

# Leptin Induces Macrophage Lipid Body Formation by a Phosphatidylinositol 3-Kinase- and Mammalian Target of Rapamycin-dependent Mechanism\*

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Leptin is an adipocyte-derived hormone/cytokine that links nutritional status with neuroendocrine and immune functions. Lipid bodies (lipid droplets) are emerging as dynamic organelles with roles in lipid metabolism and inflammation. Here we investigated the roles of leptin in signaling pathways involved in cytoplasmic lipid body biogenesis and leukotriene B<sub>4</sub> synthesis in macrophages. Our results demonstrated that leptin directly activated macrophages and induced the formation of adipose differentiation-related protein-enriched lipid bodies. Newly formed lipid bodies were sites of 5-lipoxygenase localization and correlated with an enhanced capacity of leukotriene B<sub>4</sub> production. We demonstrated that leptin-induced macrophage activation was dependent on phosphatidylinositol 3-kinase (PI3K) activity, since the lipid body formation was inhibited by LY294002 and was absent in the PI3K knock-out mice. Leptin induces phosphorylation of p70<sup>S6K</sup> and 4EBP1 key downstream signaling intermediates of the mammalian target of rapamycin (mTOR) pathway in a rapamycin-sensitive mechanism. The mTOR inhibitor, rapamycin, inhibited leptin-induced lipid body formation, both *in vivo* and *in vitro*. In addition, rapamycin inhibited leptin-induced adipose differentiation-related protein accumulation in macrophages and lipid body-dependent leukotriene synthesis, demonstrating a key role for mTOR in lipid body biogenesis and function. Our results establish PI3K/mTOR as an important signaling pathway for leptin-induced cytoplasmic lipid body biogenesis and adipose differentiation-related protein accumulation. Furthermore, we demonstrate a previously unrecognized link between intracellular (mTOR) and systemic (leptin) nutrient sensors in macrophage lipid metabolism. Leptin-induced increased formation of cytoplasmic lipid bodies and enhanced inflammatory mediator production in macrophages may have implications for obesity-related cardiovascular diseases.

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All organisms have evolved systems to sense nutrient availability and transduce this information into changes in metabolic pathways and networks. In vertebrates, a complex physiological system regulates fuel stores and energy expenditure. Leptin, a hormone/cytokine mainly secreted by adipocytes, is an important component of this system (1, 2). Leptin was described initially by its properties of inhibiting feeding and increasing energy expenditure mainly by actions on the hypothalamic axis (1, 2). In addition, leptin can act as an early acute phase reactant; it is produced in high levels during inflammatory conditions and is capable of modulating the innate and adaptive immune responses (3, 4).

Recent studies suggest that increased leptin levels may play roles in obesity-related cardiovascular diseases, including atherosclerosis (5). Indeed, leptin may exert an atherogenic effect, including endothelial dysfunction (6), proliferation of smooth muscle cells (7), inflammatory mediator production (8, 9), and modulation of platelet function (10). Formation of lipid-laden foam cells is a key event in early atherogenesis, which contributes to the formation of atherosclerotic plaques. Although leptin has demonstrated effects in lipid metabolism in adipocytes, their roles in macrophages in regulating lipid accumulation and foam cell formation are not well understood. Lipid-laden macrophage foam cell formation involves complex and multistep mechanisms that depend on different signaling pathways regulating lipid influx, metabolism, storage, and mobilization (11). Accumulating data suggest that leptin may differentially regulate intracellular lipid storage in adipocytes and macrophages. Indeed, leptin regulates key enzymes involved in lipid metabolism in macrophages, including hormone-sensitive lipase, acyl CoA: cholesterol acyl transferase, and neutral cholesteryl esterase, suggesting that leptin may favor lipid accumulation in macrophages (12).

In different cell types, including macrophages, intracellular lipids are stored and metabolized in hydrophobic organelles called lipid bodies or lipid droplets. The accumulation of lipids in these lipid bodies is of major interest for the study of atherosclerosis, obesity, and other inflammatory diseases. Cytoplasmic lipid bodies are osmiophilic structures surrounded by a phospholipid monolayer with a unique fatty acid composition and a neutral lipid rich core and contain a variable protein composition (13, 14). In addition to lipids, lipid bodies compartmentalize a diverse group of proteins. Adipose differentiation-

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related protein (ADRP)<sup>3</sup> is a major structural protein associated with lipid bodies in different cell types, including macrophages (15–17). ADRP is a member of the PAT family (perilipin, ADRP, TIP47) of proteins and plays an important role in adipocyte differentiation. More recently, it has been associated with increased lipid accumulation in lipid bodies and considered to play an active role on the lipid body assembling and lipolysis modulation (15, 18, 19). Moreover, a number of enzymes and signaling proteins were shown to be associated with lipid bodies, including fatty acid metabolic enzymes, eicosanoid-forming enzymes, specific kinases, and small GTPases (20–27). Therefore, lipid bodies are now viewed as dynamic organelles for integrating lipid metabolism, inflammatory mediator production, membrane trafficking, and intracellular signaling (28, 29).

The direct role of leptin in macrophage lipid metabolism and foam cell formation has not been well established. Here we investigated the ability of leptin to trigger lipid body biogenesis and the LTB<sub>4</sub> synthetic function of macrophage lipid bodies. Leptin effects on lipid body formation and LTB<sub>4</sub> generation are strictly dependent on PI3K and mTOR. Our results demonstrate new activities of leptin in inflammation and lipid metabolism and establish new signaling pathways for leptin in macrophages.

