An 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 the 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 on this subclinical inflammation was not yet 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.

**Materials and Methods** The effect of PD153035 was investigated on insulin sensitivity, insulin signaling and JNK and NF-κB activity in tissues of high-fat diet-fed mice, and also on infiltration and the activation state of adipose tissue macrophage (ATM) in these mice.

**Results:** PD153035 treatment for 1 day decreased the protein expression of iNOS, TNF-α and IL-6 in the stroma vascular fraction, suggesting that this drug reduces the M1 proinflammatory state in ATM, as an initial effect, in turn reducing the circulating levels of TNF-α and IL-6 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 FFAs, accompanied by an improvement in insulin signaling in liver, muscle and adipose tissue, and also a decrease in IRS-1 Ser^{307} phosphorylation, in JNK and IKKβ activation in these tissues.

**Conclusion:** Treatment with PD153035 improves glucose tolerance, insulin sensitivity and signaling and reduces subclinical inflammation in HFD mice.
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 the insulin receptor substrate 1 (IRS1) and IRS-2 (6-8). Following tyrosine phosphorylation, IRS-1/IRS-2 bind and activate the enzyme phosphatidylinositol 3-kinase (PI-3K). 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 down-regulation in this signaling pathway in insulin-sensitive tissues, in 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 JNK (9-13) and inhibitor of 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 have an important role in the increased tissue production of proinflammatory molecules and acute-phase proteins associated with obesity (13; 14). EGF receptor 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/IκB/NF-κB pathways (24; 25). Moreover, EGFR and other tyrosine 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-κB activity in liver, muscle and adipose tissue of a high-fat diet-fed mice, and also on the infiltration and activation state of adipose tissue macrophages (ATM) in these mice.

RESEARCH DESIGN AND METHODS

Materials. 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-IRβ (α-IR), anti-IRS-1, anti-Akt1/2, anti-p-JNK, anti-iNOS, anti-TNFα, anti-IL6, anti-EGFR, anti-caveolin, anti-actin, anti-IKKβ, anti-pIKKβ, anti-p-c-jun and anti-IκBα...
antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-pAkt was from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-IRS-1Ser307 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, Indiana, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), 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% purity, as determined by elemental analysis, HPLC, mass spectrometry and 1H and 13C NMR (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 - a standard rodent chow and water ad libitum; high-fat diet (HFD) group - received a HFD consisting of 55% calories from fat, 29% from carbohydrate and 16% from protein for 8 weeks; HFPD14days – received the same high-fat diet for 8 weeks, however, in the last two weeks these animals also received PD153035 (30 mg/kg) by gavage once a day. A group of HFD also received the same high-fat diet for 8 weeks, however, in the last two weeks these animals also received PD153035 (30 mg/kg) by gavage once a day. A group of HFD also received the same dose of PD153035 at 24h and 2h before the experiments and this group was denominated HFPD1day. Body weight and food intake were measured weekly. Glucose tolerance test (GTT) and insulin tolerance test (ITT) 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 levels were analyzed using the NEFA-kit-U (Wako Chemical GmbH, 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, USA). MCP-1, MCP-2 and MCP-3 ELISA kits were purchased from Antigenix America Inc. (Huntington Station, NY, USA).

Light microscopy and Morphometry. Mice were fasted for 12h and euthanized 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 Axio phot light microscope using a 40X 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 thirty randomly sorted adipocytes, per animal) were determined using the Imagelab Analysis software (version 2.4), as previously described (33).

Tissue extraction, immunoprecipitation and immunoblotting. Mice were anaesthetized 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 (3.8U/Kg ip) 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 NFκB activation.** NFκB p50 activation was determined in nuclear extracts from liver, muscle and adipose tissue by ELISA (Pierce Biotechnology-89858), 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 supplemental material available in the online appendix at http://diabetes.diabetesjournals.org.

**Arginase assay.** Arginase activity assays were performed, as previously described (35). A summary of the method is presented in supplemental material in the online appendix.

**Statistical analysis.** Data are expressed as means ± SEM and the number of independent experiments is indicated. For statistical analysis, the groups were compared using a 2-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 EGF receptor (20). In order to investigate the effect of PD153035 administration on EGFR phosphorylation we immunoprecipitated liver, muscle and adipose tissue extracts of control, HFD and HFD 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 (Figures 1A-C). High-fat diet did not change the tissue levels of EGFR in liver, muscle and epidydimal fat pad, however, there was an increase in EGFR expression (Figures 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 to the epididymal fat pad (Figures 1C). PD153035 treatment reduced EGFR tyrosine phosphorylation in a dose-dependent manner in liver, muscle and retroperitoneal tissues (Figure S1).

