J Mol Med (2013) 91:851–860
DOI 10.1007/s00109-013-1004-6

JMolMed

ORIGINAL ARTICLE

Lack of kinin B_1 receptor potentiates leptin action in the liver

Raphael Gomes Fonseca · Vicencia Micheline Sales · Eduardo Ropelle · Carlos Castilho Barros · Lila Oyama · Silvia Saiuli Iuki Ihara · Mário Jose Abdalla Saad · Ronaldo Carvalho Araújo · João Bosco Pesquero

Received: 27 February 2012 / Revised: 8 January 2013 / Accepted: 21 January 2013 / Published online: 6 February 2013

Abstract Kinins B_1 and B_2 receptors (B_1R and B_2R) are classically associated with inflammation, but our group has recently demonstrated new roles for B_1R in metabolism using a knockout model ($B_1^{-/-}$). $B_1^{-/-}$ mice display improvement on leptin and insulin sensitivity and is protected from high fat diet (HFD)-induced obesity. Here, we evaluate the hepatic effects of the B_1R ablation and its role on hepatic function. Despite no expression of hepatic B_1R, HFD-induced hepatic lipid accumulation was lower than in control animals. $B_1^{-/-}$ mice also presented lower hepatic lipogenesis and SCD1 protein content in the liver. When stimulated with exogenous leptin, $B_1^{-/-}$ mice exhibited increased hepatic pJAK2. Similarly, leptin signaling was enhanced in the liver of *ob/ob*–$B_1^{-/-}$ mice, as demonstrated by increased levels of pSTAT3 compared to *ob/ob*. Plasma concentrations of intercellular adhesion molecule 1, fetuin A, leukemia inhibitory factor, tissue inhibitor of metalloprotease-1, resistin, and oncostatin M were reduced in $B_1^{-/-}$. Finally, $B_1^{-/-}$ mice have increased gene expression of hepatic B_2 receptor, but no difference in leptin receptor expression. Our results show that $B_1^{-/-}$ mice are protected from non-alcoholic fatty liver disease (NAFLD) after HFD treatment. Since B_1R expression was not observed in the liver after HFD, we propose that the cross talk between the adipose tissue and the liver, mainly through leptin, is an important factor contributing to the observed results. Besides that, several other inflammatory mediators already correlated with NAFLD or liver function were found to be altered in our model. Taken together, our data suggest that B_1R plays an important role in hepatic steatosis development.

Keywords Kinins · Liver · Leptin · Insulin · Non-alcoholic fatty liver disease

Raphael Gomes Fonseca and Vicencia Micheline Sales contributed equally to this work.

R. G. Fonseca · V. M. Sales · C. C. Barros · R. C. Araújo · J. B. Pesquero (✉)
Department of Biophysics, Federal University of São Paulo—UNIFESP/EPM, Rua Pedro de Toledo, 669, 9° andar, Vila Clementino,
04039-032 São Paulo, São Paulo, Brazil
e-mail: jbpesquero@unifesp.br

E. Ropelle
School of Applied Science, State University of Campinas—UNICAMP, Limeira, São Paulo, Brazil

L. Oyama
Department of Physiology, Federal University of São Paulo—UNIFESP/EPM, São Paulo, São Paulo, Brazil

S. S. I. Ihara
Department of Pathology, Federal University of São Paulo—UNIFESP/EPM, São Paulo, São Paulo, Brazil

M. J. A. Saad
Department of Internal Medicine, State University of Campinas—UNICAMP, Campinas, São Paulo, Brazil

Introduction

Changes in the availability and metabolism of nutrients in the liver can lead to the development of different degrees of diseases. Non-alcoholic fatty liver disease (NAFLD) is an arising clinical finding mainly observed in obese subjects [1]. This disease encompasses a wide spectrum of changes in the hepatic tissue, ranging from steatosis, to non-alcoholic

steatohepatitis, up to the most severe cases of fibrosis or cirrhosis [2].

Kinins are classically associated with inflammatory processes [3]. Two receptors coupled to G protein are known to be involved with kinin signaling, B_1 and the B_2 receptors (B_1R and B_2R, respectively) [3, 4]. In addition to its role in inflammatory processes, our group has shown that B_1R also participates in metabolic processes and modulates leptin sensitivity [5, 6].

Leptin is a circulating hormone produced mainly by the adipose tissue and related to body weight regulation [7]. Leptin binds to its receptor in the cell membrane, leading to activation of a signaling cascade involving JAK2 and STAT3 phosphorylation. The latter dimerizes and translocates to the nucleus where it regulates transcription of target genes [8].

Leptin can improve insulin sensitivity, increasing intracellular fatty acid oxidation [9]. Mice lacking leptin receptors specifically in neurons present hepatic steatosis, although mice lacking leptin receptor in the liver do not present morphological changes [10]. Leptin also acts locally reducing stearoyl-CoA desaturase-1 (SCD1) expression [11], which adds a double bond to saturated fatty acids, channeling them to storage instead of using in oxidative processes. This enzyme levels are very high in *ob/ob* mice livers and its expression is rapidly reduced after exogenous leptin administration [9]. SCD1 knockout mice are protected from obesity and high fat diet (HFD)-induced hepatic steatosis [12].

Our group has demonstrated the involvement of B_1R in the regulation of energetic metabolism. We have shown that B_1R knockout mice ($B_1^{-/-}$) have lower circulating levels of leptin, insulin and reduced numbers of pancreatic islet [5, 13]. $B_1^{-/-}$ mouse displays increased insulin and leptin sensitivity and is also resistant to diet-induced obesity [6]. A transgenic mouse with specific expression of B_1R in adipose tissue was generated and we demonstrated that B_1R participates in the regulation of insulin circulating levels, which are normalized in this mouse, and weight gain [14]. Here, we investigate the role of B_1R in the hepatic metabolism and its effects on local leptin and insulin signaling.

Materials and methods

Animals

$B_1^{-/-}$ mice [15] originated from ten generations backcrossing of an initially mixed genetic background (129/Sv and C57Bl/6) with C57Bl/6 mice (Taconic, Germantown, NY). Therefore, C57Bl/6 animals (WT) were used as controls. The *ob/ob*–$B_1^{-/-}$ strain was generated by crossbreeding C57Bl/6-ob$^{+/-}$ mice (The Jackson Laboratories, Bar Harbor, ME) with $B_1^{-/-}$ mice. Adult males were obtained from CEDEME at the Federal University of São Paulo, Brazil. All experiments reported were approved by a local ethics committee (Process 1055/08). Animals were maintained on standard mouse chow diet at 22 °C on 12-h light/dark cycle and allowed ad libitum access to food and water.

