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Pioglitazone induces apoptosis in human vascular smooth muscle cells from diabetic patients involving the TGF-beta/ALK-4/5/7/Smad2 signaling pathway

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Abbreviations: VSMC; vascular smooth muscle cells, DP; diabetic patients, NDP; non-diabetic patients, TZD; Thiazolidinediones, PIO; pioglitazone, LDL; low density lipoprotein, HDL; high density lipoprotein, FCS; foetal calf serum.

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ABSTRACT

Aims. Alterations in vascular wall remodeling are a typical complication in type 2 Diabetes Mellitus due to an imbalance between cell proliferation and apoptosis. In this context, we have previously shown that vascular smooth muscle cells (VSMC) from diabetic patients were resistant to induced apoptosis. Thiazolidinediones, such as pioglitazone, seem to exert direct antiatherosclerotic effects on type 2 diabetes. We aimed to study whether pioglitazone was able to induce apoptosis in VSMC from diabetic patients (DP) and, if so, whether the TGF-β1/Smad-2 pathway was involved. **Methods.** Human internal mammary artery VSMC were isolated from patients who had undergone coronary-artery bypass graft. **Results.** Pioglitazone (100 μM) induced apoptosis in human VSMC from diabetic and non-diabetic patients (NDP), analyzed by DNA fragmentation and by degradation of Bcl-2, in high-glucose-containing medium (15 and 25 mM). This apoptotic effect was inhibited by the ALK-4/5/7/Smad2 inhibitor SB-431542, denoting that the TGF-β1/Smad-2 pathway was involved. Pioglitazone rapidly increased the extracellular TGF-β1 levels and concomitantly induced phosphorylation of Smad2 in VSMC from DP and NDP. **Conclusion**. We demonstrated that pioglitazone induced apoptosis in human VSMC from DP, which are strongly resistant to the induced apoptosis. This effect of pioglitazone might contribute in the treatment of alterations of vascular remodeling in type 2 Diabetes Mellitus.

INTRODUCTION

Cardiovascular complications remain as the major cause of morbidity and mortality in patients who suffer type 2 diabetes mellitus. One of these complications seems to be alterations in the normal vascular remodeling as described previously (Terry et al., 2003). This alteration has been postulated to be mediated by an imbalance in the ratio proliferation/apoptosis of vascular smooth muscle cells (VSMC) within the artery wall (Gibbons and Dzau, 1994). In fact, we reported recently that arteries and isolated VSMC from diabetic patients showed increasing levels of the antiapoptotic protein Bcl-2 and increasing levels of cell proliferation (Ruiz et al., 2006). This alteration of tissue microarchitecture may be regarded as a “failure to die” of this cell type.

Thiazolidinediones (TZD) constitute an emerging class of oral antidiabetic drugs which possess some direct vascular effects, independently of their hypoglycemic actions (Aizawa et al., 2001). In particular, pioglitazone (PIO) has been demonstrated to exert an additional benefit on the lipid profile compared to other TZD (Goke, 2002). In addition one of the most remarkable direct vascular actions of PIO is the induction of VSMC apoptosis (Bishop-Bailey et al., 2002), which might be related to the clinical finding of a decreased intima/media thickness in patients treated with PIO (Takagi et al., 2003). In this context, the pleiotropic cytokine transforming growth factor beta-1 (TGF-β1) is thought to play a major role in vascular remodeling by decreasing the ratio proliferation/apoptosis (Ivanov et al., 1998; Kannan et al., 2003). The canonical pathway of TGF-β1 in VSMC involves

activation of ALK-4/5/7 receptor-related kinases, which phosphorylates Smad2 (Ten Dijke et al., 2002). Interestingly, the importance of the TGF-β1/Smad2 pathway in PIO-mediated rat VSMC apoptosis has been demonstrated by our group (Redondo et al., 2005). We found that PIO increased TGF-β1 release in rat VSMC and this cytokine mediated the apoptosis of PIO through the Smad2 pathway. In this work, we aimed to determine whether PIO was able to overcome the human diabetic VSMC failure to die and whether TGF-β1/ALK-4/5/7 was involved.

METHODS

Description of patients

A group of patients was recruited from those undergoing coronary artery bypass graft surgery at the Cardiac Surgery Service (Hospital Clinico San Carlos, Madrid, Spain). Diabetes Mellitus was defined following the criteria established by the ADA (American Diabetes Association, 2005) as fasting serum glucose concentration ≥126mgl/dl and use of antidiabetic oral drugs or insulin. Patient data included: age, gender, active smoker, obesity, total cholesterol, cholesterol low density lipoptroteins (LDL), cholesterol high density lipoproteins (HDL), triglycerides, glucose and blood pressure (see patient details in Table 1). Internal mammary arteries were collected by the surgeons during the surgical procedure, labeled and used within the next few minutes after the operations. The study was conducted

according to the Declaration of Helsinki and we obtained informed consent from all subjects before sampling took place.

Cell Cultures and Treatments

Human internal mammary artery vascular smooth muscle cells were cultured from explants in RPMI (Life Technologies, Barcelona, Spain) containing 10% foetal calf serum (FCS). The cells exhibited typical "hill and valley" smooth muscle morphology observed by phase contrast microscopy and the cultures were stained positively with a monoclonal anti-α-actin antibody. Experiments were performed with VSMC between passages 3 and 5. For the analysis of cell death by apoptosis induced by pioglitazone (PIO), cells (diabetic and non diabetic) were treated with different concentrations of PIO (10-100 μM) for 24 h in a normo-glycemic cellular medium (5 mM glucose). Alternatively, cells from non-diabetic and diabetic patients were pretreated with 15 and 25 mM glucose (or 25 mM mannitol) for 48 h prior to treating the cells with PIO 100 μM, 24 h. Since PIO induced apoptosis under hyperglycemic conditions, the following experiments were performed in VSMC pretreated with glucose 15 mmol/l for 48 h. To determine the role of TGFβ1/ALK-4/5/7 in the apoptotic effect of PIO, cells from non-diabetic and diabetic patients were pretreated with the ALK-4/5/7 blocker (inhibitor, antagonist) SB-431542 10 μMl, 30 min prior to PIO treatment. For the analysis of TGF-β1 release and Smad2 phosphorylation, cells were pretreated with glucose 15 mM or 48 h prior to PIO treatment in RPMI medium containing PDGF 10 ng/ml and 0.2% BSA to avoid the interference of serum-contained TGF-β1.