### EXPERIMENTAL PROCEDURES

**Materials**—Murine recombinant leptin was purchased from Sigma and from Peprotech (London, UK) and was devoid of LPS contamination, as attested by the fabricants and demonstrated by LAL (<0.01 EU/ml). Calcium ionophore A23187 was obtained from Calbiochem. LY294002 and rapamycin were from Sigma.

**Animals**—C57Bl/6, C3H/HeJ, and C3H/HeN male mice were from the Oswaldo Cruz Foundation Breeding Unit and were raised and maintained under controlled housing conditions. PI3K $\gamma$ -deficient mice were obtained in sv129 mice as described (30) and were back-crossed until the 5th generation in C57Bl/6 background. The sv129xC57Bl/6 mixed wild type and C57Bl/6 were used as wild type controls. No differences between C57Bl/6 and the mixed background (sv129xC57Bl/6) in leptin response were observed (not shown). Animals were maintained and treated according to the animal care guidelines, in accordance to the National Institutes of Health recommendations. The Oswaldo Cruz Animal Welfare Committee approved all protocols used in this study.

**In Vivo Peritoneal Stimuli**—The intraperitoneal administration of leptin (0.25, 0.5, 1, or 2 mg/kg) or vehicle (sterile saline) in a final volume of 0.1 ml was used in the *in vivo* experiments. Animals were killed in a CO<sub>2</sub> chamber at different time points (1, 6, and 24 h) after leptin administration. The peritoneal cavity was rinsed with 3 ml of Hanks' balanced salt solution (HBSS). Total leukocyte counts were performed in Neubauer cham-

bers, and differential leukocyte counts were performed on cytopsin smears after May-Grünwald-Giemsa. Alternatively, animals received three intraperitoneal injections of rapamycin (15  $\mu$ g/kg) or vehicle, in a final volume of 50  $\mu$ l, 12 h and 15 min before and 12 h after the injection of leptin or saline. The peritoneal wash was analyzed at the 24 h time point of leptin administration.

**In Vitro Peritoneal Macrophage Stimulation**—Peritoneal macrophages (>90% macrophages) were collected by rinsing the peritoneal cavity from naive mice with 4 ml of cold HBSS. Macrophages (10<sup>6</sup> cells/ml) were stimulated with leptin (20 nM) or vehicle for 30 min or 4 h in RPMI medium with 2% fetal bovine serum. In designated groups, cells were pretreated with LY294002 (0.5  $\mu$ M) or rapamycin (20 nM) 30 min before the addition of the stimuli. Cell viability was always greater than 85%, as determined by trypan blue dye exclusion at the end of each experiment.

**Lipid Body Staining and Counting**—While still moist, leukocytes on cytopsin slides were fixed in 3.7% formaldehyde in HBSS (pH 7.4) and were stained with osmium tetroxide, Oil Red O, or BODIPY<sup>®</sup> 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene). For the osmium staining, the slides were rinsed in 0.1 M cacodylate buffer, incubated with 1.5% OsO<sub>4</sub> (30 min), rinsed in H<sub>2</sub>O, immersed in 1.0% thiocarbonylhydrazide (5 min), rinsed in 0.1 M cacodylate buffer, reincubated in 1.5% OsO<sub>4</sub> (3 min), rinsed in distilled water, and then dried for further analysis. The morphology of fixed cells was observed, and lipid bodies were enumerated by light microscopy with a  $\times$ 100 objective lens in 50 consecutive leukocytes in each slide. The person responsible for counting was blinded to the codes for each slide. Slides were alternatively stained with Oil Red O or BODIPY, evidencing the accumulation of neutral lipids in lipid bodies. The measurement of the area of lipid bodies was done with the BODIPY fluorescent images, obtained with a  $\times$ 60 objective (at least four fields per slide). The images were transformed into black and white pictures and analyzed with Image 2D (GE Healthcare). The spots were determined by automatic spot detection, and the total area of fluorescent lipid bodies was obtained for each field and divided by the number of cells in the respective field.

**Western Blotting**—Cell lysates were prepared in reducing and denaturing conditions and subjected to SDS-PAGE. Samples were submitted to electrophoresis in 5–15% acrylamide gradient SDS-polyacrylamide gels. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline-Tween (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Membranes were probed with anti-ADRP polyclonal antibody (Research Diagnostics Inc., Flanders, NJ), anti-phospho-p70<sup>S6K</sup> or anti-phospho-4EBP1 (Cell Signaling, Danvers, MA), or anti- $\beta$ -actin monoclonal antibody (BD Transduction Laboratories) in TBST with 1% nonfat dry milk. Proteins of interest were then identified by incubating the membrane with horseradish peroxidase-conjugated secondary antibodies in TBST, followed by detection of antigen-antibody complexes by Supersignal chemiluminescence (Pierce). The detection was done by exposing the membrane to autoradiography film, or the luminescence was captured on the Typhoon blot imager (GE Healthcare). For the

<sup>3</sup> The abbreviations used are: ADRP, adipose differentiation-related protein; 5-LO, 5-lipoxygenase; mTOR, mammalian target of rapamycin; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; PI3K, phosphatidylinositol 3-kinase; LPS, lipopolysaccharide; HBSS, Hanks' balanced salt solution; mTOR, mammalian target of rapamycin.

densitometry analysis, the Typhoon images or the images from developed films were analyzed in the software Image 2D (GE Healthcare). The spotting and the analysis parameters were performed by a colleague blind to sample identity.

