Reduced adipose tissue macrophage content is associated with improved insulin sensitivity in thiazolidinedione-treated diabetic humans

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ABSTRACT

Obesity is associated with increased adipose tissue macrophage (ATM) infiltration, and rodent studies suggest that inflammatory factors produced by ATMs contribute to insulin resistance and type 2 diabetes mellitus. However, a relationship between ATM content and insulin resistance has not been clearly established in humans. Since thiazolidinediones attenuate adipose tissue inflammation and improve insulin sensitivity, we examined the temporal relationship of pioglitazone’s effects on these two parameters. The impact of 10 and 21 days of pioglitazone treatment on insulin sensitivity in 26 diabetic subjects was assessed by hyperinsulinemic-euglycemic clamp studies. Since chemoattractant factors, cytokines, and immune cells have been implicated in regulating the recruitment of ATMs, we studied their temporal relationship to changes in ATM content.

Improved hepatic and peripheral insulin sensitivity was seen after 21 days of pioglitazone. We found early reductions in macrophage chemoattractant factors after only 10 days of pioglitazone, followed by a 69% reduction in ATM content at 21 days and reduced ATM activation at both time points. While markers for dendritic cells and neutrophils were reduced at both time points, there were no significant changes in regulatory T cells (Tregs). These results are consistent with an association between adipose macrophage content and systemic insulin resistance in humans.

Abbreviations: Thiazolidinedione (TZD), free fatty acid (FFA), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), adipose tissue macrophage (ATM), regulatory T cells (Tregs), macrophage chemoattractant protein-1 (MCP-1), chemokine (C-C motif) receptor 2 (CCR2), Clinical Research Center (CRC), stromal-vascular fraction (SVF), Confidence interval (CI), FACS (fluorescence-activated cell sorter), iNOS (inducible nitric oxide synthase)

Key words: thiazolidinediones, macrophages, adipocytes, diabetes mellitus
Obesity is an important causal factor in the global diabetes epidemic (1,2). Adipose tissue generates substantial amounts of pro-inflammatory molecules believed to contribute to insulin resistance (3). Obesity is associated with increased adipose tissue macrophage infiltration in both rodents and humans (4-6). Inflammatory cells, including macrophages, appear to be the main source of various fat derived inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β (7,8), and many rodent models suggest that increased ATM content is associated with insulin resistance (9-12). However, human studies have not universally shown a relationship between ATM content and insulin resistance, raising questions about the role of ATMs in the metabolic consequences of obesity in humans (13,14).

Increased local production of macrophage chemoattractant protein-1 (MCP-1) appears to recruit circulating monocytes/macrophages through interaction with the MCP-1 receptor chemokine (C-C motif) receptor 2 (CCR2) (9-11), and MCP-1 expression is increased in human adipose tissue from obese subjects (6). Adipocytes in obesity also appear to express increased amounts of hyaluronan and its receptor CD44, thereby recruiting more monocytes into adipose tissue (15-18). Additionally, a number of studies have suggested that regulatory T cells (Tregs) oppose recruitment of pro-inflammatory macrophages, thereby improving insulin sensitivity (19-21), although a recent study showed that Treg markers were paradoxically up-regulated and correlated with inflammation in adipose tissue of obese human subjects (22). Furthermore, rodent studies suggest a role for additional adipose inflammatory cells, including neutrophils and dendritic cells, in macrophage recruitment and insulin resistance (23,24).

Therefore, many unanswered questions remain regarding the relationships among ATMs, chemoattractant factors and insulin action in humans. Since thiazolidinediones (TZDs) have been shown to reduce insulin resistance and inflammatory factors in subjects with type 2 diabetes (25,26) and to reduce ATM content in subjects with impaired glucose tolerance (27), we used pioglitazone to
prospectively study the temporal sequence of its effects on hepatic and peripheral insulin sensitivity, subcutaneous ATM content, chemoattractant factors and immune cell populations. We conducted hyperinsulinemic-euglycemic pancreatic clamp studies with adipose tissue biopsies following 10 and 21 days of pioglitazone treatment in individuals with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Human subjects: All studies and procedures were approved by the Institutional Review Board of Albert Einstein College of Medicine. Prior to their enrollment in the study, informed, written consents were obtained from all subjects. A total of n=26 adult volunteers with type 2 diabetes were enrolled in the 10 day and/or the 21 day protocols. Three subjects participated in both 21 and 10 days studies with a ‘wash-out period’ of at least 6 months between studies. Subjects with a history of medical conditions other than type 2 diabetes and well-controlled hypertension were excluded. Women in the child-bearing age group were allowed to participate provided that they had negative pregnancy test results within a week of the studies.

Each subject was instructed to follow their usual diet and activity while participating in the studies. The subjects discontinued sulfonylureas and metformin for 3 days (the approximate half life of a typical sulfonylurea, glyburide, is 10 hours; the half life of metformin is 18 hours in blood) and long acting insulin for 24 hours prior to each clamp study. Subjects fasted overnight, but took their capsules on the morning of the study. Prior to each study, blood samples were collected for lipid profile, plasma glucose, insulin, glycerol and free fatty acid (FFA) levels.