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Measurement of cellular DNA fragmentation

Vascular smooth muscle cells from non diabetic and diabetic patients were plated on 96-well plates at a density of 7000 cells/well and allowed to attach for 24 h. Cellular DNA fragmentation was measured with a commercially available cellular DNA fragmentation ELISA kit (Roche-Boerhinger, Spain) following the manufacturer's instructions. DNA fragmentation was expressed as fold increase of the control values.

Analysis of caspase-3 activity

Human vascular smooth muscle cells (non-diabetic and diabetic) were plated on 90 mm Petri dishes and allowed to attach for 24 h. The cells were then treated with glucose 15 mM for 48h and then with PIO 100 μM for 12h. Caspase-3 activity was measured spectrophotometrically using a commercially available kit (Calbiochem) following the manufacturer's instructions. Data are represented as caspase-3 activity (pmol/min/mg of protein).

Western Blotting

To determine the expression of Bcl-2, cells from diabetic and non diabetic patients were plated onto 60 mm Petri dishes and allowed to attach for 24 h. The cells were pretreated with 5 or 15 mM glucose for 48 h prior to PIO treatment in the presence or absence of the ALK-4/5/7 blocker SB-431452. At the time of harvest, the cells were washed with ice-cold PBS, lysed on ice with 200 μl lysis buffer (10% glycerol, 2.3% SDS, 62.5 mM Tris-HCl, pH 6.8, 150 mM NaCl, 10 mM EDTA, 1 μg/ml

leupeptin, 1 μg/ml pepstatin, 5 μg/ml chymostatin, 1μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride) and boiled for 5 min. Equal amounts of protein were run on 12.5% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Amersham, Madrid, Spain) and blocked overnight at 4° C in blocking solution (5% skimmed milk in TBS-T: 25 mM Tris-HCl, 75 mM NaCl, pH = 7.4, 0.1% v/v Tween®20). For analysis of Bcl-2, the blots were incubated for 2 h with agitation at room temperature in the presence of a specific mouse monoclonal anti- Bcl-2 (Neomarker, Bionova Científica, Madrid, Spain) at 1.5 μg/ml in 0.3% bovine serum albumin in TBS-T. After washing in TBS-T solution, the blots were further incubated for 1 h at room temperature with a horseradish peroxidase conjugated anti-mouse secondary antibody diluted 1:3000 (Promega, Madison, WI, USA) in blocking solution. The blots were then washed 5 times in TBS-T and antibody-bound protein was visualized with Enhanced Chemiluminescence's (ECL) kit (Amersham Biosciences, Barcelona, Spain). Smooth muscle α-actin was used as a housekeeping protein and it was determined following the same procedure as mentioned above, using a specific anti-α-actin mouse monoclonal antibody (Sigma-Aldrich, Madrid, Spain), at 1:1000 in TBS-T.

Measurements of TGF-β1 levels

To determine TGF-β1 levels (total, acid-activable) in the culture medium of control and treated samples, we used a solid-phase TGF-β1-specific ELISA, following manufacturer instructions (R&D Systems). Cells were seeded onto 24-well plates

and allowed to attach for 24 h. The cells were pretreated with 15 mM glucose for 48 h in normal medium and then treated with pioglitazone at 100 µmol/l or control for a defined time period (30 min to 180 min) in RPMI medium containing PDGF 10ng/ml and 0.2% BSA.

Immunofluorescence staining

Subcellular location of phospho-Smad2 protein was also analyzed by confocal images of immunofluorescence-stained samples. The cells were plated onto cover slips, allowed to attach for 24 h and treated with glucose 15 mM for 48 h. Then, the cells were cultured in the presence or absence of pioglitazone at 100 µM from 30 min to 180 min. In some experiments, GW9662 (2 µM) or SB-431542 (10 µM) were added 30 min before treatment with PIO. Cells were washed with PBS and fixed for 20 min in 4% paraformaldehyde in PBS and permeabilized with 0.4% Triton-x100 for 30 min at room temperature. After blocking with 3% BSA in PBS, the cells were then incubated with goat polyclonal anti-phospho-Smad2 (1:100) for 1 h. Excess primary antibody was removed by washing with blocking solution, followed by incubation with donkey anti-goat Alexa 568 (1:100; Molecular Probes) for 1 h. The cells were washed four times with blocking buffer every 5 min. Images were captured using a Leica TCS SP2 inverted microscope. Intensity of staining was analyzed by an Image J software.

Materials

Pioglitazone was a generous gift of Takeda Chemical Industries (Osaka, Japan) and was directly diluted in cell culture media. 4-(5-benzo(1,3)dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)benzamide (SB-431542) and 2-Chloro-5-nitro-N-phenylbenzamide (GW9662) were purchased from Tocris Biosciences (Bristol, U.K.) and dissolved in DMSO at x1000 concentrated stock solution. Fluvastatin was kindly donated by Novartis Pharmaceutical Ltd. (Madrid, Spain). All other reagents were obtained from Sigma (Spain) unless otherwise stated.

Statistical analysis

The results are expressed as the mean±sd (standard deviation) and accompanied by the number of observations. A statistical analysis of the data was carried out by a Student's t test or by a one-way ANOVA when necessary, followed by Dunnett post-test where significance was detected. Differences with a *P* value of less than 0.05 were considered statistically significant.

RESULTS

Apoptotic effect of pioglitazone on human vascular smooth muscle cells

Since we reported previously that pioglitazone induced apoptosis in rat vascular smooth muscle cells, we aimed to study whether this effect was reproducible in VSMC from human patients. Moreover, since pioglitazone is used as an

antidiabetic drug, we studied specifically human VSMC isolated from diabetic patients vs. non-diabetic. As shown in Fig. 1 (Panel A), PIO at 10-100 μM did not induce apoptosis in either cells from non-diabetic or from diabetic patients when they were cultured under normoglycaemic conditions (glucose 5 mM). In addition, PIO 50 μM did not induce increase in DNA fragmentation in cells from diabetic and non-diabetic patients exposed to 10 or 15 mM glucose (data not shown). However, when cells from both non-diabetic and diabetic patients were exposed to high glucose (10 to 25 mM glucose) pioglitazone 100 μM increased DNA fragmentation in cells from non-diabetic patients (Figure 1, panel B), and more interestingly, in cells from diabetic patients (and independently of the osmotic effect of mannitol at 25 mM, Figure 1, panel C). We also tested the effect of glucose (5-25 mM) on the apoptosis and we found no effects (data not shown). To ensure that the effect of PIO was apoptosis, we tried to assess this parameter by another experimental technique. We therefore analyzed the activation of caspase-3 induced by PIO 100 μM in VSMC from diabetic and non-diabetic patients exposed to 15 mM glucose. As shown in Figure 1, Panel D, treatment with PIO 100 μM for 12 h induced an increase in caspase-3 activity in cells from diabetic and non-diabetic patients. To rule out that the effect of pioglitazone was merely an artifact, we tested the apoptotic effect of fluvastatin on human VSMC from non-diabetic and diabetic patients under different concentrations of glucose. Figure 2 shows that fluvastatin at 1μM induced apoptosis (measured as DNA fragmentation) only in human VSMC from non-diabetic patients grown in low glucose. It is also remarkable that the

apoptotic effect of fluvastatin was abolished when the cells was pretreated with glucose 15 mM or mannitol 15 mM (which suggests an osmotic effect).

