

EGFR Tyrosine Kinase Inhibitor (PD153035) Improves Glucose Tolerance and Insulin Action in High-Fat Diet-Fed Mice

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OBJECTIVE—In obesity, an increased macrophage infiltration in adipose tissue occurs, contributing to low-grade inflammation and insulin resistance. Epidermal growth factor receptor (EGFR) mediates both chemotaxis and proliferation in monocytes and macrophages. However, the role of EGFR inhibitors in this subclinical inflammation has not yet been investigated. We investigated, herein, *in vivo* efficacy and associated molecular mechanisms by which PD153035, an EGFR tyrosine kinase inhibitor, improved diabetes control and insulin action.

RESEARCH DESIGN AND METHODS—The effect of PD153035 was investigated on insulin sensitivity, insulin signaling, and c-Jun NH₂-terminal kinase (JNK) and nuclear factor (NF)-κB activity in tissues of high-fat diet (HFD)-fed mice and also on infiltration and the activation state of adipose tissue macrophages (ATMs) in these mice.

RESULTS—PD153035 treatment for 1 day decreased the protein expression of inducible nitric oxide synthase, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 in the stroma vascular fraction, suggesting that this drug reduces the M1 proinflammatory state in ATMs, as an initial effect, in turn reducing the circulating levels of TNF-α and IL-6, and initiating an improvement in insulin signaling and sensitivity. After 14 days of drug administration, there was a marked improvement in glucose tolerance; a reduction in insulin resistance; a reduction in macrophage infiltration in adipose tissue and in TNF-α, IL-6, and free fatty acids; accompanied by an improvement in insulin signaling in liver, muscle, and adipose tissue; and also a decrease in insulin receptor substrate-1 Ser³⁰⁷ phosphorylation in JNK and inhibitor of NF-κB kinase (IKKβ) activation in these tissues.

CONCLUSIONS—Treatment with PD153035 improves glucose tolerance, insulin sensitivity, and signaling and reduces subclinical inflammation in HFD-fed mice. *Diabetes* 58:2910–2919, 2009

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Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are used in the clinic to treat malignancies (1). It has recently been observed that a modest number of patients, suffering from both malignancies and type 2 diabetes, were successfully treated not only for their malignancies but also for diabetes when given some tyrosine kinase inhibitors (2–5). However, the molecular mechanisms that account for the effect of these drugs on insulin action and glucose metabolism are unknown.

Insulin stimulates a signaling network composed of a number of molecules, initiating the activation of insulin receptor tyrosine kinase and phosphorylation of insulin receptor substrates, including insulin receptor substrate (IRS)-1 and IRS-2 (6–8). Following tyrosine phosphorylation, IRS-1/IRS-2 bind and activate the enzyme phosphatidylinositol 3-kinase (PI3-K). The activation of PI3-K increases serine phosphorylation of Akt, which is responsible for most of the metabolic actions of insulin, such as glucose transport, lipogenesis, and glycogen synthesis (7,8).

In the most prevalent forms of insulin resistance, diet-induced obesity, and type 2 diabetes, there is a downregulation in this signaling pathway in insulin-sensitive tissues, parallel to a state of chronic low-grade inflammation (6). Several serine/threonine kinases are activated by inflammatory or stressful stimuli and contribute to inhibition of insulin signaling, including c-Jun NH₂-terminal kinase (JNK) (9–13) and inhibitor of nuclear factor (NF)-κB kinase (IKKβ) (12,14). In obesity, an increased macrophage infiltration in adipose tissue occurs, contributing to this low-grade inflammation (15–17), which has an important role in the increased tissue production of proinflammatory molecules and acute-phase proteins associated with obesity (13,14). EGFR has been described in monocytes and in macrophages and mediates both chemotaxis and proliferation in macrophages (18–20). However, the role of EGFR inhibitors on this subclinical inflammation of obesity was not yet investigated.

PD153035 has been shown to possess highly potent and selectively inhibitory activity against EGFR tyrosine kinase and rapidly suppresses autophosphorylation of EGFR at low nanomolar concentrations in fibroblasts and human epidermoid carcinoma cells, as well as selectively blocking EGF-mediated cellular processes, including mitogenesis and early gene expression (21–23). In addition, PD153035 has been shown to reduce JNK and IKK/κB/NF-κB pathways (24,25). Moreover, EGFR and other ty-

rosine kinase inhibitors have also been shown to inhibit the growth of monocyte/macrophages, suggesting possible mechanisms to improve insulin action (26–29).

Herein, we investigated the *in vivo* efficacy and associated molecular mechanisms by which PD153035, an EGFR tyrosine kinase inhibitor, improved diabetes control and insulin action. We studied the effect of acute (1 day) or chronic (14 days) administration of PD153035 on insulin sensitivity, insulin signaling, and JNK and NF- κ B activity in liver, muscle, and adipose tissue of high-fat diet (HFD)-fed mice and also on the infiltration and activation state of adipose tissue macrophages (ATMs) in these mice.

RESEARCH DESIGN AND METHODS

Male Swiss mice were obtained from the University of Campinas, São Paulo. The mice were bred under specific pathogen-free conditions at the Central Breeding Center of the University of Campinas. Antiphosphotyrosine (α -PY), anti-IR β (α -IR), anti-IRS-1, anti-Akt1/2, anti-p-JNK, anti-inducible nitric oxide synthase (iNOS), anti-tumor necrosis factor (TNF)- α , anti-interleukin (IL)-6, anti-EGFR, anti-caveolin, anti-actin, anti-IKK β , anti-p-IKK β , anti-p-c-Jun, and anti-I κ B α antibodies were from Santa Cruz Technology (Santa Cruz, CA). Anti-pAkt was from Cell Signaling Technology (Beverly, MA). Anti-phospho-IRS-1^{ser307} was obtained from Upstate Biotechnology (Lake Placid, NY). Human recombinant insulin was from Eli Lilly and Company (Indianapolis, IN). Routine reagents were purchased from Sigma Chemical (St. Louis, MO), unless specified elsewhere.

Compound PD153035 [4-*N*-(3'-bromo-phenyl)amino-6,7-dimethoxyquinazoline hydrochloride] was synthesized, as previously described (30). The compound was >99% pure, as determined by elemental analysis, high-performance liquid chromatography, mass spectrometry, and ¹H and ¹³C nuclear magnetic resonance (30).

