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Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis

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Gruen ML, Hao M, Piston DW, Hasty AH. Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis. *Am J Physiol Cell Physiol* 293: C1481–C1488, 2007. First published August 29, 2007; doi:10.1152/ajpcell.00062.2007.—The growing worldwide obesity epidemic is frequently linked to an increased risk of developing diseases such as diabetes, cardiovascular disease, and cancer. These diseases are associated with the infiltration of macrophages in white adipose tissue (WAT), the artery wall, and tumors, respectively; and these macrophages likely contribute to disease progression and pathogenesis. Abdominal WAT, adipose tissue surrounding the heart and artery wall, as well as carcinoma cells, secrete many factors that could induce macrophage infiltration. Leptin is an adipocyte-secreted hormone, and deficiency of either leptin or its receptor has been shown to cause morbid obesity in animals and in humans. However, what is more commonly noted in human obesity is the presence of central leptin resistance leading to hyperleptinemia. As leptin receptors are present on macrophages, we hypothesized that leptin could act as a monocyte/macrophage chemoattractant. Our current study demonstrates: *1*) leptin is a potent chemoattractant for monocytes and macrophages, inducing maximal chemotactic responses at 1 ng/ml; *2*) leptin-mediated chemotaxis requires the presence of full-length leptin receptors on migrating cells; *3*) leptin causes increased influx of intracellular calcium in macrophages; and 4) activation of janus kinase/signal transducers and activators of transduction (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways are all necessary for leptin-induced macrophage migration. Taken together, these data demonstrate that leptin is a potent monocyte/macrophage chemoattractant in vitro and that canonical cell motility machinery is activated upon macrophage exposure to leptin. These data have implications for the impact of hyperleptinemia on obesity-related pathophysiological conditions such as diabetes, cardiovascular disease, and cancer.

leptin receptor; cell motility; adipose tissue macrophages; tumor associated macrophages; migration

OBESITY has rapidly become a worldwide epidemic. The pathophysiological consequences of obesity are numerous and include insulin resistance, hypertension, and dyslipidemia which, when coincident, are referred to as the metabolic syndrome (33). Aside from these primary conditions associated with obesity, novel risk factors such as increased markers of inflammation (C reactive protein and serum amyloid A), thrombosis (plasminogen activator inhibitor 1), oxidative stress (isoprostanes), and elevated plasma leptin levels, are all thought to be associated with the pathological conditions associated with obesity (15). The combination of obesity and its associated risk factors often results in the development of diabetes and cardiovascular disease (CVD) (38). In addition, obesity has recently been found to have a significant impact on the prognosis of various cancers (4, 18). The current scientific focus on obesity has lead to much interest in understanding how obesity increases risk of these diseases.

Macrophages are cells of the innate immune system that play an important role in many disease processes. Macrophages have been shown to infiltrate white adipose tissue (WAT) in obese mice and humans, promoting chemokine and inflammatory cytokine secretion as well as insulin resistance (42, 44). Recent data suggest that perivascular adipose tissue is a metabolically active tissue, producing chemoattractant molecules that may induce macrophage migration into the artery wall (16). In addition, epicardial adipose tissue has been shown to be a source of both leptin and inflammatory mediators (26). Increased macrophage infiltration has been linked to tumor cell proliferation in breast, endometrial, and renal cancers (23), and increased numbers of tumor-associated macrophages have been reported to be associated with poor prognosis and increased motility of cancer cells (23). Thus macrophage infiltration of tissues such as visceral adipose tissue, epicardial, and perivascular adipose tissue, as well as tumors, may contribute to the increased risk and worsened prognosis of diabetes, CVD, and cancer in obese individuals.

One mechanism by which monocytes are recruited to different tissues is via chemokines. The macrophages within WAT of obese mice and humans have been shown to contribute significantly to the secretion of chemokines such as monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), and monocyte inflammatory protein-1α from WAT (5, 8). Chemokines are well established to play a significant role in the progression of both atherosclerotic disease (41) and various cancers (34). Although the resident macrophages in WAT, atherosclerotic lesions, and tumors may secrete chemokines that propagate signals to induce monocyte chemotaxis, it is likely that the initiating events derive from cells endogenous to these tissues themselves.

Leptin is an adipocyte-derived pleiotropic hormone essential to weight regulation, reproduction, endocrine function, as well as immune and inflammatory responses (1). Because obesity is associated with increased levels of circulating leptin, and adipocytes are the primary producers of leptin, it is plausible that leptin could contribute to macrophage infiltration into WAT in the obese state. Leptin is also secreted from perivascular and epicardial adipose tissue (16, 26). Furthermore, leptin and leptin receptor expression are increased in breast carcinoma cells relative to normal mammary cells (11, 13, 19). From these data, we hypothesized that leptin might be a

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monocyte/macrophage chemoattractant with potential relevance to tissue macrophage accumulation. In support of this, leptin has been identified as a chemoattractant for neutrophils (29), endothelial cells (12, 37, 43), smooth muscle cells (28), and human prostate cancer cells (9).

Knowledge of the mechanisms by which macrophages perceive and respond to increased adiposity is imperative for understanding their contribution to obesity-related diseases. In the current study, we show that leptin is a potent chemoattractant for monocytes and macrophages, that leptin-induced migration requires full-length leptin receptor, that leptin induces intracellular influx of Ca^{2+}, and that its effects are mediated through canonical activation of janus kinase/signal transducers and activators of transduction (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways.