We previously showed that VSMC from diabetic patients were more resistant to induced apoptosis since they expressed high basal levels of the antiapoptotic protein Bcl-2 (Ruiz et al., 2006). According to this, we also determined the effect of PIO on the levels of Bcl-2 measured by Western blotting. Since the apoptotic effect of PIO was observed in high-glucose conditions, independently of the osmotic effect, we performed the following experiments in a 15 mM glucose-containing medium; a serum glucose concentration typically achieved by diabetic patients. Figure 3, Panel A, shows that basal levels of Bcl-2 were highly expressed in cells from diabetic patients, as already seen. Similarly to DNA fragmentation, cells pretreated with PIO exhibited reduced Bcl-2 protein levels, which denote cell death by apoptosis. Interestingly, cells from diabetic patients also showed degradation of Bcl-2 when treated with PIO.

Role of ALK-4/5/7 on the apoptosis induced by pioglitazone

Since the TGF-β1/Smad2 pathway has been shown to be involved in the apoptotic effect of PIO in rat VSMC, we determined whether the intermediate pathway ALK-4/5/7 was also involved in human VSMC from non-diabetic and diabetic patients. Figure 3 (panel A) shows that pretreatment with SB-431542 10 μM for 30 min prior to PIO treatment abolished the effect of PIO on Bcl-2 protein levels and moreover, this inhibitor also blocked the DNA fragmentation induced by PIO in cells from both non-diabetic and diabetic patients (Figure 3, Panel B).

Pioglitazone induces the release of TGF-β1

Since TGF-β1 seems to be involved in the apoptotic effect of PIO, we studied whether this drug was able to increase the release of TGF-β1 from cultured human VSMC obtained from non-diabetic and diabetic patients pretreated with 15 mM glucose for 48 h prior to PIO treatment. Figure 4, panel A shows a rapid and transient increase in the release of total TGF-β1 induced by pioglitazone 100 μM as early as 30 min after treatment in VSMC from non-diabetic patients, as reported in rat VSMC (Redondo et al., 2005). However, VSMC from diabetic patients showed a different pattern of TGF-β1 release. Thus, TGF-β1 levels reached a peak after 60 min treatment with PIO, although levels of TGF-β1 progressively increased after 30 min treatment. Although none of the patients were taking pioglitazone, we also analyzed the concentration of TGF-β1 in sera from non-diabetic and diabetic patients and we found no differences (Table 1).

In order to corroborate that TGF-β1 itself induces apoptosis in this cell model, VSMC from non-diabetic and diabetic patients were pretreated with 15 mM glucose for 48 h and then with TGF-β1 400 pg/ml for 60 min. Twenty four hours after the exposure to TGF-ß1 we analyzed the DNA fragmentation and Bcl-2 expression. Figure 4, panel B shows that treatment with TGF-β1 increased DNA fragmentation in VSMC from both non-diabetic and diabetic patients in approximately 1.5 fold increase, a value comparable with the effect of PIO. We also found that protein levels of Bcl-2 (analyzed by Western blotting) diminished in cells from diabetic and non-diabetic patients (Figure 4, panel B) treated with TGF-β1.

Pioglitazone enhanced the phosphorylation of Smad2

We showed previously that pioglitazone at 100 μM rapidly increased the nuclear recruitment of phospho-Smad2 in rat VSMC. In human VSMC from non-diabetic and diabetic patients pretreated with glucose 15 mM, PIO 100 μM increased both cytosolic and nuclear staining of phospho-Smad2, although we did not observe a clear nuclear recruitment of phospho-Smad2 as seen in rat VSMC (Figure 5). Increase in phosphorylation of Smad2 was induced after 30 min stimulation with PIO in both non-diabetic and diabetic isolated cells. However, the level of Smad2 phosphorylation in the cytosol was higher in cells from diabetic patients. This effect of PIO was blocked by the ALK-4/5/7 blocker SB-431542 and by the PPARγ antagonist GW9662 (Figure 6).

DISCUSSION

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors involved in cellular functions such as cell differentiation, lipid metabolism and cell cycle control (Boitier et al., 2003; Fajas et al., 2001; Guan et al., 2002). Upon activation, PPARs form heterodimers with retinoid X receptors (RXRs) and bind the peroxisome proliferator response element (PPREs) in the promotor regions of responsive genes to modify gene transcription (Corton et al., 2000). Pharmaceutical ligands of PPARs are used clinically to treat chronic diseases such as diabetes and hyperlipidemia and are being investigated for

treatment of cancer, atherosclerosis and others (Fajas et al., 2001; Dunaif et al., 1996). Pioglitazone is a PPARγ agonist which is used for treatment of type 2 Diabetes Mellitus. In this manuscript we have demonstrated that the PPARγ agonist pioglitazone induced apoptosis in vascular smooth muscle cells isolated from non-diabetic and diabetic patients under hyperglycemic conditions. This effect is likely to be mediated through the PPARγ receptor, since apoptotic trigger in vascular smooth muscle cell cultures induced by thiazolidinediones has been related to PPARγ by previous reports by our group (Redondo et al., 2005) and others (Bishop-Bailey et al., 2002). Other PPARγ agonists, such as troglitazone, also induced apoptosis in vascular smooth muscle cells and (Gouni-Berthold et al., 2001; Lin et al., 2004) and in several tumor cell lines by a mechanism of action involving the decrease in the expression of the antiapoptotic protein Bcl-2 (Shiau et al., 2005; Yoshizawa et al., 2002). In this context, we also observed that PIO treatment decreased protein level of Bcl-2 in cells from both non-diabetic and diabetic patients analyzed by Western blotting.

