

Pioglitazone induces apoptosis of macrophages in human adipose tissue

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Abstract Metabolic syndrome and type 2 diabetes mellitus are associated with an increased number of macrophage cells that infiltrate white adipose tissue (WAT). Previously, we demonstrated that the treatment of subjects with impaired glucose tolerance (IGT) with the peroxisome proliferator-activated receptor γ (PPAR γ) agonist pioglitazone resulted in a decrease in macrophage number in adipose tissue. Here, adipose tissue samples from IGT subjects treated with pioglitazone were examined for apoptosis with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. TUNEL-positive cells were identified, and there was a significant 42% increase in TUNEL-positive cells following pioglitazone treatment. Overlay experiments with anti-CD68 antibody demonstrated that most of the TUNEL-positive cells were macrophages. To determine whether macrophage apoptosis was a direct or indirect effect of pioglitazone treatment, human THP1 cells were treated with pioglitazone in vitro, demonstrating increased TUNEL staining in a dose- and time-dependent manner. Furthermore, the appearance of the active proteolytic subunits of caspase-3 and caspase-9 were detected in cell lysate from THP1 cells and also increased in a dose- and time-dependent manner following pioglitazone treatment. Pretreatment with a PPAR γ inhibitor, GW9662, prevented pioglitazone induction of the apoptotic pathway in THP1 cells. Differentiated human adipocytes did not show any significant increase in apoptosis after treatment in vitro with pioglitazone. These findings indicate that PPAR γ has distinct functions in different cell types in WAT, such that pioglitazone reduces macrophage infiltration by inducing apoptotic cell death specifically in macrophages through PPAR γ activation.—Bodles, A. M., V. Varma, A. Yao-Borengasser, B. Phanavanh, C. A. Peterson, R. E. McGehee, Jr., N. Rasouli, M. Wabitsch, and P. A. Kern. Pioglitazone induces apoptosis of macrophages in human adipose tissue. *J. Lipid Res.* 2006. 47: 2080–2088.

Supplementary key words PPAR γ • diabetes • metabolic syndrome • insulin resistance

Manuscript received 10 January 2006 and in revised form 1 June 2006.

Published, JLR Papers in Press, June 23, 2006.

DOI 10.1194/jlr.M600235-JLR200

Obesity leads to insulin resistance and the subsequent development of metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disease (1). Associated with an increase in adipose tissue mass is an escalation in inflammation brought about by macrophages that infiltrate white adipose tissue (WAT) (2, 3). The accumulation of macrophages in WAT has been correlated with a rise in circulating inflammatory markers such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), which are correlated with insulin resistance and metabolic syndrome. Both adipocytes and macrophages release chemokines such as MCP-1 that can influence the further accumulation of macrophages in WAT (4). Together, the increase in numerous inflammatory markers has led to the suggestion that metabolic syndrome and diabetes are conditions characterized by a state of chronic, low-grade inflammation (5, 6).

Thiazolidinediones (TZDs) have been widely used in the treatment of diabetes mellitus and improve insulin sensitivity through a variety of mechanisms via activation of the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPAR γ). PPAR γ is found in numerous cells, but at especially high concentrations in adipocytes. PPAR α has been shown to be expressed in undifferentiated monocytes, whereas PPAR γ expression is induced upon differentiation into macrophages (7). Hence, the activation of PPAR γ by a TZD may directly affect both adipocytes and macrophages.

There have been several publications demonstrating apoptotic activities of TZDs in carcinoma cells as well as

Abbreviations: IGT, impaired glucose tolerance; IL-6, interleukin-6; PPAR, peroxisome proliferator-activated receptor; SGBS, Simpson-Golabi-Behmel syndrome; TNF- α , tumor necrosis factor- α ; TPA, tetradecanoylphorbol-13-acetate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TZD, thiazolidinedione; WAT, white adipose tissue.

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their potential to affect differentiation and proliferation. In thyroid carcinoma, troglitazone increased apoptosis while inhibiting proliferation (8). Similar effects were observed in human renal carcinoma cell lines, where TZD caused massive apoptosis with increased Bax expression and decreased proliferation (9). In other studies, TZDs inhibited vascular endothelial growth factor and basic fibroblast growth factor (10). Taken together, these studies suggest that TZDs may have anti-tumor properties through an induction of apoptosis.

On the basis of the decrease in macrophage number in adipose tissue following TZD treatment (4) and the demonstrated effects of TZDs on apoptosis, we wished to examine adipose tissue from subjects treated with a TZD for evidence of apoptosis. In addition, we performed experiments in which pioglitazone was added to macrophages and adipocytes *in vitro* to determine whether pro-apoptotic effects were primary, or secondary to some other phenomenon of treatment. Furthermore, we examined the mechanism through which pioglitazone was inducing apoptosis.

MATERIALS AND METHODS

Human subjects

Healthy subjects without diabetes but with impaired glucose tolerance (IGT) were recruited by local advertisement. All subjects provided written, informed consent under protocols that were approved by the local institutional review board, and studies were conducted at the University of Arkansas for Medical Sciences/Central Arkansas Veterans Health Care System General Clinical Research Center. Subjects with a history of coronary artery disease, or those being treated with fibrates, angiotensin-converting enzyme-inhibitors, or angiotensin receptor blockers were excluded. Subjects were included if their fasting glucose was under 126 mg/dl, and their 2 h post-challenge glucose was between 140 and 200 mg/dl as determined by an initial 75 g oral glucose tolerance test. Insulin sensitivity was measured using a frequently sampled intravenous glucose tolerance test, and subjects underwent an incisional subcutaneous adipose tissue biopsy from the lower abdominal wall. Subjects then received 30 mg pioglitazone daily for a 2 week dose escalation followed by 8 weeks at a maximum dose (45 mg of pioglitazone daily). After 10 weeks of treatment, the oral and intravenous glucose tolerance tests and the biopsies were repeated.