21 day pioglitazone studies: A total of 16 subjects participated in a pair of clamp studies following 45mg/day of pioglitazone (Pio21d) and placebo (Plc21d) treatment for 21 days each. Each participant received the experimental agents in random order, in a double blind fashion. There was a washout period of at least 3 weeks before beginning the other experimental agent. Some clamp data
from 8 of these subjects were reported previously. Adipose tissue biopsies were performed during the final 30 minutes of the clamp studies under local anesthesia as described below.

To examine the effects of 21 days of pioglitazone on both hepatic and peripheral insulin action, we used 6-h “stepped” hyperinsulinemic clamp studies (Figure 1A). The subjects were admitted the night before the study. After establishing intravenous access, insulin infusions (Novolin Regular) were begun at 3:00 A.M. and were adjusted on the basis of hourly plasma glucose measurements to gradually attain euglycemia. At 7:30 A.M. the following day, an additional intravenous cannula was inserted in the opposite arm for blood sampling and ‘stepped’ hyperinsulinemic clamps were performed as previously described (26). Briefly, a primed-continuous infusion of high-performance liquid chromatography-purified [3-3H]glucose (bolus 21.6 µCi for 5 minutes; continuous infusion of 0.15 µCi/min), was initiated at t=0 min to quantify glucose turnover. To reduce inter-study variability, individualized basal insulin replacement rates were established from 0 to 120 min by means of variable rates of insulin infusion to keep plasma glucose levels at ~90 mg/dl without the need for glucose infusion. Insulin infusion rates were then increased by 20 mU/m2/min above these basal rates to reproduce physiologic hyperinsulinemia (“low insulin” step of the clamp) from 120 to 240 min. Infusion rates were then further increased by 150 mU/m2/min above the basal rate for the final 2 h of the studies (“high insulin” step of the clamp). All experiments consisted of 360-min somatostatin (250 µg/h) infusions with replacement of glucoregulatory hormones (glucagon 1 ng/kg/min; growth hormone 3 ng/kg/min) to maintain fixed levels of these hormones throughout.

Plasma glucose was measured every 5 to 10 min and maintained at euglycemia (~90 mg/dl) by a variable infusion of [3-3H]glucose-enriched 20% dextrose. Blood samples were collected every 15 to 60 min to measure plasma insulin, C-peptide, FFA, glycerol, and [3-3H] glucose. All infusions were stopped at t=360 min. The subjects were then given a standard meal, and plasma glucose
concentrations were monitored for an additional 60 minutes. Dextrose infusion was continued for ~30 minutes after completion of the clamp to avoid hypoglycemia.

10 day pioglitazone studies: A total of 13 subjects underwent adipose tissue biopsies and/or clamp studies at baseline (B10d) and after 45mg/day (Pio10d) pioglitazone treatment for 10 days. Six of these 13 subjects participated in 3hr hyperinsulinemic clamp studies before and after pioglitazone treatment, with adipose tissue biopsies performed during the last 30 minutes of the clamp study. These shorter studies were conducted to reduce inconvenience to the subjects, since the 21 day studies had already demonstrated that pioglitazone improved both hepatic and peripheral insulin action. These 3hr clamp studies employed insulin infusion rates previously used to examine insulin sensitivity in subjects with type 2 diabetes (28). Following an overnight fast, all subjects received insulin infusions to gradually lower glucose levels to euglycemia. Insulin infusions at 50mU/m2/min were initiated at 8:00 am (t=0) and continued throughout the study duration. A primed continuous infusion of [3-3H]-glucose was initiated at t=0 min as above. In the two most recent subjects, primed continuous infusions of [6,6-2H2] glucose were used to quantify glucose turnover (29).

Plasma glucose levels were measured every 5-10 min and glucose levels were maintained at euglycemia (~90mg/dl) by a variable infusion of tracer enriched 20% dextrose for the entire study. Samples were collected every 15 to 60 min to measure plasma insulin, C-peptide, FFA, glycerol, and labeled glucose. All infusions were stopped at t=180 min. The subjects were then given a standard meal, and plasma glucose concentrations were monitored for an additional 60 minutes.

Adipose tissue biopsies: A small 0.25-cm cutaneous incision in the periumbilical region was performed under local anesthesia (Lidocaine, 1%) and 1-2 g of subcutaneous adipose tissue was obtained by aspiration (30). The biopsy specimens were immediately homogenized in TRIzol (Invitrogen) to inhibit any ribonuclease activity and subsequently stored at −80°C.
Adipose tissue separation: Adipose tissue samples from all subjects were immediately washed at least 3 times with saline to remove contaminating blood. The cells were then digested with collagenase type 1 (0.05 gm per 30ml of Hanks Balanced Salt solution with 4% BSA) (Worthington Biochemical) for 30 minutes at 37°C with intermittent shaking followed by extensive washing with Dulbecco’s phosphate buffered solution depleted of magnesium and calcium (Mediatech). The adipocytes were separated from the stromal-vascular fraction (SVF) by centrifugation at 3000 rpm for 10 minutes. Macrophages were separated from the SVF by CD14+ antibody-coated magnetic Dynabeads (Dynal Biotech) by the manufacturer’s recommended method. The separated adipocytes and macrophages were washed with PBS and stored in TRIzol and analyzed by real time reverse transcription-polymerase chain reaction (RT-PCR).