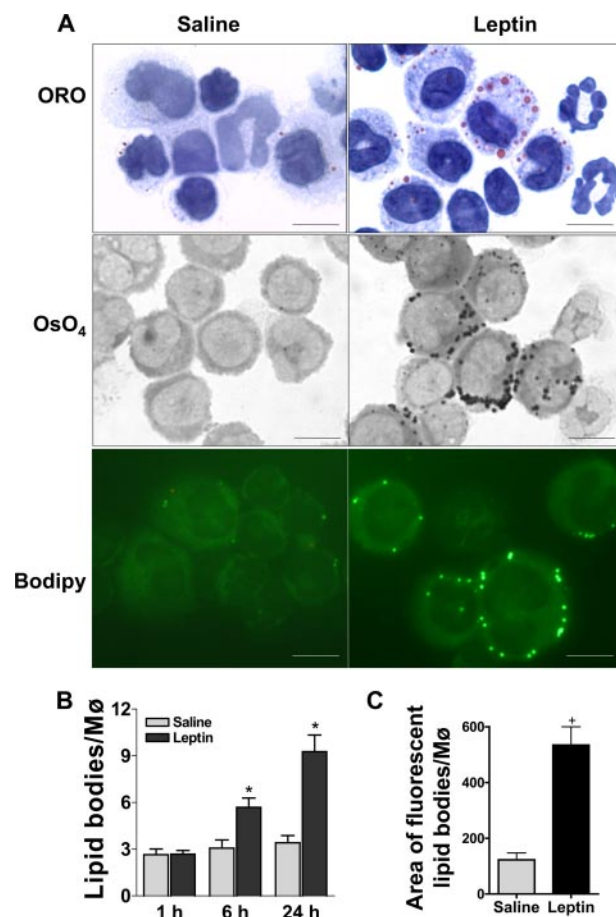
**Immunolocalization**—Cells were recovered from leptin- or saline-injected mice (24 h), and cytospin smears were immediately fixed in 3% formaldehyde for 10 min. After permeabilization in 0.2% Triton X-100 for 20 min at room temperature, slides were washed twice in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. Cell preparations were then incubated with polyclonal antibody anti-5-LO (Cayman Chemical, Ann Arbor, MI) for 30 min, followed by incubation with anti-ADRP polyclonal antibody (Research Diagnostics Inc., Flanders, NJ) antibody for an additional 30 min at room temperature. As control for the anti-5-LO, normal rabbit serum was used in separate slides. The cells were washed twice in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS for 10 min and incubated with secondary antibodies Cy3-conjugated donkey anti-guinea pig IgG and Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch). Slides were then washed with HBSS and mounted with an aqueous mounting medium (Vectashield; Vector, Burlingame, CA). Slides were viewed by both phase-contrast and fluorescence microscopy, and digital images were obtained using a Hoper Scientific digital camera with the Image-Express software.

**$\text{LTB}_4$  Assay**—The peritoneal cells were resuspended ( $10^6$  cells/ml) in HBSS containing  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$  and then stimulated with A23187 ( $0.5 \mu\text{M}$ ) for 15 min. Reactions were stopped on ice, and the supernatant was obtained after centrifugation at  $500 \times g$  for 10 min at  $4^\circ\text{C}$ .  $\text{LTB}_4$  was assayed by an enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical).

**Statistical Analysis**—Data were reported as the mean  $\pm$  S.E. and were analyzed statistically by means of analysis of variance followed by Newman-Keuls-Student's test or Student's *t* test with the level of significance set at  $p < 0.05$ .

## RESULTS

**Leptin Triggers Biogenesis of ADRP-enriched Lipid Bodies within Macrophages**—Lipid bodies in leukocytes and other cell systems are increasingly recognized as organelles with key roles in lipid metabolism, signaling compartmentalization, and inflammation (28, 29). Leptin activates cells of the immune system and modulates cellular lipid metabolism and storage (3, 4). We hypothesized a role for leptin in regulating macrophage lipid body biogenesis and functions. As shown in Fig. 1A, leptin-induced lipid body biogenesis in macrophages as seen by osmium ( $\text{OsO}_4$ ), oil red O (ORO), and BODIPY staining of the cells collected from the peritoneal cavity. The increase in lipid bodies was significant within 6 h and maximum within 24 h after peritoneal injection (Fig. 1B). In addition, BODIPY-labeled lipid bodies were quantitated by densitometric image analysis as a measure of lipid body area and neutral lipid accumulation per cell, confirming the effect of leptin in inducing lipid body biogenesis within 24 h (Fig. 1C). Although intraperitoneal administration of leptin induced significant recruitment of neutrophils to the peritoneal cavity, these neutrophils were not stimulated to form lipid bodies (Fig. 1A). Leptin also induced lipid body formation in peritoneal