Cell culture

THP1 cells are a macrophage cell line (11) and were maintained in RPMI medium (American Type Culture Collection) with 10% FBS and 1% penicillin-streptomycin. Cells were plated in serum-free medium with 1% penicillin-streptomycin and 250 nM phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 4 days to induce the differentiation of monocytes into macrophages. In some experiments, macrophages were further activated by the addition of lipopolysaccharide (LPS) for 24 h at 20 μ g/ml. Treatment with pioglitazone was carried out in serum-free medium at the dose and times indicated, but additional experiments were conducted in the presence of 125 μ M albumin, because pioglitazone circulates *in vivo* almost completely bound to albumin. The effects of pioglitazone on THP1 cells were similar in the presence of albumin (results not shown).

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes, originally derived from the stromal fraction of subcutaneous adipose tissue of an infant with SGBS, were cultured as described previously (12). Briefly, SGBS cells were maintained in DMEM:F12 (Gibco) containing 10% fetal calf serum and 1% penicillin-streptomycin. For experimental purposes, cells were plated and allowed to reach confluence before adding differentiation medium [DMEM with 25 nM dexamethasone (Sigma), 500 μ M 3-isobutyl-1-methylxanthine (Sigma), 2 μ M rosiglitazone, 0.01 mg/ml human transferrin (Sigma), 2×10^{-8} M insulin (Novo Nordisk), 10^{-7} M cortisol (Sigma), 0.2 nM T3 (Sigma), 33 mM biotin (Sigma), and 17 mM pantothenate (Sigma)] for 4 days. Cell medium was then changed to an adipogenic medium (DMEM with 0.01 mg/ml human transferrin, 2×10^{-8} M insulin, 10^{-7} M cortisol, 0.2 nM T3, 33 mM biotin, and 17 mM pantothenate) for a further 10 days or until the cells were ready for treatment. Morphologically differentiated adipocytes were obtained after 10 days. Following hormonal stimulation, >90% of these cells underwent complete differentiation into mature adipocytes as assessed by Oil Red O lipid staining and expression of adipocyte-specific mRNAs such as lipoprotein lipase, aP2 (FABP4), leptin, and GLUT4.

Oil Red O staining and extraction

SGBS cells were differentiated as described above for 10 days before assessment with Oil Red O staining (Chemicon Adipogenesis Assay Kit). Briefly, cells were fixed in 3.7% formaldehyde for 20 min at room temperature before rinsing twice with distilled water. Oil Red O staining solution was added and stained for 2 h at room temperature. The plates were aspirated and rinsed three times with distilled water before visualizing with a Nikon Eclipse E600 microscope using a 4 \times 0.50 objective. After photographing, 0.5 ml of dye extraction solution was added per well and shaken for 15–30 min. The extracted dye (200 μ l) was transferred to a 96-well plate and read at an absorbance of 540 nm. Results are the mean of three independent experiments \pm SEM.

Immunohistochemistry

Immunohistochemical detection of macrophages was performed on 5 μ m-thick human adipose paraffin sections and fixed in Bouin's solution (Statlab) for 24 h. For CD68 staining, sections were microwave-pretreated in 10 mM citric acid, pH 6.0, for 10 min. Sections were blocked for endogenous peroxidase activity by incubation in 3% H₂O₂ in methanol for 10 min at room temperature, followed by a 30 min block with normal horse serum (ImmPRESS reagents anti-mouse Ig kit; Vector Laboratories, Burlingame, CA). Primary antibody (KP1, mouse monoclonal, Abcam) diluted 1:100 in blocking solution was added for 2 h at room temperature. Sections were then incubated in secondary antibody for 1 h at room temperature, followed by treatment with a coumarin-based TSA (tyramide signal amplification) fluorescence system (PerkinElmer Life Sciences, Inc., Boston, MA) for 20 min at room temperature. Sections were visualized with a Nikon Eclipse E600 microscope using a Nikon Plan Fluor 20 \times 0.50 objective and photographed with a Photometrics CoolSnap ES camera.

Apoptosis assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was carried out according to the manufacturer's instructions (Roche). Briefly, cells (fixed in 4% paraformaldehyde for 20 min at room temperature) and human adipose tissue sections were washed thoroughly in PBS

before blocking for endogenous peroxidase activity with 3% H₂O₂ in methanol for 10 min at room temperature. Cells were permeabilized in ice-cold 0.1% triton X-100, 0.1% sodium citrate for 2 min. Human adipose tissue was permeabilized in 20 µg/ml proteinase K in 10 mM Tris-HCl pH 7.5, for 20 min at 37°C. TUNEL reaction mix was added (1:5 dilution of TUNEL reaction mix) and incubated for 1 h at 37°C in the dark under humidified conditions. TUNEL-Peroxidase (POD) converter was added and incubated at 37°C for an additional 30 min in the dark under humidified conditions. Fluorescein-based TSA fluorescence system (PerkinElmer Life Sciences) was added for 20 min at room temperature before visualizing with a Nikon Eclipse E600 microscope using a Nikon Plan Fluor 20× 0.50 objective and photographed with a Photometrics CoolSnap ES camera.