**Effect of PD153035 on body weight and fat pads in high-fat diet-fed mice.** Eight-week-old male swiss mice were placed on high-fat diet (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 chow (Figure 1D). There is a slightly reduction in body weight after 14 days of PD153035 compared to HFD or HFDPD1, 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 pads weights were higher in HFD group, and PD153035 treatment for 1 day did not change these fat pads weights, but after 14 days there was a significant reduction in retroperitoneal and mesenteric fat pads weights (Figure 1E-G).

**Effect of PD153035 on metabolic parameters in high-fat diet-fed mice.** The fasting plasma glucose levels were higher in HFD and in HFPD1 than in the other groups
(Figures 1H). PD153035 treatment reduced fasting plasma glucose levels in a dose-dependent manner (Figure S2). During the glucose tolerance test, the plasma glucose and serum insulin levels were significantly higher in HFD and HFPD1 compared to controls, and PD153035 administration for 14 days improved glucose tolerance and reduced insulin levels at all time-points studied (Figure 1I, 1J). The glucose disappearance rate was lower in HFD and in HFPD1 groups and PD153035 administration for 14 days (HFPD14) reversed these alterations (Figure 1K). Taken together, the lower insulin levels during the GTT and the increase in glucose disappearance rate during the ITT 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 PD administration (Figure 1L).

Effect of PD153035 on insulin signaling in liver, muscle and retroperitoneal adipose tissue of high-fat diet-fed mice. In liver, muscle and retroperitoneal adipose tissues, insulin-induced IR\(\beta\) (Figures 2A-C) and IRS-1 tyrosine phosphorylation (Figures 2D-F) and Akt serine phosphorylation (Figures 2G-I) were reduced by 50-70% in mice fed on a 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 (Figures 2G-I and S3). However, 14 days of treatment reversed these reductions in the three tissues studied (Figures 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 mice was dose-dependent (Figure 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 (Figure S4).

Ser\(^{307}\) Phosphorylation of IRS-1 and activation of JNK and IKK\(\beta\) in liver, muscle and retroperitoneal tissue of high-fat-fed mice treated with PD153035. IKK\(\beta\) activity was monitored using IKK\(\beta\) phosphorylation and I\(\kappa\)B\(\alpha\) protein abundance, as previously described (12). IKK\(\beta\) phosphorylation was increased and I\(\kappa\)B\(\alpha\) protein levels were reduced in liver, muscle and retroperitoneal adipose tissue of mice fed on a HFD or HFPD1, but not in these tissues of HFPD14 mice (Figures 3A-F). We also measured the nuclear NF-κB subunit p-50 activation and found an increase in the DNA binding of nuclear p50 in liver, muscle and retroperitoneal of mice on HFD and HFPD1, but there was a clear decrease in the three tissues in HFPD14 (Figures 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 WAT of mice fed on a HFD and HFPD1, and this increase was reversed by 14 days of PD153035 treatment (Figures 3J-O). We tested Ser\(^{307}\) phosphorylation of IRS-1 in liver, muscle and WAT in the four groups of mice. Ser\(^{307}\) phosphorylation was induced by HFD in the three tissues of mice and the treatment with PD153035 for 14 days reversed this alteration (Figures 3P-R).

Effect of PD153035 on retroperitoneal adipose tissue morphology and ultrastructural features in high-fat diet-fed mice. Morphometric analysis revealed that in retroperitoneal fat pad adipocytes from HFPD14 were consistently smaller than adipocytes from control mice fed on a HFD or HFPD1, with an average 40% decrease in size (Figures 4A and 4B). In mesenteric and epididymal depots, the reduction in adipocytes in HFPD14 was 30-
40% average decrease in size (Figure 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 Crown-like structures (CLS), 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 more than 200-fold (489±58) in control mice on HFD or on HFPD1 (506±66) and only ~8 fold (150±23) in HFPD14, 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 Figures 4C and 4D HFD increased F4/80+ staining and PD153035 treatment for 14 days reduced this staining suggesting less macrophage were present (Figure 4C, 4D). In epididymal and mesenteric fat pads (Figure S5, S6) the results were very similar to the retroperitoneal.

