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Resistance of Janus Kinase-2 Dependent Leptin Signaling in Natural Killer (NK) Cells: A Novel Mechanism of NK Cell Dysfunction in Diet-Induced Obesity

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Leptin acts not only as an anorexigenic hormone but also regulates cell-mediated immunity via leptin receptors (Ob-R) expressed on T and B lymphocytes. However, the impact of leptin on natural killer (NK) cells is currently elusive. We evaluated leptin effects on NK cells in relation to the body weight in rats using *in vivo* and *in vitro* approaches. Leptin was injected iv in male lean and diet-induced obese Lewis and F344 rats. NK cell numbers were analyzed in blood and spleen by fluorescence activated cell sorting and immunohistochemistry, and the activity of NK cells was measured by chromium release assay. Ob-R expression was investigated by confocal laser scanning and quantitative RT-PCR. To compare leptin-dependent intracellular signaling under basal and leptin- and tumor cell (MADB106)-stimulated conditions, intracellular target proteins of NK cells were evaluated by Western blotting. Number and distribution pattern of splenic NK cells were significantly different in lean and obese animals. Leptin administration resulted in a 4-fold higher stimulation of the NK activity in lean than obese animals. This was not due to a decreased expression of Ob-R because quantitative RT-PCR revealed significantly higher Ob-Rb mRNA levels in NK cells from obese rats. In contrast, postreceptor signaling is differentially abrogated in obese animals with significantly lower activation of postreceptor signaling components (Janus kinase-2p, protein kinase B pT308, AMPαpT172) after an *in vivo* leptin challenge. In conclusion, the results for the first time assign leptin a central role as a modulator of NK cell number and activity only in lean but not obese subjects. The differential role of leptin has important implications for the influence of body weight in the response to systemic inflammations and in the immunological defense of cancer. (*Endocrinology* 149: 3370–3378, 2008)

LEPTIN, THE PRODUCT of the *ob* gene, is primarily secreted by adipocytes, acting as a hormonal feedback signal to hypothalamic nuclei and herewith regulating energy homeostasis by decreasing energy intake and increasing energy expenditure (1, 2). Signal transduction in the leptin pathway is mediated via the full-length leptin receptor (Ob-Rb), but in addition, several alternatively spliced isoforms of the Ob-R either with a short cytoplasmic domain (Ob-Ra, c, d, f) or lacking the transmembrane and cytoplasmic domain (Ob-Re) and capable of binding leptin in the serum have been characterized (3, 4). Effects of leptin on food consumption and energy expenditure appear to be dependent on the activation of Ob-Rb linked to the Janus kinase (JAK)-2-mediated activation of signal transducer and activator of transcription (STAT) 3-3, the JAK-STAT pathway (5). Additionally, both Ob-Rb and Ob-Ra can transduce signals through MAPK (ERK-1)-dependent pathways (5, 6). Previous studies in rodents and humans demonstrated that the increase of leptin in obesity is reflected in a selective increase in free leptin and a relative deficiency in receptor-bound leptin and revealed an independent physiological role of bound leptin (7, 8). Apart from its impact on energy homeostasis, important functions of leptin for the regulation of immunocompetence have been unraveled (9, 10). Leptin receptors are expressed on T lymphocytes (11), suggesting a role for leptin in these cells. A role of leptin in immune modulation is further strengthened by the structural similarities of the hormone to cytokines namely to IL-2, IL-6, and IL-15 (12) and the high homology of Ob-Rb to members of the class I cytokine receptor family (such as IL-6) (13). Especially for the proinflammatory cytokine IL-6 interaction between both pathways have been demonstrated. Leptin increases the proliferation of naive T lymphocytes, increases Th1, and suppresses Th2 cytokine production (9). Thus, leptin deficiency or receptor mutation causes a reduction in T lymphocyte numbers and an alteration in monocyte responsiveness (14). This leptin-dependent regulation of the immune system has been confirmed in several *in vivo* models. Leptin-

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Abbreviations: AMPK, AMP-activated protein kinase; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; hrec, human recombinant; IP, immunoprecipitation; JAK, Janus kinase; LU, lytic unit; mAb, monoclonal antibody; NK, natural killer; Ob-Rb, leptin receptor; qRT-PCR, quantitative RT-PCR; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TBS, Tris-phosphatase-buffered saline.

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deficient animals have been shown to be protected in experimental autoimmune encephalitis (15), ConA-induced hepatitis (16), and several models of experimental colitis (17, 18). In contrast, leptin deficiency *in vivo* is associated with an increased susceptibility in endotoxin-induced shock (19).

Natural killer (NK) cells are an integral component of the innate immune system, in both the production of cytokines (*e.g.* interferon-γ) to stimulate other immune cells and the direct destruction of infected or transformed cells (20, 21). NK cells express a variety of activating and inhibitory receptors the composite effects of which determine their specificities for divergent targets (22). In contrast to the effect of leptin on T and B lymphocytes, only few data exist on its modulatory role in NK cell proliferation, activation, or migration. Very little information is available concerning the expression of Ob-Rs on NK cells, and it is not yet known whether body weight and herewith an altered leptin system can modulate the expression and consecutive signaling. Obese patients have an independent risk for the development of severe inflammations (14) and several types of cancer (*e.g.* colon and breast cancer) (23). Because several studies propose altered NK cell numbers and NK cell function in obese compared with normal-weight individuals (24, 25), we speculated that the increased serum leptin levels in obese subjects may directly interact with NK cells and alter their migration patterns or activity in the obese.

Materials and Methods

Animals

Male Lewis and F344 rats were obtained from Charles River GmbH (Sulzfeld, Germany) and individually housed in plastic-based cages (40 × 26 × 15 cm), in sound-proofed, air-conditioned, and artificially lighted rooms (lights on from 0700 to 1900 h) at an ambient temperature of 24.0 ± 0.5 C. The animals were kept under specific pathogen-free conditions. Standard rat chow (50% carbohydrate, 19% protein, 12% water, 4% fat, and 2.1 kcal/g) and tap water were available *ad libitum*. Animals were randomized into two groups. One group was fed a high-calorie diet containing 34% carbohydrate, 17% protein, 4% water, 35% fat, and 5.2 kcal/g for 8 wk, after which the experiments started. Figure 1A shows that rats in the obese group weighed significantly more than the lean littermates (292 g ± 18 *vs.* 397 g ± 19 g; $P < 0.01$). All research and animal care procedures had been approved by the Lower Saxony district government in Hannover, Germany.