High fat diet treatment

Twelve-week-old mice were fed standard diet (10 % kcal fat) or HFD (45 % kcal fat) (Research Diets) for 16 weeks. After treatment, mice were fasted for 12 h and sacrificed by decapitation for collection of liver samples.

Histology

Livers were collected and fixed with 10 % buffered formalin and paraffin embedded. Sections were stained with hematoxylin and eosin, and classified from 0 to 3 according to the percentage of hepatocytes with lipids droplets: 0 = 0 to 8 %, 1 = 9 to 33 %, 2 = 34 to 66 %, and 3 = 67 to 100 %.

Quantitative real-time RT-PCR

Total RNA (n=5) was isolated using TRIzol (Life Technologies) and treated with DNase I (Life Technologies). Reverse transcription was performed using M-MLV reverse transcriptase, random hexamers (Life Technologies), and 1 μg of total RNA, according to the manufacturer's protocol. Real-time PCR was performed in a 7500 Real-Time PCR System (Life Technologies) using genomic DNA-free cDNA and 0.5 μM primers. Melting curves were analyzed for the presence of nonspecific amplification. Gene expression was calculated using β-actin or GAPDH as a reference gene and the $2^{-\Delta Ct}$ formula. Primer sequences are presented in Table 1.

Lipogenesis assays

In vivo lipogenesis assay was measured by the incorporation of tritiated water (^3H_2O) into lipids. Mice (n=6 WT; n= 8 $B_1^{-/-}$) were killed by decapitation 1 h after i.p. administration of ^3H_2O. Plasma and liver samples were saponified and fatty acids extracted [16]. Tissue lipogenesis rate was expressed as micromoles of ^3H_2O incorporated into lipid/hour per gram of sample [17].

Proteome profiler array

Three-month-old WT and $B_1^{-/-}$ animals (n=3 in each group), fed chow diet, were sacrificed and blood was collected. Blood was centrifuged at 1,800×g, for 15 min at 21 °C and serum was separated. Serum cytokines were analyzed trough the proteome profiler array—Mouse adipokine array kit (R&D Systems) according to manufacturer's instructions.

Table 1 Primers sequences

Transcript	Gene symbol	Forward primer	Reverse primer
Beta-actin	Actb	5′-CTGGCCTCACTGTCCACCTT-3′	5′-CGGACTCATCGTACTCCTGCTT-3′
Acetyl-CoA carboxylase	Acaca	5′-ATCCGCCTCTTCCTGACAA-3′	5′-TGCCTGGAACCTCTTTGATT-3′
Fatty acid synthase	Fasn	5′-GCAAGCTGTCCCCTGATG-3′	5′-GAACCAGCCCCATCACAC-3′
Glycerol phosphate acyltransferase	Gpam	5′-CCCTGCCAGACTTTTTACCA-3′	5′-GCTTCTTGTCCCACTGCTG-3′
Phosphoenolpyruvate carboxykinase	Pck1	5′-CCATGATGAACCCCAGCC-3′	5′-CGTTGGTGAAGATGGTGTTT-3′
Leptin receptor	Lepr	5′-TGTGGACAGAACCAGCGCACAC-3′	5′-AGGATGACACAGCTGCTGCTCAGG-3′
Kinin B2 receptor	Bdkrb2	Assay ID Mm00437788_s1	
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Assay ID Mm99999915_g1	

Serum was conjugated with different antibodies that were spotted in duplicate on nitrocellulose membranes. Conjugates were captured by horseradish peroxidase and were detected by chemiluminescence. Membranes were photographed and analyzed using ImageJ (NIH). Data represent mean pixel density for each cytokine.

Leptin and insulin treatment

Mice (n=3 saline, n=4 treated) were anesthetized with an i.p. injection of ketamine/xylazine and were used as soon as anesthesia was assured by loss of pedal reflex. Abdominal cavity was opened, the abdominal aorta exposed, and 50 μL of normal saline (0.15 M NaCl) or 10 μM insulin or 30 μg leptin was injected. After 5 min, the liver was removed and immediately frozen in liquid nitrogen.

Western blotting

Tissues were homogenized at 4 °C in RIPA buffer [50 mM Tris, pH7.4; 1 % NP-40; 0.25 % sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 tablet/25 mL of Complete® protease inhibitors (Roche); 1 mM sodium orthovanadate; 1 mM sodium fluoride] and protein was quantified by the DC® Protein Assay kit (Bio-Rad). Twenty micrograms of lysates were submitted to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with pSTAT3 (Tyr705, Cell Signaling), pJAK2 (Tyr1007/1008, Chemicon), STAT3 (K-15, Santa Cruz), pAkt (Ser473, Cell Signaling), pIR (Tyr1162/1163, Santa Cruz), and SCD1 (S-15, Santa Cruz) antibodies. Horseradish peroxidase activity was detected by chemiluminescence using the ECL Plus® kit (GE Healthcare).

Statistical analysis

Values are expressed as mean±SEM. Statistical analyses were performed using the two-tailed Student's unpaired t test or ANOVA followed by Bonferroni's method. Significance was considered at $p<0.05$.

Results

$B_1^{-/-}$ mice are protected from HFD-induced NAFLD

Mice were fed for 16 weeks a HFD and, confirming our previously published results [6], $B_1^{-/-}$ mice presented a protection from diet-induced obesity. Liver sections stained with hematoxylin–eosin showed that the HFD induced marked lipid accumulation in WT, whereas $B_1^{-/-}$ mice developed much milder lipid accumulation (Fig. 1a). The specimens were scored in a four-graded scale according to the degree of steatosis. Livers from $B_1^{-/-}$ animals fed HFD received lower scores than WT in the same condition (Fig. 1b). These results indicate that $B_1^{-/-}$ mice are protected from diet-induced NAFLD.

Our group had previously reported that B_1R is not expressed in mouse liver under normal conditions, but its expression is highly upregulated in *ob/ob* mice livers [18]. Therefore, we investigated whether a HFD treatment was also able to induce hepatic B_1R expression in wild type mice. Even after this procedure, B_1R expression could not be detected in the livers from WT mice.

$B_1^{-/-}$ mice present reduced hepatic de novo lipogenesis

Since we observed differences in hepatic lipid accumulation, we considered the hypothesis that hepatic lipid metabolism in $B_1^{-/-}$ mice could be altered. Therefore, we performed an in vivo lipogenesis assay in 3-month-old animals. The rate of hepatic lipogenesis in $B_1^{-/-}$ mice was smaller than in WT mice (26±1 vs. 32±2 μmol/h.g, $p<0.05$, Fig. 1c). We considered that this effect could be related to the improved leptin action in this organ.