Animal care and experimental procedures. All experiments were approved by the ethics committee of the State University of Campinas. Eight-week-old male Swiss mice were divided into four groups with similar body weights and assigned to receive the following diet and/or treatment: control group received a standard rodent diet and water *ad libitum*; HFD group received an HFD consisting of 55% calories from fat, 29% from carbohydrate, and 16% from protein for 8 weeks; and HFD with PD153035 for 14 days (HFPD14days) received the same HFD for 8 weeks, but in the last 2 weeks these animals also received PD153035 (30 mg/kg) by gavage once a day. A group of HFD animals also received the same dose of PD153035 at 24 and 2 h before the experiments, and this group was called HFPD1day. Body weight and food intake were measured weekly. Glucose tolerance tests and insulin tolerance tests were performed on these mice after 8 weeks on the diets, as previously described (31,32).

Assays. Insulin, leptin, and adiponectin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (Linco). Serum free fatty acid (FFA) levels were analyzed using the NEFA-kit-U (Wako Chemical, Neuss, Germany), with oleic acid as a standard. Glucose values were measured from whole venous blood with a glucose monitor (Glucometer; Bayer). Serum concentrations of IL-6 and TNF- α were determined using mouse IL-6 ELISA and mouse TNF- α ELISA (Pierce Endogen, Rockford, IL). Monocyte chemoattractant protein (MCP)-1, MCP-2, and MCP-3 ELISA kits were purchased from Antigenix America (Huntington Station, NY).

Light microscopy and morphometry. Mice were fasted for 12 h and killed with an overdose of anesthetic (sodium thiopental). Epididymal, retroperitoneal, and mesenteric adipose tissues were dissected and assessed by light microscopy and morphometry. Tissue sections were observed with a Zeiss Axiophot light microscope using a $\times 40$ objective, and digital images were captured with a Canon PowerShot G5. Crown-like structure (CLS) density (average CLS within 10 high-power fields, per animal) and mean adipocyte surface area (average surface area of 30 randomly sorted adipocytes, per animal) were determined using the Imagemlab Analysis software (version 2.4), as previously described (33).

Tissue extraction, immunoprecipitation, and immunoblotting. Mice were anesthetized by intraperitoneal injection of sodium thiopental and were used 10–15 min later (i.e., as soon as anesthesia was assured by the loss of pedal and corneal reflexes). Five minutes after the insulin injection (3.8 units/kg *i.p.*) liver, muscle, and adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer, as described elsewhere (34). Extracts were used for immunoprecipitation with α -IR, α -IRS-1, α -EGFR, and protein A-sepharose 6MB (Pharmacia, Uppsala, Sweden). The precipitated proteins and/or whole tissue extracts were subjected to SDS-PAGE and immunoblotting as previously described (6,31).

Determination of NF κ B activation. NF κ B p50 activation was determined in nuclear extracts from liver, muscle, and adipose tissue by ELISA (89858; Pierce Biotechnology), according to the recommendations of the manufacturer.

Isolation of the stroma vascular fraction and adipocyte fraction of adipose tissue. Epididymal, retroperitoneal, or mesenteric fat pads were excised, and isolation of the stroma vascular fraction and adipocyte fraction of adipose tissue were performed, as previously described (33). A summary of the method is presented in the online appendix (available at <http://diabetes.diabetesjournals.org/cgi/content/full/db08-0506/DC1>).

Arginase assay. Arginase activity assays were performed, as previously described (35). A summary of the method is presented in the online appendix. **Statistical analysis.** Data are expressed as means \pm SE, and the number of independent experiments is indicated. For statistical analysis, the groups were compared using a two-way ANOVA with the Bonferroni test for post hoc comparisons. The level of significance adopted was $P < 0.05$.

RESULTS

Effect of PD153035 on EGFR tyrosine phosphorylation in liver, muscle, and adipose tissue of mice. The drug PD153035 was developed in 1994 as a specific tyrosine kinase inhibitor of the EGFR (20). To investigate the effect of PD153035 administration on EGFR phosphorylation, we immunoprecipitated liver, muscle, and adipose tissue extracts of controls, HFD-fed animals, and HFD-fed animals treated with PD153035 for 1 or 14 days with anti-EGFR antibody and performed immunoblotting with anti-phosphotyrosine antibody. The results showed that PD153035 administration was able to reduce EGFR tyrosine phosphorylation in the three tissues by 70–90% in a similar fashion after 1 or 14 days (Fig. 1A–C). HFD did not change the tissue levels of EGFR in liver, muscle, and epididymal fat pad; however, there was an increase in EGFR expression (Fig. 1C) and in tyrosine phosphorylation in the mesenteric and retroperitoneal fat pads. The reduction in EGFR tyrosine phosphorylation, induced by PD153035, was greater in the mesenteric and retroperitoneal fat pads compared with the epididymal fat pad (Fig. 1C). PD153035 treatment reduced EGFR tyrosine phosphorylation in a dose-dependent manner in liver, muscle, and retroperitoneal tissues (online appendix Fig. S1).

Effect of PD153035 on body weight and fat pads in HFD-fed mice. Eight-week-old male Swiss mice were placed on HFD and then supplemented, or not, with PD153035 on the last day (HFPD1) or during 14 days (HFPD14) before the experiments. Weight gain after 8 weeks was similar in HFD or HFPD groups and was higher in these groups than in the control group that received standard rodent diet (Fig. 1D). There is a slight reduction in body weight after 14 days of PD153035 compared with HFD or HFPD1, which is not statistically significant. Daily food intake was similar in HFD or HFPD, and 8-week cumulative food intake was higher for both groups on HFD (data not shown). As expected, the epididymal, retroperitoneal, and mesenteric fat pad weights were higher in the HFD group, and PD153035 treatment for 1 day did not change these fat pad weights, but after 14 days there was a significant reduction in retroperitoneal and mesenteric fat pad weights (Fig. 1E–G).