MATERIALS AND METHODS

Materials. Recombinant leptin and monocyte chemoattractant protein-1 (MCP-1) (purity >97%) were purchased from R&D Systems. Chemical inhibitors PD-98059, SP-600125, LY-294002, AG-490, wortmannin, bisindolylmaleimide 1 (GF-109203X), and U-73122 were purchased from Calbiochem. SB-203580 was purchased from Sigma. RPMI 1640 and DMEM were purchased from GIBCO-BRL. Fura-2 was from Molecular Probes.

Mouse models. C57BL/6 mice with one mutated leptin receptor allele ($LepR^{db/+}$) were purchased from Jackson Laboratories. Obese leptin receptor-deficient ($LepR^{db/db}$) and lean ($Lepr^{+/+}$) mice were either purchased or produced by intercrossing $LepR^{db/+}$ mice. Mice were genotyped by using PCR of genomic DNA from ear clips. Primers were purchased from Invitrogen and were as follows: dbF1 5′-AGA ACG GAC ACT CTT TGA AGT CTC-3′, db-R4-WT 5′-AAC CAT AGT TTA GGT TTG TTT C-3′, and db-Rb-db 5′-CAA TTC AGT GTA AAC CAT AGT TTA GGT TTG TTT A-3′. Cycling conditions were adapted from Kowalski et al. (21). Product sizes for the wild-type and leptin receptor-deficient alleles were 129 bp and 141 bp, respectively. Mice were given free access to food and water. All animal care and experimental procedures were performed with approval from and according to the rules and regulations of the Institutional Animal Care and Usage Committee of Vanderbilt University Medical Center.

RNA isolation and RT-PCR. Total THP-1 cellular RNA was extracted using TriZol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined using OD_{260}/OD_{280} reading on Eppendorf BioPhotometer. cDNA was synthesized from 1 μg/ml total RNA by reverse transcription using Qiagen Taq PCR Master Mix. Specific primers (Integrated DNA Technologies) for both the long and short form of leptin receptor (36) were used for semiquantitative PCR. Electrophoresis of PCR products were run on a 1.5% agarose gel with visualization by ethidium bromide staining.

THP-1 migration assay. Human THP-1 monocytes were purchased from ATCC and maintained in culture in RPMI 1640 supplemented with 10 mM HEPES, 10% fetal bovine serum, and penicillin-streptomycin. Cells were cultured at a density of 1×10^6 cells/ml at 37°C, and Trypan blue was used to determine cell count and viability. Migration studies were performed using a MB Series 96-well Boyden chamber (Neuroprobe). The top and bottom chambers were separated by a polycarbonate filter with 5-μm pores. The bottom wells were loaded with 390 μl of MCP-1 at a concentration of 10 ng/ml or with leptin at concentrations ranging from 0.1 pg/ml to 100 ng/ml as indicated. The top wells were loaded with 200 μl of THP-1 cells at a concentration of 2.5×10^6 cells/ml followed by incubation at 37°C for 2 h. The filter was rinsed with water, the top side was scraped, and the filter was fixed in chilled methanol for 10 min. After fixing was completed, the filter was rinsed with water then stained with 1% crystal violet for 10 min. Migrated cells were counted in five fields from images captured using an Olympus BX51 microscope with a ×20 objective lens. Although the absolute number of migrating cells differed between assays, the 96-well Boyden chamber format allows for all controls to be assessed simultaneously with the experimental conditions for each study. Each experiment was performed at least three times in triplicate wells of cells. Five high-powered fields per triplicate well were counted, as indicated in the figures.

Macrophage collections. Primary peritoneal macrophages from $LepR^{+/+}$ and $LepR^{db/db}$ mice were collected 3 days following an intraperitoneal injection of 3% thioglycollate media. Cells were washed two times and used immediately for migration studies as described for THP-1 cells.

Inhibitor assays. THP-1 cells were plated at a concentration of 2.5×10^6 cells/ml/well in a 12-well plate. MAPK, PI3K, JAK2, protein kinase C (PKC), and phospholipase C (PLC) inhibitors were reconstituted according to manufacturers′ instructions. Inhibitors of extracellular signal-regulated kinase (ERK) 1/2 (PD-98059), p38 (SB-203580), and stress-activated protein kinase (SAPK)/JNK (SP-600125) were used at 10 μM. PI3K inhibitors wortmannin and LY-294002 were used at 10 nM and 10 μM, respectively. JAK2 inhibitor AG-490 was used at 50 μM. Inhibitors of PKC (GF-109203X) and PLC (U-73122) were used at 1 μM. Concentrations for inhibitors were determined based on publications describing their use in other chemotaxis assays (2, 14) and were above the IC_{50} for each inhibitor. Control wells were treated with vehicle diluent (DMSO). THP-1 cells were pretreated with inhibitors and incubated at 37°C for 30 min before the migration assay. Migration assays were performed as described, with inhibitors added to the top and bottom wells of the Boyden chamber at equal concentrations for the duration of the study. Cells were found to be at least 95% viable after the 30-min incubation with the inhibitors listed above.

