FEBS 28951 FEBS Letters 577 (2004) 351–356

Reduced adipose tissue mass and hypoleptinemia in iNOS deficient mice: effect of LPS on plasma leptin and adiponectin concentrations

Javier Gómez-Ambrosi[a,*], Sara Becerril[a], Paula Oroz[a], Santiago Zabalza[a], Amaia Rodríguez[a], Francisco J. Muruzábal[b], Marta Archanco[b], María J. Gil[c], María A. Burrell[b], Gema Frühbeck[a,d]

[a] *Metabolic Research Laboratory, Clínica Universitaria de Navarra, Edificio CIFA, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain*
[b] *Department of Histology and Pathology, University of Navarra, Pamplona, Spain*
[c] *Department of Biochemistry, Clínica Universitaria de Navarra, University of Navarra, Pamplona, Spain*
[d] *Department of Endocrinology, Clínica Universitaria de Navarra, University of Navarra, Pamplona, Spain*

Received 17 September 2004; revised 5 October 2004; accepted 8 October 2004

Available online 21 October 2004

Edited by Robert Barouki

Abstract **The aim of this study was to evaluate the impact of the lack of inducible NO synthase (iNOS) on body weight and adipose tissue mass as well as on plasma leptin and adiponectin in basal conditions and 6 h after lipopolysaccharide (LPS) administration in mice. Body weight was not different among male, six-week-old wild-type (WT) and iNOS$^{-/-}$ animals. However, the amount of epididymal white adipose tissue (EWAT) in iNOS$^{-/-}$ mice was significantly reduced ($P < 0.05$). Circulating leptin and leptin mRNA in EWAT were decreased in iNOS$^{-/-}$ mice ($P < 0.05$ and $P < 0.01$, respectively). Plasma adiponectin and adiponectin mRNA were unchanged. LPS administration increased plasma leptin in both genotypes ($P < 0.05$). Neither genotype nor treatment changed plasma adiponectin. In summary, iNOS$^{-/-}$ mice exhibited normal body weight but reduced adipose mass accompanied by hypoleptinemia. Leptin responsiveness to LPS in iNOS$^{-/-}$ mutants is preserved, showing that the LPS-induced rise in leptin is independent of the presence of functional iNOS. In addition, iNOS deficiency or LPS does not influence expression and circulating levels of adiponectin.**

Keywords: Inducible NO synthase ; Adipose tissue; Leptin; Adiponectin; Lipopolysaccharide

1. Introduction

The increasing prevalence of obesity has drawn attention to the need for intensified research directed at the elucidation of the mechanisms regulating body weight and adipose tissue mass [1]. Current evidence for the involvement of several different factors in energy balance regulation indicates that body weight homeostasis is far more complex than initially thought. The identification of genes that cause obesity, leanness or provide resistance to obesity development has provided new clues in the understanding of body weight control [2].

Nitric oxide (NO) is a gaseous signaling molecule, which plays an important role in a wide variety of physiological functions, including inflammation, vascular tone and metabolism [3]. NO is formed in an enzymatic reaction catalyzed by three isoforms of NO synthase (NOS) cloned so far. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are calcium dependent and constitutively expressed, while inducible NOS (iNOS) is calcium independent and is synthesized in response to a variety of stimuli [4]. Evidence of NOS has been demonstrated in adipose tissue [5,6], indicating that adipocytes are a potential source of NO production. Furthermore, the involvement of NO in both rat and human lipolysis has been reported [7–9]. Stimulation of iNOS in skeletal muscle and white adipose tissue (WAT) has been proposed as a link between obesity, inflammation and insulin resistance [10]. In fact, increased iNOS expression in adipocytes of genetic and dietary models of obesity has been described [11]. Moreover, targeted disruption of iNOS has been shown to protect against diet-induced insulin resistance in muscle [11].