Effect of PD153035 on metabolic parameters in HFD-fed mice. The fasting plasma glucose levels were higher in HFD and in HFPD1 than in the other groups (Figs. 1H). PD153035 treatment reduced fasting plasma glucose levels in a dose-dependent manner (Fig. S2). During the glucose tolerance test, the plasma glucose and serum insulin levels were significantly higher in HFD and HFPD1 mice compared with controls, and PD153035 administration for 14 days improved glucose tolerance and reduced insulin

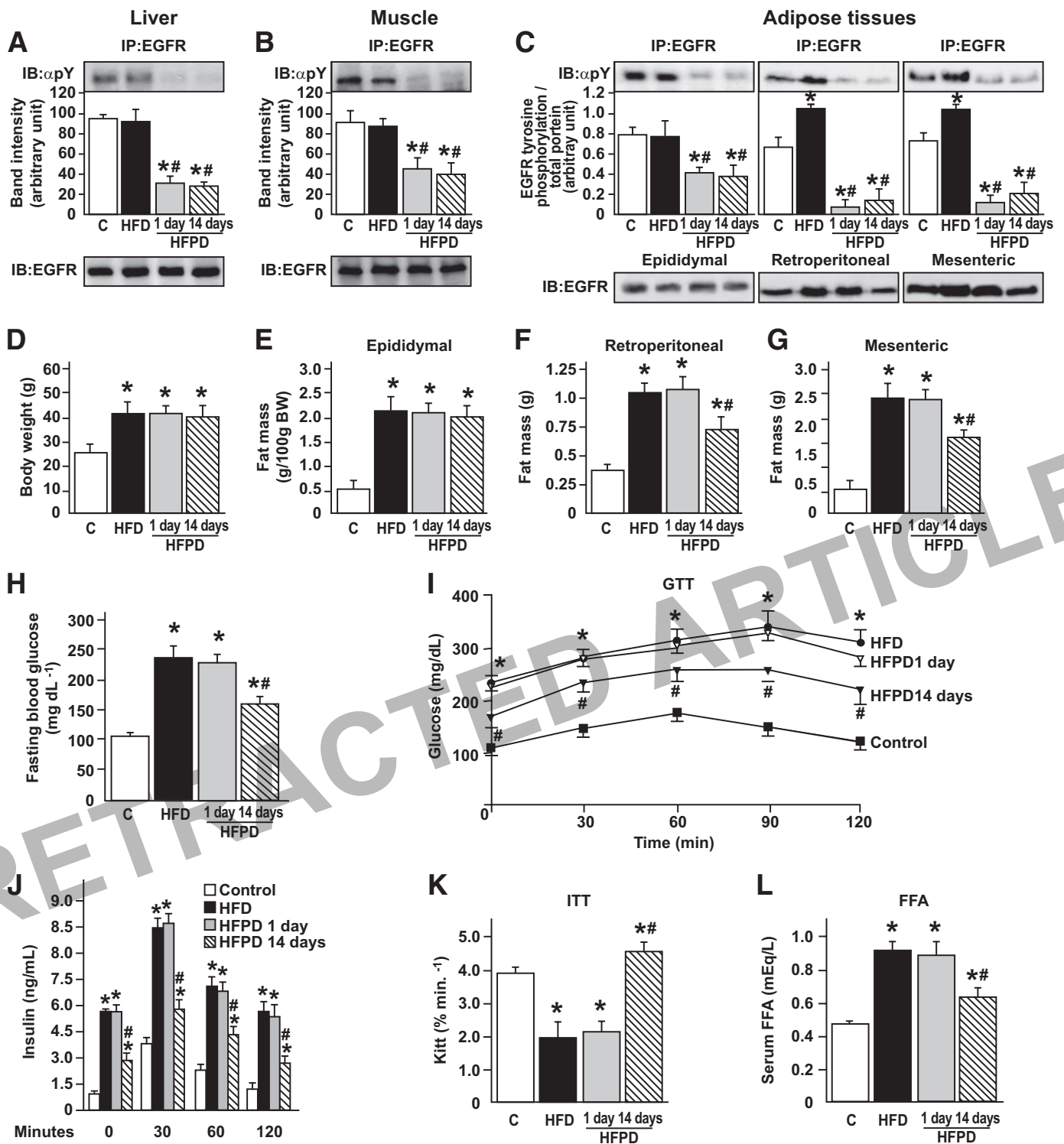


FIG. 1. Effects of acute or chronic PD153035 administration in fed mice. (A–C, upper panels). Representative blots show the tyrosine phosphorylation of EGFR of control mice, HFD mice, and HFPD 1 and 14 days in liver (A), muscle (B), and adipose (C). Total protein expression of EGFR (A–C, lower panels). D: Body weight. E: Epididymal fat pad weight. F: Retroperitoneal fat pad weight. G: Mesenteric fat pad weight. H: Fasting plasma glucose. I: Glucose tolerance test. J: Serum insulin during glucose tolerance test. K: Glucose disappearance rate. L: Serum FFAs. Data are presented as means \pm S.E.M from six to eight mice per group. * $P < 0.05$ vs. control group; # $P < 0.01$ vs. HFD. IB, immunoblot; IP, immunoprecipitate.

levels at all time points studied (Fig. 1I and J). The glucose disappearance rate was lower in HFD and in HFPD1 groups, and PD153035 administration for 14 days (HFPD14) reversed these alterations (Fig. 1K). Taken together, the lower insulin levels during the glucose tolerance test and the increase in glucose disappearance rate

during the insulin tolerance test after PD153035 treatment for 14 days suggest that this drug improves insulin sensitivity. FFA levels were significantly higher in HFD and HFPD1 and returned to levels close to those of the control group after 14 days of PD153035 administration (Fig. 1L).