Analytical Procedures: Plasma glucose levels were measured using a Beckman glucose analyzer (Fullerton, CA; glucose oxidase method). Plasma insulin was measured by radioimmunoassay, and plasma FFA levels were measured by an acyl-CoA oxidase-based colorimetric kit (Wako, Osaka, Japan). Glycerol was measured by colorimetric enzymatic methods and plasma lactate by fluorometric enzyme techniques, as previously described (31).

Fluorescence-activated cell sorter (FACS) analysis: After digestion with collagenase, the pellet consisting of stromal cells was treated with red blood cell lysing buffer for 5 min followed by incubation with saturating amounts of FITC labeled human CD14+ antibody (BD Pharmingen, San Diego, CA), and PE labeled human CCR2+ antibody (R&D Systems, Minneapolis, MN) in staining buffer (PBS containing 1 mg/ml BSA and 12 mM NaN3, pH 7.2) on ice for 20 min, washed and analyzed immediately by using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). FACS data was analyzed using FlowJo (V9.0.1) with the assistance of the Flow Cytometry Core Facility at Albert Einstein College of Medicine.
Quantitative ‘Real Time’ Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): From the adipose tissue samples, total RNA was extracted with TRIzol. cDNA was synthesized using Superscript First Strand Synthesis System for rt-PCR (Invitrogen Technologies). Gene expression was then studied by quantitative, ‘real-time’ RT-PCR using the specific protocol for the LightCycler (Roche Diagnostics, Indianapolis, IN) as previously described (26). Primer sequences are listed in Supplemental Table 3.

Reaction conditions were as follows: 40 cycles, denaturation at 95°C for 0 sec, annealing for 59°C for 5 seconds, elongation for 74°C for 12 seconds. All the reactions were performed at least three times. Results are expressed as fold change by determining the ratio of copy number of the gene of interest in a given individual after pioglitazone vs. placebo treatment or baseline, corrected for the geometric mean of housekeeping genes in the same pair of samples.

Immunohistochemistry: Adipose tissue samples were fixed for 12–16 hours at room temperature in NBS formalin and embedded in paraffin. Five-micrometer sections, cut at 50-µm intervals, were mounted on charged glass slides and deparaffinized in xylene and rehydrated. Epitope demasking was performed using Target Retrieval Solution pH 9 (Dako, Los Angeles, CA) as recommended by the manufacturer. Thereafter, endogenous peroxidase was inhibited with 3% H$_2$O$_2$ in immunohistochemistry (IHC) buffer (0.1% BSA, 0.1% Triton-X in PBS) followed by blocking nonspecific binding with IHC buffer containing 10% normal donkey serum. Subsequently slides were incubated overnight at 4°C, with anti-human Foxp3 (1:10; eBioscience clone: PCH101, Los Angeles, CA). Foxp3 immunopositive (+) cells were visualized with a peroxidase-based detection system using 3,3-diaminobenzidine (DAB) as the chromagen (Vector labs, Burlingame, CA). Slides were counterstained with Harris hematoxylin (Sigma-Aldrich, St Louis, MO), dehydrated and mounted. Sections were analyzed using a light microscope with a 40 X objective. The enumeration
of Foxp3+ cells was carried out by counting a total of 12-18 randomly selected fields per case. Data were normalized as Foxp3+ cells, percent of total nuclei per field.

**Statistical analysis:** Statistical analysis of the data over time was performed using SPSS statistical software Version 11.5 (SPSS Inc, Chicago, IL). For averaged data, paired Student’s t-tests were employed for comparisons of pioglitazone vs. placebo studies. All data were presented as mean±standard error.

**RESULTS**

**Subject characteristics:** 26 overweight or obese subjects with type 2 diabetes were studied, with the following characteristics: n=15 males; mean age 47.0±1.5 years; BMI 33.3±1.0 kg/m2; HbA1C 9.65±0.45. There were no significant differences in demographic characteristics between subjects who participated in the 10-day or 21-day pioglitazone studies (Table 1). Pioglitazone treatment did not affect liver function tests or lipid profiles (Supplemental Table 2). Of note, there were no significant differences in results when analyzed by gender; results are presented as combined data from all participants.