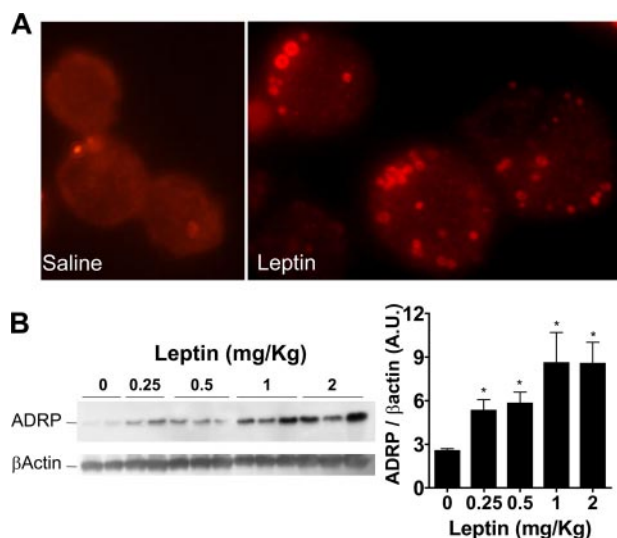
**FIGURE 1. Leptin-induced lipid body formation in peritoneal macrophages *in vivo*.** Lipid body formation was evaluated in peritoneal leukocytes 24 h after the intraperitoneal injection of leptin (1 mg/kg) or saline in C57BL/6 mice. A, microscopy images obtained from macrophages after stimulation with saline or leptin, stained with oil red O (ORO) (top), osmium-stained (middle), or labeled with BODIPY 493/503 (bottom). Bars, 10  $\mu\text{m}$ . B, kinetics of lipid body formation after stimulation (1, 6, and 24 h) with saline (gray columns) or leptin (1 mg/kg) (black columns). Lipid bodies were enumerated using osmium staining. Results were expressed as mean  $\pm$  S.E. from 6–8 animals. C, BODIPY-labeled lipid bodies were quantified by the measurement of the area of fluorescence per cell. Data on the graph correspond to the mean of four animals.

macrophages stimulated *in vitro* (control,  $2.51 \pm 0.49$ ; leptin,  $6.53 \pm 0.88$ ;  $p < 0.01$ ,  $n = 6-8$ ), pointing to a direct effect of leptin on macrophages.

TLR4-dependent signaling has been implicated in obesity and insulin resistance (31). We excluded the possibility that the observed effects of leptin on macrophage lipid body formation could involve TLR4 or was due to LPS contamination. For this experiment, we used a TLR4-defective LPS-hyporesponsive mouse strain, C3H/HeJ, that is unable to form new lipid bodies when treated with LPS (32). The intraperitoneal injection of leptin in C3H/HeJ mice led to significant macrophage lipid body formation within 24 h (control,  $3.28 \pm 1.18$ ; leptin,  $7.47 \pm 1.89$ ;  $p < 0.05$ ). Moreover leptin samples were negative for LPS contamination in the LAL test ( $<0.01$  EU/ml), indicating that LPS is not involved in the observed leptin response.

Next, we investigated the effects of leptin in ADRP expression. There is great interest in the regulation of ADRP as a key event on both adipocyte differentiation and macrophage foam cell formation. ADRP is a structural protein that sur-

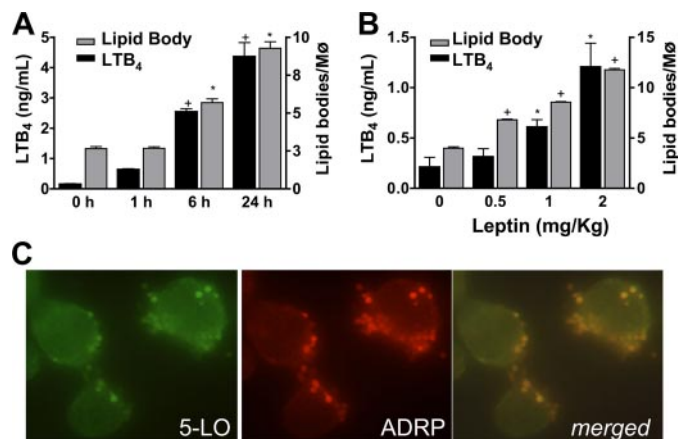
## Leptin Induces Macrophage Lipid Accumulation



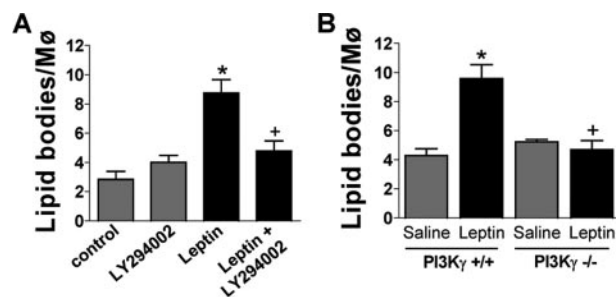
**FIGURE 2. Leptin-induced enhanced ADRP accumulation within macrophages.** Peritoneal macrophages were obtained from leptin-stimulated (0.25, 0.5, 1, or 2 mg/kg) or saline-stimulated C57BL/6 mice within 24 h after the intraperitoneal injection. *A*, peritoneal macrophages obtained from saline or leptin (1 mg/kg) were immunolabeled with anti-ADRP antibody followed by a Cy3-secondary antibody. *B*, total macrophage cell lysates ( $5 \times 10^5$  cells/lane) were separated by SDS-PAGE and submitted to Western blotting for ADRP or  $\beta$ -actin as indicated. The image is representative of at least two different blots. The graph represents the densitometric analysis (arbitrary units (A.U.)) of the Western blotting bands ( $n = 6$  for each concentration). Each bar represents the mean  $\pm$  S.E. Statistically significant ( $p < 0.05$ ) difference between control and leptin-stimulated groups are indicated by asterisks.

rounds lipid bodies in different cell types (17), including macrophages (15, 16, 33). Leptin-induced lipid body accumulation in peritoneal macrophages stimulated *in vivo* for 24 h was accompanied by an enhanced ADRP content in these cells (Fig. 2). The comparison between control and leptin by immunofluorescence images (Fig. 2A) and Western blotting (Fig. 2B) shows a concentration-dependent accumulation of ADRP on leptin-stimulated macrophages (Fig. 2B).