Measurement of caspase activity

Caspase activity was assessed by measuring the level of cleaved caspase-3 (Asp175) and cleaved caspase-9 (Asp330), which are the activated forms of these proteases. THP1 cells were harvested in M-Per Mammalian Protein Extraction reagent (Pierce), containing protease inhibitor cocktail mix (1:100) (Sigma), after treatment with pioglitazone for the times indicated. Alternatively, THP1 cells were pretreated for 30 min with the PPAR γ inhibitor GW9662 (Sigma) at 1 µM before the addition of pioglitazone at the times indicated (13). Lysates were quantified for protein by bicinchoninic acid (Pierce). Equivalent amounts of protein were resolved by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane at 100 mV for 1 h at 4°C. Membranes were blocked for 1 h at room temperature with 5% skim milk powder (MP Biomedicals) in Tris-buffered saline (10 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing 0.1% Tween-20 Tris-buffered saline (TTBS). Anti-cleaved caspase-3 or anti-cleaved caspase-9 (1:500) (Cell Signaling Technology) was diluted in TTBS containing 5% skim milk powder and applied overnight at 4°C with gentle rocking. After washing, the blot was incubated for 1 h at room temperature with alkaline-phosphatase anti-rabbit immunoglobulin G diluted in TTBS containing 5% skim milk powder. The blot was analyzed using Lumi-Light Western blotting substrate (Roche), and the chemiluminescence was recorded using the ChemiDoc XRS imager system with Quantity One 1-D analysis software (BioRad). Loading controls were detected by staining with Ponceau S (Sigma).

Total RNA isolation and real-time RT-PCR

Total RNA from SBGS or THP1 cells, was isolated using an RNeasy Lipid Tissue Mini kit from Qiagen (Valencia, CA) following the manufacturer's instructions. The quantity and quality of the isolated RNA was determined using the Agilent 2100 Bioanalyzer with RNA 6000 Nano Chips. One microgram of total RNA was reverse-transcribed using random hexamer primers with TaqMan Reverse transcription reagents (Applied Biosystems; Foster City, CA). Reverse-transcribed RNA was amplified with 1× SYBR Green PCR Master Mix (Applied Biosystems) plus 0.3 µM of gene-specific upstream and downstream primers during 40 cycles on an Applied Biosystems 7500 Real-Time Thermal Cycler. Each cycle consisted of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. Amplified 18S expression was used as standard control to normalize the differences in individual samples. All data are expressed in relation to 18S RNA, where the standard curve was generated using pooled RNA from the samples assayed. Therefore, the data represent arbitrary units, which accurately compare each set of samples to each other but do not necessarily accurately compare samples between different assays. The Ct values of the PCR reactions were generally between 20 and 30 for

all assays. The results are expressed as the sum of three independent experiments.

Statistics

All data were expressed as the mean \pm SEM, the Student's *t*-test was used for statistical analysis, and the paired *t*-test was used for paired data, with the level of statistical significance set at *P* < 0.05.

RESULTS

Macrophage apoptosis in subjects treated with pioglitazone

We have previously reported that macrophages in adipose tissue decreased following pioglitazone treatment (4). To further understand the mechanisms leading to this decrease in macrophage number, double staining of human subcutaneous adipose tissue was performed to examine cells staining for CD68 in conjunction with TUNEL-positive cells. Black arrows in **Fig. 1A** highlight CD68-positive cells, TUNEL-positive cells, and CD68-TUNEL-positive cells from the same subject before and after pioglitazone treatment. CD68-positive cells decreased by 37% following treatment with pioglitazone, as indicated in the top two panels of **Fig. 1A**, a result consistent with our previous work (4). Analysis of apoptotic cells as determined by TUNEL staining revealed a significant 42% increase in TUNEL-positive cells, as shown in the middle two panels of **Fig. 1A**. Overlay analysis of CD68 and TUNEL staining resulted in a significant 40% increase of CD68-positive cells undergoing apoptosis. Occasional TUNEL-positive nuclei were observed within CD68-negative cells (white arrow). Results shown in **Fig. 1A** are quantified in **Fig. 1B**.

Pioglitazone acts directly on macrophages

The results described above indicate that pioglitazone could have direct effects on macrophages or could have a predominant effect on adipocytes, with subsequent secondary effects on macrophages. To further elucidate the effects of pioglitazone on macrophage cells, THP1 cells, from a human monocyte cell line, were examined after treatment with pioglitazone in vitro. TPA was applied to the cells upon plating in order to stimulate their differentiation into macrophage-like cells. This treatment was continued for 4 days to ensure that nearly 100% of the cells underwent macrophage differentiation (14). Further treatment with LPS resulted in activation and stimulation of these macrophage-like cells. TUNEL staining was carried out on THP1 cells with and without LPS activation in the presence and absence of pioglitazone. As shown in **Fig. 2A**, pioglitazone treatment of differentiated macrophages resulted in an increase in the number of TUNEL-positive cells in a dose-dependent manner. Although there was no significant increase in TUNEL-positive cells at the lowest pioglitazone concentration of 0.5 µM, when compared with vehicle alone, pioglitazone significantly increased apoptosis at concentrations of 1.5 µM and 3.0 µM.