It is clearly established that adipose tissue secretes a wide variety of biologically active molecules at the same time as expressing receptors for numerous hormones and cytokines, thus representing an extremely active endocrine organ [12]. In addition, adipose tissue plays an important role in the obesity-related inflammatory process [13]. Leptin and adiponectin are pleiotropic proteins expressed and secreted mainly by adipose tissue, with a well-established participation in inflammatory processes [14,15]. Lipopolysaccharide (LPS) is a component of the gram-negative bacterial cell wall, which produces an inflammatory response and strongly induces iNOS [16]. Leptin is increased already hours later after LPS administration [17–19], and so far, the effect of LPS on adiponectin in mice is unknown. Since it is well known that LPS induces NO production by stimulating the synthesis of iNOS, we evaluated the effect of the absence of iNOS on the stimulation of leptin and adiponectin induced by LPS.

The aim of the present study, therefore, was twofold. On the one hand, to gain insight into the involvement of iNOS in body weight regulation in mice by evaluating the lack of iNOS on body weight and adipose tissue mass. On the other hand, to study whether adiponectin is acutely regulated by LPS and to evaluate the lack of iNOS on plasma leptin and adiponectin concentrations in basal conditions and after LPS administration.

* Corresponding author. Fax: +34-948-425652.
E-mail address: jagomez@unav.es (J. Gómez-Ambrosi).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.028

Table 1
Characteristics of iNOS$^{-/-}$ mice

	Body weight (g)	Epididymal WAT weight (g)	Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)	Adiponectin (μg/ml)
WT	20.2 ± 0.6	0.215 ± 0.043	96 ± 10	2.3 ± 1.8	0.67 ± 0.17	8.36 ± 2.07
iNOS$^{-/-}$	20.2 ± 1.3	0.114 ± 0.012*	164 ± 37	0.9 ± 0.4	0.23 ± 0.04*	9.46 ± 1.62

Data are means ± S.E.M. from six-week-old male mice.
* $P < 0.05$ by two-tailed unpaired t test comparing values between wild type and iNOS$^{-/-}$ groups ($n = 6$ per group).

2. Material and methods

2.1. Animals and treatments

Wild-type (WT) and iNOS deficient (iNOS$^{-/-}$) mice [20] were bred in the animal facilities of the University of Navarra. Mice were maintained at an ambient temperature of 22 ± 2 °C on a 12:12 h light–dark cycle (lights on at 08:00 h) under pathogen-free conditions and given a standard laboratory diet (Rodent Toxicology Diet, B & KUniversal Ltd., Hull, UK) and UV-irradiated tap water *ad libitum*. Twenty-four six-week-old male mice ($n = 6$ per group) were used in the study. Between 10:00 and 10:30 a.m. LPS from *Escherichia coli*, serotype 055:B5 (SIGMA, Madrid, Spain) was administered intraperitoneally at 5 mg/kg body weight to half of the mice (six WT and six iNOS$^{-/-}$), while the remaining animals were injected with vehicle (PBS). All mice were fasted with free access to water during the experiment. Six hours after LPS or vehicle administration, mice were weighed and sacrificed by CO_2 inhalation. Blood samples were collected directly from the heart. Epididymal WAT (EWAT) was excised, weighed, snap-frozen in liquid nitrogen and stored at −85 °C until extraction of RNA. Plasma was separated and stored at −85 °C for subsequent measurements. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals and the study was approved by the Ethical Committee for Animal Experimentation of the University of Navarra.

2.2. Genotyping

To confirm the iNOS genotype of the mice, genomic DNA was extracted from ear punches by using a DNeasy mini kit (QIAGEN Inc., Valencia, CA, USA). A minimal portion of the ear was cut into small pieces and incubated with proteinase K solution. The obtained genomic DNA was then eluted according to the manufacturer's instructions. To identify the presence of the WT or the disrupted iNOS allele, three primers were used. The primer 5′-ACATGCAGAATGA-