**Effects of pioglitazone on metabolic parameters and insulin action:**

**21 day studies:**

**Fasting conditions (Table 2):** Plasma glucose levels did not differ between the two experimental conditions before the insulin infusion was started at 3.00 A.M. (Plc21d=188.09±14.44 mg/dl vs Pio21d=186.67±18.35 mg/dl; p=0.56). The overnight insulin infusion rate required to maintain plasma glucose in the target range of 90-120 mg/dl was significantly lower after 21 days of pioglitazone treatment compared to that of the baseline 21-day study. (Plc21d=1.90±0.28 units/hr vs Pio21d=1.52±0.23 units/hr; p=0.03). At t=0, after an overnight fast and insulin infusion for about 4hrs, plasma insulin concentrations were significantly lower in the pioglitazone studies compared
with placebo (p=0.01), but plasma glucose levels did not differ and averaged 125.64±4.06 mg/dl for Plc21d and 129.71±5.77 mg/dl for Pio21d (p=0.38).

**Clamp conditions (Table 2):** Average plasma glucose levels were similar for both study types during the physiologic hyperinsulinemic step (low insulin; t=120-240, p=0.14) and the pharmacologic hyperinsulinemic step (high insulin; t=300-360, p=0.63). C-peptide levels were initially moderately suppressed secondary to insulin infusion prior to the clamp, and were further suppressed by somatostatin infusion for the duration of the clamp studies. There were no significant differences in FFA or glycerol levels between the study types either at the beginning of the clamp study or for the entire duration of the clamp studies. Glucose specific activity was constant after tracer equilibration during the last hour of both the low insulin (t=180–240 min) and the high insulin (t=300–360 min) steps, during which time glucose fluxes were measured. Following an initial 2 hour equilibration, specific activity remained stable throughout both the Plc21d and Pio21d studies (Figure 1B). Pioglitazone administration for 21 days markedly increased the ability of insulin to suppress EGP during the low insulin step (t=180-240min; p=0.0003; Figure 2A), but there was no significant difference in the rate of disappearance of glucose (Rd; p=0.28, Figure 2B). However, during the high insulin step of the clamp (t=300-360min), EGP was nearly completely suppressed in both the pioglitazone and placebo groups (Figure 2A), and there was a significant increase in Rd in the pioglitazone group (p =0.03, Fig2B). Neither EGP nor Rd were significantly impacted after 10 days of pioglitazone treatment, compared with baseline studies (Figure 2C, 2D).

10 day studies:

**Fasting conditions (Supplemental Table 1):** Overall there was a small but significant difference in fasting plasma glucose levels between baseline and pioglitazone studies (B10d = 221.08±26.88 mg/dl vs Pio10d = 195.31±18.69 mg/dl; p = 0.04), but insulin levels did not differ. However, among the 6 subjects who participated in hyperinsulinemic clamp studies, fasting plasma glucose and
insulin levels did not differ between the baseline and 10-day studies. There were also no differences in fasting C-peptide, FFA or glycerol levels between B10d and Pio10d.

**Clamp conditions (Supplemental Table 1):** Plasma glucose values did not differ from t=120 to t=180 min of clamp studies between the two study types (p=0.68). C-peptide levels were suppressed in response to exogenous insulin infusion. There were no significant differences in FFA or glycerol levels either at the beginning of the clamp studies or for the duration of the clamp studies between the baseline and 10-day studies. Glucose specific activity was constant after tracer equilibration during the final hour (t=120-180 min) of the clamp studies and did not differ between the two study types. Pioglitazone administration for 10 days did not alter either EGP (p=0.68, Figure 2C) or Rd (p=0.29, Figure 2D) at pharmacologic plasma insulin levels of about 80 µU/ml.

**Effects of pioglitazone on macrophage chemoattractant factors (Figure 3):**

We hypothesized that one of the mechanisms whereby pioglitazone reduces ATM content is by decreasing the expression of macrophage chemoattractant factors in adipose tissue, thus improving the inflammatory state via decreased macrophage recruitment. Expression of MCP-1, hyaluronan synthase and its receptor CD44 in whole fat were all significantly reduced after pioglitazone treatment for 10 and 21 days. Adipose macrophage CCR2 expression was also significantly reduced after both 10 and 21 days of pioglitazone, indicating that pioglitazone affected not only macrophage chemoattractant production by adipose tissue, but also expression of specific chemoattractant receptors. Quantification of CCR2+ macrophages via FACS analysis in the SVF of subcutaneous adipose tissue also showed a significant decrease in CCR2+ cells following pioglitazone treatment (Figure 4E).

**Effects of pioglitazone on adipose macrophage content (Figure 4):**
At 21 days, ATM content was reduced by 69% as measured by the number of CD14+ cells in the SVF of adipose tissue quantified by FACS. Furthermore, using expression of macrophage-specific markers as an alternate measure of ATM content, we observed a 28% reduction in CD14 expression and a 36% reduction in CD68 expression, while colony stimulating factor 1 receptor (CSF-1R) expression decreased by 45% (95%CI:31-58%). These results collectively indicate that pioglitazone treatment for 21 days reduced macrophage content in fat tissue. Conversely, there appeared to be no change in macrophage content in whole adipose tissue after 10 days of pioglitazone treatment either as quantified by FACS or by gene expression of the macrophage-specific markers CD14 and CD68.