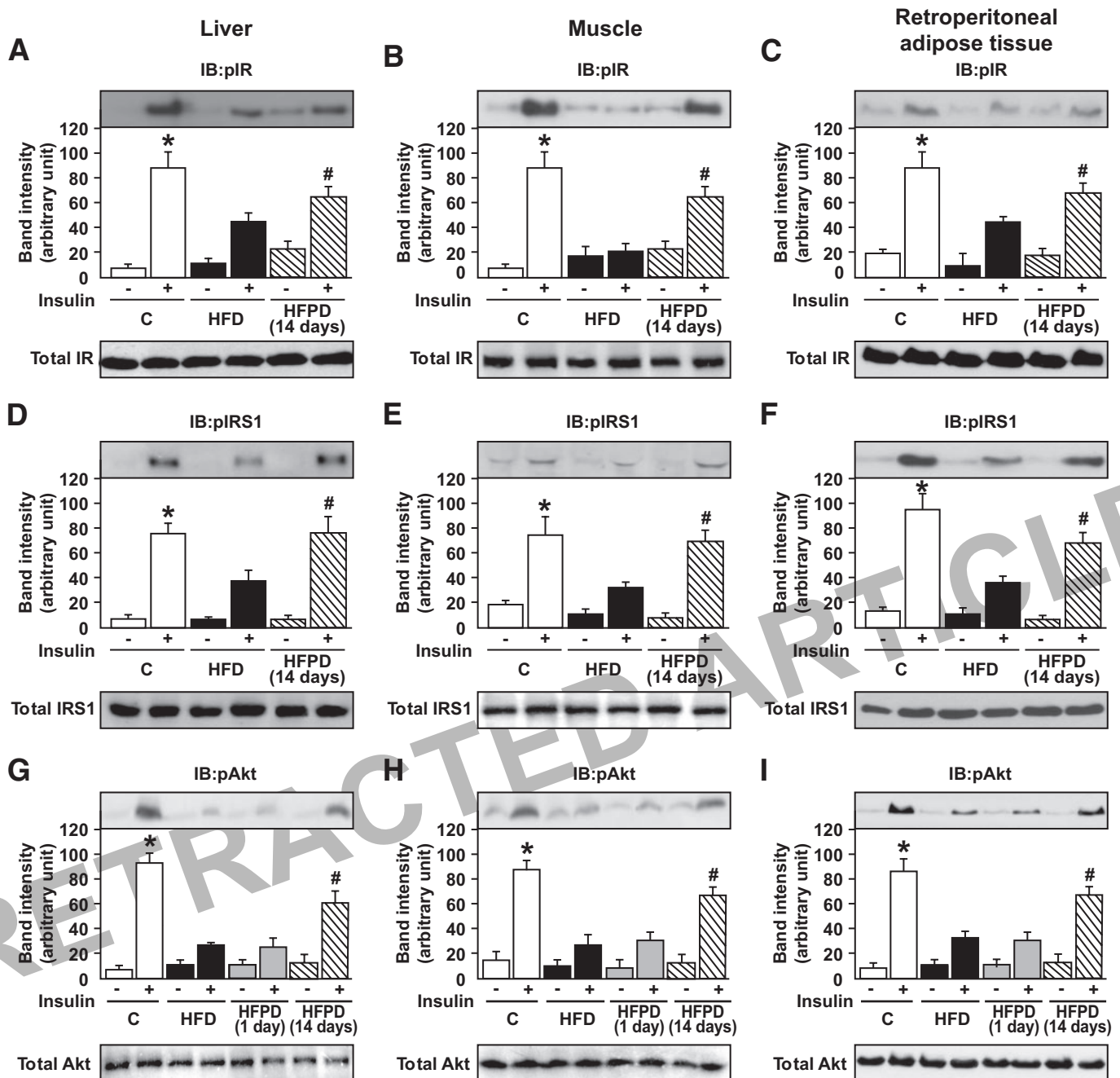


FIG. 2. Effects of PD153035 administration on insulin signaling in fed mice. Representative blots show tyrosine phosphorylation of IR β in liver (A), muscle (B), and adipose (C) of control mice, HFD mice, and HFPD during 14 days (upper panels). Total protein expression of IR β (A–C, lower panels). Tyrosine phosphorylation of IRS-1 in liver (D), muscle (E), and retroperitoneal (F) of control mice, HFD mice, and HFPD 14 days (upper panels). Total protein expression of IRS-1 (D–F, lower panels). Serine phosphorylation of Akt in liver (G), muscle (H), and adipose (I) of control mice, HFD mice, and HFPD 1 and 14 days (upper panels). Total protein expression of Akt (G–I, lower panels). Data are presented as means \pm SE from six to eight mice per group. * $P < 0.05$ control vs. HFD group; # $P < 0.05$ HFPD 14 days vs. HFD. IB, immunoblot.

Effect of PD153035 on insulin signaling in liver, muscle, and retroperitoneal adipose tissue of HFD-fed mice. In liver, muscle, and retroperitoneal adipose tissues, insulin-induced IR β (Fig. 2A–C) and IRS-1 tyrosine phosphorylation (Fig. 2D–F) and Akt serine phosphorylation (Fig. 2G–I) were reduced by 50–70% in mice fed on an HFD compared with controls. The treatment with PD153035 for 1 day did not change the insulin-induced tyrosine phosphorylation levels of IR and IRS-1 (data not shown) and also did not improve Akt serine phosphorylation levels in liver muscle and adipose tissues (Fig. 2G–I

and online appendix Fig. S3). However, 14 days of treatment reversed these reductions in the three tissues studied (Fig. 2A–I). The protein concentration of IR, IRS-1, and Akt in liver, muscle, and retroperitoneal adipose did not change between the groups.

The effect of PD153035 improving Akt phosphorylation in HFD-fed mice was dose dependent (online appendix Fig. S3). In control animals, PD153035 did not change insulin-induced Akt phosphorylation in liver, muscle, or epididymal adipose tissue or glucose uptake in isolated muscle (online appendix Fig. S4).

Ser³⁰⁷ phosphorylation of IRS-1 and activation of JNK and IKK β in liver, muscle, and retroperitoneal tissue of HFD-fed mice treated with PD153035. IKK β activity was monitored using IKK β phosphorylation and I κ B α protein abundance, as previously described (12). IKK β phosphorylation was increased and I κ B α protein levels were reduced in liver, muscle, and retroperitoneal adipose tissue of mice fed an HFD or HFDP1 diet but not in these tissues of HFDP14 mice (Fig. 3A–F). We also measured the nuclear NF- κ B subunit p50 activation and found an increase in the DNA binding of nuclear p50 in liver, muscle, and retroperitoneal of mice on an HFD and HFDP1, but there was a clear decrease in the three tissues in HFDP14 (Fig. 3G–I). JNK activation was determined by monitoring phosphorylation of JNK (Thr183 and Tyr185) and the protein levels of p-c-Jun. JNK phosphorylation and p-c-Jun were increased in liver, muscle, and white adipose tissue (WAT) of mice fed on an HFD and HFDP1, and this increase was reversed by 14 days of PD153035 treatment (Fig. 3J–O). We tested Ser³⁰⁷ phosphorylation of IRS-1 in liver, muscle, and WAT in the four groups of mice. Ser³⁰⁷ phosphorylation was induced by an HFD in the three tissues of mice, and the treatment with PD153035 for 14 days reversed this alteration (Fig. 3P–R).