This has an important medical relevance since we showed previously that VSMC from diabetic patients are resistant to induced apoptosis due, at least in part, to an increase in the protein levels of Bcl-2 within the artery wall and, in addition, this resistance of apoptosis may be acquired *in vitro* by cells from non-diabetic patients when cultured in a high-glucose medium (Ruiz et al., 2006). The apoptotic effect of PIO does not seem to be an unspecific effect since other drugs, such as fluvastatin, did not induce apoptosis in cells from diabetic patients even under high glucose conditions (Figure 2).

The current study was undertaken in an effort to determine whether pioglitazone might induce apoptosis in VSMC from diabetic patients and therefore overcome the resistance to induced apoptosis reported in those patients. We show here that the apoptotic effect of pioglitazone (measured as DNA fragmentation and Bcl-2 expression) was significantly decreased by pretreatment with the ALK-4/5/7 inhibitor SB-431542 (Figure 3) which demonstrates the role of the TGF-β1/Smad2 pathway in the apoptotic effect of pioglitazone in these cells. The compound SB-431542 we have used in the present study has been demonstrated to act as a selective pharmacologic inhibitor of ALK-4/5/7 (Inman et al., 2002). Nevertheless, since ALK-5 has been highlighted as the most important ALK isoform for TGF-β1-mediated growth arrest (Ten Dijke et al., 2004), it may be considered that the apoptotic effect of pioglitazone described in the experimental conditions of the present study is likely mediated by ALK-5.

In our previous work, we also showed that short time exposure to pioglitazone, as well as the physiologic PPARγ ligand 15-deoxy-PGJ2, induced the release of TGF-β1 into the cellular medium (Redondo et al., 2005) and that TGF-β1 was able to stimulate the phosphorylation of Smad2 and therefore its nuclear recruitment. In the present work, we also observed that pioglitazone induced the release of TGF-β1 in human VSMC from non-diabetic and diabetic patients and that TGF-β1 added exogenously also induced apoptosis in VSMC from diabetic and non-diabetic patients. It is important to point out that rat VSMC are normally grown in high-glucose medium and we found that pioglitazone only induced apoptosis in human VSMC pretreated with 15 mM or 25 mM glucose (but not with 25 mM mannitol,

which rules out an osmotic effect). This is important since diabetic patients, although well controlled, possess abnormal (usually increased) blood levels of glucose, such as those found in our cohort of patients, and therefore the observed apoptotic effect of pioglitazone only would take place in a pathological situation of hyperglycemia. Notably, the serum TGF-β1 concentration in our diabetic patients (whose mean glucose concentration is about 10 mM) does not exert any variation compared to non-diabetics (Table 1). For that reason, *in vitro* glucose concentration was increased up to 15 and 25 mM in order to assess whether variations in the TGF-β1 pathway took place. Our approach is based on the conclusion from the UKPDS study, which establishes that future complications in type 2 Diabetes Mellitus (such as macrovascular disease) are related to a poor glycaemic control (United Kingdom Prospective Diabetes Study Group, 1998), where postprandial peaks of 15 mM glucose can be achieved.

The role of TGF-β1 in atherosclerosis and type 2 Diabetes Mellitus is controversial. Although an increase in the expression of TGF-β1 in atherosclerotic clinical specimens has been found (Panutsopulos et al., 2002) and the serum levels of TGF-β1 were reported to be increased in diabetic patients (Pfeiffer et al., 1996), we analyzed the serum concentration of TGF-β1 in our diabetic and non-diabetic patients and we found no statistical differences in both groups of patients (39.94 pg/ml, $CL_{95\%}$: 35.87-44.01 in non diabetic and 44.91 pg/ml $CL_{95\%}$: 41.11-48.71 in diabetic, P=0.07, n=96). Moreover, the bioavailability of TGF-β1 is thought to be reduced in atherosclerotic patients (O´Neil et al., 2004; Byrne et al., 1998) as well as Smad2/3 signaling (Kalinina et al., 2004). Moreover, current cardiovascular risk

factors such as the antifibrinolytic PAI-1 or a high fat diet have been reported to sequester active TGF-β1 (Ten Dijke and Hill, 2004).

In accordance with our previous work, pioglitazone also induced Smad2 phosphorylation. This effect was due to the effect of pioglitazone on the PPARγ receptor since it was blocked by the PPARγ antagonist GW9662 (Figure 5).

Concerning the timing of Smad2 phosphorylation, in both non-diabetic and diabetic patients pioglitazone induced phosphorylation of Smad2 early after 30 min treatment (which correlates with an increase of TGF-β1 in both types of patients).

In relation with the concentration of pioglitazone used in this study, we have found that pioglitazone induces VSMC apoptosis when incubated at a concentration of 100 μM. A clinical study on the pharmacokinetics of pioglitazone reported average peak serum concentrations of 3.38 μM for 45 mg dose (Budde et al., 2003). However, the existence of active liver metabolites (Budde et al., 2003) makes the relationship between the concentrations found in vivo and the one used in our cell culture studies an interesting topic.

Apoptotic effects which have been related to pioglitazone incubation in the present study may be related to some in vivo and clinical results. This drug has been proven to decrease experimental balloon angioplasty in animal models (Aizawa et al., 2001). In addition, a pioglitazone-mediated decrease of intima-media thickness has been reported in retrospective studies (Koshiyama et al., 2001). Moreover, in type 2 diabetics, pioglitazone has been reported to decrease cardiovascular risk markers independently of glycaemic control at the dose of 45 mg (Puftzner et al.,

2005) and prevent macrovascular complications in doses ranging 15-45 mg (Dormandy et al., 2005).

An increased PPARγ expression in neointimal VSMC has been described (Bishop-Bailey et al., 2002). It is noteworthy that the decisive role of high-glucose and PPARγ for the apoptotic effect of pioglitazone might mediate a molecular correlation for pioglitazone selectivity.

The clinical relevance of these findings concerns the fact that pioglitazone induced apoptosis in VSMC from diabetic patients and under high glucose concentrations. The fact that pioglitazone exhibits this effect is important since this drug is extensively used in the treatment of type 2 Diabetes Mellitus. It is also important to point out that the effect only occurred under abnormal glucose conditions, which are comparable to those found in diabetic patients. Although this is an in vitro study, we can argue that treatment with pioglitazone might ameliorate the vascular remodeling and abnormal intima/media thickening after angioplastia that occurs in arteries from diabetic patients, which seems to be due to an imbalance between proliferation and apoptosis of vascular smooth muscle cells.