As shown in Figure 4E treatment with PD153035 significantly impaired the migration of THP1 in a dose response manner (methods related to this essay are presented in supplemental material in the 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 (SVF).** In retroperitoneal adipose tissue, separation of the SVF from adipocytes of lean, HFD, HFPD1 and HFPD14 indicated that there was a modest increase in TNF-α protein expression in adipocytes from HFD animals compared to controls, and that PD153035 reduced the expression of this cytokine only after 14 days of treatment (Figure 5A). In adipocytes, the expressions of IL-6 and iNOS were higher in mice that received the HFD, these expressions 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 (Figures 5B, 5C). Similar results were observed in liver and muscle (Figure S7). Treatment with PD153035 for 1 or 14 days reduced EGFR tyrosine phosphorylation in adipocytes (Figure 5D) In SVF, the expressions of TNF-α, IL-6 and iNOS were also higher in HFD animals compared to controls. Different from adipocytes, PD153035 administration for just 1 day was able to reduce the SVF expression of TNF-α, IL-6 and iNOS, which were normalized after 14 days administration of this drug (Figure 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 (Figures 5I). Similar results were observed in adipocytes and SVF from epididymal (Figures 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 HFPD14 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 (Figure 5K). However, arginase activity was significantly reduced in the SVF of rats on a 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 (Figure 5K).

Adiponectin levels were reduced in control mice on a HFD and HFPD1, but increased significantly after 14 days of PD153035 administration. (Figure 5L). Serum leptin levels were higher in the HFD group,
and PD153035 administration did not change these levels (Figure 5N). Serum TNF-α and IL-6 levels were higher in mice on a HFD and, interestingly, PD153035 administration for 1 day reduced the levels of these cytokines. After two weeks of PD153035 treatment, TNF-α and IL-6 returned to normal levels (Figures 5M and O).

The protein levels of MCP-1 and MCP-3 were significantly increased in adipose tissue of HF 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 (Figures 5P-R).

DISCUSSION

Our results show that the use of PD153035 (EGFR tyrosine kinase inhibitor) in high-fat diet-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 the 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 one 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 stromal vascular fraction. These data suggest that the first effect observed with this drug is a change in macrophage activation. Macrophage activation has been defined across 2 separate polarization states, M1 and M2 (35; 40; 41). M1 or “classically activated” macrophages are induced by proinflammatory mediators such as LPS and IFN-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 (42). In summary, M2 macrophages are believed to participate in the blockade of inflammatory responses and in the promotion of tissue repair (40). Our data showing that PD153035 treatment for just 1 day reduced the expression of IL-6, TNF-α and iNOS in the stroma vascular fraction, and in parallel induced an increase in arginase activity suggest that PD153035 may lead to a shift in the activation state of ATM, reducing the M1 proinflammatory state that contributes to insulin resistance. Since EGFR tyrosine phosphorylation was increased in SVF of HFD mice, it is possible that the primary action of PD153035 is on ATM, but direct relation between EGFR and macrophage activation deserves further investigation.

In mice treated with PD153035 for 14 days, the high-fat diet 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 demonstrated that EGFR and/or other tyrosine-kinase inhibitors, inhibits the growth and/or activation of some non-malignant hematopoietic cells, including monocyte/macrophages (21-23). Interestingly, another study has shown that a reduction in macrophage infiltration and/or resident alternatively-activated macrophages can decrease local inflammation in white adipose tissue (43). In accordance with this, our data show that in the adipose tissue of high-fat diet-fed mice treated with PD153035 for 14 days, in parallel with a reduction in...
macrophage infiltration, there were lower expressions of TNF-\(\alpha\), IL-6 and iNOS, indicating that this drug decreases local inflammation in white adipose tissue 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 Ser\(^{307}\) 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. Ser\(^{307}\) is reported to be a phosphoacceptor of JNK and IKK\(\beta\) (10; 47) and, as previously described, (44; 46; 48) 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\(\beta\) 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 FFA, TNF-\(\alpha\) and IL-6. However, we can not exclude the possibility of a direct effect of PD153035 on JNK and IKK\(\beta\)/NF\(\kappa\)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.

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 (49; 50). Our data showed that HFD increased EGFR expression and basal tyrosine phosphorylation in mesenteric and retroperitoneal (internal fat depot) but not in epididymal fat pad, 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-\(\alpha\) and IL-6 in SVF. We can, thus, suggest that PD153035 inhibits EGFR tyrosine kinase activity in ATM, reducing the M1 proinflammatory state as an initial effect. This reduces the circulating levels of TNF-\(\alpha\) 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-\(\alpha\), 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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**FIGURE LEGENDS**

**Figure 1.** Effects of acute or chronic PD153035 administration in high-fat diet-fed mice. (A-C, upper panels) Representative blots show the tyrosine phosphorylation of Epidermal Growth Factor Receptor (EGFR) of control mice, HFD mice and HFD+PD 1 and 14 days in liver (A), muscle (B) and adipose (C). Total protein expression of EGF receptor (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 free fatty acids. Data are presented as means +/- S.E.M from 6-8 mice per group. *P<0.05, versus control group; #P<0.01, versus HFD. IB, immunoblot; IP, immunoprecipitate. HFD: high-fat diet; HFPD1: high-fat diet treated with PD153035 for 1 day; HFPD14: high-fat diet treated with PD153035 for 14 days.