Effect of PD153035 on retroperitoneal adipose tissue morphology and ultrastructural features in HFD-fed mice. Morphometric analysis revealed that in retroperitoneal fat pad, adipocytes from HFDP14 were consistently smaller than adipocytes from control mice fed on an HFD or HFDP1, with an average 40% decrease in size (Fig. 4A and B). In mesenteric and epididymal depots, the reduction in adipocytes in HFDP14 was 30–40% average decrease in size (online appendix Fig. S5). In addition, the frequency and distribution of mature macrophages in fixed WAT differed between the groups. As previously described (15), macrophages were aggregated in CLSs, which contained up to 15 macrophages surrounding what appeared to be individual adipocytes. CLS formation was a rare event in control mice (24 ± 9) but was increased >200-fold (489 ± 58) in control mice on HFD or on HFDP1 (506 ± 66) and only ~8-fold (150 ± 23) in HFDP14, indicating a much lower macrophage infiltration in the WAT of the latter group. To analyze if PD153035 was able to reduce macrophage infiltration in retroperitoneal adipose tissue, immunohistochemical staining using specific macrophage marker F4/80+ was performed. As shown in Fig. 4C and D, HFD increased F4/80+ staining, and PD153035 treatment for 14 days reduced this staining, suggesting less macrophage were present (Fig. 4C and D). In epididymal and mesenteric fat pads (online appendix Figs. S5 and S6) the results were very similar to the retroperitoneal. As shown in Fig. 4E, treatment with PD153035 significantly impaired the migration of human monocytic leukemia cell line (THP1) in a dose-response manner (online appendix).

Effect of PD153035 on tissue protein levels of TNF- α , IL-6, and iNOS and arginase activity in adipocytes and stroma vascular fraction. In retroperitoneal adipose tissue, separation of the stroma vascular fraction (SVF) from adipocytes of lean, HFD, HFDP1, and HFDP14 animals indicated that there was a modest increase in TNF- α protein expression in adipocytes from HFD animals compared with controls and that PD153035 reduced the expression of this cytokine only after 14 days of treatment (Fig. 5A). In adipocytes, the expressions of IL-6 and iNOS were higher in mice that received the HFD; these expres-

sions were not significantly affected by PD153035 treatment for 1 day. However, after 14 days of PD153035 administration, there was a clear decrease in the expression of these proteins in adipose tissue (Fig. 5B and C). Similar results were observed in liver and muscle (online appendix Fig. S7). Treatment with PD153035 for 1 or 14 days reduced EGFR tyrosine phosphorylation in adipocytes (Fig. 5D). In SVF, the expressions of TNF- α , IL-6, and iNOS were also higher in HFD animals compared with controls. Different from adipocytes, PD153035 administration for just 1 day was able to reduce the SVF expressions of TNF- α , IL-6, and iNOS, which were normalized after 14 days administration of this drug (Fig. 5F–H). There was a significant increase in EGFR tyrosine phosphorylation in SVF of HFD group, and the treatment with PD153035 for 1 or 14 days induced a marked reduction in EGFR tyrosine phosphorylation levels in SVF (Fig. 5I). Similar results were observed in adipocytes and SVF from epididymal (online appendix Fig. S8) and mesenteric (data not shown) fat depots.

An important characteristic of the alternative macrophage activation state is the increased arginase activity (35). Arginase activity was measured in adipocytes and SVF samples from controls, HFD, and HFD rats treated with PD153035 for 1 or 14 days. Results showed that the activity of this enzyme did not differ between the isolated adipocytes from the four groups of animals (Fig. 5K). However, arginase activity was significantly reduced in the SVF of rats on an HFD, and a significant increase was observed after just 1 day of PD153035 administration. After 14 days of treatment, arginase activity was similar to that of control animals (Fig. 5K).

Adiponectin levels were reduced in control mice on an HFD and HFDP1 but increased significantly after 14 days of PD153035 administration. (Fig. 5L). Serum leptin levels were higher in the HFD group, and PD153035 administration did not change these levels (Fig. 5N). Serum TNF- α and IL-6 levels were higher in mice on an HFD; interestingly, PD153035 administration for 1 day reduced the levels of these cytokines. After 2 weeks of PD153035 treatment, TNF- α and IL-6 returned to normal levels (Fig. 5M and O).

The protein levels of MCP-1 and MCP-3 were significantly increased in adipose tissue of HFD mice, and treatment with PD153035 for 14 days significantly reduced these chemokines. MCP-2 protein levels were not influenced by high fat as previously described (36) or PD153035 (Fig. 5P–R).

DISCUSSION

Our results show that the use of PD153035 (EGFR tyrosine kinase inhibitor) in HFD-fed mice for 14 days induced a marked improvement in glucose tolerance; a reduction in insulin resistance; a reduction in macrophage infiltration in adipocytes and in low-grade inflammation, accompanied by an improvement in insulin signaling in liver, muscle, and adipose tissue; and also an increase in serum adiponectin levels.