Calcium signaling. Primary peritoneal macrophages from $LepR^{+/+}$ were collected as previously described and plated onto 35-mm glass bottom microwell dishes (Mat Tek). Cells were loaded in Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS) buffer containing 10 mM HEPES. Assay buffer consisted of loading buffer with 1 mM Ca^{2+}. A concentrated stock of 2 mM fura-2 was made by dissolving fura-2 in DMSO and used at a 2 μM concentration. Cells were loaded at room temperature while being shaken for 10 min and then washed five times with loading buffer. Cells were incubated in assay buffer at room temperature while being shaken for another 10 min, followed by extensive washing and addition of fresh assay buffer. Ca^{2+} signals from fura-2-loaded cells were obtained by using a fura-2 filter set (Chroma Technology). The cells were placed on a stage enclosed in Plexiglas and kept in humidified air at 37°C. All images were acquired on a Nikon TE300 inverted wide-field microscope equipped with a ×40 1.3 numerical aperature plan Apochromat objective driven by MetaMorph imaging software (Universal Imaging). The 340-to-380 nm intensity ratio of fura-2 was obtained from the entire image after background correction.

Statistical analysis. Student's *t*-tests or ANOVA with a Bonferonni post hoc tests were used to compare either two or multiple groups, respectively, as described in the figures.

RESULTS

Effect of leptin on THP-1 monocyte migration. The presence of leptin receptor on human THP-1 monocytes was confirmed by RT-PCR. Both long form (Ob-Rb) and short form (Ob-Rt) leptin receptor PCR products were detected by using specific primers for each isoform (Fig. 1).

Leptin has been shown to act as a chemoattractant for various cell types such as neutrophils, smooth muscle cells, endothelial cells, and cancer cells at doses ranging from 10 pg/ml to 100 ng/ml (9, 12, 28, 29, 37, 43). We hypothesized

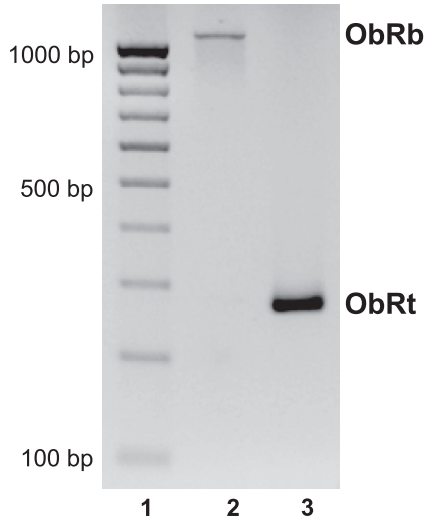

Fig. 1. Leptin receptor expression in THP-1 monocytes. Total RNA was extracted from human THP-1 monocytes and analyzed for the presence of the long- (ObRb, 1071 bp) and short- (ObRt, 273 bp) forms of the leptin receptor using RT-PCR as described in MATERIALS AND METHODS. *Lane 1*, molecular weight ladder; *lane 2*, long form; *lane 3*, short form.

that leptin could also be a chemoattractant for monocyte/macrophages, and we performed migration studies using the 96-well Boyden chamber system. Human THP-1 monocytes were allowed to migrate through 5-μm pores to increasing concentrations of leptin (0.1 pg/ml to 100 ng/ml) for 2 h. Leptin induced migration of human THP-1 monocytes at concentrations as low as 1 pg/ml with maximal responses at 1 ng/ml (Fig. 2). Migration of THP-1 cells to DMEM alone was nearly undetectable. The chemotactic response for leptin at 1 ng/ml was similar to that of MCP-1 at a concentration of 10 ng/ml.

When equal concentrations of leptin (1 ng/ml) were added to the top and bottom chambers, effectively eliminating the leptin gradient, THP-1 cell migration was completely blocked, indicating that leptin functions in a chemoattractant rather than a chemokinetic manner (Fig. 3*A*). The combination of leptin and MCP-1 in the bottom chamber did not have additive effects on monocyte migration (Fig. 3*A*).

Competition studies were performed to determine whether MCP-1 and leptin possessed antagonizing chemoattractant potential. When both chemoattractants were used at their maximal concentrations (1 ng/ml leptin and 10 ng/ml MCP-1), MCP-1 was able to counteract the chemotactic potential of leptin; however, leptin had no impact on the chemotactic potential of MCP-1 (Fig. 3*B*). When leptin was placed in the bottom well at 1 ng/ml and MCP-1 was placed in the top well at an equimolar concentration (62.5 pM), MCP-1 retained its ability to counteract the chemoattractant potential of leptin (Fig. 3*C*). When MCP-1 was placed in the bottom well at 10 ng/ml and leptin was placed in the top well at an equimolar concentration (1.14 nM), leptin was not able to counteract the chemoattractant potential of MCP-1 (Fig. 3*C*).