Interestingly, Smad-dependent pathways have been described to mediate TGF-β1-induced apoptosis of VSMC, an effect exerted by high concentrations of this cytokine (Ten Dijke et al., 2002). According to the present study, high concentrations of TGF-β1 would activate these mediators and trigger a subsequent VSMC apoptosis.

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FOOTNOTES

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E. R. and S. R. have contributed equally to this work.

LEGENDS FOR FIGURES

Figure 1. *Apoptosis induced by pioglitazone in human vascular smooth muscle cells*. Panel A: Vascular smooth muscle cells from non-diabetic and diabetic patients were treated with pioglitazone (PIO, 10-100 μM) for 24 h in cellular medium containing 5 mM glucose. Panel B: Cells from non-diabetic and diabetic patients were pretreated with glucose (5-25 mM) or mannitol (25 mM) 48 h prior to PIO treatment (100 μM). DNA fragmentation was measured according to Methods. Panel C: The same experiments were performed with cells from diabetic patients. Panel D: Effect of PIO 100 μM on caspase-3 activation in VSMC from non-diabetic and diabetic patients exposed to glucose 15 mM. Bar graphs show the mean±sd of n=4 patients, *P<0.05; **P<0.01; ***P<0.001, NS=Non-significant.

Figure 2. *Effect of fluvastatin on apoptosis*. Human VSMC from non-diabetic and diabetic patients were pretreated with glucose (5 or 15 mM) or mannitol (15 mM) 48h and then with fluvastatin (0.1 or 1μM for further 24 h. NA fragmentation was measured according to Methods. Bar graphs show the mean±sd of n=6 patients.

Figure 3. *Role of ALK-4/5/7 on the apoptotic effect of pioglitazone*. Panel A: Effect of PIO on Bcl-2 protein levels: VSMC from non-diabetic and diabetic patients were pretreated with 15 mM glucose for 48 h prior to treating the cells with PIO 100 μM for a further 18h in the presence or absence of SB-431542 (10 μM, 30 min). Bcl-2 expression was determined by Western blotting. Panel B: Human VSMC from non-

diabetic or diabetic patients were pretreated with glucose 15 or 25 mM for 48 h and then with the ALK-4/5/7 blocker SB-431542 (10 μM, 30 min) prior to PIO treatment (100 μM). DNA fragmentation was measured according to methods. Bar graphs show the mean±sd of n=6 patients, **P<0.01; ***P<0.001.

Figure 4. *Pioglitazone increased TGF-β1 levels in cell medium*. Panel A: PDGF-stimulated proliferating human VSMC from non-diabetic (left) or diabetic patients (right) were pretreated with glucose 15 mM for 48 h and then treated with pioglitazone (100 μM) for 30 to 180 min. Each bar shows the mean±sd of three separate experiments, each in quadruplicate and expressed as pg of TGF-β1/mg of total cell protein content. *P<0.05; **P<0.01. Panel B: Apoptosis induced by TGF-β1 in VSMC from non-diabetic and diabetic patients pretreated with glucose 15 mM was analyzed by DNA fragmentation (left) and changes in Bcl-2 protein levels by Western blotting (right). Each bar shows the mean±sd of three independent experiments, each in quadruplicate. *P<0.05. C=control, T= TGF-β1 at 400 pg/ml.

Figure 5. *Phosphorylation of Smad2 induced by pioglitazone.* Panel A: Representative confocal images of phospho-Smad2 in cells from non-diabetic (ND) or diabetic (D) patients pretreated with glucose 15 mM for 48 h and then treated with pioglitazone for the time indicated. Panel B: Upper: Graph shows analysis of the intensity in phospho-Smad2 signal in the cytosol of human VSMC. Bottom: Graph shows analysis of the intensity in phospho-Smad2 signal in the nuclei of

human VSMC. Each graph shows the mean±sd of three separate experiments. *P<0.05; **P<0.01; ***P<0.001.

Figure 6. *Phosphorylation of Smad2 was prevented by the ALK-4/5/7 blocker and by the PPARγ antagonist.* Panel A: Representative confocal images of phospho-Smad2 in in cells from non-diabetic (ND) or diabetic (D) patients pretreated with glucose 15 mM for 48 h and then treated with pioglitazone for 30 min in the presence or absence of the ALK-4/5/7 blocker SB-431542 (10 μM) or the PPARγ antagonist GW9662 (2 μM). Panel B: upper: Graph shows the analysis of the intensity in the phospho-Smad2 signal in the cytosol of human VSMC. Bottom: Graph shows the analysis of the intensity in phospho-Smad2 signal in the nuclei of human VSMC. Each graph shows the mean±sd of three separate experiments. *P<0.05; **P<0.01; ***P<0.001.

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TABLES

	Non diabetic	diabetic	P
N	65	51	
Age	63.4 ± 9.5	66.4 ± 8.2	0.07 *
Women (%)	8 (12%)	9 (18%)	0.42 §
BMI (kg/m^2)	27 ± 3.2	27.6 ± 3.7	0.37 *
Total cholesterol (mg/dl)	158 ± 51	148 ± 40	0.221 §
LDL (mg/dl)	89 ± 40	73 ± 32	0.019 §
HDL (mg/dl)	42 ± 14	39 ± 10	0.238 §
Triglycerides (mg/dl)	125 ± 42	154 ± 87	0.005 §
Glucose (mg/dl)	93 ± 19	136 ± 50	< 0.001 *
Hb1ac (%)	5.6 ± 0.4	7.4 ± 1.1	< 0.001 *
Hypertension (N/%)	18 /35%	33/56%	0.06 §
Smokers (N/%)	25 /38%	13/25%	0.13 §
TGF-β1 (pg/ml) (log)	39.94± 13.6	44.91± 12.8	0.86 *
TGF-β1/No.platelet	0.16± 0.64	0.18± 0.65	0.21 *

Table 1. Clinical and biochemical characteristics of the patients studied.

BMI= body mass index. ¶ Fisher test, § Chi-squared, * Student T-test.