**Leptin Induces Time- and Concentration-dependent Priming for  $LTB_4$  Production and 5-LO Localization in Lipid Bodies**—Macrophage lipid bodies are sites of intracellular localization of leukotriene-forming enzymes, store arachidonic acid, and are sites for eicosanoid synthesis (22, 32, 33). We analyzed whether leptin-induced increase in lipid body is associated with  $LTB_4$  production (Fig. 3). After macrophages were elicited *in vivo* with leptin, lipid bodies were enumerated, and the isolated leukocytes were stimulated with the calcium ionophore, A23187 (0.5  $\mu$ M). Leptin-induced priming for enhanced  $LTB_4$  generation was concordant with the increases in lipid body numbers and presented an identical temporal sequence ( $r = 0.97$ ;  $p < 0.001$ ) (Fig. 3A). The capacity for synthesizing  $PGE_2$  was also enhanced by leptin (saline,  $9.4 \pm 1.7$  ng/ml; leptin,  $26.7 \pm 4.2$  ng/ml). Leptin-induced lipid body formation and priming for  $LTB_4$  generation occur in a dose-dependent manner, significant with 0.5 mg/kg and maximum after 2 mg/kg of leptin within 24 h (Fig. 3B). We investigated the capacity of leptin-induced macrophage lipid bodies to compartmentalize the leukotriene-synthesizing enzyme 5-lipoxygenase. We analyzed the intracellular compartmentalization of 5-LO by immunofluorescence, employing conditions of leukocyte fixation and per-



**FIGURE 3. Leptin-induced priming for  $LTB_4$  production and 5-LO localization within macrophage lipid bodies.** *A*, kinetics of leptin-induced parallel enhancement of lipid body formation (gray column) and priming for  $LTB_4$  production (black column). Peritoneal macrophages were obtained at different times after intraperitoneal leptin administration (1 mg/kg). *B*, dose-dependent effect of leptin-induced lipid body formation (gray column) and enhanced  $LTB_4$  production (black column). Peritoneal macrophages were obtained 24 h after intraperitoneal leptin administration (0.5–2 mg/kg). Macrophages ( $1 \times 10^6$  cells/ml) were resuspended in HBSS containing  $Ca^{2+}/Mg^{2+}$  and restimulated with A23187 (0.5  $\mu$ M) for 15 min, and  $LTB_4$  was measured in the supernatants by an enzyme immunoassay. Each bar represents the mean  $\pm$  S.E. from at least six animals. The statistically significant differences between control and leptin-stimulated animals are indicated for  $LTB_4$  (+) or lipid body (\*). *C*, leptin-stimulated leukocytes (24 h) were immunolabeled with anti-ADRP for lipid body detection (left) and with anti-5-LO (middle). The merged image is shown on the right.



**FIGURE 4. Leptin-induced macrophage lipid body formation depends on PI3K.** *A*, lipid body counting in peritoneal macrophages after *in vitro* incubation for 4 h with leptin (20 nM) alone or in the presence of LY294002 (0.5  $\mu$ M) as indicated. \*, statistically significant differences between leptin and control; +, statistically significant difference between LY294002-treated and leptin-stimulated groups,  $n = 3$ . *B*, the analyses of macrophage lipid body formation *in vivo* in the PI3K $\gamma^{-/-}$  or PI3K $\gamma^{+/+}$  (wild type) mice were performed 24 h after leptin (1 mg/kg) or saline intraperitoneal administration. \*, statistically significant differences between saline and leptin on PI3K $\gamma^{+/+}$ ; +, statistically significant difference between leptin-treated PI3K $\gamma^{-/-}$  and PI3K $\gamma^{+/+}$  (wild type) mice,  $n = 4-6$ .

meabilization that prevent dissolution of lipid bodies. Macrophages stained with rabbit polyclonal antibody to 5-LO showed punctate cytoplasmic localizations fully consistent in size, form, number, and distribution of lipid bodies (Fig. 3C). Leptin-induced 5-LO localization within cytoplasmic lipid bodies was confirmed by co-localization of 5-LO with ADRP (Fig. 3C). Only ADRP was detected when control nonimmune rabbit serum was used as control for 5-LO antibody (not shown).

**Leptin-induced Macrophage Activation Is Dependent on the PI3K Pathway**—As shown in Fig. 4A, LY294002 completely abrogated leptin-induced lipid body formation in peritoneal macrophages stimulated *in vitro*. To determine the role of PI3K $\gamma$  in the macrophage activation induced by leptin, we

examined lipid body formation in wild type mice or mice genetically deficient in PI3K $\gamma$  24 h after leptin intraperitoneal administration. Leptin-induced macrophage lipid body formation *in vivo* in the peritoneal cavity was completely abolished in PI3K $\gamma^{-/-}$  mice in comparison with wild type control animals (Fig. 4B). These *in vivo* and *in vitro* results identify PI3K $\gamma$  as a critical signaling pathway in leptin-induced macrophage activation.