**Effects of pioglitazone on adipose tissue inflammatory markers and macrophage activation (Figure 5):**

Adipose tissue in obese subjects has been shown to contain increased numbers of classically activated (M1) macrophages, a major source of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (32). Alternatively activated (M2) macrophages are associated with increased production of arginase-1 and IL-10, and may provide protection from obesity and insulin resistance, at least in part via PPAR-γ activation (32,33). We therefore examined multiple markers of inflammation and macrophage activation in adipose tissue. After 10 days of pioglitazone treatment, gene expression of IL-6 and IL-1β was decreased in whole fat, with a downward trend in TNF-α. There was no change in expression of inducible nitric oxide synthase (iNOS) after this duration of treatment. However, all of these markers were decreased in whole fat following 21 days of pioglitazone. Adipose tissue macrophage activation was also significantly reduced after 10 and 21 days of pioglitazone treatment, as shown by the expression of TNF-α, IL-6, and iNOS. Interestingly, after 21 days of pioglitazone expression of arginase-1 and IL-10 increased significantly in ATMs, consistent with a shift toward the M2 phenotype. Furthermore, pioglitazone induced a ~65% reduction in the quantity of cells with positive fluorescence for both
iNOS and CD68, indicating a substantial reduction in the amount of iNOS produced by macrophages in adipose tissue. Of note, the more pronounced decrease in inflammatory markers at 21 days corresponds with an observed reduction in ATM content at 21 days.

**Effects of pioglitazone on adipose tissue regulatory T lymphocyte, neutrophil and dendritic cell content (Figure 6):**

After both 10 and 21 days of pioglitazone treatment, expression of the dendritic cell markers DEC-205 and DC-SIGN were significantly decreased in whole fat. In addition, expression of the neutrophil marker myeloperoxidase significantly decreased at both time points. There were no significant changes in expression of the Treg specific markers FOXP3 or CD25 following 10 or 21 days of pioglitazone. Furthermore, the content of FOXP3 positive cells in adipose tissue as analyzed by IHC did not differ with treatment of any duration.

**DISCUSSION**

To explore the relationship between adipose inflammation and insulin resistance in humans, we designed studies examining early effects of treatment with the PPAR-γ agonist pioglitazone on insulin action, macrophage chemoattractants, and adipose inflammatory cell content in subjects with type 2 diabetes. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a nuclear receptor which regulates fatty acid storage and glucose metabolism, and is known for its insulin sensitizing and anti-inflammatory effects (34,35). In humans these receptors are most highly expressed in adipocytes (36), but are also present in other cells types, including macrophages (37,38). After 21 days of pioglitazone treatment, a substantial decrease in macrophage content coincided with improved hepatic and peripheral insulin action. Although no significant changes were seen in either macrophage content or insulin action after 10 days of pioglitazone, significant decreases in whole fat macrophage chemoattractant factors and their receptors, neutrophils, and dendritic cells
at 10 days preceded the subsequent reduction in ATM content at 21 days. Conversely, there were no changes in FOXP3 or CD25 expression at 10 or 21 days, suggesting that changes in Tregs were not responsible for the observed changes in macrophage content.

Of note, consistent data are lacking to substantiate a relationship between ATM content and insulin sensitivity in humans. Cancello et al reported significant improvements in fasting glucose and insulin levels and quantitative insulin sensitivity check index 3 months after gastric bypass surgery in obese subjects, in association with ~18% decrease in body weight (6). The number of subcutaneous ATMs was about 20% higher in obese subjects, and drastic post-operative weight loss resulted in a significant decrease (~11%) in macrophage number. Expression of a number of factors involved in macrophage attraction was also higher in the obese subjects, and significantly decreased after surgery. Additionally, Makkonen et al reported an inverse correlation between whole body insulin sensitivity (M value) and adipose tissue expression of CD68 in overweight and obese humans (4).

By contrast, Tam et al reported that 28 days of feeding a high fat diet to healthy individuals failed to increase adipose tissue macrophage number, T-cell number, circulating immune cell number or expression of their surface activation markers, despite an 11% decrease in insulin sensitivity as measured by hyperinsulinemic euglycemic clamp (14). The fact that the subjects in this study group had only a modest weight gain (<3 kg) may explain the lack of increase in adipose tissue macrophage content and MCP-1 expression, while the decreased insulin sensitivity may have been explained by a significant increase in liver fat content. Finally, a study of nondiabetic Pima Indians found that subcutaneous ATM content did not correlate with insulin action independent of adiposity, although subjects had a fairly wide range of BMI and different relationships may have applied in the overweight vs. morbidly obese subjects (13).

Our studies assess the effects of pioglitazone on macrophage content, chemoattractant factors, cytokine expression and immune cell activation at an early time point in treatment course, before
glucose levels are impacted, and before decreases in adipose macrophage content become significant. This allows us to carefully study early factors that could contribute to the ultimate ~70% drop in macrophage content after 21 days. After only 10 days, pioglitazone therapy resulted in decreased expression of macrophage chemoattractant factors in whole fat, including MCP-1, hyaluronan, and hyaluronan’s receptor CD44, as well as decreased expression of the MCP-1 receptor CCR2 in macrophages.