Role of leptin receptor in mediating leptin-induced macrophage migration. To determine the ability of primary macrophages to migrate to leptin and whether expression of full-length leptin receptor is required, we performed Boyden chamber migration studies using primary peritoneal macrophages from C57BL/6 ($LepR^{+/+}$) and leptin receptor-deficient ($LepR^{db/db}$) mice (Fig. 4). The $LepR^{db/db}$ mice express short forms of leptin receptor but do not express the full-length, signal-transducing form (39). Macrophages from $LepR^{+/+}$ and $LepR^{db/db}$ mice both migrated to MCP-1, indicating that primary peritoneal macrophages are capable of chemotaxis and that leptin receptor deficiency does not impair the general migratory ability of macrophages. $LepR^{+/+}$, but not $LepR^{db/db}$ macrophages, were able to chemotax to leptin, indicating that expression of full-length leptin receptor is required for leptin-mediated macrophage migration.

Role of JAK2/STAT3, PKC, and PLC in leptin-induced THP-1 cell migration. The involvement of the JAK/STAT pathway has previously been shown to be required for leptin to promote invasiveness of endometrial cancer cells as well as epithelial cells (3, 36). To determine the involvement of JAK/STAT in leptin-mediated monocyte chemotaxis, THP-1 cells were pretreated with the JAK2 tyrosine kinase inhibitor AG490. Inhibition of JAK2 resulted in the prevention of migration to leptin (Fig. 5). PKC and PLC are downstream of the leptin receptor (10) and are also modulators of chemotactic response and cell motility. Inhibition of PKC and PLC with GF109203X and U-73122, respectively, resulted in a complete absence of leptin-induced monocyte migration (Fig. 5).

Leptin induces intracellular calcium influx. It is well established that an influx of Ca^{2+} is stimulated by various chemoattractants via PLC and precedes cell motility (20). To determine whether leptin could induce Ca^{2+} mobilization, we loaded murine primary peritoneal macrophages with the Ca^{2+} indicator fura-2, stimulated the cells with either MCP-1 or leptin, and quantified intracellular Ca^{2+} levels over a 15-min time course. The Ca^{2+} curves shown in Fig. 6 are representative of at least eight wells with the integrated signal from 50 to 75 cells per field. In this experiment, intracellular Ca^{2+} levels were induced by 15% in MCP-1-treated macrophages and reached maximal levels within 2.5 min of treatment, whereas leptin induced a 10% rise at 5 min posttreatment.

Role of MAPK pathways in leptin-induced monocyte migration. MCP-1-induced macrophage migration is mediated by MAPK family members: ERK1/2, p38 MAPK, and SAPK/

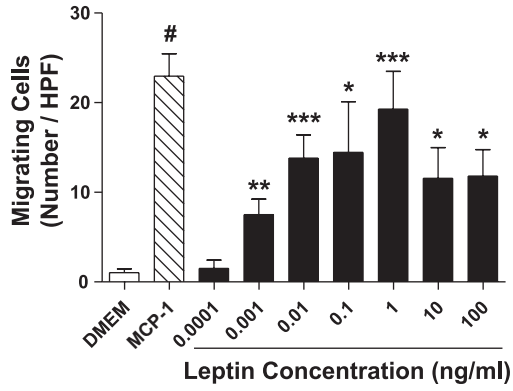

Fig. 2. THP-1 monocyte migration to leptin. The ability of leptin to act as a chemoattractant was assayed using the 96-well Boyden chamber system. Leptin was added to the bottom chamber at concentrations ranging from 0.1 pg/ml to 100 ng/ml. Monocyte chemoattractant protein-1 (MCP-1, 10 ng/ml) was used as a positive control. Cells were allowed to migrate for 2 h at 37°C. Results represent counts from five high-power fields from four independent experiments performed in triplicate wells. Statistics were performed using Students *t*-test to compare each condition with DMEM control. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $\#P < 0.001$ vs. DMEM.

JNK (2, 14). To determine whether the chemoattractant properties of leptin are also mediated through these same pathways, THP-1 cells were pretreated with inhibitors of ERK 1/2 (PD-98059), p38 (SB-203580), and SAPK/JNK (SP-600125). Whereas inhibition of ERK1/2 and p38 completely abrogated leptin-induced monocyte migration, inhibition of SAPK/JNK only partially diminished the chemoattractant potential of leptin (Fig. 7).

Role of PI3K in leptin-induced monocyte migration. PI3K has been implicated in the migration of monocytes and endothelial cells to MCP-1 (2). Furthermore, leptin-induced chemotaxis of endothelial cells and prostate cancer cells is dependent on the PI3K pathway (9, 12). To determine the contribution of PI3K to leptin-mediated chemotaxis of monocytes, THP-1 cells were preincubated with two different PI3K inhibitors wortmannin and LY-294002. As shown in Fig. 8, both PI3K inhibitors abolished leptin-induced migration of THP-1 monocytes to MCP-1 and leptin. These results confirm the involvement of PI3K in leptin-mediated monocyte migration.