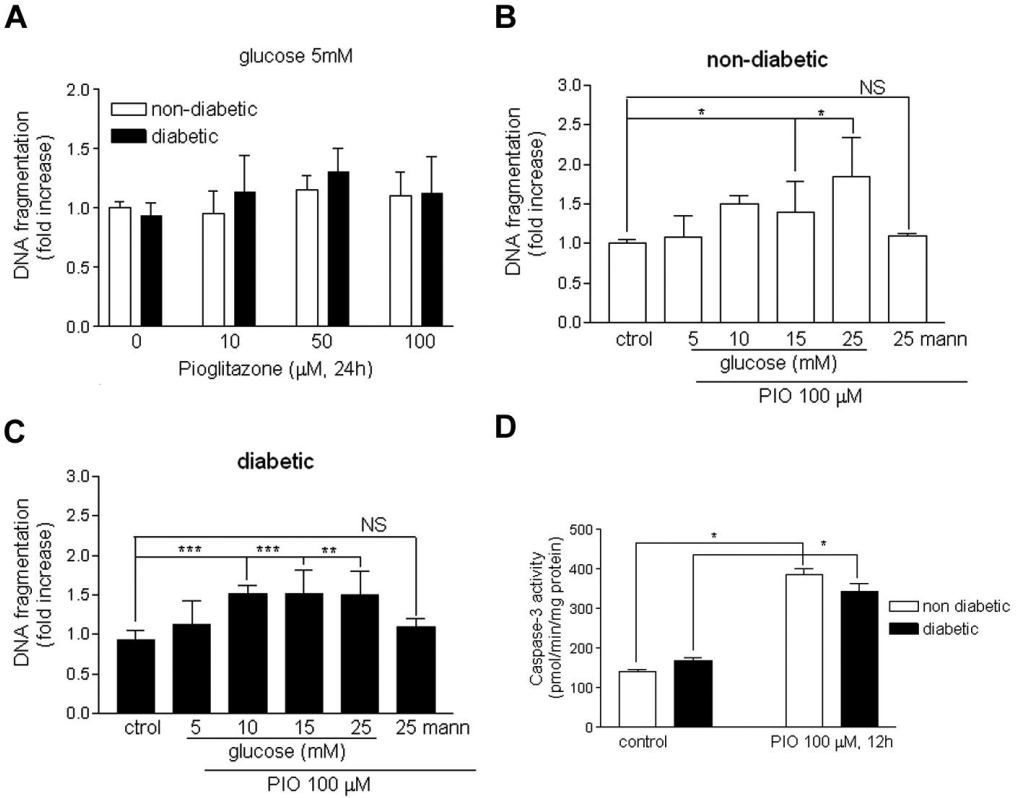
A
glucose 5mM
non-diabetic
diabetic
DNA fragmentation (fold increase)
2.0
1.5
1.0
0.5
0.0
0
10
50
100
Pioglitazone (μM, 24h)
B
non-diabetic
NS
*
*
DNA fragmentation (fold increase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ctrol
5
10
15
25
25 mann
glucose (mM)
PIO 100 μM
C
diabetic
NS
***
***
**
DNA fragmentation (fold increase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ctrol
5
10
15
25
25 mann
glucose (mM)
PIO 100 μM
D
*
*
Caspase-3 activity (pmol/min/mg protein)
500
400
300
200
100
0
non diabetic
diabetic
control
PIO 100 μM, 12h

Figure 1

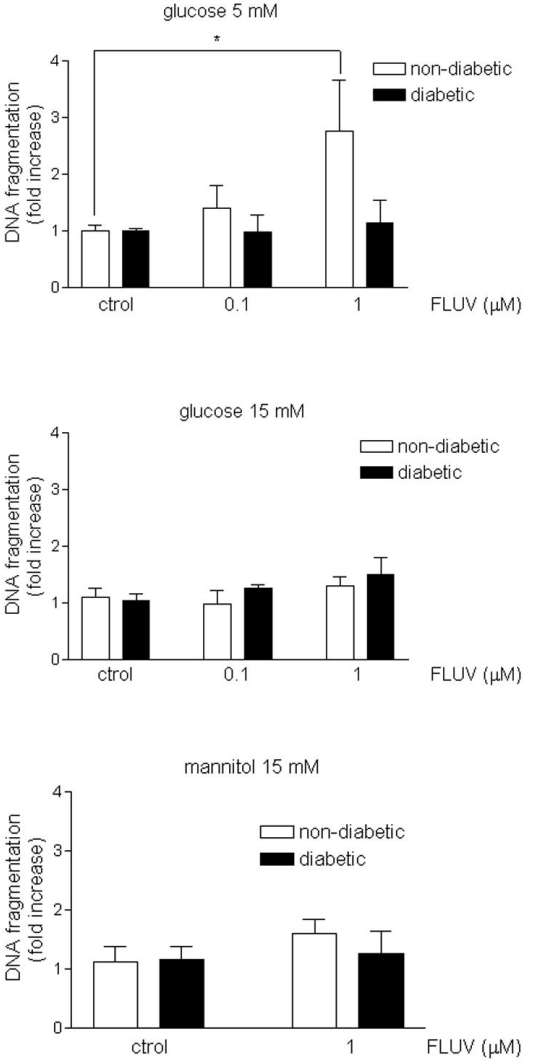
glucose 5 mM
*
non-diabetic
diabetic
DNA fragmentation (fold increase)
0
1
2
3
4
ctrol
0.1
1
FLUV (μM)
glucose 15 mM
non-diabetic
diabetic
DNA fragmentation (fold increase)
0
1
2
3
4
ctrol
0.1
1
FLUV (μM)
mannitol 15 mM
non-diabetic
diabetic
DNA fragmentation (fold increase)
0
1
2
3
4
ctrol
1
FLUV (μM)