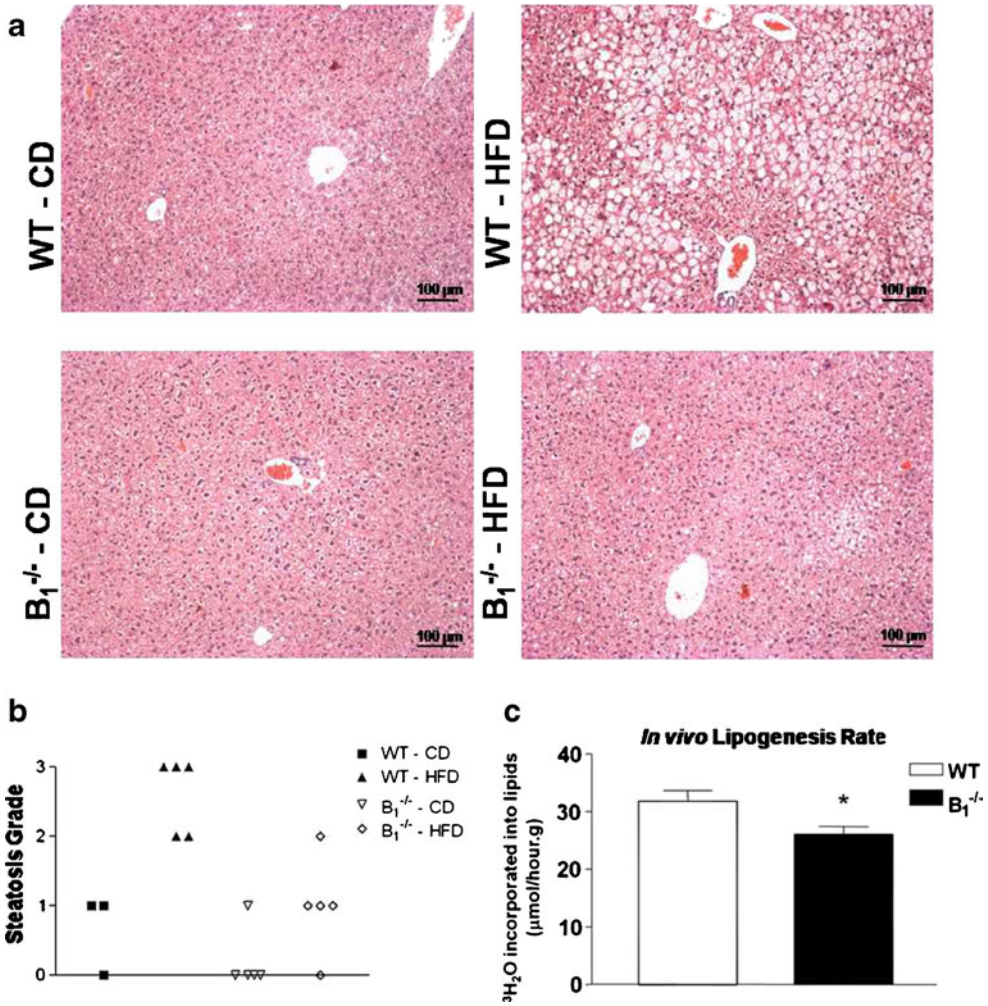

Fig. 1 $B_1^{-/-}$ mice are protected from non-alcoholic fat liver disease (NAFLD) induced by high fat diet. **a** Twelve-week-old mice were fed high fat diet (*HFD*) or control diet (*CT*) for 16 weeks. After diet, liver sections were collected for histological evaluation and hematoxylin–eosin staining was performed. $B_1^{-/-}$ mice accumulate much less lipids in the liver when compared with the WT. **b** After a pathological evaluation of hepatic steatosis grade, $B_1^{-/-}$ mice fed a HFD received scores from 0 to 3 according to the percentage of hepatocytes with lipids droplets: 0 = 0–8 %, 1 = 9–33 %, 2 = 34–66 %, 3 = ≥67 %. $B_1^{-/-}$ mice received lower scores than WT mice. **c** Twelve-week-old animals received an intraperitoneal injection of ^3H_2O and lipids were extracted from livers. The radiation present in the extracted lipids was counted and used as a measure of the rate of de novo synthesis of lipids. The incorporation of ^3H_2O into lipids in $B_1^{-/-}$ mice was 18 % lower than in WT. Values are means±SEM of ^3H_2O incorporated into lipids. *$p<0.05$; $n=6–8$

$B_1^{-/-}$ mice are more metabolically adaptable

To analyze the differences in gene expression regulation in the liver of $B_1^{-/-}$ mice, 3-month-old male mice were fasted overnight. To investigate the metabolic flexibility of the livers from $B_1^{-/-}$ mice, we performed real-time PCR to assess the expression of genes related to metabolic processes. As expected, fasting caused a reduction in the expression of lipogenic enzymes mRNA acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The $B_1^{-/-}$ presented increased expression of both enzymes when fed, but not when starved (Fig. 2a, b). The expression of glycerol-3-phosphate acyltransferase (GPAT) was not only significantly different among groups but also showed the same trend (Fig. 2c). Also as expected, there was an increase in the expression of genes related to β-oxidation of lipids (carnitine palmitoyltransferase I; CPT-1) and gluconeogenesis (phosphoenolpyruvate carboxykinase; PEPCK) after fasting (Fig. 2d, f). Changes induced by fasting in $B_1^{-/-}$ mice were more pronounced than in WT mice, suggesting that $B_1^{-/-}$ mice might be more metabolically adaptable to nutrient availability changes.

$B_1^{-/-}$ mice livers are more sensitive to leptin, but not to insulin

To assess the hepatic sensitivity to both hormones, we performed Western blot to analyze the phosphorylation of proteins involved in their signaling pathways. From the insulin pathway, hepatic Akt and insulin receptor (IR) phosphorylation was similar in both groups after an i.p. injection of insulin, which shows that insulin action in hepatic tissue is not altered (Fig. 3a, b). Conversely, after i.p. injection of leptin, there was a marked increase in JAK2 phosphorylation in $B_1^{-/-}$ mice (Fig. 3c) compared to WT mice.