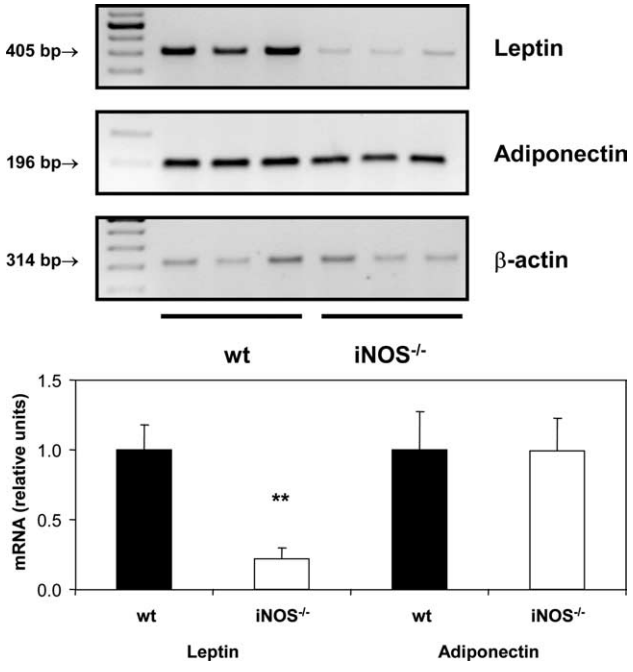

Fig. 1. Leptin and adiponectin mRNA expression in EWAT of WT and iNOS$^{-/-}$ mice. Photographs show representative sets of PCR products from individual mice. Bars represent means ± S.E.M. of the ratio between leptin and adiponectin to β-actin. The expression of leptin and adiponectin in WT mice was assumed to be 1. ** $P < 0.01$ vs. WT mice by two-tailed unpaired t test; $n = 6$ per group.

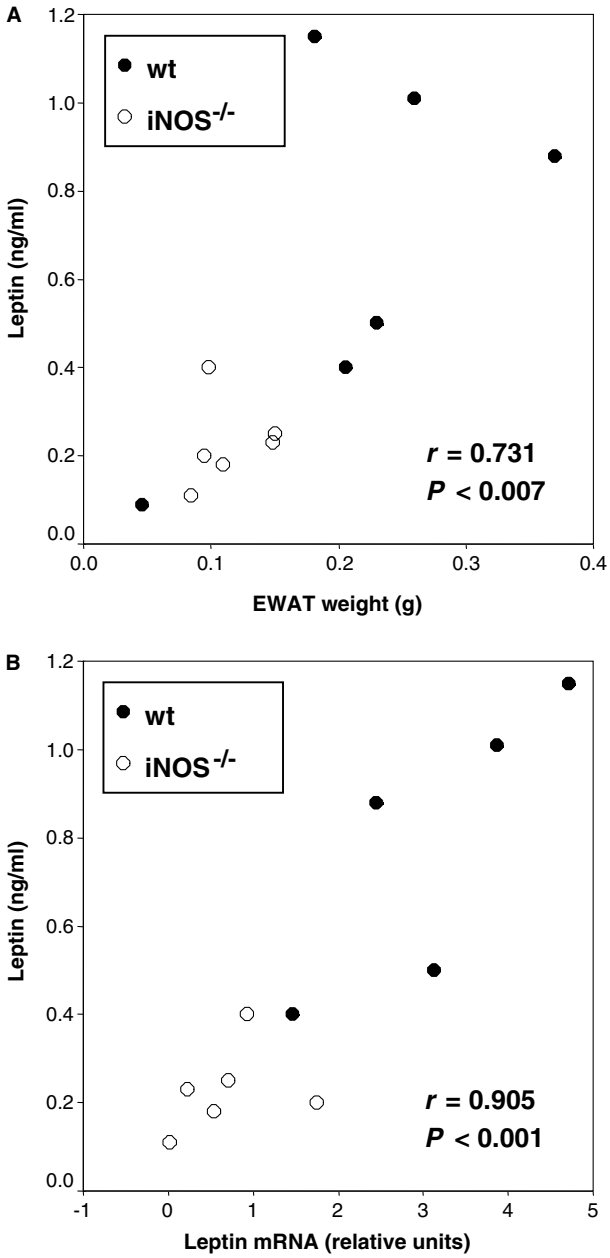

Fig. 2. Scatter diagrams showing the positive correlation found between circulating concentrations of leptin and the weight of the epididymal fat pads (A) and between concentrations of leptin and leptin mRNA expression in EWAT (B) of WT (●) and iNOS$^{-/-}$ (○) mice. Pearson's correlation coefficient and P values are indicated.