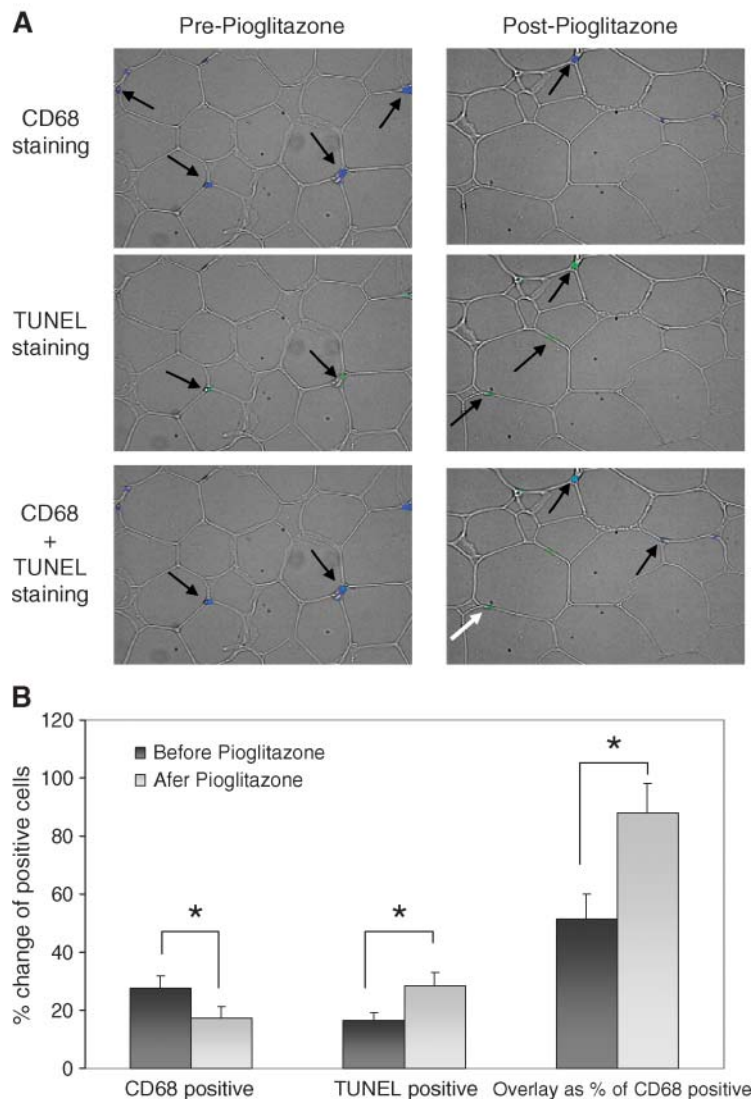


Fig. 1. Immunohistochemistry of pre- and post-pioglitazone-treated human adipose tissue for CD68 and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Human adipose tissue samples were fixed and stained with antibodies for macrophage markers and for apoptosis using TUNEL application. Positive cells are stained blue for CD68 and green for apoptotic nuclei. Representative positive staining is indicated by black arrows. A: Top panels show CD68 staining, TUNEL staining, and CD68 plus TUNEL staining, respectively, of a representative subject with impaired glucose tolerance before and following pioglitazone treatment for 10 weeks. Positive cells are indicated by black arrows. The white arrow indicates a TUNEL-positive, CD68-negative cell. B: Summary graph of CD68-positive cells, TUNEL-positive cells, and calculated overlay of CD68/TUNEL as a percent of total CD68-positive cells pre- and post-pioglitazone treatment. Results shown are the percentage mean \pm SEM from seven independent paired samples, with at least four fields counted in each sample. *, Differences in each case between pre- and post-pioglitazone are significant at $P < 0.05$.

The addition of LPS to differentiated macrophages also tended to increase the percentage of TUNEL-positive cells, and the combination of pioglitazone plus LPS further increased macrophage apoptosis (Fig. 2B). In the presence of LPS, the levels of pioglitazone-induced apoptosis appear to reach a maximum at 1.5 μ M.

To examine the time course of apoptosis with pioglitazone, we analyzed TUNEL staining in differentiated THP1 cells at 24, 48, and 72 h following treatment *in vitro* with pioglitazone at 1.5 μ M. As shown in Fig. 3A, pioglitazone induced apoptosis in a time-dependent manner in both the presence and the absence of added LPS. At 48 h and 72 h, the numbers of apoptotic cells were significantly higher under all conditions, compared with the respective 24 h time point. Again, as described above, the addition of LPS increased the number of TUNEL-positive cells; this can be visualized by comparing the top three panels of Fig. 3A with the bottom three panels. As shown in Fig. 3B, the addition of 1.5 μ M pioglitazone to differentiated THP1 cells resulted in a significant increase in apoptosis at 48 h. In addition, there was a significant increase in apoptosis in cells treated

with TPA plus LPS, compared with those treated with TPA alone (Fig. 3B).

To further confirm that these pioglitazone-treated macrophage cells were undergoing apoptosis, the levels of caspase-3, a major component of the apoptosis signaling pathway, and caspase-9, an initiator caspase, were analyzed by Western blotting. Cell lysates of THP1 cells (100 μ g) were applied to a 15% SDS-PAGE gel and probed with an antibody to the active forms of caspase-3 and caspase-9. Pioglitazone increased the amount of cleaved caspase-3 and cleaved caspase-9 in THP1 cells treated with TPA in a dose-dependent manner after 72 h of treatment (Fig. 4A). Densitometric analysis confirmed these findings (Fig. 4B).

PPAR γ activators are known to induce a number of genes, among them CD36. To establish this expected pattern of gene expression, CD36 mRNA was quantified using real-time RT-PCR before and after pioglitazone treatment. THP1 and SGBS cells, at day 8 of differentiation, were treated with pioglitazone at 1.5 μ M for 48 h. Compared with control cells, pioglitazone-treated THP1 and SGBS cells