These studies also examined whether pioglitazone could affect activation of macrophages in adipose tissue. Indeed, after only 10 days of pioglitazone treatment, ATM activation was significantly reduced in adipose tissue of subjects with type 2 diabetes, as demonstrated by reductions in expression of iNOS, IL-1b and IL-6. Additionally, pioglitazone impacted the quantity of iNOS associated with ATMs after 21 days of treatment, as quantified by immunofluorescence. Furthermore, while reductions in markers of adipose tissue activation were already observed after 10 days of pioglitazone, a shift toward the alternatively activated M2 phenotype was noted subsequently at 21 days. Given the likelihood that classically and alternatively activated macrophages represent a continuum (39-41), we have expressed our results in terms of degrees of inflammatory activation.

In addition to macrophages and adipocytes, T lymphocytes neutrophils, and dendritic cells have recently received attention (22-24,42). In mice, high-fat feeding causes early infiltration of neutrophils into adipose tissue (23), and dendritic cells have also been implicated in the development of obesity-associated inflammation by inducing macrophage infiltration into adipose tissue (24,42). Furthermore, several studies have shown that both CD8+ and CD4+ T cells are increased in obese adipose tissue (17), and may recruit and assist adipose tissue macrophages in promoting inflammation and insulin resistance (39,43). In our studies, significant decreases in neutrophil and dendritic cell markers were observed at both 10 and 21 days, but there was no effect on Treg content,
suggesting that decreases in regulatory T cell content are not required for the changes in macrophages observed with pioglitazone treatment. Consistent with our inability to demonstrate a negative association between Tregs and ATM infiltration in humans, Zeyda et al. reported that regulatory T cells were significantly elevated in visceral as well as subcutaneous adipose tissue of morbidly obese subjects compared to that of lean control subjects (22). This does not exclude the possibility that Tregs play a role in the regulation of macrophages in fat.

To conclude, these are the first studies in humans to demonstrate a temporal relationship between ATM content and insulin sensitivity. Our studies confirm that improved insulin sensitivity is evident after only 21 days of pioglitazone treatment in subjects with type 2 diabetes. This effect may be mediated, at least in part, by decreased ATM content. Given pioglitazone’s effects on adipose tissue macrophage chemoattractant factor expression, it is likely that the decrease in adipose macrophage content is mediated by decreased chemoattractant production. We also showed decreased expression of inflammatory markers of adipose tissue macrophage activation, along with decreases in adipose tissue neutrophils and dendritic cells, though adipose Treg content did not change. Thus, results from this early time point of PPAR-γ activation provide further insights into mechanisms of macrophage recruitment in adipose tissue in humans. These extensive studies highlight the important role of macrophage chemoattractants and multiple inflammatory cells in adipose tissue inflammation, and strongly suggest a role for ATMs in the metabolic consequences of obesity in humans.

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Figure legends

**Fig1A**: Schematic depiction of “stepped clamp” protocol. Plasma glucose values were clamped at 90 mg/dl for the duration of each study. Glucose fluxes were measured by infusing tritiated glucose. Somatostatin was infused for the entirety of the clamp to inhibit pancreatic hormone secretion, with concomitant replacement of glucagon and growth hormone. Basal insulin infusion rates (---) were established for the first 2 h, and rates were then increased by 20 mU/m2/min to reproduce 2 h of physiologic hyperinsulinemia, the “low insulin” step of the clamp (•••). During the final 2 h of the clamp, the insulin infusion rate was increased by 150 mU/m2/min above basal, the “high insulin” step of the clamp (•••).

**Fig1B**: Glucose specific activity (SA) (cpm/mg) during the steady state of “stepped clamp” shown after 21 days of placebo (Plc21d) vs. pioglitazone (Pio21d).

**Fig2**: Endogenous glucose production (EGP) (A) and rate of disappearance of glucose (Rd) (B) after 21 days of placebo (Plc21d) vs. pioglitazone (Pio21d) in response to low insulin (180-240 min) or high insulin (300-360 min) steps of the clamp. EGP (C) and Rd (D) at baseline (B10d) and after 10 days of pioglitazone (Pio10d) during last hour (120-180 min) of the clamp study. *p<0.05.