**Leptin-induced Lipid Body Formation Depends on Mammalian Target of Rapamycin (mTOR)**—The mTOR pathway integrates insulin and nutrient signaling in numerous cell types and the main upstream signaling mechanism for mTOR activation involves PI3K pathway (34). Leptin levels are regulated by nutritional status and are reduced during starvation (1). Recent studies show that activation of the mTOR pathway is decreased during starvation and increased in high fat-fed obese animals and that mTOR is involved with the leptin effects on hypothalamus (35, 36). Although inflammatory responses are activated within these conditions, the role of mTOR in leptin-induced effects on leukocyte function has never been addressed.

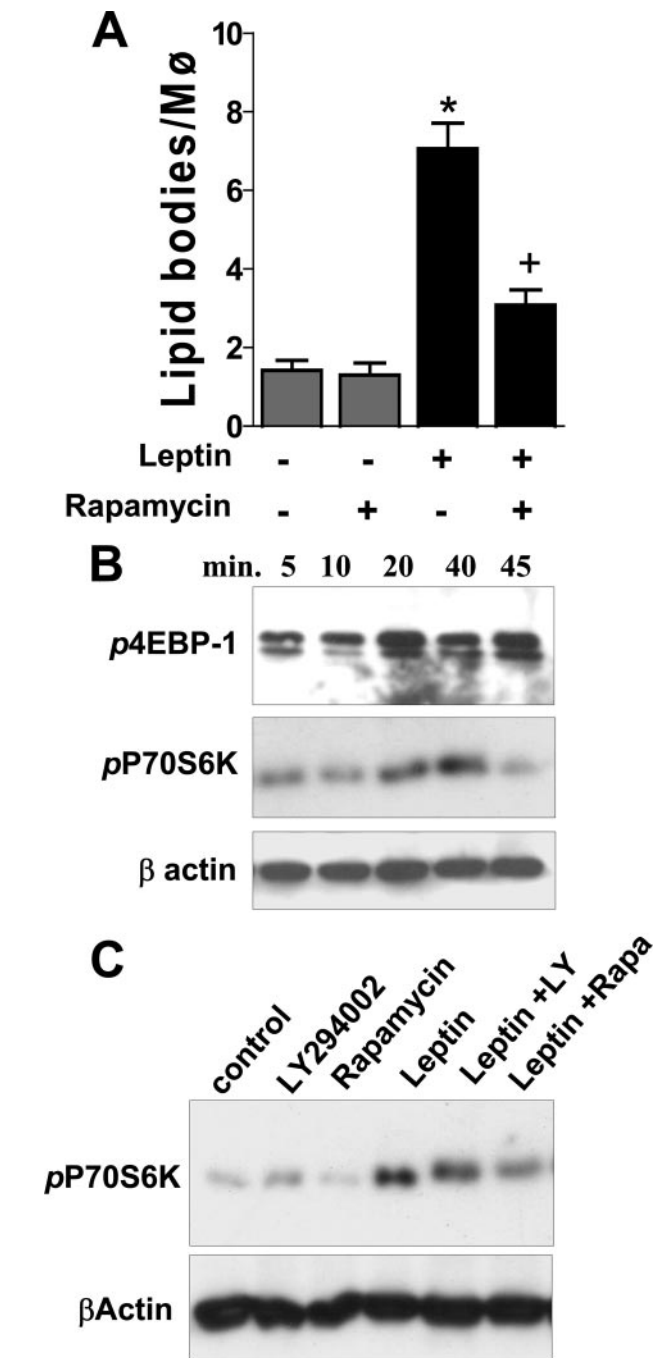
To investigate the functional role of mTOR activation in leptin-induced macrophage activation, we employed rapamycin, a specific inhibitor and probe for mTOR activity (37). We performed both *in vitro* and *in vivo* experiments using the previous described protocols for leptin stimuli in the presence of rapamycin. Rapamycin pretreatment inhibited lipid body formation in peritoneal macrophage stimulated by leptin *in vitro* (Fig. 5A). The p70<sup>S6K</sup> and 4EBP1 are the main downstream proteins that are phosphorylated by mTOR and activate the translation initiation by activation of the S6 protein and release of the eIF4E, respectively (38). As shown in Fig. 5B, leptin induced a time-dependent phosphorylation of p70<sup>S6K</sup> and 4EBP1 in macrophages *in vitro*. Leptin-induced p70<sup>S6K</sup> phosphorylation *in vitro* was inhibited by the PI3K inhibitor LY294002 and by rapamycin (Fig. 5C).

The intraperitoneal administration of rapamycin also completely inhibited lipid body formation after 24-h treatment with leptin *in vivo* (Fig. 6A). This effect was accompanied by an inhibition in the capacity for LTB<sub>4</sub> production (Fig. 6B), reinforcing the importance of the mTOR pathway on the eicosanoid production upon leptin stimuli. Moreover, Western blotting of macrophage lysates obtained from *in vivo* leptin-stimulated animals showed an inhibition of ADRP protein accumulation by rapamycin (Fig. 6, C and D). These data suggest that leptin-mediated biogenesis of ADRP-enriched lipid body is partly attributable to a rapamycin-sensitive increase in ADRP content.

Together, these experiments show that leptin induced mTOR activation and regulated macrophage activation and lipid accumulation, providing novel links between nutrient regulatory pathways and macrophage responses and lipid metabolism.