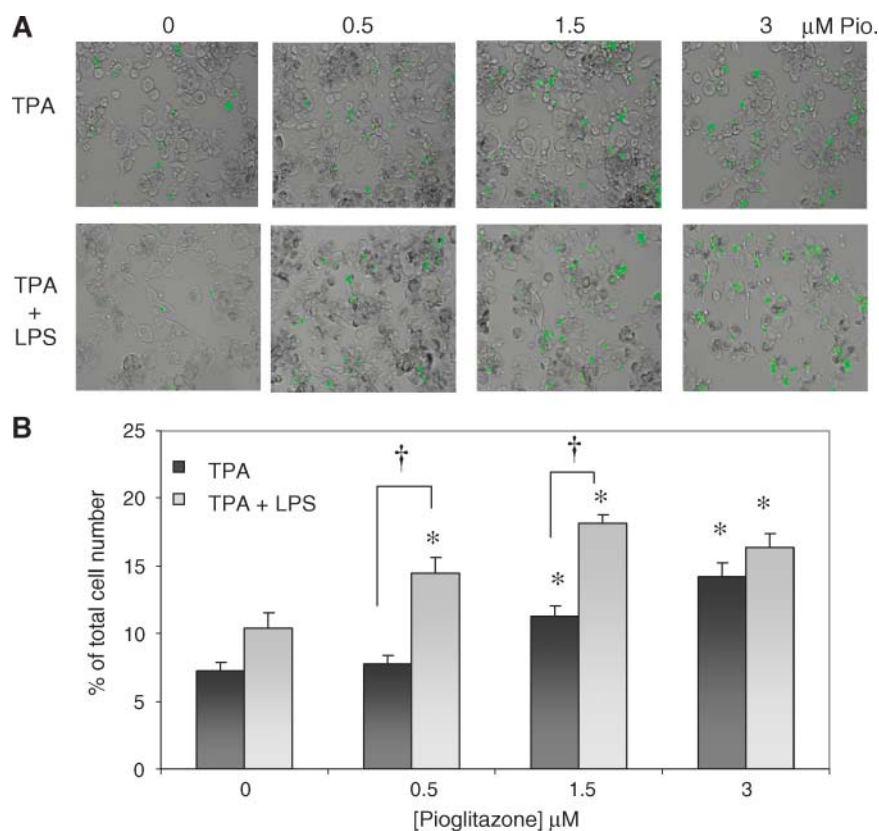


Fig. 2. THP1 cells undergo apoptosis following treatment of pioglitazone in a dose-dependent manner. THP1 cells were plated at 200,000 cells per well in an 8-well chamber slide, treated for 4 days with tetradecanoylphorbol-13-acetate (TPA; 250 nM), with and without 20 $\mu\text{g}/\text{ml}$ LPS for an additional 24 h, before treatment with pioglitazone at concentrations from 0 to 3 μM for 72 h. A: Representative images showing positive TUNEL staining (green) after pioglitazone treatment for 72 h at the concentrations indicated. B: Graph of TUNEL-positive THP1 cells after pioglitazone treatment at the concentrations indicated for 72 h, as a percentage of the total THP1 cell number. Data are expressed as the percent TUNEL-positive cells normalized to the control (0 μM pioglitazone) cells from three independent experiments, with at least four fields counted in each sample. *, Differences compared with respective controls are significant at $P < 0.05$. †, Differences between TPA and TPA + LPS at each concentration are significant at $P < 0.05$.

both showed a significant increase in CD36 gene expression (Fig. 4C).

Pioglitazone does not induce apoptosis in adipocytes

The immunohistochemical experiments using adipose tissue from patients with IGT, described in Fig. 1, revealed that some TUNEL-positive cells did not stain with CD68, as indicated by the white arrow in the bottom right panel of Fig. 1A. This could be because some apoptotic macrophages were no longer expressing macrophage markers, but could also be due to the induction of apoptosis in adipocytes. To confirm that adipose cells were not undergoing significant cell death with pioglitazone treatment, we examined the effect of pioglitazone on apoptosis in human SGBS adipocytes (12). A low level of adipocyte apoptosis was noted, as indicated by the white arrow in Fig. 5A. However, no significant increase in apoptosis was noted in differentiated adipose cells with pioglitazone at any of the concentrations examined over

a 72 h period, suggesting that pioglitazone does not induce apoptosis in adipocytes under these conditions (Fig. 5B).

Pioglitazone-induced macrophage apoptosis is PPAR γ dependent

Pioglitazone could have induced macrophage apoptosis through PPAR γ activation or through other pleiotropic effects. To establish whether the pioglitazone-induced macrophage cell death was occurring in a PPAR γ -dependent manner, GW9662, a potent, irreversible, and functionally selective PPAR γ antagonist, was used. As a control for the effectiveness of GW9662 in blocking the well-characterized function of PPAR γ in promoting adipogenic differentiation, SGBS cells were differentiated in the presence of 1 μM pioglitazone for 10 days, with or without GW9662, and adipogenic differentiation was assessed. Compared with untreated cells, GW9662-treated cells did not differentiate robustly, as shown by reduced Oil Red O staining