Figure 2

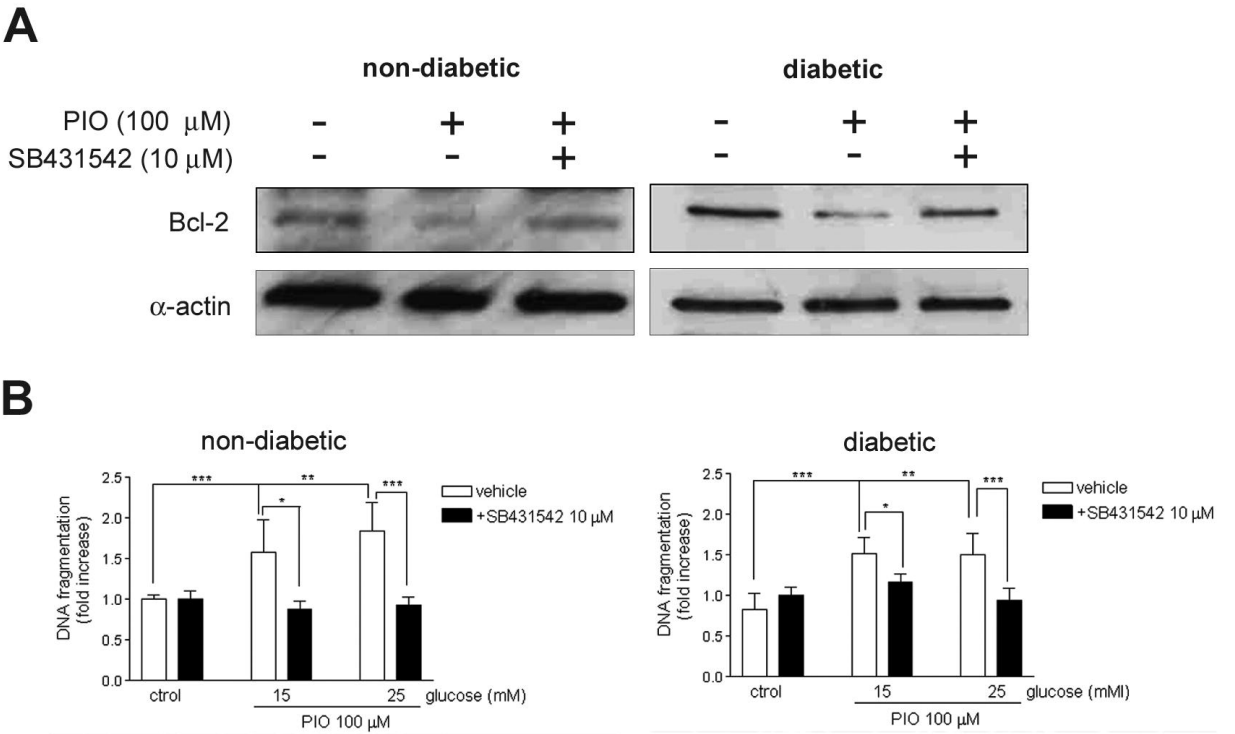
A
non-diabetic
diabetic
PIO (100 μM)
SB431542 (10 μM)
Bcl-2
α-actin
B
non-diabetic
diabetic
DNA fragmentation (fold increase)
vehicle
+SB431542 10 μM
ctrol
15
25
glucose (mM)
PIO 100 μM

Figure 3

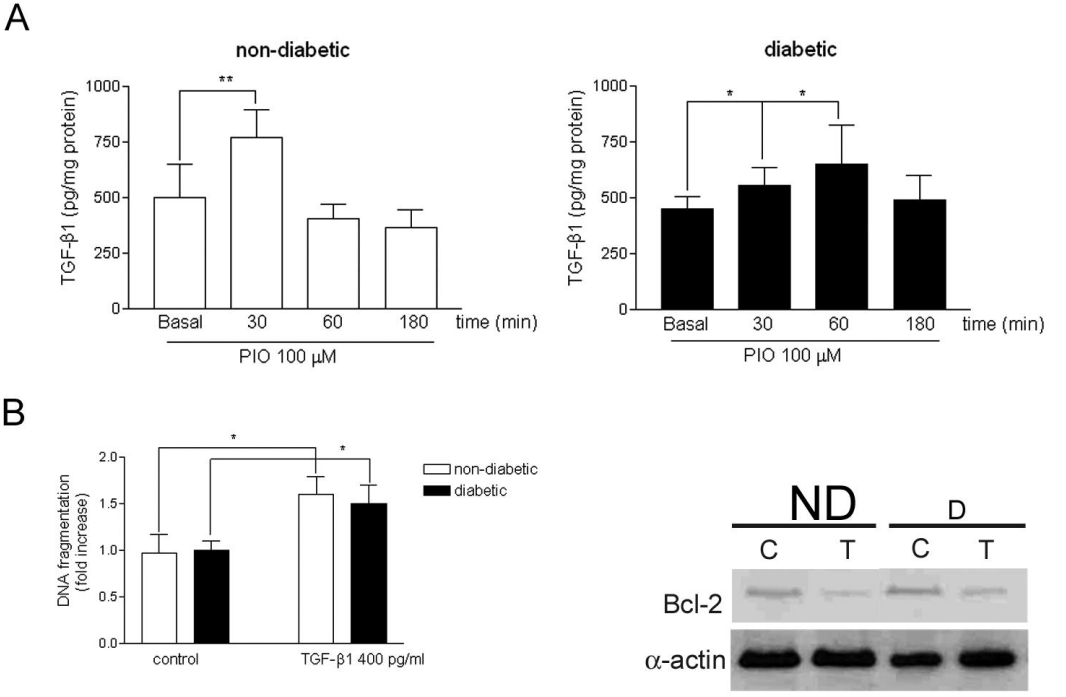
A
non-diabetic
diabetic
TGF-β1 (pg/mg protein)
1000
750
500
250
0
**
*
*
Basal
30
60
180
time (min)
PIO 100 μM
B
DNA fragmentation (fold increase)
2.0
1.5
1.0
0.5
0.0
*
*
non-diabetic
diabetic
control
TGF-β1 400 pg/ml
ND
D
C
T
C
T
Bcl-2
α-actin