SCD1 expression is reduced in the liver of $B_1^{-/-}$ mice

SCD1 inhibition is considered an important mechanism of the antisteatotic effect of leptin [9]. Since $B_1^{-/-}$ mice showed reduced accumulation of lipids after HFD and lower hepatic lipogenesis rates, we investigated the possibility that the lack of B_1R could cause a reduction in SCD1 expression in the liver. Western blots showed that there was a marked

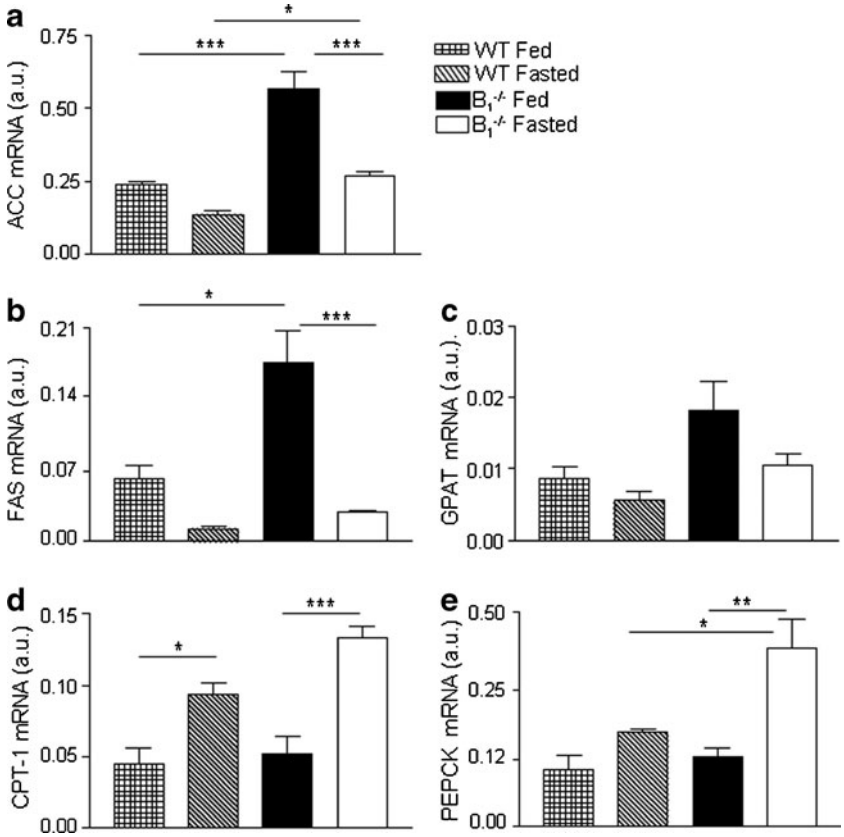

Fig. 2 Fasting-induced changes in the expression of metabolic genes. **a**–**c**. Genes involved in lipogenesis (ACC, FAS, and GPAT, respectively). **d** A gene involved in β-oxidation of lipids (CPT-1). **e** An enzyme from the gluconeogenesis pathway (PEPCK). *$p<0.05$; **$p<0.01$; ***$p<0.001$; $n=5$. *ACC* acetyl-CoA carboxylase, *FAS* fatty acid synthase, *GPAT* glycerol phosphate acyltransferase, *CPT-1* carnitine palmitoyltransferase 1, *PEPCK* phosphoenolpyruvate carboxykinase

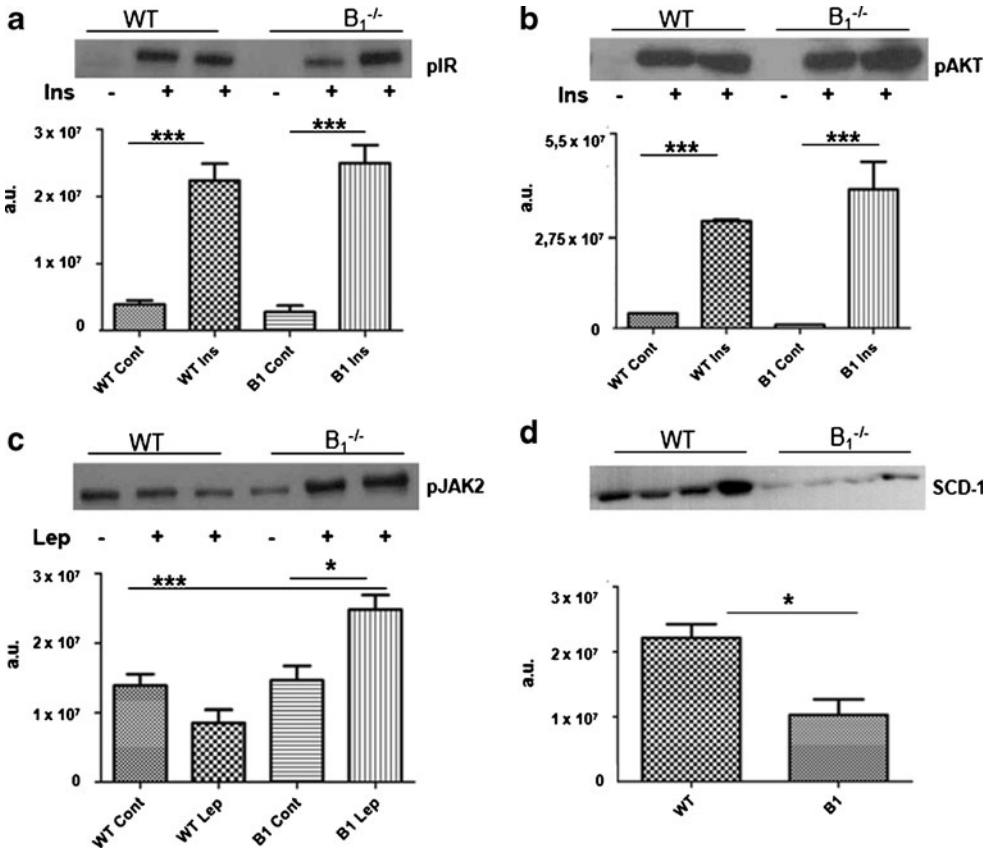

Fig. 3 $B_1^{-/-}$ mice livers are more sensitive to leptin, but not to insulin. Three-month-old mice were stimulated intraperitoneally with leptin or insulin injection. After this treatment, livers were collected for protein extraction. Western blots were performed to detect protein phosphorylation. **a** Insulin-induced phosphorylation of the insulin receptor. $n=3$ (controls) and $n=4$ (stimulated). **b** Insulin-induced phosphorylation of Akt. $n=3$ (control) and $n=4$ (stimulated). **c** Hepatic phosphorylation of JAK2 after IP injection of leptin. $n=3$ (controls) and $n=4$ (stimulated). *$p<0.05$; ***$p<0.001$. **d** Three-month-old mice were killed and livers were collected for protein extraction. Western blot was performed to detect SCD1 protein expression. $n=8$ (WT) and $n=8$ ($B_1^{-/-}$). *$p<0.05$

reduction in SCD1 protein expression in the knockout animals fed a control diet (Fig. 3d).