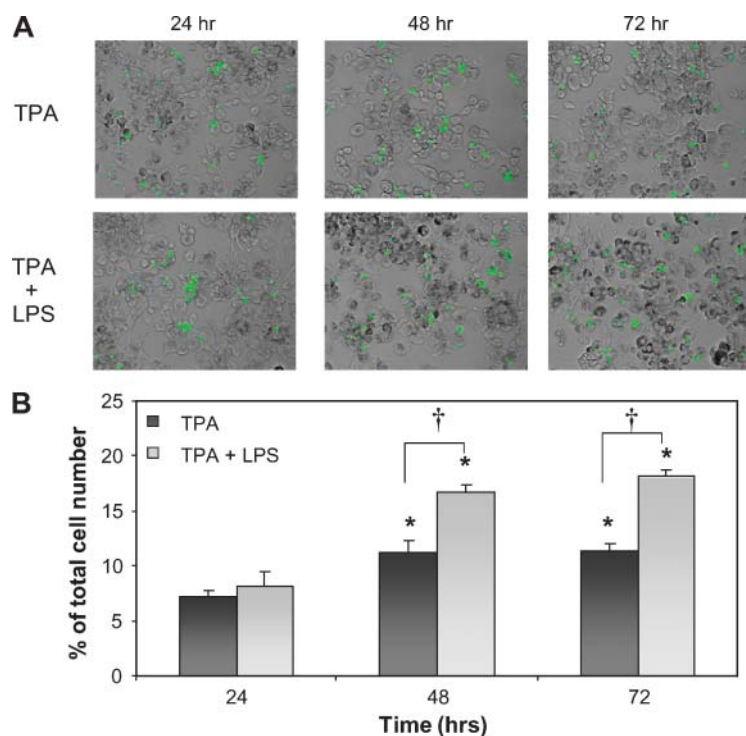


Fig. 3. THP1 cells undergo apoptosis with treatment of pioglitazone in a time-dependent manner. THP1 cells were plated at 200,000 cells per well in an 8-well chamber slide, treated for 4 days with TPA (250 nM) with and without 20 $\mu\text{g}/\text{ml}$ LPS for an additional 24 h, before treatment with pioglitazone at 1.5 μM for 24, 48, and 72 h. A: Representative images showing positive TUNEL staining (green) after pioglitazone (1.5 μM) treatment for 24, 48, and 72 h, as indicated. B: TUNEL-positive THP1 cells as a percentage of the total THP1 cell number after pioglitazone treatment (1.5 μM) at the times indicated. Data are expressed as the mean \pm SEM from three independent experiments, with at least four fields counted in each sample. *, Differences compared with respective 24 hr time points are significant at $P < 0.05$. †, Differences between TPA and TPA + LPS at 48 and 72 h are significant at $P < 0.05$.

(Fig. 6A, B). THP1 cells pretreated with GW9662 for 30 min before the addition of pioglitazone for 72 h were then analyzed by caspase-3 and caspase-9 Western blotting. Pioglitazone, in the presence of GW9662, did not induce apoptosis, as indicated by the absence of cleaved caspase-3 and caspase-9 (Fig. 6C).

DISCUSSION

Adipose tissue consists of various cell types, including adipocytes, stromal cells, endothelial cells, macrophages, and other blood cells. The characterization of macrophages in obese adipose tissue is relatively recent, with studies showing that an increased number of circulating monocytes derived from bone marrow become resident WAT macrophages in obese rodents and humans (2–4). There have also been many human studies showing a link between obesity, insulin resistance, and cytokine levels (15, 16). Recent studies have indicated that much of the TNF α and IL-6 secreted by adipose tissue is derived from the macrophage fraction (2), and monocyte chemoattractant protein-1 (MCP-1) is expressed by both macrophages and adipocytes (4). Treatment with TZDs results in a decrease in inflammatory cytokine levels and a reduction in MCP-1 (17), and appears to reduce the inflammatory milieu overall.

In a previous study, we demonstrated that human adipose tissue contained macrophages and that macrophage number was positively correlated with TNF α and IL-6 levels and inversely correlated with insulin sensitivity. Improved insulin sensitivity with pioglitazone treatment in subjects

with IGT resulted in a decrease in macrophage number (4). The present study was designed to determine whether this decrease in macrophage number was due, at least in part, to apoptosis. Fat biopsies subjected to TUNEL staining from subjects with IGT before and after pioglitazone treatment showed a significant increase in the total number of TUNEL-positive cells. Although TUNEL-positive cells increased, the number of CD68-positive cells decreased and the percentage of CD68/TUNEL-positive cells increased, indicating that the decrease in macrophages following pioglitazone treatment was due to macrophage apoptosis.

Both adipocytes and macrophages express PPAR γ (7, 18), and therefore the increase in macrophage apoptosis could have been due to a direct effect on the macrophage, or could have been due to an effect on adipocytes, with secondary effects on macrophages. To assess the direct effects of pioglitazone on macrophages, the drug was added to cultures of THP1 cells, a human macrophage cell line. The addition of pioglitazone resulted in an increase in TUNEL staining in both a dose- and time-dependent fashion. Confirmation of apoptosis was obtained by demonstrating an increase in caspase activity in the cells. For both the dose and time course experiments, the concentrations of pioglitazone were consistent with in vivo plasma levels after administration of pioglitazone (14). LPS, a known endotoxin that is the major structural component of the outer wall of Gram-negative bacteria, is a potent activator of macrophage cytokine production and was used to activate THP1 cells, and treatment with LPS followed by pioglitazone showed an increased tendency of cells to undergo apoptosis. During