Leptin and MADB106 tumor cells

For both rat strains, a bolus injection of human recombinant (hrec)-leptin was used to stimulate leptin-dependent signaling. hrec-leptin (Natutec, Frankfurt, Germany) was dissolved in 0.9% NaCl, and a dose of 500 μg/kg in 0.2 ml NaCl was applied.

MADB106 tumor cells are a selected cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma chemically induced in the inbred F344 rat. In the present study, the challenge with these tumor cells was used to further activate NK cells. MADB106 tumor cells were stored in 5% CO_2 at 37 C in monolayer cultures in RPMI 1640 medium (Life Technologies, Inc., Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum, 45 U/ml penicillin, 0.045 mg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Immediately before starting the experiments, cells, derived from the log phase of tumor growth, were separated using 0.25% trypsin, washed, and resuspended in RPMI 1640. For injection 1×10^6 cells per 300 g were used.

Intravenous cannulation

Under im ketamine-hydrochloride (10%, 0.35 ml) and medetomidine hydrochloride (0.001%, 0.05 ml) anesthesia, Lewis rats received a central venous catheter in the right external jugular vein as described before (26). The animals were allowed to recover for 4 d postoperatively to avoid interference with surgical stress. The F344 rats were anesthetized and the right external jugular vein was short-term cannulated.

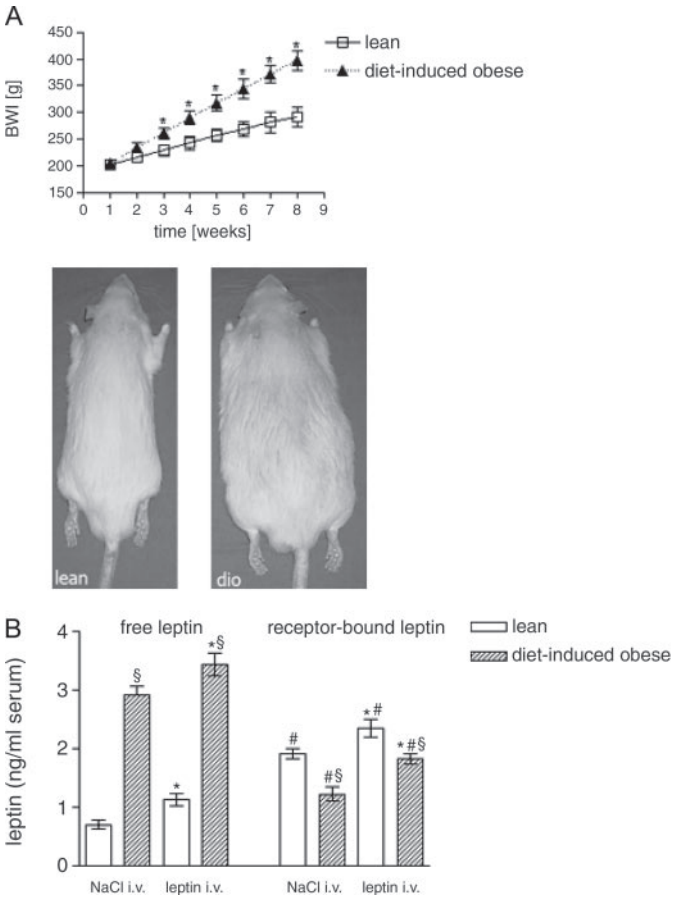

FIG. 1. Serum levels of free and receptor-bound leptin are significantly different in obese animals. A, Changes in body weight (BW) were monitored (n = 15/group). Significant *post hoc* effects between obese and lean animals are indicated by an *asterisk*. *, $P < 0.001$. The photo shows representative Lewis rats at 8 wk. B, Both the basal and leptin stimulated (4 h after iv injection of hrec-leptin) levels of leptin measured by RIA are significantly increased (free leptin) and decreased (receptor bound leptin) in diet-induced obese animals, compared with lean littermates (n = 5 in each group). Significant *post hoc* effects *vs.* the corresponding NaCl controls within the lean or the obese animal group are indicated by an *asterisk*. *, $P < 0.001$. Significant *post hoc* effects of receptor-bound *vs.* free leptin in corresponding groups are indicated by a *rhomb*. #, $P < 0.001$. Significant *post hoc* effects of free or receptor-bound leptin of obese animals *vs.* the corresponding lean littermates in the same treatment group (NaCl or leptin) are indicated by a *paragraph sign*, §, $P < 0.001$. Data are expressed as mean ± SEM.

Experimental design

Lewis rats were divided into six groups (n = 5/group): groups 1 + 2, NaCl-treated lean and obese animals (killed 4 h after the injection); groups 3 + 4, leptin-treated lean and obese animals (killed 4 h after the injection); groups 5 + 6, lean and obese animals exsanguinated without surgical intervention [for the quantitative RT-PCR (qRT-PCR) analysis].

For the immunoblot analyses, an additional NK cell challenge with MADB106 tumor cells was performed. Because these tumor cells activate only NK cells from F344 rats, the experimental animal strain was changed. F344 were randomized into eight groups (n = 1–3/group). Experiments started with the first iv injection. Five minutes later, the second injection was performed. Animals were killed 15 min later:

groups 1 + 2, NaCl + NaCl-treated lean and obese animals; groups 3 + 4, leptin + NaCl-treated lean and obese animals; groups 5 + 6, NaCl + MADB106-treated lean and obese animals; groups 7 + 8, leptin + MADB106-treated lean and obese animals.

Blood and tissue sampling

Four hours (Lewis rats) and 15 min (F344 rats) after injection, rats were anesthetized, the left heart ventricle was punctured, and blood was collected. One part of the spleen of the Lewis rats was processed for the *in vitro* NK cell assay. For the immunohistological investigations, Lewis rats were perfused transcardially with 100 ml PBS. The spleen and the intermediate lobe of the liver were removed, and one part was immediately frozen in liquid nitrogen and stored at −80 C for immunohistological analysis.

Flow cytometry

Leukocyte numbers were determined using a Coulter counter. Fluorescence activated cell sorting (FACS) analysis (FACS-Scan; Becton Dickinson, Heidelberg, Germany) was performed using the monoclonal mouse antirat antibodies (mAb) 10/78 (NK cells/NKR-P1A$^+$/CD161^{bright}; Serotec GmbH, Düsseldorf, Germany) and R73 (T cell receptor, CD3; Serotec). Two-color staining (data not shown) using either mAb ED9/10/78 to exclude monocytes or mAb R73/10/78 to exclude T cells, revealed that NKR-P1A^{bright} events reflect only NK cells.