**Figure 2.** Effects of PD153035 administration on insulin signaling in high-fat diet fed mice. Representative blots show tyrosine phosphorylation of Insulin Receptor β (IRβ) in liver (A), muscle (B) and adipose (C) of control mice, HFD mice and HFD plus PD during 14 days (upper panels). Total protein expression of IRβ (A-C, lower panels). Tyrosine phosphorylation of Insulin Receptor Substrate 1 (IRS1) in liver (D), muscle (E) and retroperitoneal (F) of control mice, HFD mice and HFD+PD 14 days (upper panels). Total protein expression of IRS1 (D-F, lower panels). Serine phosphorylation of Akt in liver (G), muscle (H) and adipose (I) of control mice, HFD mice and HFD+PD 1 and 14 days (upper panels). Total protein expression of Akt (G-I, lower panels). Data are presented as means +/- S.E.M from 6-8 mice per group, *P<0.05, control versus HFD group; #P<0.05, HFPD 14 days versus HFD. IB, immunoblot; HFD: high-fat diet; HFPD1: high-fat diet treated with PD153035 for 1 day; HFPD14: high-fat diet treated with PD153035 for 14 days.

**Figure 3.** Effects of PD153035 administration on modulators of insulin signaling. Representative blots show the expression of IκBα phosphorylation in liver (A), muscle (B) and retroperitoneal (C) of control mice, HFD mice and HFD+PD 1 and 14 days (upper panels). Total protein expression of IκBα (A-C, lower panels). IκBα in liver (D), muscle (E) and adipose (F) of control mice, HFD mice and HFD+PD 1 and 14 days. NFκB 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 HFD+PD 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 HFD+PD 1 and 14 days. IRS1 serine 307 phosphorylation in liver (P), muscle (Q) and adipose (R) of control mice, HFD mice and HFPD 1 and 14 days (upper panels). Total protein expression of IRS-1 (P-R, lower panels), IB, immunoblot. Data are presented as means +/- S.E.M from 6 mice per group, *P<0.05, versus control group and #P<0.05, versus HFD. HFD: high-fat diet; HFPD1: high-fat diet treated with PD153035 for 1 day; HFPD14: high-fat diet treated with PD153035 for 14 days.

**Figure 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 HFD plus PD after 1 or 14 days, 50µm 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 white adipose
tissue using the specific macrophage marker F4/80+. (D) F4/80-positive cells (+ cells/total cells) of all above groups. Data are presented as means +/- S.E.M from 6 mice per group, *P<0.05, versus control group and # P<0.05, versus 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 chemotatic agent RANTES. * P<0.05 versus DMSO alone; #P<0.05 versus DMSO/RANTES. Values represent the average of 5 different assays: high-fat diet; HFPD1: high-fat diet treated with PD153035 for 1 day; HFPD14: high-fat diet treated with PD153035 for 14 days.

**Figure 5.** Effect of PD153035 on tissue protein levels of TNF-α, IL-6 and iNOS, and arginase activity in adipocytes and stromal vascular fraction from retroperitoneal adipose tissue. Representative blots show the tissue levels of TNF alpha, IL-6, iNOS, EGRF tyrosine phosphorylation, EGRF, Caveolin and Cd68 protein expression in adipocytes (A-D) and TNF alpha, IL-6, iNOS, EGRF tyrosine phosphorylation, EGRF, Cd68 and actin protein expression in the stromal vascular fraction (F-J). (K) Arginase activity of adipocytes and stromal vascular fraction from control mice, HFD mice and HFD+PD 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 +/- S.E.M of 6-8 mice per group. *P<0.05 versus control group; #P<0.05 versus HFD group. HFD: high-fat diet; HFPD1: high-fat diet treated with PD153035 for 1 day; HFPD14: high-fat diet treated with PD153035 for 14 days.
FIGURE 2

Liver

A. IB:pIR

B. IB:pIR

C. IB:pIR

Muscle

D. IB:pIRS1

E. IB:pIRS1

F. IB:pIRS1

Retroperitoneal adipose tissue

G. IB:pAkt

H. IB:pAkt

I. IB:pAkt
FIGURE 4

A

B

C

D

E

Retropitoneal

Adipocyte size ($\mu$m$^2$)

Retention

F4/80 positive cells (+ cells/total cells)

THP-1 migration (cells x 10$^6$)

DIAGO, DIABETES, PD 1/200, PD 1/200, PD 5/200, PD 5/200