Absence of kinin B_1 improves leptin action in the liver of *ob/ob*–$B_1^{-/-}$ mice

To demonstrate the importance of leptin to B_1R action in the liver, mice lacking both leptin and B_1R (*ob/ob*–$B_1^{-/-}$) were generated [6]. Liver samples from 3-month-old *ob/ob* and *ob/ob*–$B_1^{-/-}$ mice fed a control diet were collected for histological analysis. Hematoxylin–eosin staining was used to assess hepatic morphology, and no histological difference between genotypes was observed (data not shown). We analyzed the phosphorylation of STAT3 after an i.p. injection of leptin (Fig. 4). The Western blot showed a striking increase in the phosphorylation of this protein involved in the signaling pathway of leptin.

Serum adipokines correlated with steatosis are reduced in $B_1^{-/-}$ mice

Three-month-old lean mice were sacrificed, blood was collected, and several adipokine levels analyzed. The analysis showed reduction of the following adipokines: intercellular adhesion molecule 1 (ICAM-1), fetuin A, leukemia inhibitory factor (LIF), tissue inhibitor of metalloprotease-1 (TIMP-1), resistin, and oncostatin M (OSM) (Fig. 5). Increased levels of all these proteins have been related with steatosis development and they are all reduced in $B_1^{-/-}$ mice.

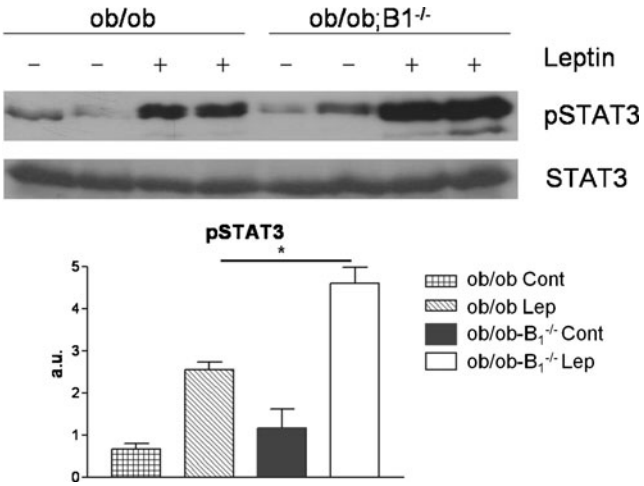

Fig. 4 Leptin induced STAT3 phosphorylation in livers of obese mice. Three-month-old mice were stimulated by IP injection of leptin. After this, mice were killed and livers were collected for protein extraction. Western blot was performed to detect protein expression. Hepatic phosphorylation of STAT3 after IP injection of leptin was evaluated. In the *upper panel*, Western blot was used to detect pSTAT3 and STAT3. In the *lower panel*, a graphic representing the ratio of the intensity of phosphorylated bands over the non-phosphorylated. $n=2$, $^*p<0.05$

Liver expression of leptin receptor and B_2R in $B_1^{-/-}$ mice

Livers were collected from 2-month-old, chow-fed mice for RNA extraction and leptin receptor and B_2R gene expression analysis. The $B_1^{-/-}$ mice presented no change in expression of leptin receptor in liver. On the other hand, these animals presented increased expression of B_2R (Fig. 6).

Discussion

$B_1^{-/-}$ mice present diverse altered phenotypes related to inflammatory processes, as expected from an animal in which an inflammation-related receptor is missing [15, 19]. However, recent studies have demonstrated a role for the kinin B_1 receptor in metabolic processes [5, 6, 13, 14, 18, 20, 21]. Our group has already demonstrated that kinin B_1 receptor stimulation modulates leptin homeostasis [5] and that $B_1^{-/-}$ mice are resistant to diet-induced obesity and are more sensitive to leptin centrally [6]. In addition, a role for kinin B_1 receptor in insulin homeostasis and pancreatic islet function was proposed [13]. Since the liver is a key organ in the regulation of metabolism of nutrients and is much affected by insulin and leptin homeostasis, we decided to investigate possible metabolic alterations in the livers of $B_1^{-/-}$ mice. Here, we report that NAFLD development in $B_1^{-/-}$ mice is much milder, another evidence for the participation of B_1R in metabolic processes.

Our group has previously shown that B_1R is expressed in liver from *ob/ob* mice [18]. In this study, however, we could not detect the expression of this receptor in wild type mice even after HFD treatment. Therefore, hepatic alterations observed in our knockout model may derive from systemic factors arising from different tissues. We propose that adipose tissue-derived adipokines and other cytokines are the main factors affecting liver function in our knock model, protecting it from NAFLD.

B_1R is constitutively expressed in mouse adipocytes and its activation improves insulin sensitivity in these cells [6, 14]. Adipose tissue in $B_1^{-/-}$ is protected from HFD-induced increase fat accumulation and in leptin expression [6]. Besides that, leptin hypersensitivity was observed in these mice as seen by the stronger inhibitory effect of exogenous leptin on food intake [5]. Restoration of B_1R expression specifically in the adipocytes reverses $B_1^{-/-}$ mouse protection from obesity [14]. Therefore, B_1R absence has important outcomes in adipocyte function and leptin homeostasis and these effects may be closely linked with the changes in hepatic function observed here.

