative stress signaling pathways in the liver was performed after four weeks of treatment. Mn-SOD was overexpressed in diabetic rats ($p < 0.05$) and insulin-treated diabetic rats ($p < 0.01$) in comparison with the control group (Figure 4(a)). In contrast, CAT expression was comparable in all three groups (Figure 4(b)). Expression of p67phox, a subunit of the pro-oxidant enzyme NADPH oxidase, was significantly higher in non-treated diabetic rats than that in control group ($p < 0.05$) (Figure 4(c)). Moreover, p38-MAPKinase activation was observed in untreated diabetic rats only ($p < 0.05$) (Figure 4(d)).

Table 1 General metabolic characteristics of rats at sacrifices

	Body weight (g)	Tail vein glycemia (mg/dL)	Plasmatic fructosamine (μmol/L)	Total cholesterol (μmol/L)
W1 C	266.9 ± 6.56	126 ± 5	135.33 ± 12.44	0.795 ± 0.01
W1 DIAB	185.6 ± 8.99***	587 ± 13	245 ± 4.29***	1.095 ± 0.26
W1 INS	218.8 ± 11.93	548 ± 33***	222 ± 12.17***	0.825 ± 0.025
W4 C	361.9 ± 10.93	115 ± 3	130.25 ± 4.96	0.763 ± 0.04
W4DIAB	171.3 ± 25.61***	584 ± 16***	312 ± 6.56***	1.547 ± 0.28***
W4 INS	263.8 ± 8.05***###	477 ± 51***###	249.25 ± 12.23***###	0.709 ± 0.04##

Note: Effects of diabetes on body weight, tail vein glycemia, plasmatic fructosamine, and total cholesterol.
Values are mean ± SEM of five different rats.
* Significant results versus age-matched control rats (C) and # versus age-matched diabetic- rats (DIAB) or insulin treated rats (INS). *$p < 0.05$, ***$p < 0.001$; ##$p < 0.01$, ###$pp < 0.001$ (A color version of this table is available in the online journal.)

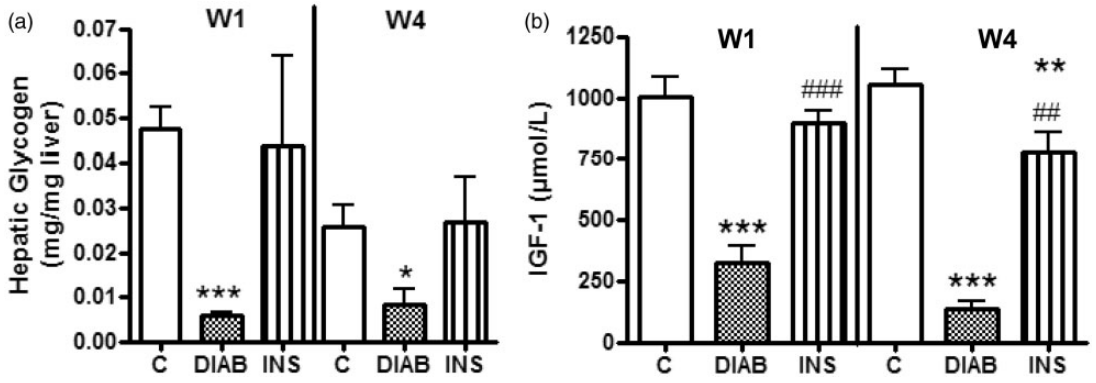

Figure 2 Measurement of hepatic glycogen content (a) and plasma IGF-1 levels (b). Results are given as mean ± SEM for control rats (C), non-treated diabetic rats (DIAB), and insulin-treated rats (INS) after one week (W1) and four weeks (W4), n = 5; mean ± SEM *p > 0.05, **p < 0.01, ***p < 0.001 compared with C-rats, ##p < 0.01, ###p < 0.001 compared with DIAB rats