It is important to emphasize that administration of PD153035 for 1 day did not change insulin sensitivity/signaling or macrophage infiltration in adipose tissue but reduced the circulating levels of IL-6 and TNF- α , probably as a consequence of reduced activation of macrophage, as shown by a reduction in the expression of these cytokines in the SVF. These data suggest that the first effect observed

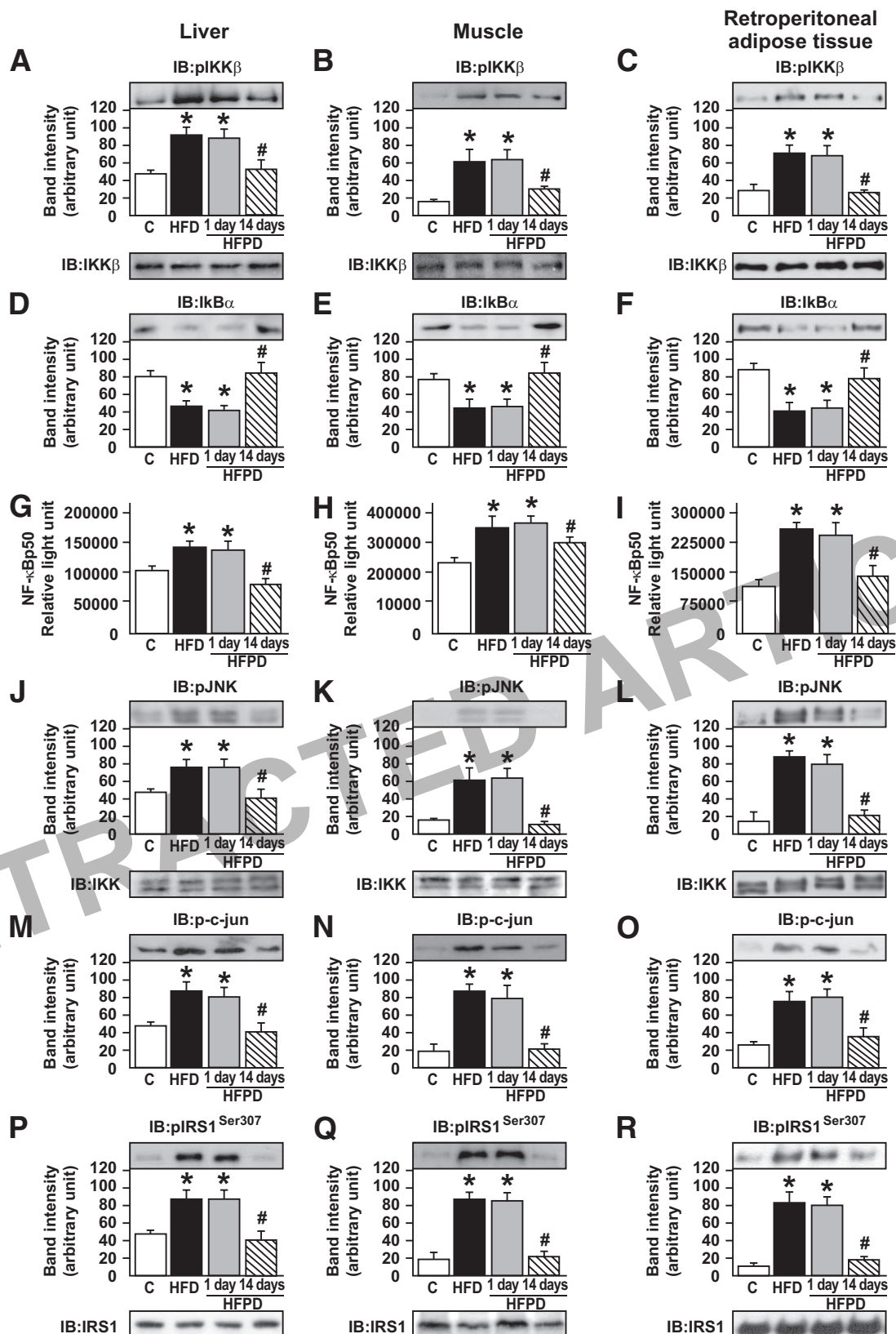


FIG. 3. Effects of PD153035 administration on modulators of insulin signaling. Representative blots show the expression of IKK β phosphorylation in liver (A), muscle (B), and retroperitoneal (C) of control mice, HFD mice, and HFDPD 1 and 14 days (upper panels). Total protein expression of IKK β (A–C, lower panels). I κ B α in liver (D), muscle (E), and adipose (F) of control mice, HFD mice, and HFDPD 1 and 14 days. NF κ B p50 activation was determined in nuclear extracts from liver (G), muscle (H), and adipose (I) tissue by ELISA. JNK phosphorylation in liver (J), muscle (K), and adipose (L) of control mice, HFD mice, and HFDPD 1 and 14 days (upper panels). Total protein expression of JNK (J–L, lower panels). c-Jun phosphorylation in liver (M), muscle (N), and adipose (O) of control mice, HFD mice, and HFDPD 1 and 14 days. IRS1 serine 307 phosphorylation in liver (P), muscle (Q), and adipose (R) of control mice, HFD mice, and HFDPD 1 and 14 days (upper panels). Total protein expression of IRS-1 (P–R, lower panels). Data are presented as means \pm SE from six mice per group, * P < 0.05 vs. control group and # P < 0.05 vs. HFD. IB, immunoblot.