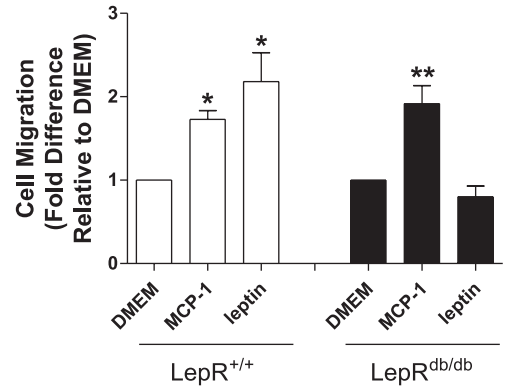

Fig. 4. Requirement of leptin receptor for leptin-mediated macrophage chemotaxis. Primary peritoneal macrophages from $LepR^{+/+}$ and $LepR^{db/db}$ mice were allowed to migrate to leptin (1 ng/ml). DMEM with no chemoattractant was used as a negative control, and MCP-1 (10 ng/ml) was used as a positive control. Data are plotted as the fold increase of migrating macrophages relative to DMEM for each cell type. Results represent counts from five high-power fields from three independent experiments performed in triplicate wells. All conditions for each experiment were performed simultaneously; however, statistics were performed separately for $LepR^{+/+}$ and $LepR^{db/db}$ cells using ANOVA. $*P < 0.05$ compared with DMEM; $**P < 0.05$ compared with DMEM and $P < 0.01$ compared with leptin.

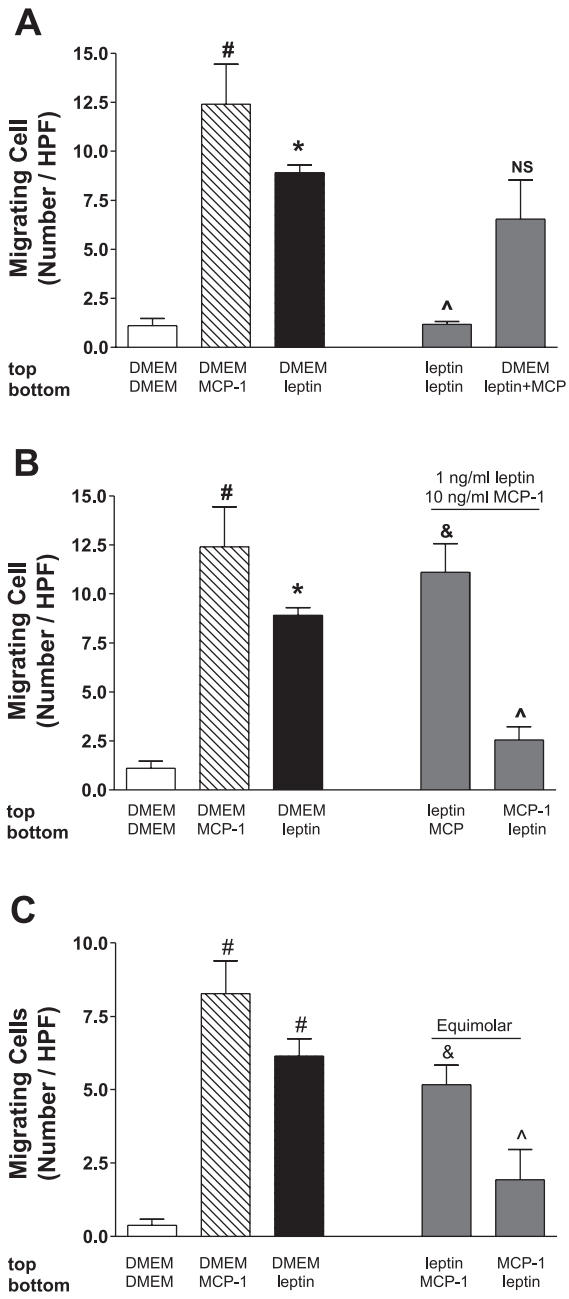

Fig. 3. Competitive interaction of MCP-1 and leptin in monocyte chemotaxis. Migration studies were performed with THP-1 cells as described in MATERIALS AND METHODS. MCP-1 only and leptin only were used in the bottom chamber as positive controls for all experiments. *A* and *B*: experimental conditions were performed simultaneously; thus, the DMEM, leptin, and MCP-1 controls are identical. *C*: experiments were performed on a separate occasion. Addition of MCP-1 and leptin to the top and bottom chambers is indicated. *A*: an equal concentration of leptin (1 ng/ml) was placed in the top chamber, thus eliminating the leptin gradient. Both leptin (1 ng/ml) and MCP-1 (10 ng/ml) were placed together in the bottom chamber. *B*: competition studies were performed by placing MCP-1 (10 ng/ml) in the bottom well and leptin (1 ng/ml) in the top well, or leptin (1 ng/ml) in the bottom well and MCP-1 (10 ng/ml) in the top well. *C*: competition studies with equimolar concentrations of leptin and MCP-1 were performed by placing MCP-1 (10 ng/ml = 1.14 nM) in the bottom well and leptin (1.14 nM) in the top well or leptin (1 ng/ml = 62.5 pM) in the bottom well and MCP-1 (62.5 pM) in the top well. Results represent counts from five high-power fields from four independent experiments performed in triplicate wells. Statistics were performed using ANOVA to compare each experimental condition with the three control conditions (DMEM, MCP-1, and leptin) on the far left of each panel. NS, not significant. $*P < 0.01$ vs. DMEM; $\#P < 0.001$ vs. DMEM; $\wedge P$ = NS vs. DMEM; $P < 0.05$ vs. leptin, and $P < 0.001$ vs. MCP-1; $\&P < 0.01$ vs. DMEM, P = NS vs. leptin, and P = NS vs. MCP-1.