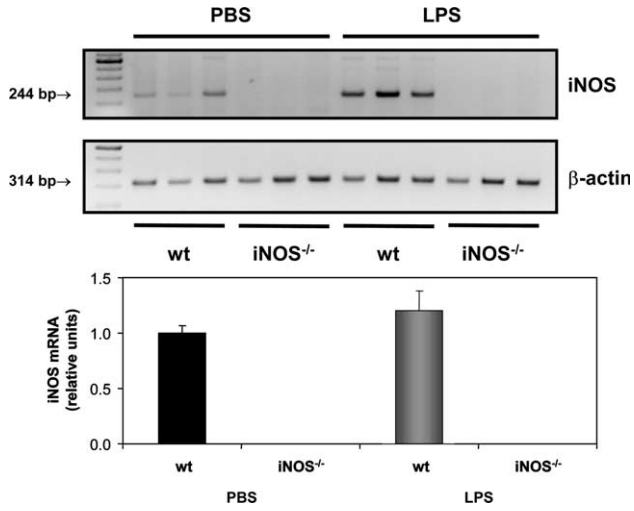

Fig. 3. iNOS mRNA expression in EWAT. WT or iNOS deficient ($iNOS^{-/-}$) mice received an intraperitoneal injection of either LPS (5 mg/kg) or PBS. Photographs show representative sets of PCR products from individual mice. Bars represent means ± S.E.M. of the ratio between iNOS and β-actin. The expression of iNOS in WT mice was assumed to be 1; $n = 6$ per group.

GTACCGG-3′ was complementary to both the genomic and disrupted alleles and amplified a 108-bp fragment with the primer 5′-TCAACATCTCCTGGTGGAAC-3′ specific for the genomic sequence and a 275-bp fragment with the primer 5′-AATATGCGAAGTGGACCTCG-3′ complementary to a region of the neomycin resistance insert specific to the disrupted iNOS allele [21]. The PCR was performed as described elsewhere [21]. Two hundred nanograms of DNA was used per reaction. The PCR products were then separated on 1.5% agarose gel and visualized with ethidium bromide staining.

2.3. Plasma measurements

Blood samples were collected from the heart. Plasma glucose was analyzed by an automated analyzer (Roche/Hitachi Modular P800), with quantification being based on enzymatic colorimetric reactions. Plasma insulin was measured by means of an Ultra Sensitive Rat Insulin ELISA kit using mouse insulin as standard (Crystal Chem Inc., Chicago, IL, USA). Intra- and inter-assay coefficients of variation (CV) were 5.5% and 4.8%, respectively. Circulating concentrations of leptin were measured by ELISA (Crystal Chem Inc.). Intra- and inter-assay CV were 5.4% and 6.9%, respectively. Plasma adiponectin was quantified by ELISA (B-Bridge International, Inc., San José, CA, USA). Intra- and inter-assay CV were 4.2% and 5.9%, respectively. NO was determined spectrophotometrically in plasma by its oxidation products nitrite and nitrate (NO_x) using a colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA). Nitrate present in samples is reduced to nitrite utilizing nitrate reductase. The addition of the Griess Reagent converts nitrite into a deep purple azo compound. Finally, the measurement of the absorbance at 540 nm accurately determines NO_2^- concentration [22]. Concentrations were determined compared with a standard curve of sodium nitrite. The detection limit of the assay was 2.5 μM nitrite.