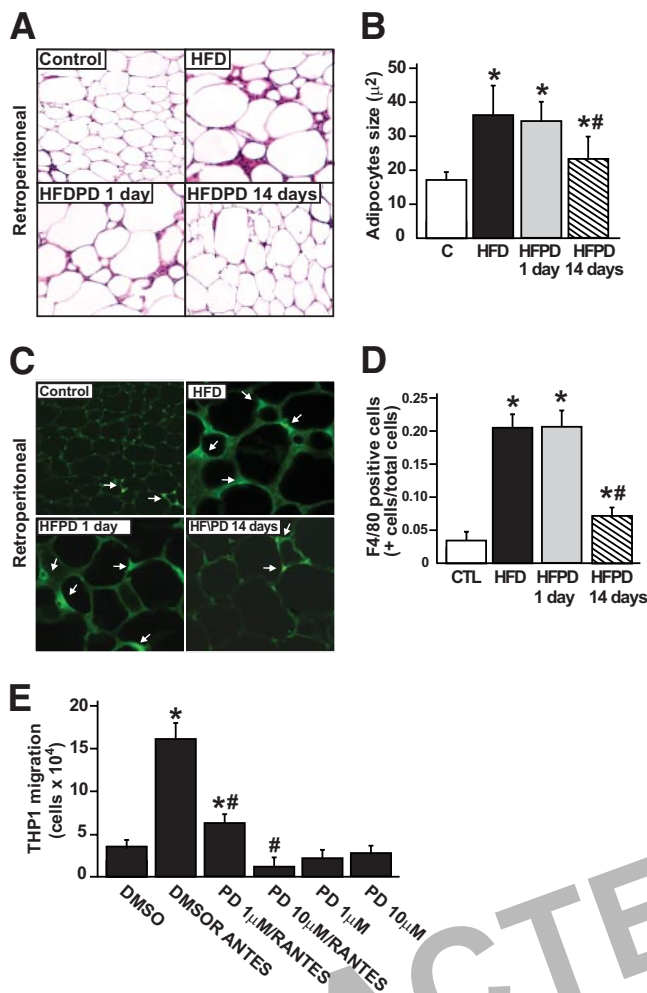


FIG. 4. Effects of PD153035 on adipocyte morphology and activation and migration of macrophages in adipocyte tissue. **A:** Histological sections of retroperitoneal fat pads from control, HFD, and HFDPD after 1 or 14 days, 50- μ m scale bar for all pictures. **B:** Quantification of adipocyte size. About 100 cells were measured in each group, and the average adipocyte was calculated. **C:** Representative immunohistochemical staining of WAT using the specific macrophage marker F4/80+. **D:** F4/80-positive cells (+ cells/total cells) of all above groups. Data are presented as means \pm SE from six mice per group, * P < 0.05 vs. control group and # P < 0.05 vs. HFD. **E:** Bar graph indicates the number of THP1 cells that migrate from the top to the bottom level of the Boyden blindwell chamber stimulated or not with chemotactic agent RANTES. * P < 0.05 vs. DMSO alone; # P < 0.05 vs. DMSO/RANTES. Values represent the average of five different assays. (A high-quality color digital representation of this figure is available in the online issue.)

with this drug is a change in macrophage activation. Macrophage activation has been defined across two separate polarization states, M1 and M2 (35,37,38). M1 or “classically activated” macrophages are induced by proinflammatory mediators, such as lipopolysaccharide and interferon- γ , and have enhanced cytokine production (IL-6 and TNF- α) and generate reactive oxygen species such as NO via activation of iNOS. M2 or “alternatively activated” macrophages have low proinflammatory cytokine expression and, instead, generate high levels of the anti-inflammatory cytokines IL-10 and IL-1 decoy receptor. In addition, in these macrophages, arginase production (an enzyme that blocks iNOS activity) is increased (39). In summary, M2 macrophages are believed to participate in the blockade of inflammatory responses and in the promotion of tissue repair (37). Our data show that

PD153035 treatment for just 1 day reduced the expression of IL-6, TNF- α , and iNOS in the SVF and, in parallel, induced an increase in arginase activity, suggesting that PD153035 may lead to a shift in the activation state of ATMs, reducing the M1 proinflammatory state that contributes to insulin resistance. Since EGFR tyrosine phosphorylation was increased in the SVF of HFD mice, it is possible that the primary action of PD153035 is on ATMs, but a direct relation between EGFR and macrophage activation deserves further investigation.

In mice treated with PD153035 for 14 days, the HFD induced a less marked macrophage infiltration in adipose tissue, accompanied by an attenuated increase in TNF- α , IL-6, and FFAs. This decrease in macrophage infiltration may be a direct effect of EGFR tyrosine kinase inhibition. In agreement, our data show that PD153035 reduces monocyte migration. Recent studies (21–23) demonstrated that EGFR and/or other tyrosine kinase inhibitors inhibit the growth and/or activation of some nonmalignant hematopoietic cells, including monocyte/macrophages. Interestingly, another study (40) has shown that a reduction in macrophage infiltration and/or resident alternatively activated macrophages can decrease local inflammation in WAT. In accordance with this, our data show that in the adipose tissue of HFD-fed mice treated with PD153035 for 14 days, in parallel with a reduction in macrophage infiltration, there were lower expressions of TNF- α , IL-6, and iNOS, indicating that this drug decreases local inflammation in WAT of HFD mice. In addition, in HFD mice treated with PD153035 for 14 days there was also a decrease in MCP-1 and MCP-3 in adipose tissue, which may have a role in the reduced macrophage infiltration. These results lead us to suggest that this decrease in inflammation in WAT may have an important role in the effect of PD153035, improving insulin resistance and glucose tolerance in HFD mice.

The improvement in insulin action induced after 14 days of PD153035 administration was also demonstrated at the tissue level in the insulin signaling pathway. The blunted insulin-stimulated IR tyrosine phosphorylation and phosphorylation of Akt and the increase of IRS-1 Ser³⁰⁷ in liver, muscle, and WAT of HFD mice was prevented by treatment with PD153035, providing a biochemical correlate for the increase in in vivo insulin sensitivity. Ser³⁰⁷ is reported to be a phosphoacceptor of JNK and IKK β (10,41); as previously described (42–45), our results also show that these kinases are activated in tissues of HFD mice. Our data demonstrated that PD 153035 administration for 14 days prevents the activation of IKK β and JNK in liver, muscle, and WAT, which may be a consequence of the reduction in inflammation in WAT and in the circulating levels of FFAs, TNF- α , and IL-6. However, we cannot exclude the possibility of a direct effect of PD153035 on JNK and IKK β /NF- κ B pathways as previously described in cell culture (24,25), although our data show that acute administration of PD153035 did not have this effect.

It is unlikely that PD153035 improved insulin action by a direct effect on glucose transport in muscle because the administration of this drug to isolated muscle did not increase insulin-induced glucose uptake. Another mechanism that may have contributed to the effect of PD153035 on glucose homeostasis is the reversal of the decreased adiponectin levels observed in HFD mice. It is possible that the reduced inflammatory state in adipose tissue and smaller adipocytes in HFPD14 may have allowed the restoration or even an increase in adiponectin secretion.