DISCUSSION

In the current study we sought to determine whether leptin is a monocyte/macrophage chemoattractant. We show not only that leptin is a chemoattractant at low concentrations, but that full-length leptin receptor and canonical signaling pathways are required for leptin to mediate monocyte/macrophage motility and chemotaxis. Mechanisms by which obesity increases the risk of diseases such as diabetes, cardiovascular disease, and cancer are at the forefront of recent scientific research. Our data are consistent with the hypothesis that obesity-related changes in interstitial leptin gradients may induce monocyte

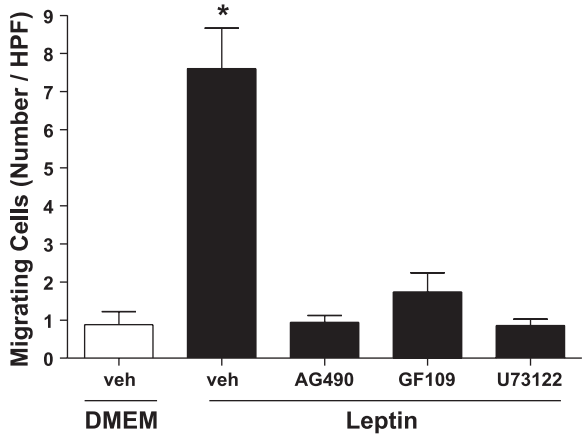

Fig. 5. Effect of inhibition of leptin receptor signaling pathway intermediates on leptin-induced THP-1 cell migration. THP-1 cells were preincubated for 30 min with inhibitors to activation of janus kinase 2 (JAK2), protein kinase C (PKC), and phospholipase C (PLC) (AG-490, 50 μM; GF-109, 1 μM; and U-73122, 1 μM; respectively). Migration of cells to 1 ng/ml of leptin was assessed using the Boyden chamber system. Results represent counts from five high-power fields per triplicate wells from five independent experiments. veh, Vehicle control. *$P < 0.001$ compared with all.

infiltration of various tissues, leading to pathophysiological effects.

Our data conclusively demonstrate that leptin is a chemoattractant at concentrations as low as 1 pg/ml, with maximal effects at 1 ng/ml (Fig. 2). By comparison, the concentration of leptin required for monocyte chemotaxis is at least 10-fold lower than that for other cell types reported (3, 9, 12, 28, 29, 43). Conventionally, macrophage motility is characterized by the cell's ability to respond to gradients emanating from a chemokine source, with differences as little as 2% in concentration capable of inducing migratory responses (25). Both gradients and absolute concentrations are important to evaluate when characterizing chemoattractant molecules. For example, in our studies, as the leptin gradient increased by 10-fold at each concentration used, the chemotaxis of monocytes was also increased. Yet, when concentrations of 10 and 100 ng/ml were reached, chemotaxis tended to decline (Fig. 2). These data indicate that high concentrations are not required for leptin-mediated monocyte chemotaxis; rather, a change in gradient ranging from 1 pg/ml to 1 ng/ml is sufficient. From a physiological standpoint this has important implications for pathophysiological consequences of small leptin gradients. Whereas plasma leptin levels may reflect increased overall secretion, they do not provide detailed information regarding local concentration gradients formed in specific tissues. Thus, although 1 ng/ml of leptin is a physiologically low plasma concentration, our data indicate that slight fluctuations in the interstitial concentration of leptin could have potent effects on recruitment of monocytes to leptin secreting tissue such as adipose and breast. Further studies are needed to characterize the leptin gradients that exist in these tissues and whether obesity induces changes in these gradients.

The apparent hierarchical relationship between leptin and MCP-1 is also noteworthy (Fig. 3, *B* and *C*). It is known that different chemokines can have additive or competitive effects on migration of individual cells. In fact, leptin has been shown to competitively inhibit the migration of neutrophils to *N*-formyl-methionyl-leucyl-phenylalanine, IL-8, and C5a (29). Through the use of competition studies in the Boyden chamber, the relative ability of MCP-1 and leptin (at their individual maximal doses and at equimolar doses) to induce monocyte/macrophage migration was compared. It was noted that when MCP-1 was placed in the top chamber, THP-1 cells could no longer migrate toward leptin. The absence of monocyte migration toward leptin in the bottom chamber when MCP-1 was placed in the top chamber indicates that MCP-1 can counteract the chemoattractant potential of leptin. These data are supported by our observation that when MCP-1 was used as the chemoattractant in the bottom chamber, the presence of leptin in the top chamber did not blunt migration of THP-1 cells. The data from these studies provide evidence that MCP-1 may be a higher-order chemoattractant for monocytes compared with leptin. It is also interesting that the combination of both leptin and MCP-1 in the bottom chamber did not produce an additive migratory response. In fact, monocyte migration trended toward a reduction compared with either leptin or MCP-1 alone,