**Fig3**: Gene expression in whole adipose tissue of macrophage chemoattractant factors. MCP-1 expression in adipose tissue at baseline (B10d) vs. after 10 days of pioglitazone (Pio10d; A, left panel) and after 21 days of placebo (Plc21d) vs. pioglitazone (Pio21d; A, right panel). MCP-1 decreased by 30% (95%CI: 16-44%) following 21 days of pioglitazone. CCR2 expression in macrophages at baseline vs. after 10 days of pioglitazone (B, left panel) and after 21 days of placebo vs. pioglitazone (B, right panel). CCR2 decreased by 34% (95%CI: 6-62%) following 21 days of pioglitazone. Hyaluronan synthase expression in adipose tissue at baseline vs. after 10 days of pioglitazone (C, left panel) and after 21 days of placebo vs. pioglitazone (C, right panel). Hyaluronan expression decreased by 37% (95%CI: 22-52%) following 21 days of pioglitazone. Expression of the hyaluronan receptor CD44 in adipose tissue also decreased significantly following both 10 and 21 days of pioglitazone (D), with a 42% decrease (95%CI: 23-61%) at 21 days (D, right panel). *p<0.05; **P<0.01

**Fig4**: FACS analysis of % of CD14 positive cells in whole adipose tissue at baseline (B10d) vs. after 10 days of pioglitazone (Pio10d; A, left panel) and after 21 days of pioglitazone (Pio21d) vs. placebo (Plc21d; A, right panel). CD14+ cells decreased by 69% (95%CI: 38-97%). Gene expression in whole adipose tissue of CD14 (B) and CD68 (C) after 10 and 21 days of pioglitazone. At 10 days no significant reductions in CD14 expression or CD68 expression were observed by FACS or by gene expression. At 21 days a 28% reduction in CD14 expression (95%CI:20-37%) and a 36% reduction (95%CI:24-37%) in CD68 expression were observed. Quantification of CD14+ and CCR2+ cells in the stromal vascular fraction of subcutaneous adipose tissue from one diabetic subject by flow cytometry before and after 21 days of pioglitazone is shown (D and E). FITC labeled CD14+ antibody and PE labeled CCR2+ antibody were used. Results were analyzed using FACS Calibur flow cytometer. *p<0.05.

**Fig5**: Percent change in cytokine gene expression in whole fat following 10 (A) and 21 (B) days of pioglitazone. Percent change in cytokine gene expression in adipose tissue macrophages following 10 (C) and 21 (D) days of pioglitazone. * indicates significance by p<0.05 or CI. Double-immunofluorescence stains for iNOS expression (red), CD68 (green) and iNOS and CD68
co-expression (yellow, merged panel) in a placebo-treated subject. Bottom panels show adipose tissue from a subject treated with pioglitazone for 21 days (E). Note reduced iNOS (red), CD68 (green) and iNOS/CD68 co-expression (overlay) following pioglitazone treatment. iNOS positive CD68 co-expression was significantly reduced in 21 day pioglitazone treated group (F) compared to placebo; *$P=0.03$ (n=5).

**Fig6:** Gene expression in whole adipose tissue of DEC-205 and DC-SIGN at baseline (B10d) vs. after 10 days of pioglitazone (Pio10d; A and B, left panels) and after 21 days of placebo (Plc21d) vs. pioglitazone (Pio21d) (A and B, right panels). Gene expression in whole adipose tissue of myeloperoxidase (MPO) following 10 (C, left panel) and 21 (C, right panel) days of pioglitazone. Gene expression in whole adipose tissue of FOXP3 and CD25 following 10 (D and E, left panels) and 21 (D and E, right panel) days of pioglitazone. Percentage of total nuclei per field of FOXP3 cells in whole adipose tissue samples of 21 days of placebo (plc21d) vs. pioglitazone (Pio10d) as assessed by immunohistochemistry (F). Representative histological staining for FoxP3 (G).
Table 1: Subject Characteristics

<table>
<thead>
<tr>
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<th>10 day studies</th>
<th>21 day studies</th>
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<tr>
<td>N</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Age</td>
<td>45.85±2.18</td>
<td>47.88±2.13</td>
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<tr>
<td>Sex (M/F)</td>
<td>8M/5F</td>
<td>10M/6F</td>
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<tr>
<td>BMI</td>
<td>34.12±1.41</td>
<td>32.89±1.29</td>
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<tr>
<td>Weight (B or Plc)</td>
<td>97.75±4.72</td>
<td>93.44±3.27</td>
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<tr>
<td>Weight (Pio)</td>
<td>96.29±4.89</td>
<td>93.75±3.28</td>
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<tr>
<td>Medication group (S, M, I, M+S, I+M)</td>
<td>1,4,3,4,1</td>
<td>1,4,3,7,1</td>
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<tr>
<td>HbA1C</td>
<td>8.97±0.68</td>
<td>9.87±0.44</td>
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</table>

Data are means ± SE.
Weight (B), weight at baseline study before 10 day pioglitazone treatment; weight (Plc), weight after 21 days of placebo treatment; Weight (Pio), weight after 10 or 21 days of pioglitazone treatment; S, sulfonylurea alone; M, metformin alone; I, insulin alone; M+S, metformin plus sulfonylurea; I+M, metformin plus insulin

Table 2: Plasma hormones and substrate levels after 21 days of placebo or pioglitazone therapy