Quantification of NK cells in the spleen in situ

Cryostat sections (5–7 μm) were obtained, and immunohistochemical characterization of NK cells was performed with the alkaline phosphatase antialkaline phosphatase technique. In brief, sections were immersion fixed with acetone for 10 min, rehydrated with Tris-phosphatase-buffered saline (TBS) and incubated with the primary mouse mAb 10/78 (NKR-P1A$^+$; 1:250) overnight. Binding of the primary mAbs was revealed by mouse IgG (1:50 in 5% rat serum in PBS) for 30 min. To visualize the binding, the alkaline phosphatase antialkaline phosphatase complex (1:50 in TBS for 30 min; DakoCytomation, Hamburg, Germany) was used, followed by the repetition of the last two steps for 15 min. The sections were stained with Fast Red for 25 min, counterstained with hematoxylin (1:5 in PBS) for 90 sec, and mounted in glycergel (DakoCytomation). Method specificity was tested by omission of the primary antibody and by corresponding isotype controls. Each experimental group was composed of five animals, and for the quantitative analysis, five sections and six visual fields/section (resulting in 150 mm^2 investigated area/group) were examined for each animal by two observers blinded to the treatment conditions.

In situ detection of Ob-R expression of NK cells by confocal laser-scanning microscopy

Cryostat sections (6–8 μm) were obtained and NK cells and Ob-R were detected with immunofluorescence. Sections were immersion fixed with acetone for 10 min and rehydrated with TBS. Nonspecific protein binding was blocked by treating the sections for 60 min at room temperature with 5% donkey serum diluted in PBS. Both primary and secondary antibodies were simultaneously present in the incubation solutions. Incubation with the primary antibodies (10/78, NKR-P1A$^+$, 1:100; M18, Ob-R, 1:150; Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted in PBS was performed overnight at 4 C in a humid chamber. After rinsing in PBS sections were incubated likewise with donkey antimouse Cy2 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and donkey antigoat Cy3 diluted 1:50 in PBS. Thereafter nuclei were stained with To-Pro-3 (Molecular Probes, Eugene, OR) 1:300 in PBS for 30 min. Sections were mounted in fluorescent mounting medium (DakoCytomation). Confocal laser-scanning microscopy was performed with an LSM 510 META microscope equipped with an Axiovert 200 M (Carl Zeiss, Jena, Germany), an argon laser (30 mW maximal power), and a He/Ne-Laser (1 mW maximal power). Images were acquired with the LSM 510 Image Browser software (version 3.5; Carl Zeiss).

Chromium release assay

For detection of splenic NK cell mediated lysis, one part of the spleen was minced immediately after removing and mashed through a cell strainer, yielding single-cell suspensions of splenocytes. The lymphocyte fractions in spleen suspension and blood were isolated by a Ficoll gradient. The murine lymphoma cell line YAC-1 was labeled for 60 min with 100 μCi sodium chromate-51 (Amersham, Braunschweig, Germany). Cytotoxicity assays were performed using V-bottomed microtiter plates with 5000 ^{51}Cr-labeled YAC-1 cells at various effector to target ratios in triplicates. Release of ^{51}Cr into the supernatant was measured after 4 h at 37 C. Maximal release was determined by addition of 1% Triton X-100. Spontaneous release was always less than 10%. Specific cytotoxicity was calculated according to a standard method and transformed into lytic units (LU; $LU_{20}/10^7$ effector cells) as described before (27). This value was then divided by the actual NK cell proportion in each assay, which is expressed as LU/NK cells.

Receptor-bound leptin measurement

The amount of receptor-bound leptin in the blood was measured as described before (28). In short, antibodies to the N-terminal portion of the protein (leptin amino acid number 26–38) were generated by coupling to hemocyanine by the carbodiimide method. N-terminal antibodies selectively detect protein bound leptin immunoreactivity.

Sorting of NK cells

For qRT-PCR and immunoblotting, blood cells were incubated consecutively with phycoerythrin-labeled anti-CD161 (NKR-P1A) and fluorescein isothiocyanate-labeled anti-CD3 (T cell receptor) for 60 min at 4 C. NK$^+$CD3$^-$ cells were sorted and collected with a FACSAria (Becton Dickinson).

qRT-PCR analysis for Ob-Rb

Total RNA from NK-cells from diet-induced obese and lean rats was extracted with Trifast Gold reagent (Peqlab, Erlangen, Germany) and thereon reverse transcribed using oligo (deoxythymidine)$_{12–18}$-primers (Invitrogen, Karlsruhe, Germany) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a volume of 77.5 μl at 37 C for 55 min. cDNA (1 μl) was subjected to real-time PCR analysis using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 5 pmol of gene-specific primers in a total volume of 20 μl. The following primer sequences were used for detection of Ob-Rb receptor transcripts: forward, 5′-CAC CCA GGG AAC CTG TGA GG-3′, reverse, 5′-GGA ATG TTT CCT GGC GAT GC-3′. PCR was performed on a Lightcycler (Roche, Basel, Switzerland) using the following protocol: 2 min *Taq*-polymerase activation at 95 C, 50 cycles of 5 sec denaturation at 94 C, 20 sec primer annealing at 60 C, and 10 sec extension at 72 C followed by a melt point analysis. For quantification serial dilutions of a cDNA generated from total RNA extracted from the brain of a Wistar rat were coamplified. Gene-specific mRNA expression was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which proved to be unaltered between obese and lean rats. GAPDH primer sequences: forward, 5′-TGC ATC CTG CAC CAC CAA CT-3′, reverse, 5′-ACG CCA CAG CTT TCC AGA GG-3′. All values were expressed as the ratio of Ob-Rb and GAPDH expression.

Immunoblotting

Frozen NK cells were suspended in buffer L [25 mM Tris/HCl (pH 7.4), 250 mM sucrose, 100 mM NaF, 10 mM $NaPP_i$, 1 mM Na_3VO_4, 1 mM EDTA, 20 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 100 μM 3-isobutyl-1-methylxanthine; 100 μl per 100,000 cells] containing 1% Nonidet P-40 and 1% sodium dodecyl sulfate at 4 C. After ultrasonic treatment (Branson B-12, microtip, output 4, 3 × 20 sec with 30 sec intervals, on ice), the lysates were centrifuged (1500 × *g*, 5 min, 4 C). The supernatants were removed and recentrifuged. The supernatants were removed and used for immunoprecipitation.