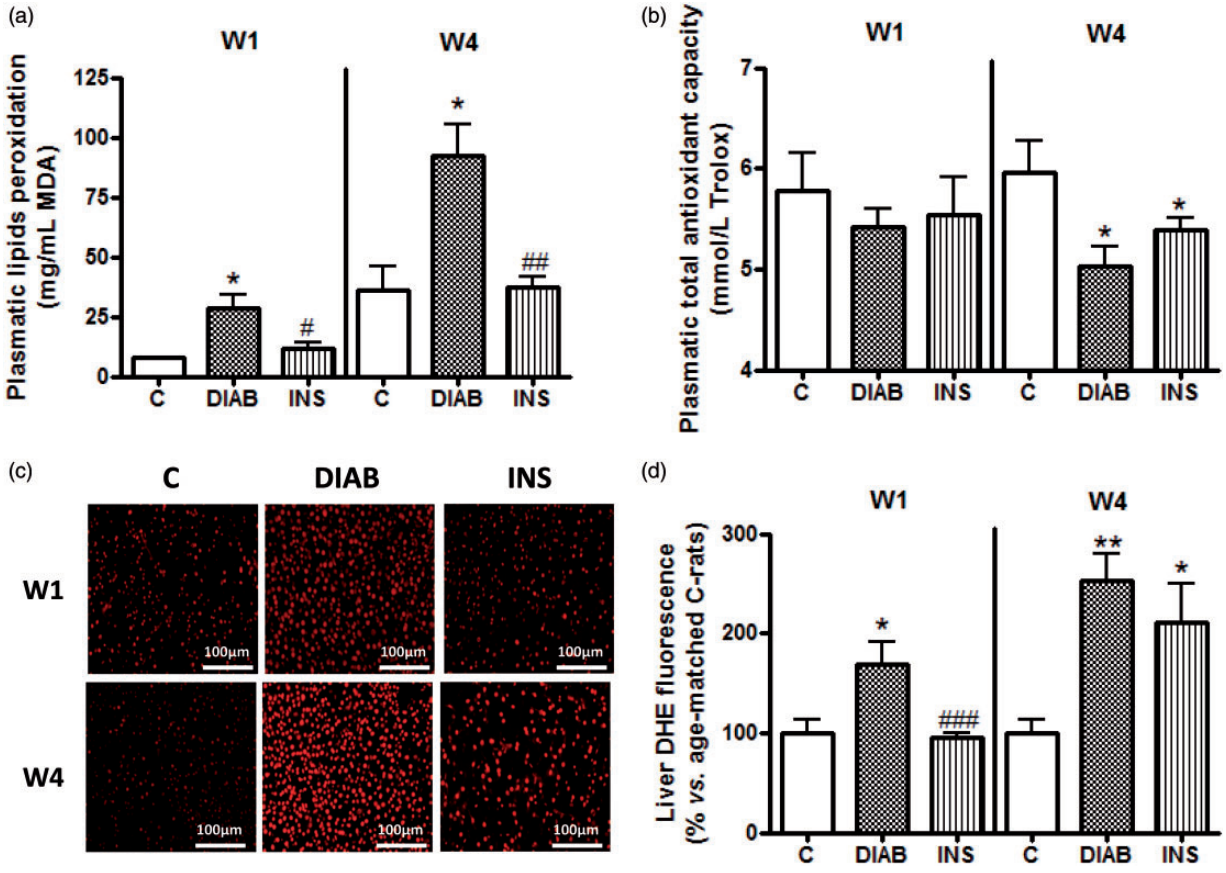

Figure 3 Measurement of plasma oxidative stress as defined by (a) lipid peroxidation, (b) total antioxidant capacity and tissue oxidative stress with the hepatic ROS production (c, d). (a and b) Results are given as mean ± SEM. (c) Similar results have been observed for four additional rats in each group. (d) Corresponding cumulative data. For all experiments: n = 5 for control rats (C), non-treated diabetic rats (DIAB), and insulin-treated rats (INS) after one week (W1) and four weeks (W4) of treatment. *p > 0.05, **p < 0.01, ***p < 0.001 compared with C-rats, #p < 0.05, ##p < 0.01, ###p < 0.001 compared with DIAB rats

Subcutaneous insulin treatment did not lead to improvements in liver inflammatory status

α2-macroglobulin, a protein characteristic of acute inflammation in rats, was measured (Figure 5(a)). We observed that after one and four weeks of insulin treatment, the level of α2-macroglobulin was comparable to that of untreated diabetic rats, and was higher than that of the control group (Figure 5(a)). At four weeks, α2-macroglobulin concentration in control group was 11.87 ± 1.2 µg/mL, compared to 24.65 ± 4.11 µg/mL in diabetic rats, and 27.64 ± 3.73 µg/mL in insulin-treated diabetic rats ($p < 0.05$).

Analysis of liver inflammation was carried out through the measurement of macrophage infiltration. This infiltration was higher after one week in diabetic rats with or without insulin treatment ($p < 0.05$) and was maintained after four weeks (Figure 5(b) and (c)).