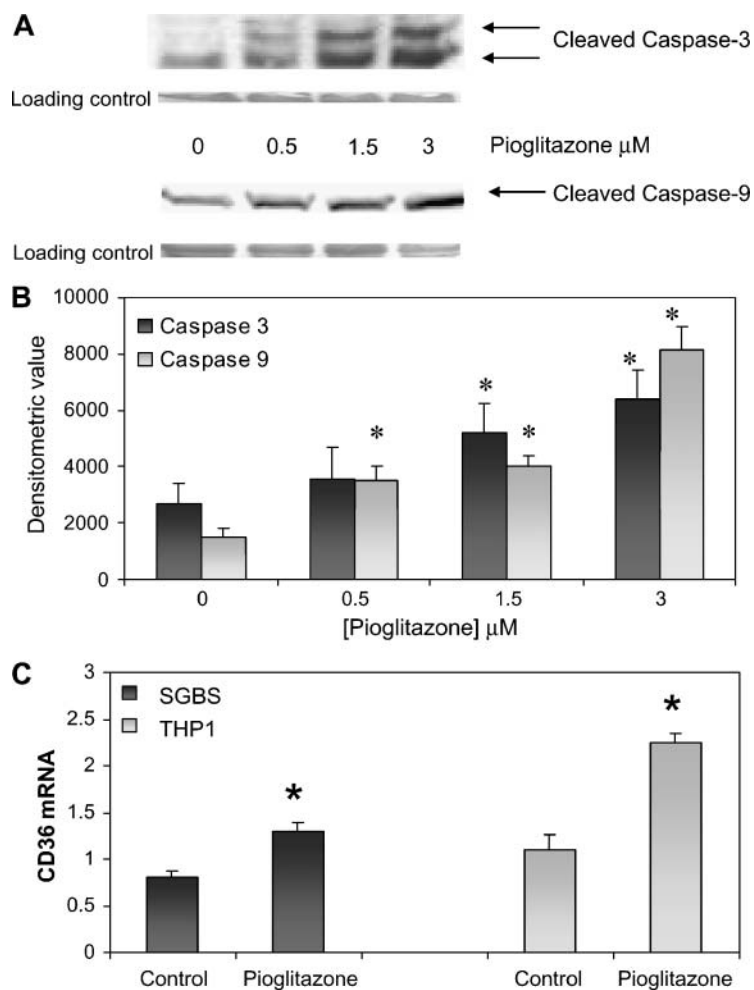


Fig. 4. Apoptotic THP1 cells express cleaved caspase-3. A: THP1 cells, stimulated with TPA for 4 days, were treated with pioglitazone for 72 h at 0, 0.5, 1.5, and 3 μM . Equal amounts of protein were subjected to Western blot with an anti-cleaved caspase-3 antibody (17 and 19 kDa) and an anti-cleaved caspase-9 antibody (37 kDa) as indicated by arrows. B: Densitometric analysis of Western blots for caspase-3 and caspase-9, respectively. *, Differences are significant at $P < 0.05$ and are the mean of at least three independent experiments. C: CD36 expression level in Simpson-Golabi-Behmel syndrome (SGBS) and THP1 cells after 48 h of treatment with pioglitazone. *, Differences are significant at $P < 0.05$ compared with control and are the mean \pm SEM of at least three independent experiments.

the time course experiment, we observed more apoptosis at 48 h- and 72 h-time points for the LPS-treated cells, when compared with cells not treated with LPS, suggesting that LPS activation induced macrophage cell apoptosis (19) and that the addition of pioglitazone further increased apoptosis.

Induction of apoptosis stimulates a cascade of events that ultimately leads to cell death. In these studies, pioglitazone-induced apoptosis in THP1 cells occurred via caspase-9 and caspase-3. These findings confirm our TUNEL staining and begin to elucidate further the apoptotic pathway involved. However, to further understand the exact mechanism involved, a PPAR γ inhibitor was utilized. SGBS cells treated with GW9662 did not undergo robust differentiation, indicating that PPAR γ is essential for adipocyte differentiation. Similarly, THP1 cells treated with GW9662 did not undergo apoptosis upon treatment with pioglitazone, indicating that PPAR γ activation is essential for pioglitazone-induced apoptosis in these cells. Thus, although PPAR γ may have some similar functions in adipocytes and macrophages, as indicated by induction of CD36 gene expression in both cell types by pioglitazone, PPAR γ clearly has distinct functions within different cell types in WAT. Further studies will be needed to better understand other

downstream mechanisms for PPAR γ -mediated macrophage apoptosis.

Pioglitazone has previously been shown to induce apoptosis in a variety of cancer cells and to inhibit proliferation (8–10). In addition, there is evidence linking TZDs and inflammation. Patients with diabetic nephropathy had a significant reduction in pro-inflammatory markers after treatment for 16 weeks with pioglitazone (20). In nondiabetic obese individuals, it was found that troglitazone also had the ability to suppress inflammatory markers, such as C-reactive protein, IL-6, and TNF- α . Moreover, intraperitoneal inflammation, associated with endometriosis, has been shown in mice to be reduced by TZD administration by significantly diminishing the number of macrophages (21). TZDs have also been shown to increase production of IL-1 receptor antagonist, an anti-inflammatory cytokine, in THP1 cells, although there was no significant effect on the levels of proinflammatory cytokines (22).

Overall, our data indicate for the first time that at least one of the mechanisms leading to the pioglitazone-induced reduction in macrophage cells is apoptotic cell death. The increasing number of apoptotic macrophage cells explains the observed decrease in overall macro-