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
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<th>Placebo</th>
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<tbody>
<tr>
<td></td>
<td>Pre-clamp</td>
<td>180-240</td>
<td>300-360</td>
<td>Pre-clamp</td>
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<tr>
<td>Glucose</td>
<td>188.09±14.44</td>
<td>96.43±1.92</td>
<td>89.99±1.06</td>
<td>186.67±18.35</td>
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<tr>
<td>Insulin</td>
<td>53.73±9.83</td>
<td>83.13±8.26</td>
<td>433±30.37</td>
<td>34.91±7.61*</td>
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<tr>
<td>FFA</td>
<td>198.46±29.91</td>
<td>93.40±16.11</td>
<td>31.09±3.92</td>
<td>148.24±29.72</td>
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<tr>
<td>Glycerol</td>
<td>47.80±7.97</td>
<td>64.06±10.46</td>
<td>50.46±6.86</td>
<td>42.14±5.77</td>
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<tr>
<td>C-peptide</td>
<td>0.54±0.08</td>
<td>0.16±0.02</td>
<td>0.08±0.01</td>
<td>0.50±0.06</td>
</tr>
</tbody>
</table>

Data are means ± SE. * p<0.05 Placebo vs Pioglitazone.
Fig1

A

Variable Glucose Infusion
- Glucose Tracer
- Somatostatin, GH, Glucagon
- Basal Insulin
- Low Insulin
- High Insulin

Plasma glucose (mg/dL)
- 0
- 50
- 100
- 150
- 200
- 300
- 400
- 500
- 600
- 700
- 800
- 180 195 210 225 240 300 315 330 345 360

Plasma insulin (µU/mL)
- 0
- 100
- 200
- 300
- 400

Time (minutes)

B

SA (cpm/mg)
- 100
- 200
- 300
- 400
- 500
- 600
- 700
- 800

Plc21d
- 180 195 210 225 240

Pio21d
- 300 315 330 345 360

Time (min)
C

Relative copy number

CD68

B10d
Plo10d

CD68

Plc21d
Pio21d

D

CD14+ Pre Treatment
9.26%

CD14+ Post Treatment
3.57%

E

CCR2+ Pre Treatment
5.38%

CCR2+ Post Treatment
2.15%
Fig 5

A

Cytokine Gene Expression (% Change)

IL-6  TNF-a  iNOS  IL-1B

B

Cytokine Gene Expression (% Change)

IL-6  TNF-a  iNOS  IL-1B
Supplemental Table 1: Plasma hormones and substrate levels at baseline or after 10 days of pioglitazone therapy

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Pio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre-clamp</td>
<td>120-180</td>
</tr>
<tr>
<td>Glucose</td>
<td>227.67±38.92</td>
<td>91.80±1.59</td>
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<tr>
<td>Insulin</td>
<td>35.76±10.30</td>
<td>79.85±6.45</td>
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<tr>
<td>FFA</td>
<td>177.25±38.86</td>
<td>104.38±31.8</td>
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<tr>
<td>Glycerol</td>
<td>59.00±15.00</td>
<td>52.25±17.25</td>
</tr>
<tr>
<td>C-peptide</td>
<td>0.21±0.10</td>
<td>0.11±0.01</td>
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</tbody>
</table>

Data are means ± SE.

Supplemental Table 2: Lipid Panel and Liver Function Tests after 21 days of placebo or pioglitazone therapy

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Pioglitzone</th>
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<tbody>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>185.13±6.14</td>
<td>185.25±5.57</td>
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<tr>
<td>LDL (mg/dl)</td>
<td>114.00±5.44</td>
<td>113.80±5.59</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>143.93±29.11</td>
<td>146.07±27.20</td>
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<tr>
<td>Aspartate Aminotransferase (mg/dl)</td>
<td>18.08±1.33</td>
<td>18.53±1.46</td>
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<tr>
<td>Alanine Aminotransferase (mg/dl)</td>
<td>19.25±1.47</td>
<td>18.27±2.13</td>
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<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.61±0.09</td>
<td>0.57±0.08</td>
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Data are means ± SE.
## Supplemental Table 3: Primer Sequences

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<th>Primers</th>
<th>Forward</th>
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<td>hCCR2</td>
<td>TGGCTGTGTTTGCTTCTGTC</td>
<td>TCTCACTGCCCTATGCTTCT</td>
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<td>hCD14</td>
<td>GCCCTGAACCTCCCTCAAATCT</td>
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<td>hCD68</td>
<td>ACCAAGAGCCACAAAACCAC</td>
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<tr>
<td>hCCL2(MCP-1)</td>
<td>ATGCAATCAATGCCGCGACTC</td>
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<tr>
<td>hFOXP3</td>
<td>CATGATCAGCCTCATACACACAC</td>
<td>CCACCTGAGACACAAGATT</td>
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<td>hCD25</td>
<td>GCAGAATAAAAAAGCGGGTCA</td>
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<td>hGAPDH</td>
<td>TCGGAGTCAACCGGTTTGG</td>
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<td>h18S</td>
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<td>hBeta-actin</td>
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<td>hHAS</td>
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