Cell lysates (20 μl) were diluted in 500 μl of immunoprecipitation (IP) buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 6 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 100 mM NaF, 10 μg/ml aprotinin, 1 mM benzamidine, and 100 μM phenylmethylsulfonyl fluoride] and then precleared with protein G/A-Sepharose (20 μl of 50 mg/ml IP buffer). After addition of antibodies [anti-JAK-2, 1:200; Upstate (Charlottesville,

VA); anti-PKB, 1:500; BIOMOL (Hamburg, Germany); antiglycogen synthase kinase (GSK)-3β, 1:100; anti-ERK-1, 1:50, Santa Cruz; anti-ERK-2, 1:50; Rockland (Gilbertsville, PA); anti-AMP-activated protein kinase (AMPK)α-pan, 1:50, Upstate; caveolin-1, 1:100; Transduction Laboratories, Heidelberg, Germany], each preadsorbed to 20 μl of protein G/A-Sepharose (50 mg/ml IP buffer), the mixtures were incubated (16 h, 4 C, under rotation) and then centrifuged (12,000 $\times$ *g*, 2 min, 4 C). The collected immune complexes were washed four times with 1 ml each of IP buffer, then two times with IP buffer containing 0.1% Nonidet P-40 and 500 mM NaCl, then two times with IP buffer lacking Nonidet P-40, and finally two times with IP buffer lacking Nonidet P-40 and NaCl. After dissolution of the immune complexes in 50 μl of 2-fold Laemmli sample buffer (5 min, 95 C) and centrifugation (12,000 $\times$ *g*, 2 min), the supernatant proteins (15 μl portions) were analyzed by SDS-PAGE (Novex 8–14%, Tris-glycine, precast gels with morpholinopropanesulfonic acid-sodium dodecyl sulfate running buffer) and immunoblotting.

Immunoblotting using chemiluminescent detection with primary antibodies against phosphotyrosine, JAK-2, PKBpT308, PKB, GSK-3βpS9, GSK-3β, AMPKαpT172, AMPKα-pan, phospho-ERK-1/2 (Upstate), ERK-1, and ERK-2 (Rockland) was performed as described previously (8). The amounts of protein recovered by the immunoprecipitation were evaluated by homologous immunoblotting and used for correction. Lumiimages were processed and quantified by computer-assisted video densitometry using the Lumiimager system (Roche).

cAMP levels were determined quantitatively from 5-μl portions of the lysates using a commercially available competitive enzyme-linked immunoassay kit according to the instructions of the manufacturer (Biotrend, Cologne, Germany). Because of the competition between cAMP in the sample and the cAMP alkaline phosphatase tracer for a limited amount of cAMP antiserum, the signal obtained with the assay will be inversely proportional to the amount of cAMP in the sample. This equilibration is performed in the wells of a 96-well plate precoated with mouse antirabbit IgG, which binds all of the cAMP antiserum added to the well. After the equilibration step, the plate is washed, and a solution of the alkaline phosphatase substrate, *para*-nitrophenyl phosphate, is added. Product formation of this enzymic reaction is monitored at 412 nm. The assay permits cAMP measurements within the range of 3–3000 pmol/ml, typically with a limit of quantification of 10 nM, intraassay and interassay precisions of 5–20%, and cross-reactivities with AMP and adenosine of less than 0.05%.

Statistics

Data are expressed as means + SEM. To analyze the effect of the leptin administration and the body weight on the different measured parameters, one-way-ANOVA was applied with treatment being the first factor and the measured parameter being the second factor. Least significant differences *post hoc* analysis was implemented to determine significant differences in the case of main treatment effects. Differences were considered significant if $P < 0.05$.

Results

An excess of total leptin but decreased serum levels of receptor-bound leptin in obese rats cannot be reversed by exogenous leptin

In the serum, the adipose tissue-derived hormone leptin circulates as a free hormone and in high-molecular-weight complexes formed with its soluble receptor (Ob-Re). Recent evidence reported by our group suggests that free and bound leptin are differentially regulated in various pathophysiological conditions and that bound leptin may serve independent functions (4, 29). Because binding of leptin to its soluble receptor might be essential for the bioavailability and the stabilization of the ligand, we performed RIAs and measured total and receptor-bound leptin fractions in the sera of chronically iv cannulated lean and diet-induced obese Lewis rats 4 h after an iv bolus injection of hrec-leptin (500 μg/kg) or vehicle. In the sera of obese rats, an excess of total leptin was observed. Figure 1B depicts the high serum levels of total leptin in obese control animals (410% of lean values) with only a small influence of the bolus injection of hrec-leptin on this leptin component. The challenge with hrec-leptin slightly increased the amount of bound leptin in lean and obese rats. However, obese animals still had significantly lower levels, compared with lean rats (64% *vs.* lean rats before and only 78% after the leptin administration). These data document the deficiency in producing receptor-bound leptin of diet-induced obese subjects and the lack of an effect of iv leptin administration in obese animals.

Leptin administration enhances blood NK cell numbers

Recent data strongly suggest that apart from its potent weight-reducing effects leptin can act as an immunomodulatory peptide *in vitro* and in normal weight subjects. The present study confirmed that leptin components and levels are differentially regulated by the nutritional status. However, no data exist showing the influence this has on numbers of immunocompetent NK cells in the blood of lean and obese subjects. To investigate whether NK cell numbers in the peripheral blood are modified in diet-induced obesity and whether they can be modulated by an exogenous leptin challenge, NK cell numbers were determined by monoclonal antibodies against the NKR-P1A and the T cell receptor in FACS analyses (Fig. 2, A and B). Leptin administration caused a marked increase in blood NK cells both in lean and obese animals (Fig. 2, A–C). However, under both conditions (control and leptin challenged animals), no significant differences between lean and obese animals were detectable.