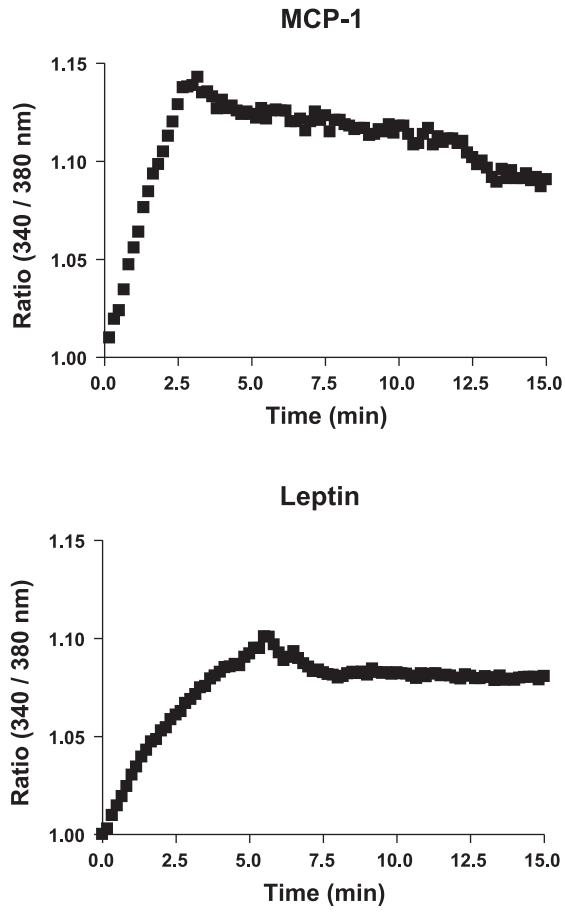

Fig. 6. Intracellular calcium levels in MCP-1 and leptin-treated primary peritoneal macrophages. Primary peritoneal macrophages from C57BL/6 mice were plated onto MatTek dishes and allowed to adhere overnight. Cells were washed in Ca^{2+}, Mg^{2+}-free buffer (1× PBS, 10 mM HEPES) and then loaded with 2 μM fura-2 for 10 min at 25°C while being shaken. After extensive washing was completed, cells were incubated with Ca^{2+}-containing media for 10 min. MCP-1 (10 ng/ml) or leptin (1 ng/ml) were added to cells, and Ca^{2+} signals were obtained by using a fura-2 filter set. The fura-2 intensity ratio of 340 to 380 nm was obtained from the entire image of 50 to 75 cells after background correction. Both MCP-1 and leptin plots shown are a representative curves of at least 8 separate wells.

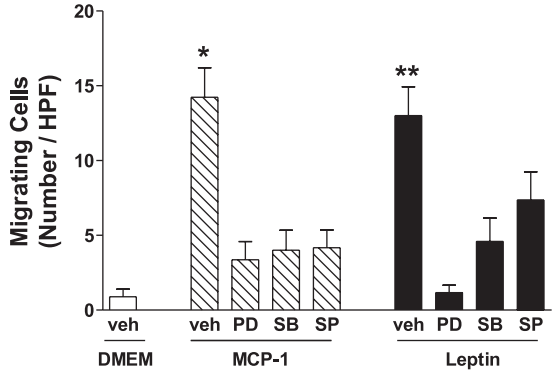

Fig. 7. Effect of mitogen-activated protein kinase (MAPK) inhibition on migration of THP-1 monocytes to leptin. THP-1 cells were pretreated for 30 min with inhibitors of kinases that phosphorylate ERK1/2 (PD-98059, 10 μM), p38 MAPK (SB-203580, 10 μM), and SAPK/JNK (SP-600125, 10 μM). Cells were allowed to migrate for 2 h to MCP-1 or leptin in the presence or absence of inhibitors. Results represent counts from five high-power fields from three independent experiments performed in triplicate wells. All experimental conditions were performed simultaneously; however, statistics were performed separately for MCP-1 and leptin-treated cells. *$P < 0.001$ compared with DMEM, and $P < 0.01$ compared with PD-98095, SB-203580, and SP-600125 treatment groups. **$P < 0.001$ compared with DMEM and PD-98059, $P < 0.05$ compared with SB-203580.

suggesting that intracellular signaling mechanisms within the cell could become overstimulated leading to desensitization. Future studies may shed light on the interactions of leptin and MCP-1 in vitro and in vivo.

Leptin-mediated monocyte chemotaxis requires full-length and functional leptin receptor. This is evidenced by the fact that primary peritoneal macrophages from $LepR^{db/db}$ mice, which express only the short forms of leptin receptor, were not able to migrate to leptin (Fig. 4). Upon binding of leptin to its full-length receptor, *trans*-autophosphorylation results in activation of the JAK2/STAT3 signal transduction pathway (35). Additional evidence for a role of leptin receptor signaling in leptin-induced monocyte chemotaxis stems from the observation that inhibition of JAK2 activity completely blocked THP-1 cell migration to leptin (Fig. 5). Canonical receptors for monocyte chemotaxis such as the CC and CXC receptors are G protein-coupled receptors (6, 24); however, the leptin receptor is a tyrosine protein kinase receptor. The mechanism by which tyrosine protein kinase receptors mediate chemotaxis can occur independently or dependently of G protein-coupled receptors. Macrophage colony-stimulating factor-1 receptor is a tyrosine protein kinase receptor and directly mediates the chemotaxis of monocytes to colony-stimulating factor (20, 31). In other cases, sequestering of G protein-coupled receptors has been shown to be involved in tyrosine protein kinase receptor-mediated chemotactic responses (17, 27, 40). For example, platelet-derived growth factor acts through a tyrosine kinase but requires the G protein-coupled receptor EDG-1 to promote migration of fibroblasts (17). More studies are needed to determine whether leptin receptor acts on its own or in cooperation with G protein-coupled receptors.