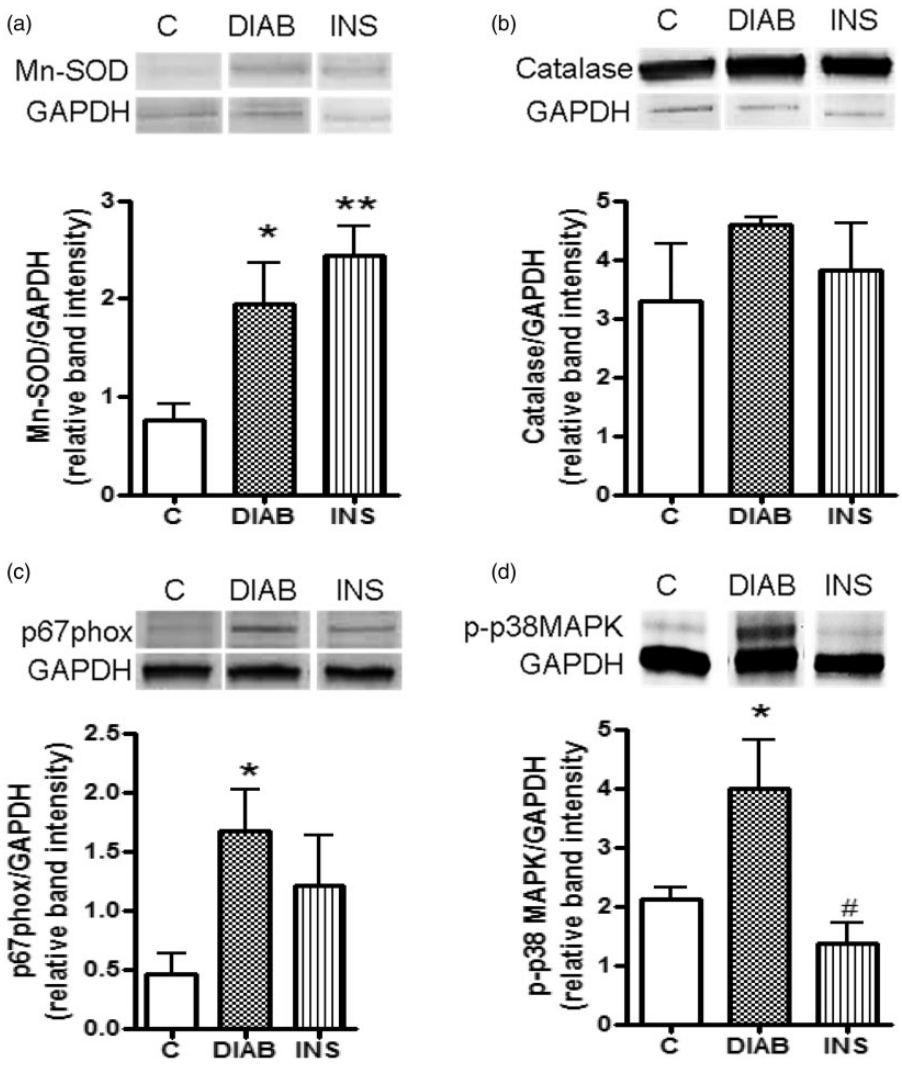

Figure 4 Hepatic oxidative stress signaling pathways after four weeks of treatment. Based on expression of manganese superoxide dismutase (Mn-SOD) (a), catalase (CAT) (b), p67phox (a subunit of NADPH oxidase) (c), and p38-MAPKinase (d). The relative intensities of the bands were determined using Image J software $n = 5$; mean ± SEM for control rats (C), non-treated diabetic rats (DIAB), and insulin-treated rats (INS). *$p > 0.05$, **$p < 0.01$ compared with C-rats, #$p < 0.05$ compared with DIAB rats

Finally, no macroscopic or physiopathological modifications, in particular signs of steatosis of the liver, were observed for animals undergoing Insuplant® treatment, as determined by hematoxylin/eosin coloration (data not shown).

Discussion

In this work, we have confirmed that hyperglycemia induced by diabetes is the pivotal mechanism leading to plasma and liver oxidative stress, and an increase in the production of ROS in diabetes. The control of blood glucose levels through intensive subcutaneous insulin administration led to a decrease in plasma oxidative stress but not to a reduction in the oxidative or inflammatory status of the liver.