Splenic NK cell numbers are increased and differently distributed in diet-induced obesity

To determine whether the leptin administration exerts comparable effects on tissue NK cells, the number of splenic NK cells in the four animal groups was investigated via immunohistological staining (with the same antibody used for FACS analyses) and consecutive counting by two scientists blinded to the treatment conditions. In lean animals the administration of hrec-leptin resulted in an increase of cells in the spleen. Interestingly, the number of splenic NK cells was significantly higher in obese *vs.* lean control animals (Fig. 3, A and B). The administration of leptin could not further increase the cell number in obese rats (Fig. 3C). As expected, the majority of NK cells were diffusely distributed in the red pulp. Nevertheless, in the sections of the obese animals, various NK cells could also be found in the white pulp. Figure 3 shows micrographs of representative spleen sections taken from lean (Fig. 3A) and diet-induced obese control animals (Fig. 3B). The significant difference of NK cell numbers between lean and obese control rats was also seen in the liver of the experimental animals. Leptin treatment further enhanced NK cell count in livers of lean and obese rats, resulting in significantly higher amounts of liver NK cells in obese leptin-challenged animals compared with corresponding lean littermates (Fig. 3D).

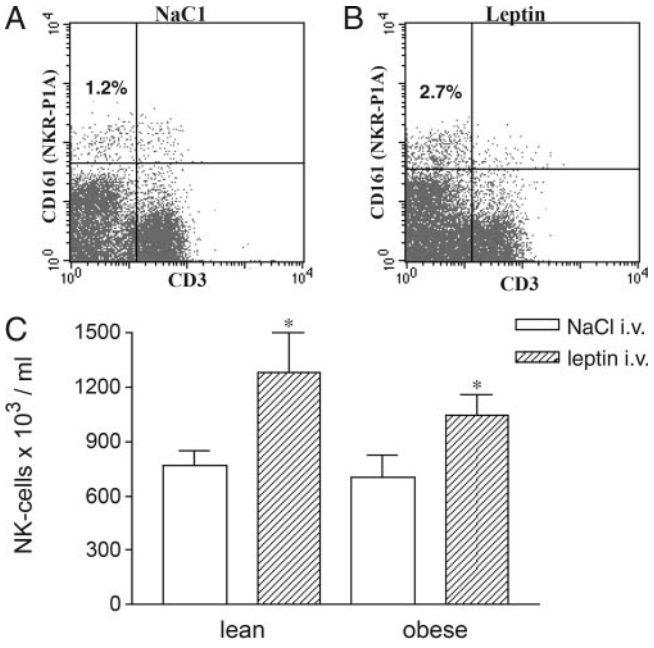

FIG. 2. Leptin enhances blood NK cells. Effect of hrec-leptin iv injections on the number of blood NK cells in chronically iv-cannulated lean and diet-induced obese Lewis rats 4 h after injection (n = 5 in each group). Representative dot plots show the NKR-P1A$^+$ (CD161)^{bright} events in a lean control (A), compared with a lean leptin-treated animal (B). C, Significant *post hoc* effects *vs.* the corresponding NaCl controls are indicated by an *asterisk*. *, $P < 0.001$. Data are expressed as mean + SEM.

Leptin activates NK cells in vivo exclusively in lean subjects

To evaluate whether the increased accumulation of NK cells after leptin treatment and the higher amount of NK cells in the spleen and liver of obese subjects also result in an increased activity, we measured the specific lysis of target (YAC-1) cells by blood and splenic NK cells of lean and diet-induced obese animals. Figure 4 delineates the lytic activity dependent on the body weight and treatment of rats. The iv leptin challenge had negligible effects on the NK cell activity in the obese experimental animals. However, leptin administration resulted in a 4-fold increase of NK cell activity in the blood of lean animals, compared with vehicle-treated lean animals, and a 2.8-fold enhancement of activity, compared with the corresponding obese littermates (Fig. 4A). The NK cell activity in the spleen of the obese subjects was likewise extremely low, resulting in an increase of the lytic activity of splenic NK cells from lean animals after leptin challenge by 405%, compared with the corresponding diet-induced obese rats (Fig. 4B).

Splenic NK cells of lean and obese animals express Ob-Rs in vivo

We next asked whether the leptin effects on NK cells solely seen in lean animals could be direct receptor-mediated effects. Therefore, we performed confocal laser-scanning microscopy of spleen tissue stained with a polyclonal antibody raised against all Ob-R isoforms and a monoclonal antibody against NKR-P1A. As expected, immunolabeling showed single positive cells for both antibodies (Fig. 5, A and B). It has been demonstrated that human NK cell lines express Ob-Rs (30). However, no data exist evaluating the *in vivo* Ob-R expression depending on nutritional status. We found numerous double-positive cells (NK cells expressing Ob-Rs) in the spleen tissue of both lean and diet-induced obese rats. Figure 5C demonstrates an NK cell positive for Ob-R from a diet-induced obese animal.

The expression of Ob-Rb is dramatically increased in diet-induced obesity

Because the extracellular domain of all Ob-R isoforms is identical, a determination of the subtype by antibody staining is not feasible. Thus, to evaluate the Ob-Rb expression quantitatively in blood NK cells, quantitative RT-PCR comparing the Ob-Rb mRNA expression in NK cells from lean and diet-induced obese animals was performed. As shown in Fig. 6, a significant 3.5-fold up-regulation of Ob-Rb mRNA was demonstrated in the obese rats when compared with lean littermates.

NK cells of obese animals are leptin resistant

To further test for a possible mechanism to explain the discrepancy between higher circulating leptin levels and increased leptin receptor expression in obese rats and the decreased activity of the cells in the obese, we measured leptin receptor-dependent intracellular signaling under basal conditions and after *in vivo* stimulation with leptin or using MADB106 tumor cells. Basally JAK-2, PKB, AMPKα, ERK2–2, GSK3β, and ERK-1 expression were evaluated by Western blotting (Fig. 7, A–F). Expression values compared well between lean and obese rats. *In vivo* stimulation with hrec-leptin resulted in a significant increase of JAK-2p, PKBpT308, and AMPKα-pT172 (Fig. 7, A–C) in NK cells from lean animals. However, NK cells from obese animals were markedly resistant to leptin stimulation, suggesting a functional desensitization of leptin signaling in NK cells of obese animals. The observed resistance affects distinct signaling components: JAK-2p, PKBpT308, AMPKα-pT172, and ERK-2p. Leptin-induced enhancement of GSK3β-pS9 and ERK-1p (Fig. 7, E and F) was comparable in both groups of NK cells.

Neither leptin nor tumor cells affected levels of c-AMP (data not shown). Sensitivity/resistance was not markedly altered by an additional challenge with tumor cells (supplemental Fig. 8, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org).