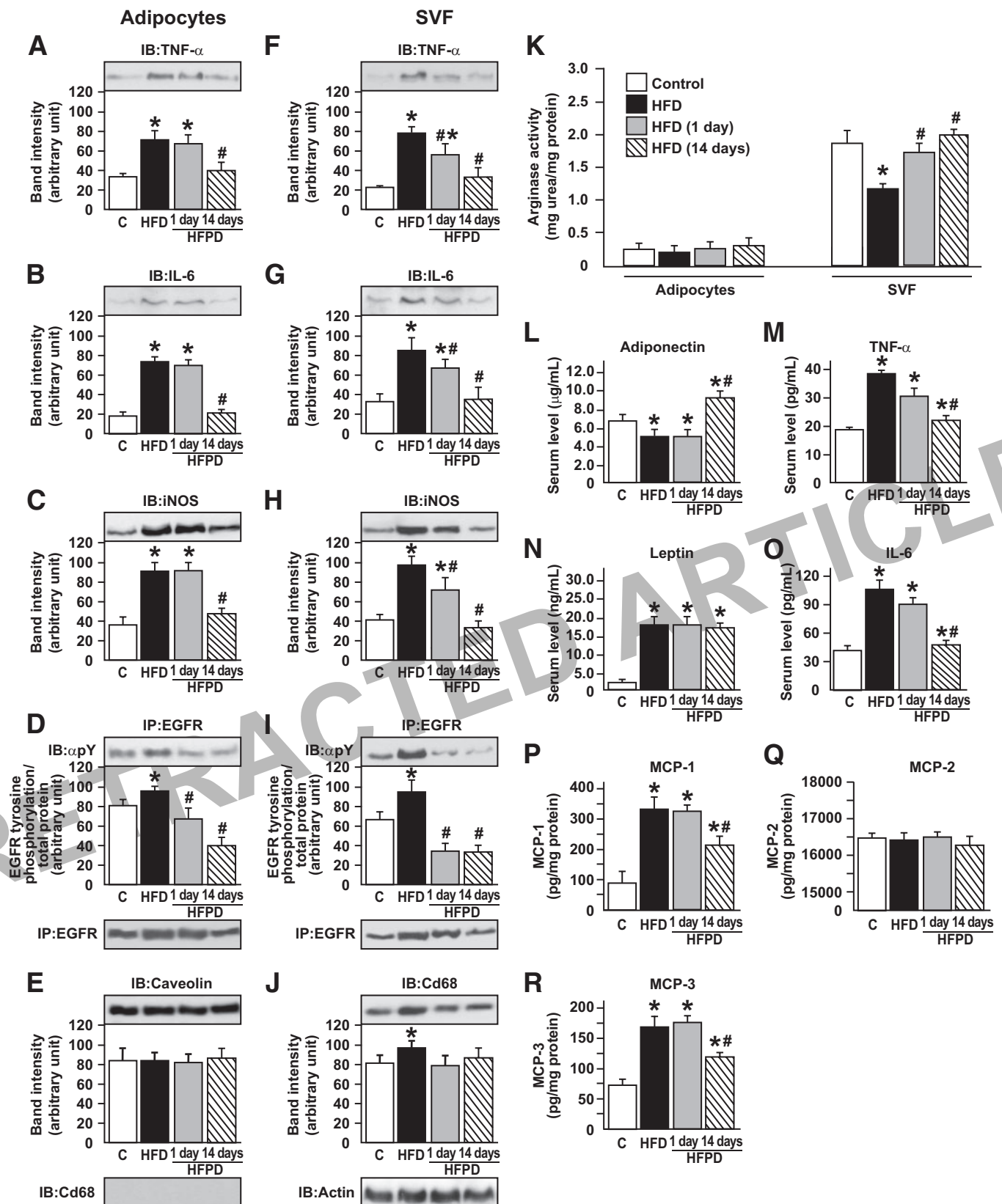


FIG. 5. Effect of PD153035 on tissue protein levels of TNF- α , IL-6, and iNOS and arginase activity in adipocytes and SVF from retroperitoneal adipose tissue. Representative blots show the tissue levels of TNF- α , IL-6, iNOS, EGFR tyrosine phosphorylation, EGFR, Caveolin, and Cd68 protein expression in adipocytes (A–D) and TNF- α , IL-6, iNOS, EGFR tyrosine phosphorylation, EGFR, Cd68, and actin protein expression in the SVF (F–J). K: Arginase activity of adipocytes and SVF from control mice, HFD mice, and HFD 1 and 14 days. Serum levels of adiponectin (L), TNF- α (M), leptin (N), and IL-6 (O) and MCP-1 (P), MCP-2 (Q), and MCP-3 (R) protein expression were obtained using ELISA assay. Data are presented as means \pm SE of six to eight mice per group. * P < 0.05 vs. control group; # P < 0.05 vs. HFD group.

The distribution of body fat appears to be even more important than the total amount of fat. The adverse metabolic impact of visceral fat has been attributed to distinct biological properties of adipocytes in this depot, including variations in the metabolic activity of fat cells and in the expression of cytokines, hormones, and polypeptides (46,47). Our data showed that HFD increased EGFR expression and basal tyrosine phosphorylation in mesenteric and retroperitoneal (internal fat depot) but not in epididymal fat pads, suggesting a role of this receptor in the development of central obesity and/or its metabolic consequences. Moreover, the more marked decrease in EGFR tyrosine phosphorylation after PD153035 treatment in the internal fat depots accompanied the significant reduction in the weight of these fat depots. It is possible that the decrease in fat depots may contribute to the improvement in glucose tolerance and insulin sensitivity in animals treated with PD153035 for 14 days. In this regard, the regulation of EGFR in macrophages and in mesenteric and retroperitoneal fat pads in HFD suggests that this receptor and/or signaling pathway may have a role in the insulin resistance of obesity and diabetes and deserves further exploration.

In summary, our results show that the use of PD153035 for just 1 day was able to reduce the protein expressions of iNOS, TNF- α , and IL-6 in SVF. We can thus suggest that PD153035 inhibits EGFR tyrosine kinase activity in ATMs, reducing the M1 proinflammatory state as an initial effect. This reduces the circulating levels of TNF- α and IL-6, initiating an improvement in insulin signaling and sensitivity. After 14 days of the drug administration, there was a marked improvement in glucose tolerance; a reduction in insulin resistance; a reduction in macrophage infiltration in adipocytes and in TNF- α , IL-6, and FFAs; accompanied by an improvement in insulin signaling in liver muscle and adipose tissue. We, therefore, suggest that PD153035 presents an attractive opportunity for the treatment of insulin resistance and type 2 diabetes.

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