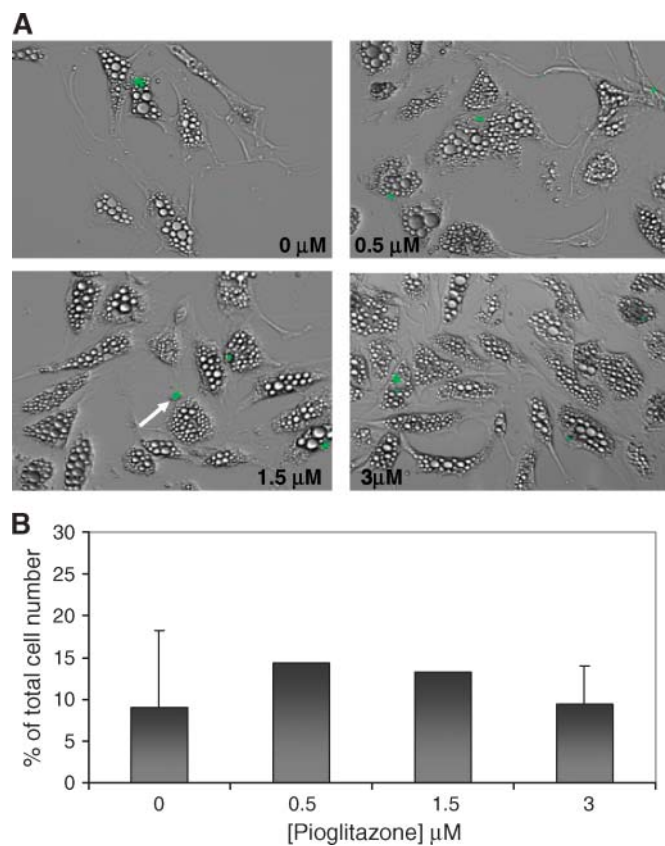


Fig. 5. SGBS cells do not undergo apoptosis with pioglitazone treatment. **A:** SGBS cells were plated and allowed to reach confluence before differentiating into adipose cells. At day 8 of differentiation, cells were treated with pioglitazone at the concentrations indicated for 72 h before TUNEL staining. The white arrow indicates a rare TUNEL-positive cell. **B:** Graph of TUNEL-positive adipose cells as a percentage of the total SGBS cell number after pioglitazone treatment at the concentrations indicated for 72 h. Data are expressed as the mean \pm SEM from three independent experiments, with at least four fields counted in each sample. For 0.5 and 1.5 μM pioglitazone treatments, the error bars were too narrow to be detected on the bar graph.

phage cell number. Although there were some apoptotic cells that were not CD68 positive, we believe that these may be adipose, endothelial, or some other cell type that is undergoing natural apoptosis but that this cell death is not related to the pioglitazone treatment, because it was not significantly different pre- and post-treatment. These results identify macrophage cell death as an important function of pioglitazone treatment that may play an essential role in the management of diabetes mellitus and metabolic syndrome. **■**

The authors would like to thank Takeda Pharmaceuticals for providing Pioglitazone for the in vivo experiments and Leslie Miles for her recruitment of patients, as well as the nurses of the General Clinical Research Center. This work was supported by a merit review grant (P.A.K.) and a Veterans Integrated Service Network career development award (N.R.) from the Veterans

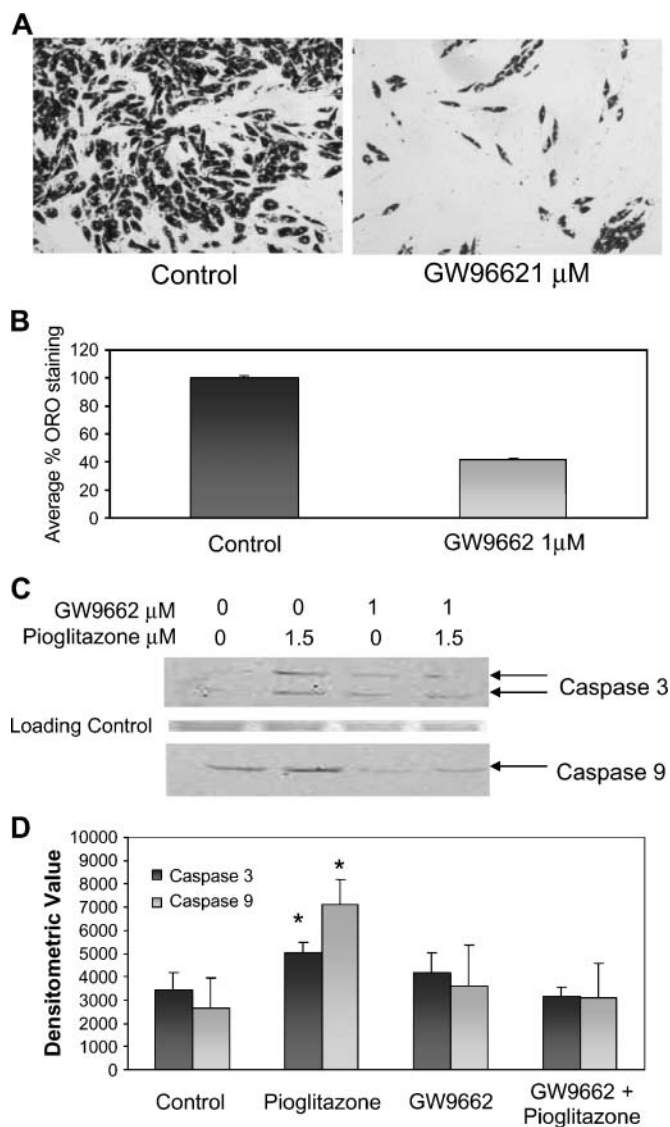


Fig. 6. Peroxisome proliferator-activated receptor γ inhibitor blocks SGBS differentiation and THP1 apoptosis. **A:** SGBS cells were plated and allowed to reach confluence before differentiating into adipose cells in the presence of 1 μM GW9662. At day 10 of differentiation, cells were assessed by Oil Red O lipid staining. Representative images at 4 \times magnification are shown for control and GW9662-treated cells. **B:** Graph showing Oil Red O lipid extraction from SGBS cells treated with GW9662 compared with control. Results are the mean \pm SEM of three independent experiments. **C:** THP1 cells stimulated with TPA for 4 days were pretreated with GW9662 at 1 μM for 30 min followed by pioglitazone for 72 h at 1.5 μM . Equal amounts of protein were subjected to Western blot with an anti-cleaved caspase-3 antibody (17 and 19 kDa) and an anti-cleaved caspase-9 antibody (37 kDa) as indicated by arrows. **D:** Densitometric analysis of Western blots for caspase-3 and caspase-9, respectively. *, Differences are significant at $P < 0.05$ and are the mean \pm SEM of at least three independent experiments.

Administration, Grant M01RR14288 from the General Clinical Research Center, and Grants DK-039176 and DK-071277 (P.A.K.), AG-12411 and DK-071349 (C.A.P.), and DK-071346 (R.E.M.) from the National Institutes of Health.

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