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EPC adhesion to arteries from diabetic and non-diabetic patients: effect of pioglitazone

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1. ABSTRACT

Endothelial progenitor cell (EPC) dysfunction is an important mediator of vascular disease in diabetes. We aimed to elucidate the mechanism of adhesion of EPC to diabetic and non-diabetic arteries and to study the effect of the anti-diabetic drug pioglitazone. Peripheral blood mononuclear cells were isolated from healthy donors. Human internal mammary arteries (HIMA) were isolated from patients who underwent coronary artery bypass surgery. EPC were labelled with ^{111}In-oxine and perfused to HIMA in a perfusion chamber. Stromal derived factor-1 (SDF-1) and cyclooxygenase-2 (COX-2) were assessed by immunohistochemical analysis. CXCR-4 expression was assessed by flow cytometry. Adhesion of EPC was increased in HIMA from diabetic patients and was reduced after preincubation with 15 mM glucose for 72 h. EPC adhesion and CXCR-4 expression were inversely correlated. COX-2 and SDF-1 immunostaining in HIMA were positively correlated. Pioglitazone (1 μM) increased the adhesion of EPC to HIMA and the expression of CXCR-4 in EPC. Therefore, EPC-recruiting capability is increased in diabetic arteries, although EPC adhesion is notably impaired by high glucose concentrations. Interestingly, pioglitazone treatment enhances EPC adhesiveness.

2. INTRODUCTION

Coronary artery disease (CAD) is the leading cause of death in diabetic patients (1, 2). Endothelial dysfunction plays a key role in the onset, progression and clinical complication of atherosclerosis, the pathological condition underlying CAD (3). In this context, an increasing body of evidence highlights the importance of endothelial progenitor cells (EPC) in endothelial function and inflammatory vascular self-repair (4-7). The number of EPC is lower in diabetic patients (4) and the functionality of EPC obtained from diabetic patients is impaired compared to non-diabetic controls (5). These facts agree with the widely accepted point of view which considers maintenance of EPC number and function as a protective factor against CAD (6, 7). Given these premises, the possibility to amplify EPC *in vitro*, in order to re-inject them to improve ischemia, as shown in experimental animal models (8) is intriguing. However, the clinical evidence shows a very discrete benefit after these procedures are done in coronary disease (9, 10).

Pioglitazone is a PPARγ agonist prescribed as an oral anti-diabetic agent in the management of type 2 diabetes mellitus that has demonstrated improved survival in a cohort of diabetic patients (11). Moreover, beyond its

Table 1. Clinical characteristics of the patients studied.

Variable	Complete Group	non- diabetic	Diabetic	P
Number of patients	20	11	9	-
Age (years)	64.4 ± 6	63.5 ± 8.5	64.8 ± 6.2	0.07 [1]
Women (%)	4 (20%)	2 (20%)	2 (21%)	0.42 [†]
BMI	27.3 ± 3.4	27.2 ± 2.2	27.6 ± 3.7	0.37 [1]

$P< 0.05$: [1] = Student's t-test; [†] = Chi-squared.

Table 2. Biochemical characteristics of the patients studied

Variable	Complete group	non-diabetic	Diabetic	P
Glucose (mg/dl)	111 ± 42	90 ± 19	138 ± 48.9	< 0.001 [†]
$Hb1_{ac}$ (%)	6.4 ± 1.2	5.6 ± 0.4	7.4 ± 1.1	< 0.001 [†]
Total cholesterol (mg/dl)	154 ± 47	158 ± 51	148 ± 40	0.221 [1]
HDL-cholesterol (mg/dl)	43 ± 12	42 ± 14	39.5 ± 10	0.238 [1]
LDL-cholesterol (mg/dl)	82 ± 37	89 ± 40	71 ± 32.3	0.019 [1]
non-HDL cholesterol (mg/dl)	113 ± 41	116 ± 43	116 ± 38	0.230 [1]
Triglycerides (mg/dl)	137 ± 68	122 ± 42	154 ± 87	0.005 [1]
Creatinine (mg/dl)	1.08 ± 0.2	1.08 ± 0.2	1.08 ± 0.2	0.530 [1]

$P< 0.05$: [1] = Student's T-test; † = Welch's t-test (Beherens-Fisher)

effect on glucose homeostasis this drug has shown anti-atherosclerotic effects in both experimental and clinical studies (12, 13), and is able to increase neoangiogenesis and prevent apoptosis of ECP in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (14). EPC, rapidly mobilized in a process regulated by the SDF-1/CXCR-4 axis, are critical for endothelial recovery (15, 16), but in the diabetic vascular disease scenario this process is impaired due to a decreased adhesion capability of these cells (5). Interestingly, incubation of human EPC with pioglitazone (1 μM for 72 h) enhanced the adhesion of early EPC to a fibronectin matrix under flow conditions (17). Nevertheless, although the defective role of the diabetic EPC ("the seed") has been shown in this context, the role of the diabetic vessels ("the soil") is still not well understood. Thus, the aims of the present study were: first, to elucidate the adhesion of EPC from human donors on blood vessels from diabetic and non-diabetic patients who underwent bypass surgery; second, to assess the role of pioglitazone in this context, as well as the underlying mechanism of action.