2.4. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 100 mg of EWAT samples by homogenization with an ULTRA-TURRAX® T 25 basic (IKA® Werke GmbH, Staufen, Germany) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RNA concentration determined from absorbance at 260 nm. One μg of RNA was used to synthesize first-strand cDNA after treatment with DNase I (Roche, Basel, Switzerland). The RT-PCRs were carried out as previously described [23]. Primers used to amplify the cDNA of leptin (GenBank U22421) were 5′-CCAGGATGACACCAAAACCC-3′ and 5′-TCCAACTGTTGAAGAATGTCC-3′, adiponectin (GenBank AF304466) 5′-TGTTCCTCTTAATCCTGCCC-3′ and 5′-TCTCCTTTCTCTCCCTTCTC-3′, iNOS (GenBank NM_010927) 5′-TCATGGACCACCACACAGCC-3′ and 5′-CGGATCTCTCTCCTCCTGGG-3′, and β-actin (GenBank X03672) 5′-TCTACAATGAGCTGCGTGTG-3′ and 5′-GGTCAGGATCTTCATGAGGT-3′. The pair of primers designed to analyze iNOS mRNA expression amplified a 244-bp fragment of cDNA codified within a region of the iNOS gene that was not included in the target construct and, therefore, was absent in $iNOS^{-/-}$ mice. Primers for leptin, adiponectin, and iNOS were designed using the *Oligo® 4.05 Primer Analysis Software* (National Biosciences, Inc., Plymouth, MN, USA). cDNA was amplified for 33 (leptin), 26 (adiponectin), 33 (iNOS) and 25 (β-actin) cycles, using the following parameters: 94 °C for 30 s, 56 °C (leptin), 57 °C (adiponectin), 58 °C (iNOS) and 59 °C (β-actin) for 30 s and 72 °C for 30 s, with a final extension step at 72 °C for 7 min. Amplifications were linear under these conditions and were carried out in a GeneAmp® PCR System 2400 (Perkin–Elmer, Norwalk, CT, USA). All PCRs for each gene were performed at the same time and with the same batch of Taq polymerase in order to reduce variations in the efficiency of PCR. The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Levels of mRNA were expressed as the ratio of signal intensity for each gene relative to that of β-actin. The ratio of each gene/β-actin in the WT group was assumed to be 1. The intensity of the PCR bands was determined by densitometric analysis with the *Gel Doc 1000 UV fluorescent gel documentation system* and *Molecular Analyst 1.4.1* software for quantitation of images (Bio-Rad, Hercules, CA, USA).

2.5. Statistical analysis

Data are presented as means ± S.E.M. Differences between WT and $iNOS^{-/-}$ mice were analyzed by two-tailed unpaired *t* tests. The effect of LPS in both genotypes was analyzed by means of a two-way (genotype and treatment) ANOVA. Correlations between two variables were studied by Pearson's correlation coefficients. A P value lower than 0.05 was considered statistically significant. The calculations were performed using the SPSS/Windows version 11.0.1 statistical package (SPSS, Chicago, IL, USA).

3. Results

3.1. $iNOS^{-/-}$ mice show reduced adipose mass and hypoleptinemia

Body weight of mice lacking iNOS was not different from that of WT mice. However, $iNOS^{-/-}$ showed reduced adipose tissue mass as evidenced by the smaller amount of EWAT, which weighed less than EWAT of WT littermates ($P < 0.05$). There were no changes in circulating concentrations of either glucose or insulin. Plasma leptin concentrations were significantly reduced ($P < 0.05$) as expected after the reduced adipose tissue mass. Despite having reduced amounts of body fat, adiponectin concentrations remained unchanged in $iNOS^{-/-}$ mice as compared to WT controls (Table 1).

3.2. Adipokines expression in $iNOS^{-/-}$ mice

Leptin expression was significantly reduced in $iNOS^{-/-}$ mice as compared to WT mice ($P < 0.01$). Similar to the finding in plasma concentrations, no effect of the lack of iNOS on adiponectin expression in EWAT was observed (Fig. 1). According to the circulating values, a significant correlation between leptin concentrations and the weight of epididymal fat pads was shown (Fig. 2A). Likewise, a positive correlation between leptin concentrations and leptin expression was observed (Fig. 2B).

3.3. Effect of LPS on iNOS expression in WAT and plasma NO_x concentrations

As expected, iNOS mRNA expression was undetectable in adipose tissue of $iNOS^{-/-}$ mice and was slightly increased by LPS treatment in WT animals, although these differences felt out of significance (Fig. 3). However, LPS significantly

increased NO_x in the plasma of WT mice, but had no effect on iNOS$^{-/-}$ mice, showing that there is no induction of NO production by LPS in iNOS deficient mice (Fig. 4).