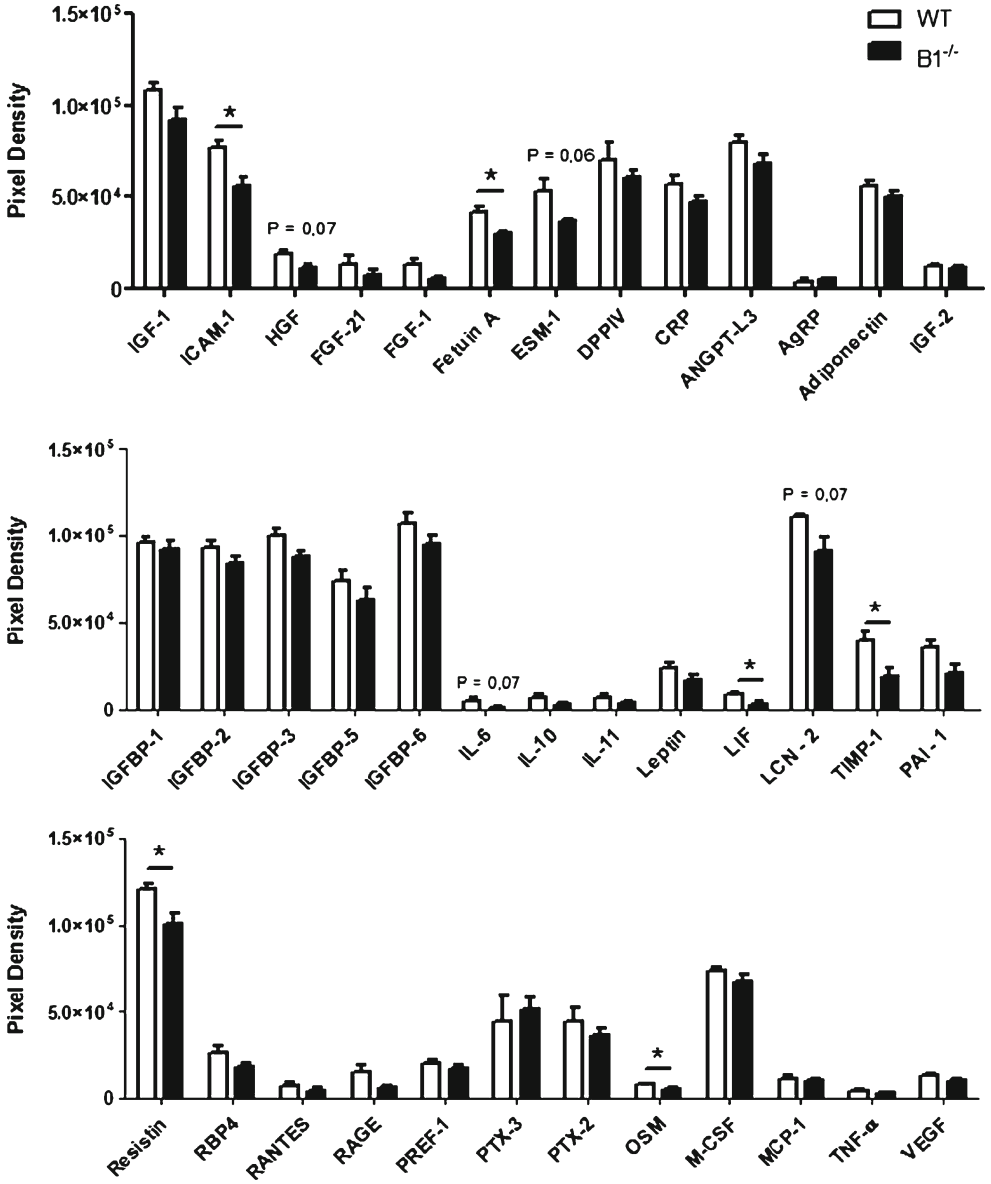

Fig. 5 Adipokines related to steatosis are reduced in $B_1^{-/-}$ mice. Adipokine proteome array was performed using serum from WT and $B1^{-/-}$ mice (n=3). Circulating level of cytokines was accessed and expressed as mean pixel density. *p<0.05. *IGF-1* insulin-like growth factor 1, *ICAM-1* intercellular adhesion molecule 1; *HGF* hepatocyte growth factor, *FGF-21* fibroblast growth factor 21, *FGF-1* fibroblast growth factor 1, *ESM-1* endothelial cell-specific molecule 1, *DPP IV* dipeptidyl peptidase 4, *CRP* C-reactive protein, *ANGPT-L3* angiopoietin-like 3, *AgRP* agouti-related protein, *IGF-2* insulin-like growth factor 2, *IGFBP-1* insulin-like growth factor-binding protein 1, *IGFBP-2* insulin-like growth factor-binding protein 2, *IGFBP-3* insulin-like growth factor-binding protein 3, *IGFBP-5* insulin-like growth factor-binding protein 5, *IGFBP-6* insulin-like growth factor-binding protein 3, *IL-6* interleukin 6, *IL-10* interleukin 10, *IL-11* interleukin 11, *LIF* leukemia inhibitory factor, *LCN-2* lipocalin 2, *TIMP-1* tissue inhibitor of metalloproteinase 1, *PAI-1* plasminogen activator inhibitor-1, *RBP4* retinol binding protein 4, *PREF-1* preadipocytes factor 1, *PTX-3* pentraxin 3, *PTX-2* pentraxin 2, *OSM* oncostatin M, *M-CSF* macrophage colony-stimulating factor, *MCP-1* monocyte chemotactic protein 1, *TNF-α* tumor necrosis factor α, *VEGF* vascular endothelial growth factor

Leptin, an adipocyte-derived cytokine, has been considered the main regulator of hepatic triglyceride content [22]. It can reduce, by central or direct mechanisms, hepatic stores of triglycerides by modulating the expression of genes involved in the synthesis and oxidation of lipids [23, 24]. We observed an increased signaling of leptin in $B_1^{-/-}$ mice livers, as assessed by phosphorylation of JAK2. Similar results were observed in livers of *ob/ob*–$B_1^{-/-}$ mice, where leptin induced higher levels of STAT3 phosphorylation. Conversely, we found no evidence of increased hepatic insulin sensitivity, as assessed by the phosphorylation of IR and Akt after i.p. injection of insulin.

Cohen et al. have proposed that the regulation of hepatic triglyceride content by leptin is mainly due to the

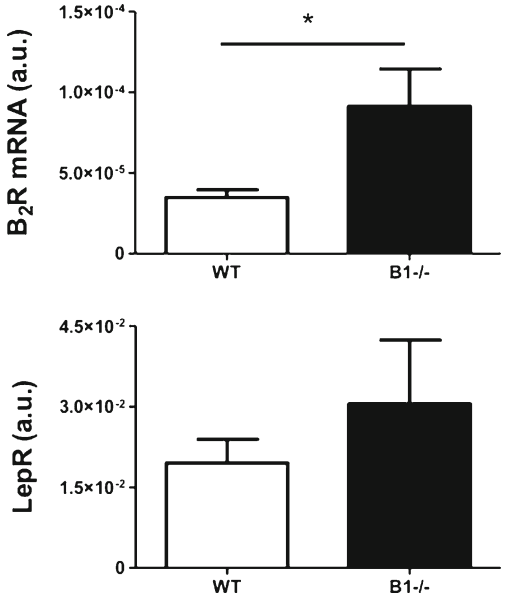

Fig. 6 Leptin receptor and kinin B2 receptor gene expression in liver. Livers were collected from 2-month-old mice. Leptin receptor (*LepR*) and kinin B2 receptor (*B_2R*) gene expression were analyzed. $B_1^{-/-}$ mice present increased expression of B2R, but not LepR. n=4, *p<0.05

modulation of the expression of SCD1 [9]. It is known that leptin can reduce hepatic SCD1 expression in mice and that *ob/ob* mice lacking the SCD1 gene are less obese and present normal hepatic histology [9]. In addition, SCD1 deficiency in lean mice is sufficient to increase lipid oxidation and reduce lipid synthesis and storage [12]. In our model, we have found that the hepatic expression of this protein is reduced. Therefore, this result corroborates the hypothesis of a more efficient leptin signaling in $B_1^{-/-}$ mice.