3. MATERIAL AND METHODS

3.1. Description of patients

A group of patients was recruited from those undergoing coronary artery bypass graft (CABG) surgery at the Cardiac Surgery Service (Hospital Clinico San Carlos, Madrid, Spain). Diabetes mellitus was defined following the criteria established by the American Diabetes Association as fasting serum glucose concentration ≥126 mg/dl and use of anti-diabetic oral drugs or insulin. All our patients had type 2 diabetes mellitus. Patient data included: age, gender, active smoker, obesity, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose and blood pressure. Exclusion criteria were: patients older than 80 years of age or with pathologies that affect the inflammatory status (renal failure, liver disease, immunological disease), cancer, recent myocardial infarction, surgery or vascular intervention (≤7 days). Internal mammary arteries were collected by the surgeons during the surgical procedure within the next few minutes after the operations and frozen at -70º C. Before freezing, the endothelial lining was gently removed using a scalpel blade. This method has been previously confirmed to denude endothelium completely (18). In addition, to ensure the absent of residual endothelial cells non-perfused arteries were immunostained and used as negative controls. The study was conducted according to the Declaration of Helsinki (revised in 2000). We obtained informed consent from all subjects before sampling took place and the responsible ethics committee has given approval. Clinical and biochemical characteristics of our cohort of patients are shown in Table 1 and 2, respectively.

3.2. Isolation and culture of human endothelial progenitor cells

Buffy coat preparations of healthy donors were separated using Biocoll separating solution (Biochrom) in order to obtain peripheral blood mononuclear cells (PBMC) by density gradient centrifugation. PBMC were seeded (10×10^6/well) on cell culture flasks coated with 10 μg/ml human fibronectin (Tebu-Bio) in microvascular endothelial growth medium with 5% FCS and supplements (MV-2; PromoCell; glucose content: 5 mM, PromoCell technical service, Heidelberg, Germany). Medium was carefully changed on day 4. All experiments were performed with EPC at day 7. Therefore, our cell culture model was an "early outgrowth" EPC culture, which has been related to the early phase of endothelial repair (10, 15) and to initiate local angiogenesis by involving paracrine factors (19, 20). Each set of experiments was done using cells from at least 4 different donors.

3.3. EPC characterization by flow cytometry

For uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled ac-LDL (DiI-ac-LDL;), adherent cells were incubated at 37°C with 4 μg/ml DiI-ac-LDL in culture medium. After two hours the cells were washed twice in PBS, trypsinized and fixed in 2% paraformaldehyde (PFA) following incubation with 10 μg/ml FITC-labelled *Ulex europaeus* agglutinin-I (lectin; Sigma-Aldrich) for one hour at room temperature (RT) protected from light. For KDR (kinase insert domain-containing receptor) and CD31 positive cell assessment, the

cells were detached with a commercial cell detachment enzymatic solution (Accutase® PAA laboratories, Linz, Austria) and incubated in PBS/0.5% BSA with 5 μg/ml of anti-KDR-phycoerythrin (PE) (R&D Systems) or with 10 μg/ml anti-CD31-FITC (Caltag-Invitrogen). Respective IgG isotype controls from the same manufacturers were used. After 30 min cells were washed twice and fixed in 2% PFA. Measurement was performed using an appropriate setting in a *FACSCalibur* flow cytometer (Becton Dickinson) on at least 10,000 gated cells. Remaining cells and debris were gated out. Analysis was subsequently done on the respective dot of histogram plots by manual gating and subtraction of background fluorescence (*Cell-QuestPro* software, Becton Dickinson).