3.4. Effect of LPS on adipokines plasma concentrations

Body weight was not different among the groups receiving PBS (Table 1) and LPS (WT: 20.0 ± 0.4, iNOS$^{-/-}$: 20.0 ± 0.9; NS). However, the iNOS$^{-/-}$ groups receiving either PBS (Table 1) or LPS (WT: 0.193 ± 0.020, iNOS$^{-/-}$: 0.158 ± 0.020; $P < 0.05$ main effect of genotype by ANOVA) showed a reduced adipose mass. Plasma leptin concentrations were significantly reduced in iNOS$^{-/-}$ mice ($P = 0.035$) as mentioned before, but were significantly increased by LPS treatment ($P = 0.018$) in both genotypes, while there was no statistically significant interaction ($P = 0.091$), meaning that the effect of LPS is independent of the presence of the mutation (Fig. 5). Neither genotype nor LPS treatment changed plasma adiponectin concentrations, showing that circulating adiponectin is not affected by either lack of iNOS or LPS administration (Fig. 6).

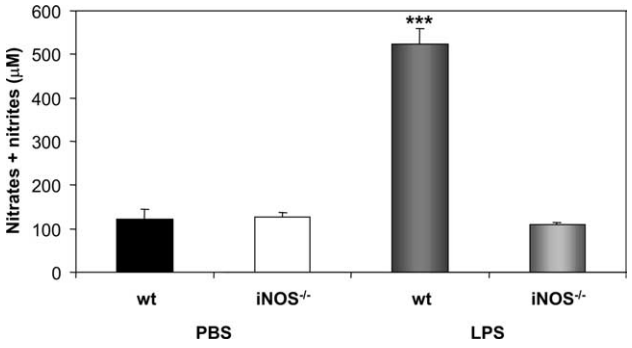

Fig. 4. Effect of LPS on NO production. WT or iNOS deficient (iNOS$^{-/-}$) mice received an intraperitoneal injection of either LPS (5 mg/kg) or PBS. Six hours after administration, plasma nitrate and nitrite concentrations were determined using the Griess reaction. ***$P < 0.001$ vs. other groups by ANOVA. Values are means ± S.E.M.; $n = 6$ per group.

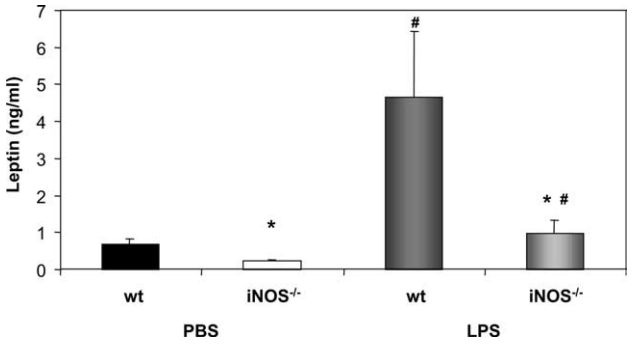

Fig. 5. Effect of LPS on plasma leptin concentrations. WT or iNOS deficient (iNOS$^{-/-}$) mice received an intraperitoneal injection of either LPS (5 mg/kg) or PBS. Six hours after administration, plasma leptin was determined by ELISA. *$P < 0.05$ main effect of genotype and # $P < 0.05$ main effect of LPS by ANOVA. Values are means ± S.E.M.; $n = 6$ per group.

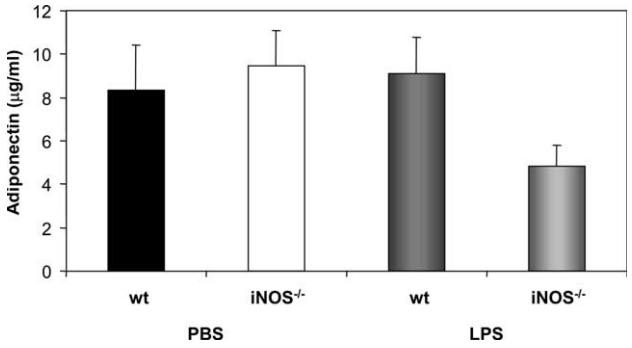

Fig. 6. Effect of LPS on plasma adiponectin concentrations. WT or iNOS deficient (iNOS$^{-/-}$) mice received an intraperitoneal injection of either LPS (5 mg/kg) or PBS. Six hours after administration, plasma adiponectin was determined by ELISA. Values are means ± S.E.M.; $n = 6$ per group.