The administration of STZ is a well-established experimental tool in the induction of type 1 diabetes. Its selective toxicity toward insulin-secreting β-cells leads to hyperglycemia and hypoinsulinemia.[36,37] As expected in our study, diabetic animals exhibited higher blood glucose levels and lower body weight at the end of the experiment in comparison to the onset. Good control of diabetes, which often requires intensive insulin therapy, is desirable in type 1 diabetes.[38] The intensive subcutaneous insulin administration performed using a mini pump in our study led to an improvement in the metabolic control of diabetic rats, as confirmed by a decrease in fructosamine levels and an increase in body weight after four weeks of treatment. Moreover, blood insulin concentration was maintained at the same level throughout the study attesting to the efficiency of this therapy. The improvement in the metabolic status of diabetic rats is comparable to results from studies in humans.[39] Moreover, plasma cholesterol increased dramatically in diabetic rats, and similar elevated cholesterol levels have been reported for diabetic human patients.[40] It is well known that insulin deficiency leads to inactivation of the lipoprotein lipase promoting hepatic conversion of free

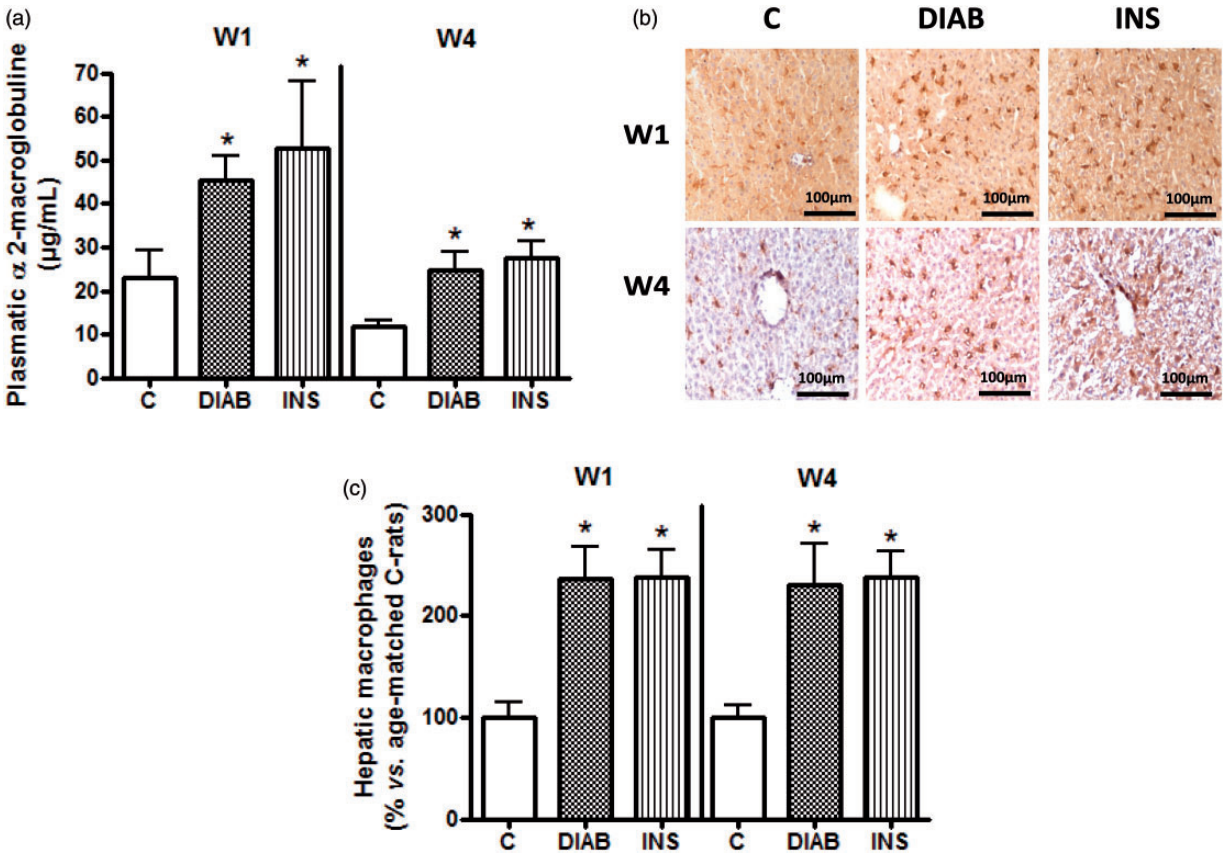

Figure 5 Measurement of plasma inflammation as defined by α2-macroglobulin level (a), and tissue inflammation as measured by the visualization and quantification of macrophage infiltration in the liver (b and c). (a) Results are given as mean ± SEM. (b) Similar results have been observed for four additional rats in each group. (c) Corresponding cumulative data. In all experiments: n = 5 for control rats (C), non-treated diabetic rats (DIAB), and insulin-treated rats (INS) after one week (W1) and four weeks (W4) of treatment. *$p > 0.05$ compared with C-rats