## DISCUSSION

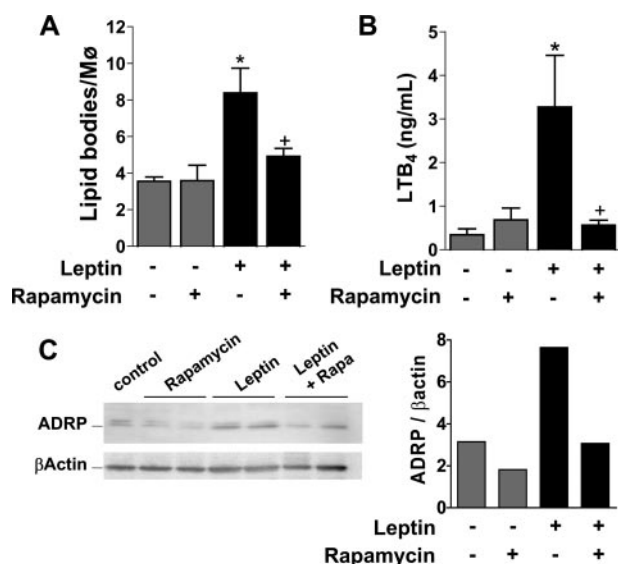
Leptin is a key intermediary between energy homeostasis and the immune system and may play roles in inflammation and obesity-related diseases, including atherosclerosis (4, 5). The characterization of leptin functions in regulating lipid metabo-



**FIGURE 5. *In vitro* leptin-induced macrophage lipid body biogenesis depends on mTOR downstream signaling.** A, effect of rapamycin (20 nM) in leptin-induced (20 nM) lipid body formation in peritoneal macrophages *in vitro* within 4 h. B, time-dependent phosphorylation of 4EBP-1 and p70<sup>S6K</sup> in peritoneal macrophages stimulated *in vitro* with leptin (20 nM) analyzed by Western blotting. C, effect of preincubation (30 min) LY294002 (0.5  $\mu$ M), rapamycin (20 nM), or vehicle p70<sup>S6K</sup> phosphorylation induced 30 min after leptin stimulation.

lism, leukotriene generation, and lipid body biogenesis in macrophages is of importance for understanding the roles of leptin in the pathogenesis of atherosclerosis and other inflammatory diseases. Here we show that leptin directly activates resident macrophages to form ADRP-enriched lipid bodies and enhance leukotriene production, in a mechanism dependent on activation of the PI3K/mTOR pathway.

## Leptin Induces Macrophage Lipid Accumulation



**FIGURE 6. Leptin-induced macrophage lipid body formation, LTB<sub>4</sub> production, and ADRP accumulation *in vivo* are requisitely dependent on mTOR activity.** Shown is lipid body formation (A) and priming for LTB<sub>4</sub> production (B) after intraperitoneal injection of leptin (1 mg/kg) or saline (24 h) in animals treated with rapamycin (15 μg/kg, each 12 h) or vehicle as described under "Experimental Procedures." Each bar represents the mean ± S.E. from at least six animals. \*, statistically significant differences between leptin and saline. †, statistically significant differences between rapamycin-treated and leptin-stimulated groups. C, one representative Western blotting for ADRP or β-actin as indicated with its corresponding densitometry graph, from total peritoneal cell lysates (5 × 10<sup>5</sup> cells/lane) obtained after the *in vivo* leptin or saline stimulation.

Proteins of the PAT family are associated with lipid bodies and have previously been implicated in lipid body assembling and biogenesis (reviewed in Ref. 39). To our knowledge, this is the first demonstration that leptin directly regulates the increase in ADRP cell content and accumulation of lipids within ADRP-enriched lipid bodies in peritoneal macrophages, thus suggesting that leptin has roles in foam cell formation. Increased ADRP expression by itself has been directly related to the enhanced capacity of neutral lipid storage, since ADRP promotes triglycerides and cholesterol storage and reduces cholesterol efflux (15). ADRP may act also as a nucleation center for the assembly of lipid to form nascent lipid bodies and to enhance droplet stability upon lipolytic conditions (18, 19). Our work is in accordance with that of Kjerrulf *et al.* (40), which showed reduced cholesterol accumulation in ob/ob mouse macrophages. Further implications of leptin in lipid-laden foam cell formation come from observations that vascular smooth muscle cells treated with an adipocyte differentiation medium accumulate lipids and express both leptin and leptin receptor ObRb (41). It should be pointed out, however, that leptin in fact diminishes lipid accumulation in liver, kidney, and adipose tissue (42–44). In addition, increased ADRP expression and lipid accumulation has been observed in the kidney of leptin receptor-deficient animals, FVB<sup>db/db</sup> mice (45). These data point to different roles for leptin, depending on the tissue.

Recent studies have demonstrated a role for leptin in prostaglandin and leukotriene synthesis by leukocytes and suggest physiological activities for leptin in inflammation (9, 46). Leukotriene synthesis is reduced in macrophages from leptin-deficient mice following bacterial infection (47). Moreover, leptin