Figure 4

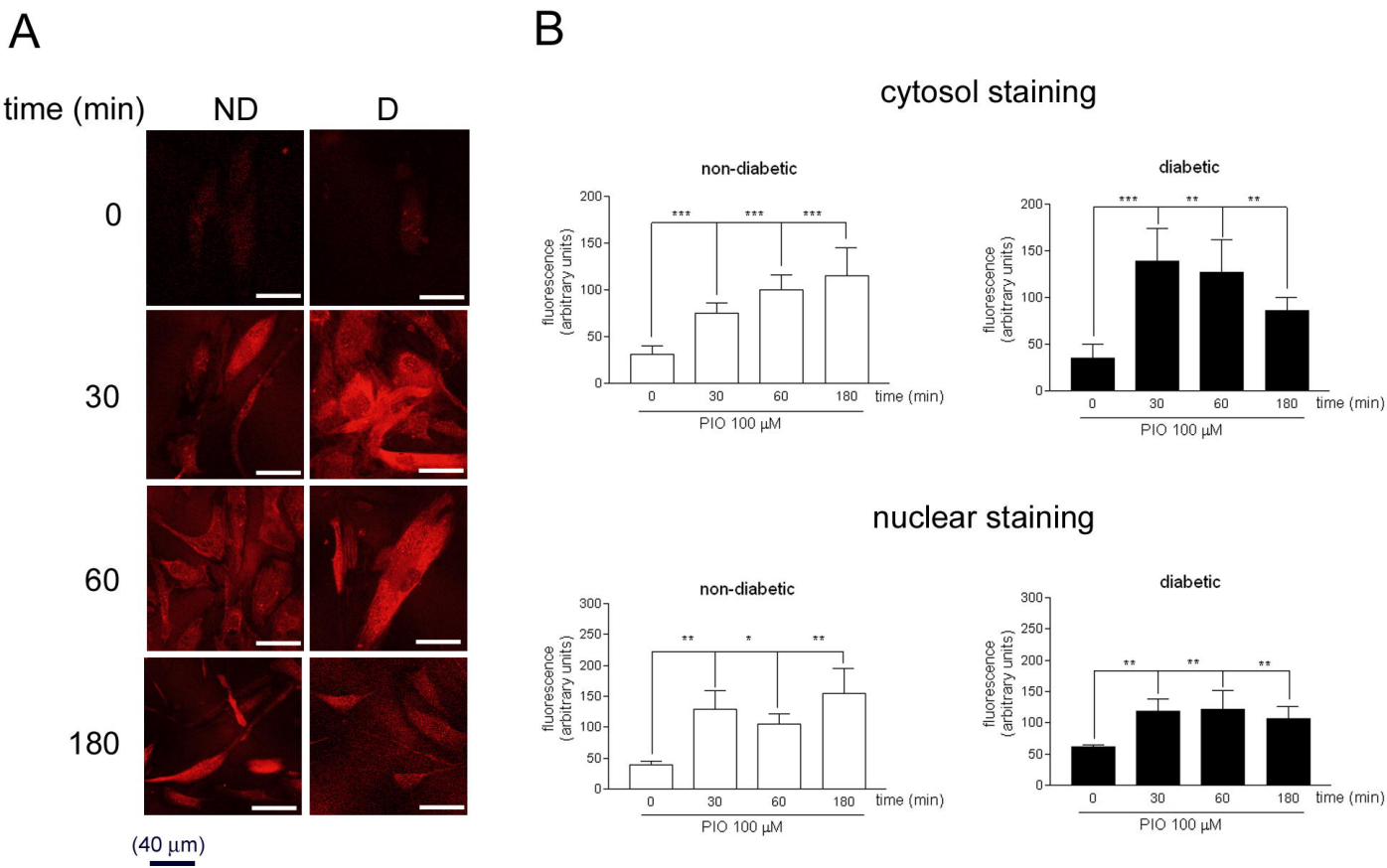
A
time (min)
ND
D
0
30
60
180
(40 μm)
B
cytosol staining
non-diabetic
diabetic
fluorescence (arbitrary units)
time (min)
PIO 100 μM
nuclear staining
non-diabetic
diabetic
fluorescence (arbitrary units)
time (min)
PIO 100 μM

Figure 5

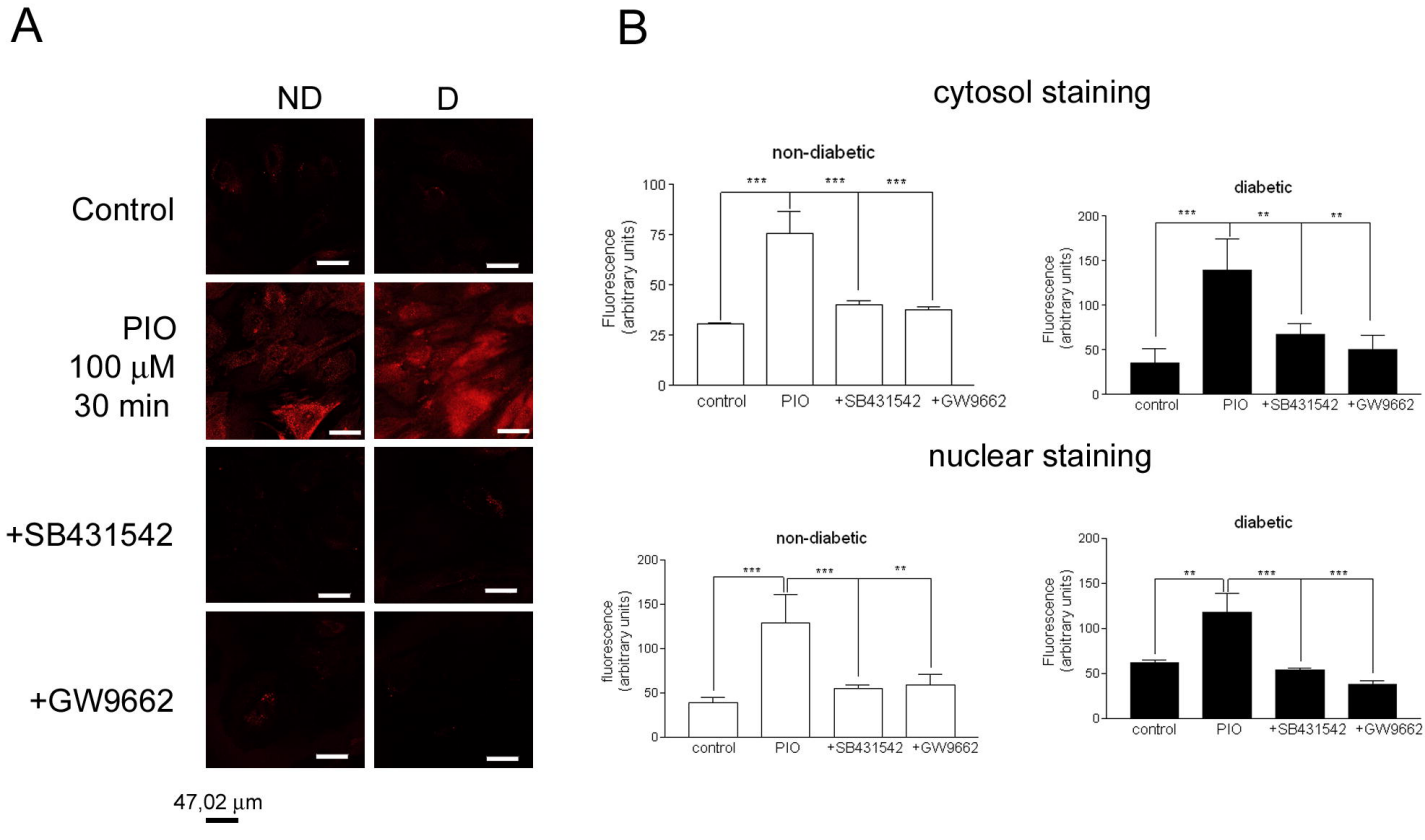

Figure 6