fatty acids into phospholipids and cholesterol which are then discharged into the blood resulting in elevated serum phospholipid levels.[41] In insulin-treated rats, cholesterol concentration was maintained at a level comparable to that of control rats. In fact, insulin can activate the action of lipolytic hormones on peripheral fat which hydrolyses triglycerides and prevents the mobilization of free fatty acids.[42]

In addition to metabolic parameters, the evaluation of plasma oxidative status in diabetic rats showed an increase in lipid peroxidation; again, the administration of insulin restored lipid peroxidation levels to those of control rats. These data confirm that hyperglycemia causes oxidative stress,[43] and the regulation of glycemic control during insulin therapy can reduce this stress.

TAOC, which reflects the collective reducing contribution of individual non-protein antioxidants or electron donating components,[44] was evaluated. In our study, the level of TAOC decreased in insulin-treated rats after four weeks of treatment and was comparable to that of untreated diabetic rats, suggesting that even if lipid peroxidation is reduced after insulin therapy, oxidative stress was not reduced sufficiently to improve TAOC status. In accordance with these results, ROS production in the liver was significantly higher in untreated diabetic rats after one week and four weeks but insulin therapy did not result in a decrease in ROS production after four weeks. ROS production was associated with elevated SOD levels, and MAPKinase and NADPH oxidase activation in STZ-induced diabetic rats was comparable to control rats. The administration of insulin led to a decrease in MAPKinase activation in diabetic rats. In fact, tissue antioxidant status was altered with diabetes as previously described in the literature;[22] however, insulin treatment partially reversed these changes. These effects were not due to the experimental model of diabetes used as STZ is rapidly metabolized.[45] Moreover, no alterations in the oxidative status of the livers of STZ-injected animals that failed to develop diabetes were detected (data not shown). In agreement with our results, Palma *et al.*[30] recently demonstrated that insulin therapy can prevent an increase in TBARS levels in serum but does not lead to an improvement in the antioxidant defenses of the liver.

It is well known that type 1 diabetes is considered an inflammatory process, and the effects of hyperglycemia are mediated by elevations in the levels of pro-inflammatory proteins.[46] Indeed, the levels of α2-macroglobulin, a typical acute-phase protein in rats[47–49] that is comparable to C-reactive protein in humans,[50] suggest an increase in inflammation after one week, which is maintained after four weeks in diabetic rats. Moreover, insulin therapy did not lead to a reduction in this acute inflammation.

Oxidative stress and inflammation have been reported to cause direct organ damage in diabetic rats[51] and humans.[52] In this study, macrophage infiltration in the liver was observed in diabetic rats and, once again, insulin therapy did not lead to a decrease in this infiltration. Infiltrating macrophages are a source of pro-inflammatory cytokines in the liver.[53] Our study showed that intensive insulin therapy cannot reduce the systemic and hepatic inflammation induced by diabetes.

The poor regulation of glycemia in diabetic rats with insulin therapy was not the cause of these results as insulin therapy restored glycogen storage in the liver and IGF-1 levels in the serum. In fact, some previous studies show that glycogen levels in the liver are lower in diabetic animals; however, accumulation of glycogen in the livers of insulin-treated animals is similar to that of control rats[54–56] and is often accompanied by an increase in serum IGF-1 levels toward normal values.[31,57–59]

In summary, we have explored the effects of intensive subcutaneous insulin administration using a subcutaneous pump in a type 1 diabetic rat model. We found that insulin treatment could restore metabolic control in diabetic rats, and this was associated with a reduction in plasma oxidative stress. However, the important outcome of this study is that insulin treatment did not lead to a decrease in the acute inflammation associated with oxidative stress and macrophage infiltration in the livers of these rats. As liver inflammation affects insulin sensitivity and can induce systemic insulin resistance,[60,61] targeting oxidative stress and/or inflammation in diabetic patients could improve their therapeutic options.

Authors' contribution: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; SD, ES, WB, CP, and SS conducted the experiments, SD, NJD, and SS wrote the manuscript.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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