Discussion

Our detailed *in vitro* and *in vivo* analyses show for the first time the leptin resistance of NK cells from obese rats. NK cells accumulate in the spleen and liver of obese control animals. A leptin challenge increased the number of splenic and liver NK cells in lean animals without reaching the amount in obese littermates. We demonstrate that the shift from bound to free leptin observed in obese subjects cannot be modulated by leptin treatment. Leptin activates NK cells exclusively in lean animals. Confocal laser-scanning microscopy showed the expression of Ob-Rs on NK cells from both lean and obese animals. However, qRT-PCR revealed significantly higher Ob-Rb mRNA levels in

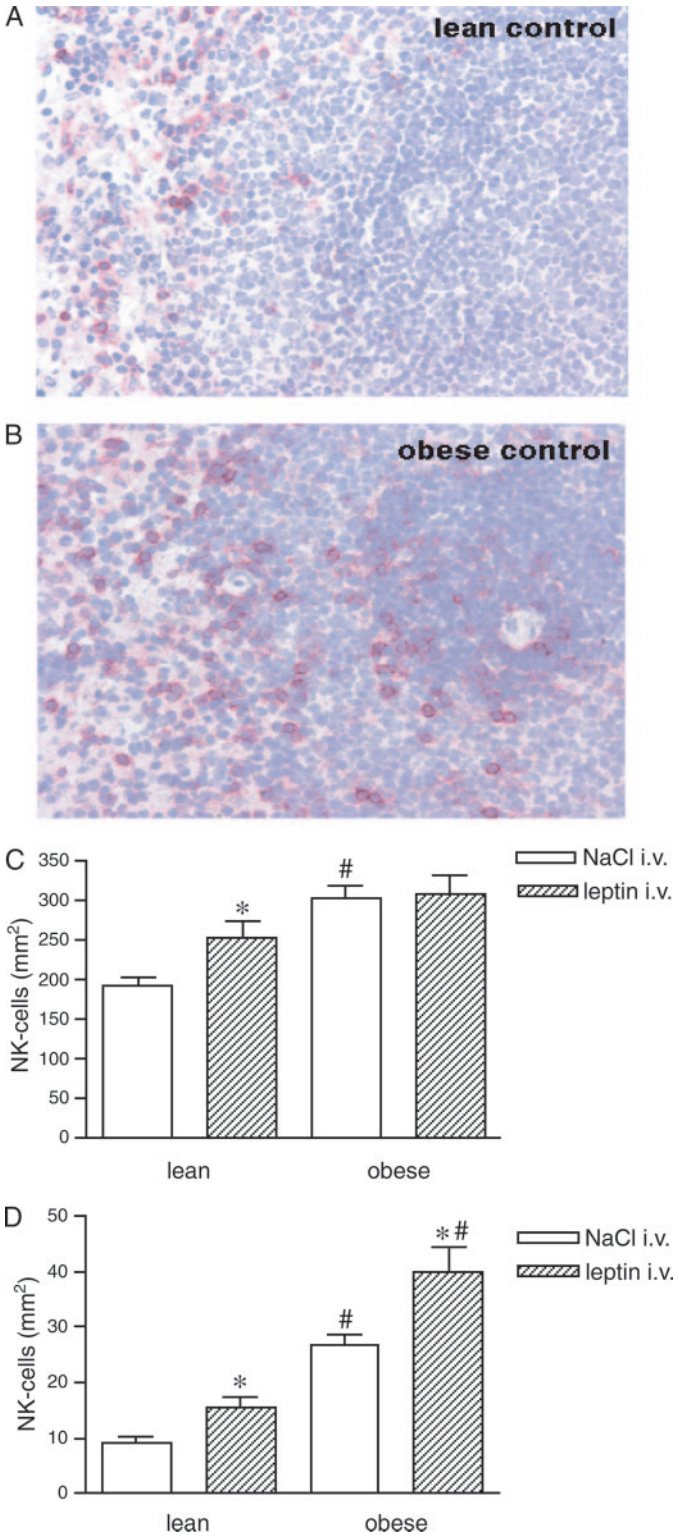

FIG. 3. Differential distribution of splenic NK cells is dependent on iv leptin treatment and body weight in Lewis rats. A and B, Representative immunohistological detection of NK cells (NKR-P1A$^+$, *red*) in cryostat sections of the spleen of a lean (A) and an obese (B) control animal. In B, several NK cells have infiltrated the white pulp. C and D, Numbers per square millimeter (mean + SEM) of splenic (C) or liver (D) NK cells 4 h after iv injection of NaCl or hrec-leptin (n = 5 in each group). Significant *post hoc* effects *vs.* the corresponding NaCl controls are indicated by an *asterisk*. *, $P < 0.001$. Significant *post hoc* effects of obese *vs.* lean animals within one treatment group (NaCl or leptin) are indicated by a *rhomb*. #, $P < 0.001$.

NK cells from obese than from lean rats. All these observations point toward an increased activity of innate immunity in obesity. However, mechanistically, after receptor signaling is abrogated in obese animals with significantly lower activation of postreceptor signaling components (JAK-2p, PKBpT308, AMPKα-pT172) after an *in vivo* leptin challenge.

Excess body weight is directly associated with risk of cancer at several organ sites, such as colon, breast (in postmenopausal women), and lung (23, 25, 31). However, the mechanisms by which obesity promotes the development of cancer are still unknown. The role of leptin in the modulation of the innate and acquired immune system is evident (10, 32). Because NK cells are the central active component of the host's immune system in the early phase of cancer development and metastasis, we focused on NK cell function in obesity.

Leptin was shown to increase the growth of human colon and breast cancer cell lines by up-regulation of MAPK and STAT3 signaling pathways (33, 34). In contrast, several reports delineate that leptin has opposite effects *in vivo* (35). Because it is known that leptin influences both adaptive and innate immune functions, *i.e.* by interaction with its specific receptors expressed on T lymphocytes (32), it can be assumed that leptin may also influence NK cells, the multifunctional effector cells against a variety of neoplastic diseases. Our data concerning the increase of NK cell activity in normal-weight animals parallel investigations by Tian and colleagues (30, 36) and a study of Dovio *et al.* (37) showing a comparable enhanced activity of NK cells from lean and obese humans after IL-2 stimulation. Induction of NK cell proliferation and activation may be suppressed by leptin, which has been linked to inhibition of hepatocellular carcinoma cells *in vitro* (38). However, our data suggest that this stimulatory effect of leptin on NK cells activity is lacking in diet-induced obese rats.