4. Discussion

The present study provided evidence that iNOS deficient mice show a significant decrease in body fat. The weight of epididymal fat pads in mutants was about half of that of WT, while body growth was normal, as has been reported in other mice models of leanness [24–26]. In iNOS$^{-/-}$ mutants, leanness is not a consequence of decreased food intake. On the contrary, these mice exhibit increased food intake compared to WT controls [11]. Increased food intake is in agreement with the reduced leptin concentrations found in our study. However, the hyperphagia described seems insufficient to maintain normal adiposity and due to some unknown mechanisms these mice fail to accumulate fat under a normal diet. Therefore, it may be suggested that iNOS$^{-/-}$ animals have an increased energy expenditure. In this sense, it has been reported that NO downregulates uncoupling protein-2 (UCP2) expression in 3T3F442A adipocytes [27], and UCPs are markedly involved in energy expenditure regulation [2]. Although iNOS$^{-/-}$ mice do not show a reduced systemic NO production, as evidenced by NO_x concentrations, the lack of iNOS may decrease NO production in adipose tissue in an autocrine/paracrine fashion, thereby increasing UCP2 expression and hence energy expenditure. On the other hand, a role of iNOS in the regulation of sympathetically mediated blood flow in brown adipose tissue has also been described [6,28], arguing against an increased energy expenditure in this tissue in iNOS$^{-/-}$ mice. Furthermore, eNOS deficient mice show reduced energy expenditure and increased body weight [29], suggesting that NO involvement in the regulation of energy expenditure is complex and may exhibit NOS-specific differences. Increased energy expenditure associated to increased locomotor activity can be excluded given the fact that NO has been reported to induce locomotor activity in mice [30] and eNOS deficient mice show normal locomotor activity [29]. Another possibility explaining the observed reduced adiposity in iNOS$^{-/-}$ mice is related to an increased lipolytic activity. This reasoning is supported by the observation that blockade of NO production with an inhibitor of NOS increases the lipolytic rate in humans, indicating an inhibitory effect of NO on lipolysis [8]. Reduced adipose mass of iNOS$^{-/-}$ mice might also be due to impaired adipogenesis. In this sense, NO has been shown to promote differentiation of rat white preadipocytes in culture [31]. Fur-

ther analyses are required to unravel the underlying causes of reduced adipose mass in iNOS deficient mice.

Hypoleptinemia of iNOS$^{-/-}$ mice is accompanied by reduced leptin expression in EWAT. Leptin has been shown to induce the release of NO [9,32,33], but to our knowledge, no data regarding the effect of NO on leptin expression and secretion have been reported so far. The reduced expression and circulating concentrations of leptin found in our study may well reflect the reduced adipose mass [34] as evidenced by the positive correlations found, although a direct effect of iNOS derived NO on leptin expression cannot be discarded.

Plasma concentrations of glucose and insulin were not significantly modified by the lack of iNOS. iNOS$^{-/-}$ mice have been shown to exhibit normal glycemia and insulinemia under a chow diet, while being resistant to developing hyperinsulinemia under a high-fat diet as a consequence of their protection against insulin resistance in skeletal muscle [11]. Circulating concentrations of adiponectin were unaltered in iNOS$^{-/-}$ mice, which is in agreement with the absence of significant changes in the concentrations of glucose and insulin, given the insulin-sensitizing nature of this adipokine [35]. On the other hand, adiponectin increases NO production in human and bovine aortic endothelial cells [36–38], but the effect of NO on adiponectin is unknown so far. Our data show that adiponectin expression in EWAT and plasma concentrations is not regulated by iNOS-derived NO as evidenced by the studies performed in iNOS$^{-/-}$ and WT mice receiving LPS.