alone or in combination with other agonists increases prostaglandin and leukotriene production in macrophages (9, 46). Mechanisms involved in leptin-induced priming for eicosanoid production are not fully defined and may involve different processes. Indeed, it has been demonstrated that leptin increases arachidonic acid availability, increases phospholipase A<sub>2</sub> protein expression and activity, and induces COX-2 expression (9, 46, 48). An additional mechanism that may contribute to the enhanced eicosanoid production is the compartmentalization of eicosanoid production at specialized sites, as in lipid bodies. The compartmentalization of arachidonate substrate, cytosolic phospholipase A<sub>2</sub>, and eicosanoid-forming enzymes in lipid bodies provides a spatially localized domain to regulate arachidonate release and direct its metabolism to eicosanoid formation (for a review, see Ref. 29). Moreover, macrophage lipid bodies formed *in vivo* by ox-LDL-derived lipids and LPS-induced inflammation or bacterial infection compartmentalize 5-LO and COX-2 enzymes and are involved in the enhanced eicosanoid synthesis during these inflammatory conditions (32, 33, 49, 50). It has been shown that 5-LO is enhanced in the atherosclerotic plaque and that both higher 5-LO expression and increased LTB<sub>4</sub> production or activity are associated with complications of these lesions (51–53). In this study, we showed that stimulation of macrophages *in vivo* or *in vitro* with leptin induces a dose- and time-dependent lipid body formation in macrophages, localization of 5-LO in this organelle, and enhanced generation of leukotrienes. Collectively, these findings indicate that leptin may contribute to atherogenesis by inducing lipid-laden macrophages with increased leukotriene synthetic ability.

The molecular mechanisms of leptin-induced leukocyte activation were investigated. Similar to insulin and other growth factors and cytokines, leptin receptor activation leads to tyrosine kinase phosphorylation and further activation of MAPK and STAT pathways in parallel to the activation of PI3K (3, 4). Leptin signaling through the PI3K pathway and Akt was described in different cells, including macrophages (54, 55). Here we used *in vivo* and *in vitro* approaches to show that PI3K activity is requisitely involved in macrophage activation to induce lipid body biogenesis.

Macrophage activation to form lipid bodies *in vivo* was largely dependent on PI3K, as demonstrated by pharmacological PI3K inhibition, using the LY294002, and by the absence of leptin effect on the PI3Kγ knock-out mice. When studying signaling by leptin *in vivo*, direct peripheral effects and effects mediated via the central nervous system must be distinguished. Direct PI3K signaling effects were supported by the *in vitro* effect of the PI3K inhibitor, LY294002, which significantly inhibited leptin-induced lipid body formation. Interestingly, studies using immunocytochemistry and subcellular fractionation demonstrated that PI3K localizes in part into leukocyte cytoplasmic lipid bodies. In addition, PI3K p85 was also shown to co-localize with phosphorylated downstream kinases in lipid bodies of stimulated leukocytes, suggesting that this lipid kinase may have signaling functions within lipid bodies (23).

The activation of PI3K leads to activation of several other downstream pathways involved on cell activation and prolifer-

eration. The mTOR kinase is activated on the PI3K downstream pathway and regulates protein synthesis, cell metabolism, and growth (34). It has been recently demonstrated that the leptin-induced hypothalamic effect on food intake was dependent on the mTOR pathway (36). In adipose tissue, mTOR is involved with the regulation of lipid metabolism (56–58). The mTOR activity has been implicated in regulation of inflammatory and thrombotic processes by regulating signal-dependent translation in platelets, monocytes, and neutrophils (59–61). Therefore, we hypothesized that leptin might modulate specialized macrophage responses through the mTOR pathway. Indeed, in macrophages, leptin induced a time-dependent phosphorylation of 4EBP1 and p70<sup>S6K</sup> in a rapamycin-sensitive mechanism. Moreover, rapamycin drastically inhibited macrophage lipid body formation induced by leptin, suggesting that mTOR activity, downstream of PI3K, is crucial to leptin effects. In support to our finding, activation of mTOR has been associated with other cytokine and hormone receptor signaling, as is the case for interferon and insulin in different systems (62, 63). Our results add a new knowledge that mTOR is critically involved in the leptin signaling to activation of macrophage, leading to intracellular lipid accumulation. Moreover, the increased ADRP protein content in macrophages observed after leptin stimulation was also dependent on mTOR activation. Accordingly, insulin-driven mTOR activation leads to adipocyte differentiation with increased lipid storage (56, 57). In addition, the mTOR inhibitor rapamycin up-regulates the translation of proteins that promote fatty acid oxidation while down-regulating the ones participating in fatty acid synthesis (64). Although indirect inhibition of mTOR by leptin-induced AMP-dependent kinase activation has been suggested (65, 66), we show here that leptin directly induces macrophage mTOR activation with lipid accumulation, suggesting that AMP-dependent kinase is not being activated or, at least, is not inhibiting mTOR in macrophages. Indeed, leptin-induced AMP-dependent kinase-dependent signaling may vary according to tissue and stimulation conditions (67, 68).

There is accumulating evidence that hyperleptinemia and the combination of hyperinsulinemia and hyperglycemia observed in metabolic syndrome play roles in the development of atherosclerosis and are markers of cardiovascular risk (69–71). Our observation that leptin induced neutral lipid and ADRP accumulation in macrophages is in agreement with the work suggesting that leptin may induce an increase in cholesterol ester within macrophages (12), which may suggest that leptin-induced modulation of macrophage lipid metabolism may have roles in obesity-enhanced cardiovascular diseases. Collectively, our data suggest a role for leptin through a PI3K/mTOR-dependent pathway in regulating macrophage lipid metabolism and intracellular accumulation, and it may contribute to the study of inflammatory lipid disorders, such as foam cell differentiation and atherosclerosis development. Furthermore, our results demonstrate a previously unrecognized pathway for leptin cell signaling in macrophages, linking leptin-dependent extracellular nutrient sensing and the intracellular sensor mTOR in inflammation.

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**Leptin Induces Macrophage Lipid Body Formation by a Phosphatidylinositol 3-Kinase- and Mammalian Target of Rapamycin-dependent Mechanism**  
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