We have also found reduced rates of hepatic lipogenesis in our model. It is important to point out that this process is strongly influenced by leptin. Despite reduced rates of lipogenesis, we have found that the expression of lipogenesis-related genes ACC and FAS were increased in $B_1^{-/-}$ mice. We hypothesize that increased leptin sensitivity may have some effect on hepatic lipid beta-oxidation or even in the secretion of lipids out of hepatocytes that could account for these conflicting results. As a matter of fact, we have observed an increase in CPT-1 gene expression, which catalyzes the rate-limiting step in the mitochondrial import of long-chain fatty acids prior to entering the β-oxidation cycle [25]. Despite that ACC and FAS gene expression is increased in $B_1^{-/-}$ mice when fed, when starved, the drop in expression of both genes is pronounced in $B_1^{-/-}$ mice and this difference disappears, showing the higher metabolic adaptability of animals lacking B_1R.

Interestingly, it has been recently shown that pharmacological blockage of B_1R with the SSR240612 antagonist could reverse the increase in plasma fatty acids, glucose, and insulin induced by long-term high-glucose diet. In addition, B_1R antagonist also reversed the enhancing effect of glucose feeding on whole body and epididymal fat mass and on the expression of inflammatory markers in retroperitoneal adipose tissue [26]. The results from this pharmacological model of B1R blockage are in agreement with our results in a knockout model, both showing the importance of B_1R in metabolic processes.

Since B_1R is classically regarded as an important player in inflammatory processes [3, 27], we also evaluated other circulating inflammatory mediators in our model. The analysis of serum cytokine profile showed that several factors, which are correlated with steatosis, have decreased levels in $B_1^{-/-}$ mice, even in the basal state without any diet intervention. Among these molecules, we can highlight ICAM-1, fetuin A, LIF, TIMP-1, OSM, and resistin. ICAM-1 is an important molecule involved in the inflammatory process in steatosis [28], as well as TIMP-1, whose inhibition promotes antifibrotic effects [29]. In this sense, fetuin A was shown to cause insulin resistance and liver lipid accumulation in humans [30]. Resistin, a cytokine produced by the adipose tissue, is reduced in the $B_1^{-/-}$. Its deficiency reduces steatosis in obese and lean mice [31, 32]. LIF is increased in NAFLD and related to atherosclerosis [33]. OSM is very important for liver development, regeneration, and metabolism. It also induces LDL receptor expression in mature hepatocytes and regulates TIMP-1 expression and matrix remodeling and ICAM-1 expression in endothelial cells [34]. OSM was recently proposed to be produced in response to prostaglandin E2 (a product of B_1R activation) and can also contribute to steatosis [35]. In addition, OSM was shown to stimulate B_1R in rabbit aorta [34]. OSM and LIF are in the same chromosomal location and they are members of IL-6 superfamily, as well as leptin [36, 37]. Downregulation of LIF and OSM in the absence of B_1R could lead to a downregulation of its targets, such as TIMP-1 and ICAM-1, which could help to explain the decreased steatosis in the $B_1^{-/-}$ mouse.

Besides all the circulating factors altered in $B_1^{-/-}$ mice, at least one local factor could be responsible for the hepatic phenotypes in our model. It has been shown that deletion of one kinin receptor could lead to the modulation of the other kinin receptor expression [38, 39]. In our work, we detected an increase in B_2R gene expression in the liver of $B_1^{-/-}$ mice, despite no change in leptin receptor. B_2R could be acting in a compensatory fashion due to the lack of B_1R, as seen in other models [38, 39]. Interestingly, our group has recently shown that intraportal injection of B_2R agonist bradykinin in lean mice reduces hepatic expression of PEPCK and also that *ob/ob* mice with depletion of B_2R exhibit increased gluconeogenesis [40]. These results are in line with the observed increase in PEPCK shown here in $B_1^{-/-}$ mice.

In conclusion, we have shown that after a HFD treatment, $B_1^{-/-}$ mice are protected from the development of NAFLD. We propose that a cross talk between the adipose tissue and the liver in $B_1^{-/-}$ mice, mainly through adipokines such as leptin and resistin, is an important factor contributing to these observations. In this sense, we have found evidences for increased hepatic leptin signaling in $B_1^{-/-}$ mice, as well as other metabolic alterations that seem to be mainly related to the action of leptin, such as reduced expression of SCD1, reduced rates of hepatic lipogenesis, and improved response to gene expression changes during fasting. Other circulating immune mediators (ICAM-1, fetuin A, LIF, TIMP-1, and OSM) were found to be altered in our model, all of them previously related to steatosis development or liver function. These results strengthen the importance of B_1R in metabolic processes and suggest that it should be further investigated as a target for the treatment of NAFLD.

Acknowledgments This work was supported by grants of Fapesp Processo 2008/06676-8, CNPq, and CAPES.

Conflict of interest No potential conflicts of interest relevant to this article were reported.