To further clarify the apparent difference between high circulating leptin levels but lack of response to a leptin challenge, we investigated leptin signaling pathways in both lean and diet-induced animals. Several immune cells, such as T and B lymphocytes and monoyctes express the Ob-R(a+b). Thus, it is likely that NK cells also express isoforms of the Ob-R, so the above-mentioned induction of proliferation and activation of NK cells may be direct receptor-mediated effects of leptin. It was shown that the Ob-Rb isoform is the crucial one for leptin-mediated changes in the immune system (9). Both RT-PCR and immunofluorescence analyses clearly support that Ob-Rb (mRNA) is expressed on NK cells *in vivo*. In contrast to expectations, no receptor down-regulation was found in NK cells from obese experimental animals, leading to a reduced responsiveness upon leptin challenge, but Ob-Rb was substantially up-regulated in diet-induced obesity.

To further explain the discrepancy to the reduced biological activity of NK cells, we speculated that downstream leptin signaling is desensitized. Levels of JAK-2p, PKBpT308, and AMPKα-pT172 were significantly increased after a bolus injection of hrec-leptin in lean, but no effect could be detected in obese rats. However, JAK-2-

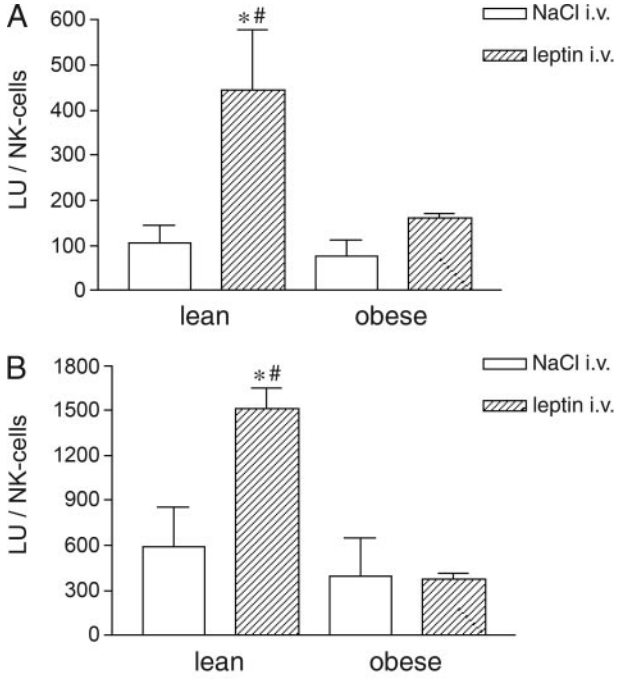

FIG. 4. Leptin activates NK cells only from lean subjects. NK cell cytotoxicity assays reveal high lytic activities in the blood (A) and spleen (B) of lean Lewis rats 4 h after an iv injection of hrec-leptin, compared with the three other groups (n = 5 in each group). Leptin treatment produces no effect in obese animals. Significant *post hoc* effects *vs.* the corresponding NaCl controls are indicated by an *asterisk*. *, $P < 0.001$. Significant *post hoc* effects of the lean *vs.* the obese leptin-treated animals are indicated by a *rhomb*. #, $P < 0.001$. Data are expressed as mean + SEM.

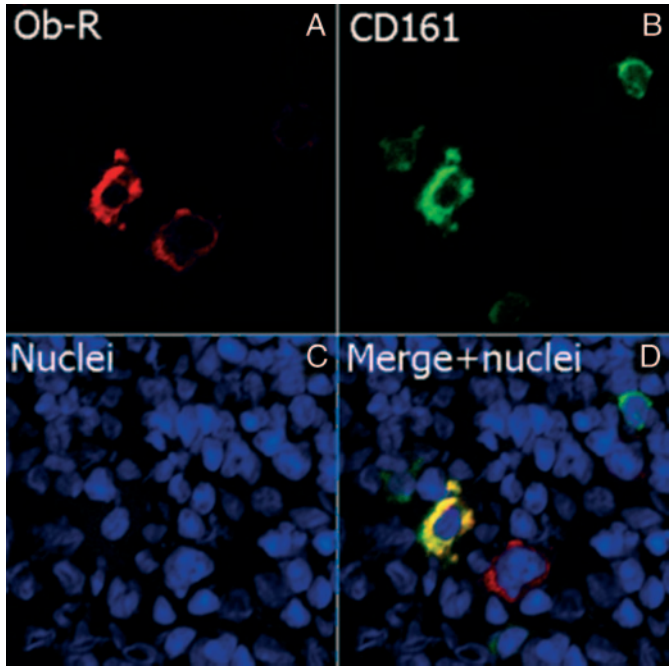

FIG. 5. Splenic NK cells of both lean and obese Lewis rats express the Ob-R *in vivo*. Immunofluorescent staining for Ob-R (*red*, A) and NK cells (NKR-P1A$^+$, CD161, *green*, B) in a representative spleen section of an obese animal. C, Nuclei are stained *blue* with To-Pro-3. D, Besides single-positive cells confocal analyses clearly show various double-positive cells (NK cells expressing Ob-Rs). Single-positive cells for Ob-R are predominantly T lymphocytes. NK cells expressing Ob-Rs are also found in lean animals.

independent signaling components (*e.g.* ERK-1) were not altered in NK cells of obese animals, suggesting that an abrogated JAK/STAT pathway may be the underlying mechanism for the leptin-resistant NK cells of the obese experimental animals. Because proliferation and migration of immune and cancer cells appear to be dependent on ERK-1/2 signaling (39), our results of a comparable level of blood NK cells in lean and obese animals are in line with an unstimulated ERK pathway in obesity. The mechanism underlying this dissociation of intracellular leptin signaling pathways is unclear. In liver-specific insulin receptor knockout mice, hyperinsulinemia is combined with high leptin levels and greatly increased levels of Ob-R. These mice exhibit, in contrast to animals with diet-induced obesity, a normal or even increased leptin sensitivity. Dysregulation of suppressor of cytokine signaling (SOCS)-3 appears to be the most important regulator because SOCS-3 is increased in diet-induced obesity as a mediator of leptin resistance (40), whereas in liver-specific insulin receptor knockout mice, SOCS-3 levels were not affected. Our model with increased leptin receptor expression and a slightly increased formation of leptin binding fit to such regulatory pattern. The dissociation of leptin-dependent signaling pathways in our model fit, however, better to the up-regulation of SOCS-3 found in diet-induced obesity as a basis of SOCS-3-dependent leptin insensitivity (41, 42).