The involvement of the MAPKs ERK1/2, p38, and JNK/SAPK has been demonstrated for MCP-1-mediated macrophage migration (2, 14). Likewise, it has been reported that MAPK pathways are involved in leptin-induced migration, proliferation, and invasion of other cell types, including neutrophils (29) and cancer cells (3, 9, 36). Our current study showed that monocyte migration to leptin is also dependent on the MAPK signaling pathways. PLC and PKC have been shown to be involved in classical cell migratory responses via Ca^{2+} mobilization and microtubule stabilization, respectively (20, 27), and our data demonstrate that they have a role in leptin-mediated monocyte chemotaxis (Fig. 5). Furthermore, a role for PLC is indicated by the increase in intracellular Ca^{2+} in macrophage induced by leptin (Fig. 6). GF-109203X is highly selective for typical PKC members α, βI, βII, γ, δ, and ε. However, the atypical PKC, PKCζ has been shown to be involved in the chemotaxis of neutrophils to IL-8 and CD34+ cells to SDF-1 (22, 30). It is not yet known whether atypical PKCs are also involved in leptin-mediated monocyte/macrophage migration.

PI3K activation is associated with cell motility response and function. Activation of PI3K can promote migration through Akt phosphorylation and through downstream activators Cdc42, Rac, and Rho (31). These three molecules are required for filopodia formation and cell polarization, lamellipodia and focal complex formation, and actin cable formation, respectively (20). PI3K has been shown to be involved in MCP-1-induced monocyte and endothelial cell migration (2). Similar to MCP-1, leptin-induced chemotaxis requires PI3K in cancer cells, smooth muscle cells, and endothelial cells (3, 9, 12, 28, 36). In addition, Attoub et al. reported that PI3K was necessary for leptin-induced invasion of kidney and colonic epithelial cells, and that this effect was independent of the PI3K downstream effector Rac (3). Our data indicate that blocking PI3K activation results in complete inhibition of leptin-induced monocyte migration. The roles of Akt and/or Cdc42, Rac, and Rho in leptin-induced monocyte migration require further study. Taken together, our inhibitor studies demonstrate that all intracellular migratory signaling pathways are required to work in concert for leptin to induce monocyte chemotaxis.

The pathophysiological consequences of leptin-induced monocyte chemoattraction may have physiological relevance

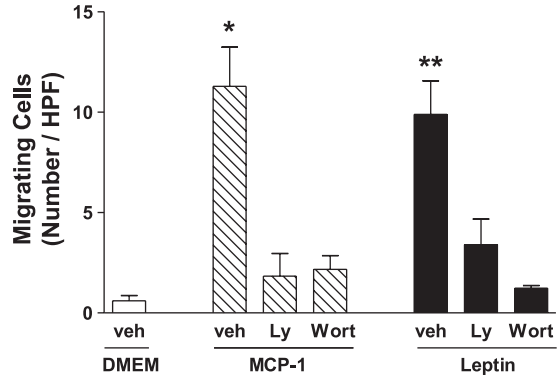

Fig. 8. Effect of phosphatidylinositol 3-kinase (PI3K) inhibition on leptin-induced THP-1 monocyte migration. Monocytes were preincubated with PI3K inhibitors wortmannin (10 nM) and LY294002 (10 μM) for 30 min before and during Boyden chamber migration assay. Cells were allowed to migrate to DMEM with either MCP-1 (10 ng/ml) or leptin (1 ng/ml) added to the bottom chamber. Results represent counts from five high-power fields from three independent experiments performed in triplicate wells. All conditions were performed simultaneously; however, statistics were performed separately for MCP-1 and leptin treated cells. *$P < 0.001$ compared with DMEM, $P < 0.05$ compared with LY294002, and $P < 0.01$ compared with wortmannin. **$P < 0.001$ compared with DMEM, $P < 0.01$ compared with LY294002 and wortmannin.

to various diseases. Risk for diseases such as atherosclerosis and breast cancer has been shown to be increased in obesity and is related to plasma leptin concentrations (11, 19, 32, 38). With respect to atherosclerotic lesion formation, it has been shown that both epicardial and perivascular WAT are increased in obesity and are responsible for the secretion of many factors, including leptin, which could promote monocyte transmigration into the intima (16, 26, 32). In fact, Curat et al. (7) reported that leptin promotes monocyte transmigration by activating endothelial cell adhesion molecule expression, demonstrating that leptin could play a role in atherosclerotic disease by not only increasing monocyte recruitment, but also by increasing adhesion to endothelial cell layers. It has also been shown that breast tissue leptin secretion is correlated with tumor-associated macrophage accumulation, resulting in adverse prognosis for breast cancer in obese patients (13). Thus leptin-mediated macrophage infiltration of various tissues may play a role in several pathophysiological consequences related to obesity.

Taken together, our data demonstrate that leptin is a potent chemoattractant for monocyte/macrophages. Our data have implications for understanding the pathophysiology of obesity-associated disease and, in particular, macrophage infiltration of various tissues in response to leptin.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

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