RT-PCR experiments studying iNOS expression in adipose tissue confirmed the presence of iNOS mRNA in adipose tissue of WT mice and the lack of expression in iNOS$^{-/-}$ mice. Although iNOS is a protein considered to be inducible, some degree of basal expression can be detected in white adipose tissue under physiological circumstances by both RT-PCR [11,16] and Western blot [5,39]. iNOS mRNA was slightly increased by LPS treatment in WT animals, although these differences felt out of significance. Previous studies have shown that LPS administration induces a strong increase in iNOS mRNA expression in EWAT [16,40]. However, in those experiments, which were performed in rats receiving a higher dose of LPS (15–20 mg/kg), the peak of induction of iNOS mRNA was observed 4 h after administration and was undetectable after 8 h [16]. The apparent lack of induction found in our study may relate to several factors, including the animal model used and the time point (6 h) at which iNOS mRNA expression was measured, since the peak effect of LPS on iNOS mRNA expression may be missed. However, LPS significantly increased plasma NO_x of WT mice, with no effect on iNOS$^{-/-}$ mice. This indicates that the induction of iNOS by LPS and, therefore, the increase in NO production was notable in WT mice and totally absent in iNOS deficient mice.

The well described increase in leptin concentrations after LPS administration could be mediated by corticoids [19], IL-1β [41], TNFα [42], or neurally mediated [43]. This effect takes place apparently by NO-independent mechanisms, because the effect is equally observed after blocking NO production with L-NAME [19]. To explore whether LPS was able to induce leptin in the absence of functional iNOS, the effect of intraperitoneal LPS in iNOS deficient mice was tested. Despite having reduced leptinemia, LPS increased plasma leptin concentrations in iNOS deficient mice to a similar extent than in normal mice. This finding confirms the independence of NO in endotoxemia-derived hyperleptinemia, showing that the LPS-induced rise in leptin circulating concentrations is independent of the presence of functional iNOS.

Adiponectin is an adipokine with anti-inflammatory properties, in addition to its well-known insulin sensitizing effects [15,44]. Anti-inflammatory actions of adiponectin are mediated in part by suppression of NFκB signaling and ERK1/2 activity [45]. Moreover, adiponectin suppresses LPS-induced TNFα mRNA expression in macrophages [46]. The present study demonstrates that LPS does not change plasma concentrations of adiponectin with independence of the presence of functional iNOS. Experiments in humans have shown that adiponectin concentrations are reduced during a resting and fasting state, and that this reduction is slightly reversed by endotoxin [47]. However, the effect was very small and differences between species in the regulation of adiponectin expression may explain the different response observed, given the fact that adiponectin in mice is not affected by fasting [48]. In addition, it has been recently described that adiponectin is not acutely regulated by endotoxemia in pigs [49]. Our data suggest that, at least in mice, adiponectin is not involved in the acute inflammatory response to LPS.

In summary, mice lacking iNOS exhibit a normal body weight but reduced adipose mass accompanied by hypoleptinemia. The deficiency of iNOS does not affect adiponectin expression and circulating concentrations. In addition, the LPS-induced increase in leptin concentrations is independent of iNOS and LPS does not influence circulating concentrations of adiponectin. Our data support the involvement of NO and iNOS in adipose mass regulation.

Acknowledgements: The authors are grateful to Dr. Victor E. Laubach from the University of Virginia for kindly providing the initial breeding pairs of the iNOS knockouts. We also thank all the staff of the animal housing facilities for their technical expertise in animal care and handling and, in particular, to Javier Guillén, Juan Percaz, and Eneko Elizalde. This work was supported by grants SAF2003-09225 from Plan Nacional de I+D+I, Ministerio de Ciencia y Tecnología (to G.F.), PI030381 from Instituto de Salud Carlos III. Fondo de Investigación Sanitaria (FIS) del Ministerio de Sanidad y Consumo (to J.G.-A.), 96/2000 from the Departamento de Educación del Gobierno de Navarra (to J.G.-A.), 48/2003 from the Departamento de Salud del Gobierno de Navarra (to J.G.-A.), and 2001–14 from PIUNA (to G.F.).

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