In conclusion, our data are compatible with a model in which leptin levels increase acutely as part of an acute-phase response to inflammation (43). This increases through ERK signaling pathway NK cell numbers but due to the dissociation of leptin signaling with desensitization of JAK/STAT-dependent pathways, NK cell activity is stimulated only in lean but not the obese animals. Thus, as in cancer, the proin-

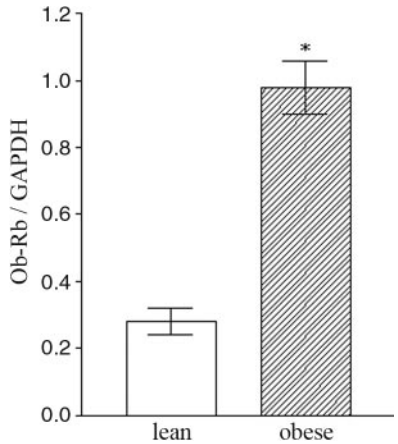

FIG. 6. Ob-Rb mRNA expression on blood NK cells is dramatically increased in diet-induced obesity. NK cells from lean and obese Lewis rats (n = 5 in each group) were purified, subjected to RNA isolation, and quantitative RT-PCR was performed as described in detail in the *Materials and Methods*. Values are expressed as the ratio of Ob-Rb and GAPDH expression. Significant *post hoc* effects of the obese *vs.* the lean animals are indicated by an *asterisk*. *, $P < 0.001$. Data are expressed as mean ± SEM.

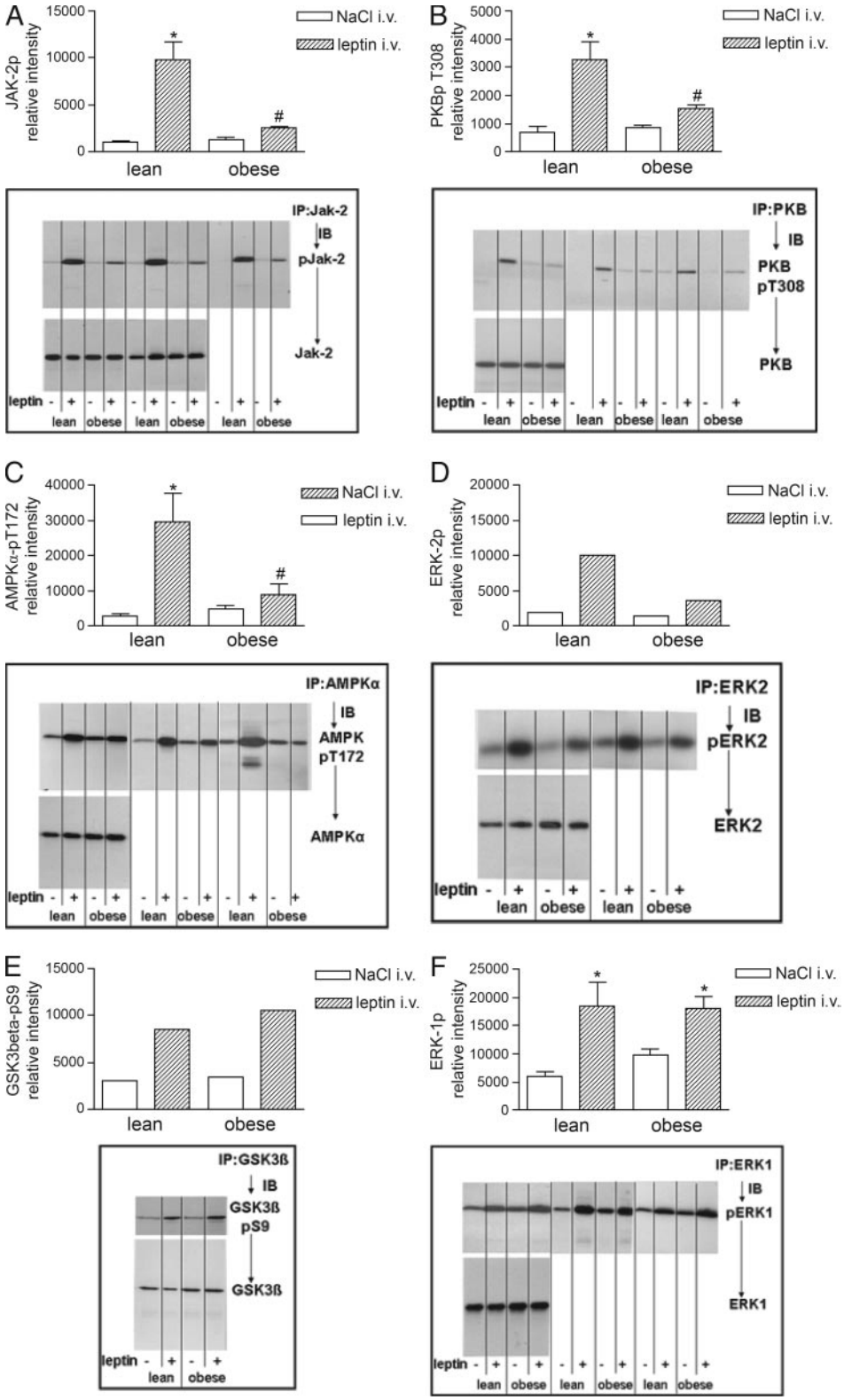

FIG. 7. Resistance of JAK-2-dependent leptin signaling in NK cells of obese F344 rats. JAK-2p (A), PKBpT308 (B), AMPKα-pT172 (C), ERK-2p (D), GSK3β-pS9 (E), and ERK-1p (F) were immunoprecipitated (IP) with appropriate antibodies (see *Materials and Methods*) from NK cells of lean and obese rats that had been pretreated with hrec-leptin or NaCl. Quantitative data are presented in means + SEM corrected for amount of immunoprecipitated protein actually applied onto gel. For the analysis of ERK-2p (D) and GSK3β-pS9 (E), number of animals were lower than n = 3 per group; thus, no SEM could be calculated. Significant *post hoc* effects *vs.* the corresponding NaCl controls are indicated by an *asterisk*. *, $P < 0.001$. Significant *post hoc* effects of the obese leptin-treated animals *vs.* the corresponding lean animals are indicated by a *rhomb*. #, $P < 0.001$. Data of animals receiving an additional challenge with MADB106 tumor cells are presented in supplemental Fig. 8.

flammatory state of obesity may induce an attenuated response of immune-competent cells to an acute endogenous or exogenous leptin challenge and inhibit the promigratory and activating effects of a leptin challenge on NK cells.

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