References

1. Tilg H, Moschen AR (2008) Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 19:371–379
2. Alba LM, Lindor K (2003) Review article: non-alcoholic fatty liver disease. Aliment Pharmacol Ther 17:977–986
3. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev 57:27–77
4. Bhoola KD, Figueroa CD, Worthy K (1992) Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol Rev 44:1–80
5. Mori MA, Araujo RC, Pesquero JB (2008) Kinin B1 receptor stimulation modulates leptin homeostasis. Evidence for an insulin-dependent mechanism. Int Immunopharmacol 8:242–246
6. Mori MA, Araujo RC, Reis FC, Sgai DG, Fonseca RG, Barros CC, Merino VF, Passadore M, Barbosa AM, Ferrari B et al (2008) Kinin B1 receptor deficiency leads to leptin hypersensitivity and resistance to obesity. Diabetes 57:1491–1500
7. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543–546
8. Carvalheira JB, Ribeiro EB, Folli F, Velloso LA, Saad MJ (2003) Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver. Biol Chem 384:151–159
9. Cohen P, Miyazaki M, Socci ND, Hagge-Greenberg A, Liedtke W, Soukas AA, Sharma R, Hudgins LC, Ntambi JM, Friedman JM (2002) Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. Science 297:240–243
10. Cohen P, Zhao C, Cai X, Montez JM, Rohani SC, Feinstein P, Mombaerts P, Friedman JM (2001) Selective deletion of leptin receptor in neurons leads to obesity. J Clin Invest 108:1113–1121
11. Kakuma T, Lee Y, Unger RH (2002) Effects of leptin, troglitazone, and dietary fat on stearoyl CoA desaturase. Biochem Biophys Res Commun 297:1259–1263
12. Ntambi JM, Miyazaki M, Stoehr JP, Lan H, Kendziorski CM, Yandell BS, Song Y, Cohen P, Friedman JM, Attie AD (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. Proc Natl Acad Sci U S A 99:11482–11486
13. Araujo RC, Mori MA, Merino VF, Bascands JL, Schanstra JP, Zollner RL, Villela CA, Nakaie CR, Paiva AC, Pesquero JL et al (2006) Role of the kinin B1 receptor in insulin homeostasis and pancreatic islet function. Biol Chem 387:431–436
14. Mori MA, Sales VM, Motta FL, Fonseca RG, Alenina N, Guadagnini D, Schadock I, Silva ED, Torres HA, Dos Santos EL et al (2012) Kinin b(1) receptor in adipocytes regulates glucose tolerance and predisposition to obesity. PLoS One 7:e44782
15. Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva JA Jr, Walther T, Oliveira SM, Pesquero JL, Paiva AC, Calixto JB et al (2000) Hypoalgesia and altered inflammatory responses in mice lacking kinin B1 receptors. Proc Natl Acad Sci U S A 97:8140–8145
16. Stansbie D, Brownsey RW, Crettaz M, Denton RM (1976) Acute effects in vivo of anti-insulin serum on rates of fatty acid synthesis and activities of acetyl-coenzyme A carboxylase and pyruvate dehydrogenase in liver and epididymal adipose tissue of fed rats. Biochem J 160:413–416
17. Robinson AM, Williamson DH (1978) Control of glucose metabolism in isolated acini of the lactating mamary gland of the rat. Effects of oleate on glucose utilization and lipogenesis. Biochem J 170:609–613
18. Abe KC, Mori MA, Pesquero JB (2007) Leptin deficiency leads to the regulation of kinin receptors expression in mice. Regul Pept 138:56–58
19. Araujo RC, Kettritz R, Fichtner I, Paiva AC, Pesquero JB, Bader M (2001) Altered neutrophil homeostasis in kinin B1 receptor-deficient mice. Biol Chem 382:91–95
20. Barros CC, Haro A, Russo FJ, Schadock I, Almeida SS, Ribeiro RA, Vanzela EC, Lanzoni VP, Barros FC, Moraes MR et al (2012) Altered glucose homeostasis and hepatic function in obese mice deficient for both kinin receptor genes. PLoS One 7:e40573
21. Merino VF, Todiras M, Mori MA, Sales VM, Fonseca RG, Saul V, Tenner K, Bader M, Pesquero JB (2009) Predisposition to atherosclerosis and aortic aneurysms in mice deficient in kinin B1 receptor and apolipoprotein E. J Mol Med (Berl) 87:953–963
22. Fishman S, Muzumdar RH, Atzmon G, Ma X, Yang X, Einstein FH, Barzilai N (2007) Resistance to leptin action is the major determinant of hepatic triglyceride accumulation in vivo. FASEB J 21:53–60
23. Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, DePaoli AM, Taylor SI, Gorden P et al (2002) Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. J Clin Invest 109:1345–1350
24. Huang W, Dedousis N, Bhatt BA, O'Doherty RM (2004) Impaired activation of phosphatidylinositol 3-kinase by leptin is a novel mechanism of hepatic leptin resistance in diet-induced obesity. J Biol Chem 279:21695–21700
25. McGarry JD, Woeltje KF, Kuwajima M, Foster DW (1989) Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. Diabetes Metab Rev 5:271–284
26. Dias JP, Couture R (2012) Blockade of kinin B(1) receptor reverses plasma fatty acids composition changes and body and tissue fat gain in a rat model of insulin resistance. Diabetes Obes Metab 14:244–253

27. Schulze-Topphoff U, Prat A, Bader M, Zipp F, Aktas O (2008) Roles of the kallikrein/kinin system in the adaptive immune system. Int Immunopharmacol 8:155–160
28. Soderberg C, Marmur J, Eckes K, Glaumann H, Sallberg M, Frelin L, Rosenberg P, Stal P, Hultcrantz R (2011) Microvesicular fat, inter cellular adhesion molecule-1 and regulatory T-lymphocytes are of importance for the inflammatory process in livers with non-alcoholic steatohepatitis. APMIS 119:412–420
29. Parsons CJ, Bradford BU, Pan CQ, Cheung E, Schauer M, Knorr A, Krebs B, Kraft S, Zahn S, Brocks B et al (2004) Antifibrotic effects of a tissue inhibitor of metalloproteinase-1 antibody on established liver fibrosis in rats. Hepatology 40:1106–1115
30. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Krober SM, Machicao F, Fritsche A, Haring HU (2006) Alpha2-Heremans-Schmid glycoprotein/fetuin-A is associated with insulin resistance and fat accumulation in the liver in humans. Diabetes Care 29:853–857
31. Singhal NS, Patel RT, Qi Y, Lee YS, Ahima RS (2008) Loss of resistin ameliorates hyperlipidemia and hepatic steatosis in leptin-deficient mice. Am J Physiol Endocrinol Metab 295:E331–E338
32. Ailhaud G (2006) Adipose tissue as a secretory organ: from adipogenesis to the metabolic syndrome. C R Biol 329:570–577, discussion 653-575
33. Sookoian S, Gianotti TF, Rosselli MS, Burgueno AL, Castano GO, Pirola CJ (2011) Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease. Atherosclerosis 218:378–385
34. Levesque L, Larrivee JF, Bachvarov DR, Rioux F, Drapeau G, Marceau F (1995) Regulation of kinin-induced contraction and DNA synthesis by inflammatory cytokines in the smooth muscle of the rabbit aorta. Br J Pharmacol 116:1673–1679
35. Henkel J, Gartner D, Dorn C, Hellerbrand C, Schanze N, Elz SR, Puschel GP (2011) Oncostatin M produced in Kupffer cells in response to PGE2: possible contributor to hepatic insulin resistance and steatosis. Lab Invest 91:1107–1117
36. Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD, Furman TC, Hale JE, Hsiung HM et al (1997) Crystal structure of the obese protein leptin-E100. Nature 387:206–209
37. Tanaka M, Miyajima A (2003) Oncostatin M, a multifunctional cytokine. Rev Physiol Biochem Pharmacol 149:39–52
38. Duka A, Kintsurashvili E, Duka I, Ona D, Hopkins TA, Bader M, Gavras I, Gavras H (2008) Angiotensin-converting enzyme inhibition after experimental myocardial infarct: role of the kinin B1 and B2 receptors. Hypertension 51:1352–1357
39. Marcon R, Claudino RF, Dutra RC, Bento AF, Schmidt EC, Bouzon ZL, Sordi R, Morais RL, Pesquero JB, Calixto JB (2013) Exacerbation of DSS-induced colitis in mice lacking kinin B1 receptor through compensation of up-regulation of kinin B2 receptors: the role of tight junctions and intestinal homeostasis. Br J Pharmacol 168:389–402
40. Barros CC, Haro A, Russo FJ, Schadock I, Almeida SS, Reis FC, Moraes MR, Haidar A, Hirata AE, Mori M et al (2012) Bradykinin inhibits hepatic gluconeogenesis in obese mice. Lab Invest 92:1419–1427