3.4. ^{111}In-oxine cell radiolabelling and EPC adhesion assay to human internal mammary arteries

To analyze the adhesion of EPC on human internal mammary arteries, the cells were radiolabelled with ^{111}In-oxine and flew through a longitudinally-opened human internal mammary artery after endothelium removal (from diabetic and non-diabetic patients). EPC cultures were detached with Accutase®, resuspended (1.0×10^5/ml) in PBS and loaded for 20 min at room temperature with ^{111}In-oxine (148 MBq/1.0×10^5 cells) (21). The EPC were measured for radioactivity after labelling with ^{111}In-oxine and the labelling efficiency was calculated. To remove unbound radioactivity, the cells were washed twice with PBS buffer. Flow adhesion assays of EPC to human arteries were performed in a perfusion chamber extensively described elsewhere (22, 23). Briefly, it consists of a cylindrical flow channel (1 mm diameter and 2.5 cm length) where the artery is opened longitudinally and therefore allows the cell suspension to flow over the arterial substrate. Sections of HIMA (0.8 cm^2) were set into the perfusion chamber and were perfused at 37ºC with radiolabelled cells suspended in HHMC medium (HEPES-buffered HBSS, 1 mM Mg^{2+}/Ca^{2+}, 0.5% BSA) at 0.3 ml/min for 5 to 15 min (for time point experiments) and for 15 min in the following experiments. EPC were also cultured in a cell medium containing glucose at a concentration related to *in vivo* blood glucose concentrations (15 mM) (1) or mannitol (15 mM) for 72 h prior to the adhesion experiments. In another set of assays, EPC were pre-incubated for 24 hours with pioglitazone (1 and 10 μM). These concentrations of pioglitazone were selected from pilot studies and according with the literature (17, 24, 25). In a third experimental scenario, prior to pioglitazone treatment, EPC were pre-treated for 30 min with the pharmacologic PPARγ antagonist 2-chloro-5-nitrobenzanilide (GW9662) at a concentration (2 μM) that efficiently blocks PPARγ in cell culture as has been shown previously (2, 17). In another set of experiments, the cells were incubated with the endogenous non-thiazolidinedione PPARγ agonist 15d-PGJ_2 5 μM for 24 h.

3.5. Assessment of CXCR-4 expression in EPC by flow cytometry

CXCR-4 is a chemokine family receptor of EPC involved in early EPC adhesion (15, 16). To assess a potential involvement of this receptor in the pioglitazone-mediated increase in EPC-adhesion, CXCR-4 expression (anti-CXCR-4-PE monoclonal antibody; eBioscience) was analyzed by flow cytometry in EPC incubated in the presence or absence of pioglitazone (1 μM) in glucose (5 mM or 15 mM).

3.6. Immunostaining of EPC

To visualize the adhesion of EPC on human internal mammary arteries, small segments of internal mammary arteries were fixed with 4% PFA for 2 h immediately after the adhesion experiment or after additional incubation of 24 h at 37ºC. In this kind of experiments, three diabetic and three non-diabetic arteries were used and several sections were analyzed. They were washed with PBS, immersed in PBS 0.1 mM + sucrose 30% at 4°C for 3 h and embedded in OCT (OCT-tissue tek, Bayer) for 30 min. Cross sections (7 μm thick) were obtained (Cryostat HM500, Microm international GmbH, Düsseldorf), dried at 37°C and washed with PBS/0.3% Tween20® (PBS-T). Unspecific binding was blocked by incubating the samples for 1 h in goat serum. Cross sections were incubated with mouse monoclonal anti-CD34 antibodies at 1:100 (BD Transduction Labs, Madrid, Spain) for 1 h at room temperature, according to a method previously described (19).

3.7. Immunohistochemical analysis of SDF-1 and COX-2

We also analyzed the expression of stromal derived factor-1 (SDF-1) and cyclooxygenase-2 (COX-2) in cross sections of fixed human internal mammary arteries. After the fixation procedure, Cross sections 7 μm thick were obtained (Cryostat HM500, Microm International GmbH, Düsseldorf, Germany), dried at 37°C and washed with PBS + 0.3% Tween20® (PBS-T). Unspecific binding was blocked by incubating the samples for 1 h in goat serum. Cross sections were incubated with mouse monoclonal anti-COX-2 or rabbit polyclonal anti-SDF-1 antibodies at 1:100 (BD Transduction Labs, Madrid, Spain) for 1 h at room temperature and after washing, with Alexa-546 goat anti-mouse or Alexa-546 goat anti-rabbit secondary antibodies (Molecular Probes, USA) at 1:500 for 1 h at 37°C. Images were captured using a Leica TCS SP2 inverted microscope. Semiquantitative measure of protein expression was performed by using the Image J 1.33 software (NIH, USA). Data are presented as fold increase of protein expression with respect to each negative control.

3.8. 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. For variables with different variances, Welch's t-test was used. Correlation between two quantitative continual variables was done using Pearson's correlation. Differences with a P value of less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Adhesion of EPC to human internal mammary arteries

To analyze the adhesion of EPC isolated from healthy subjects to a physiological substrate, we performed

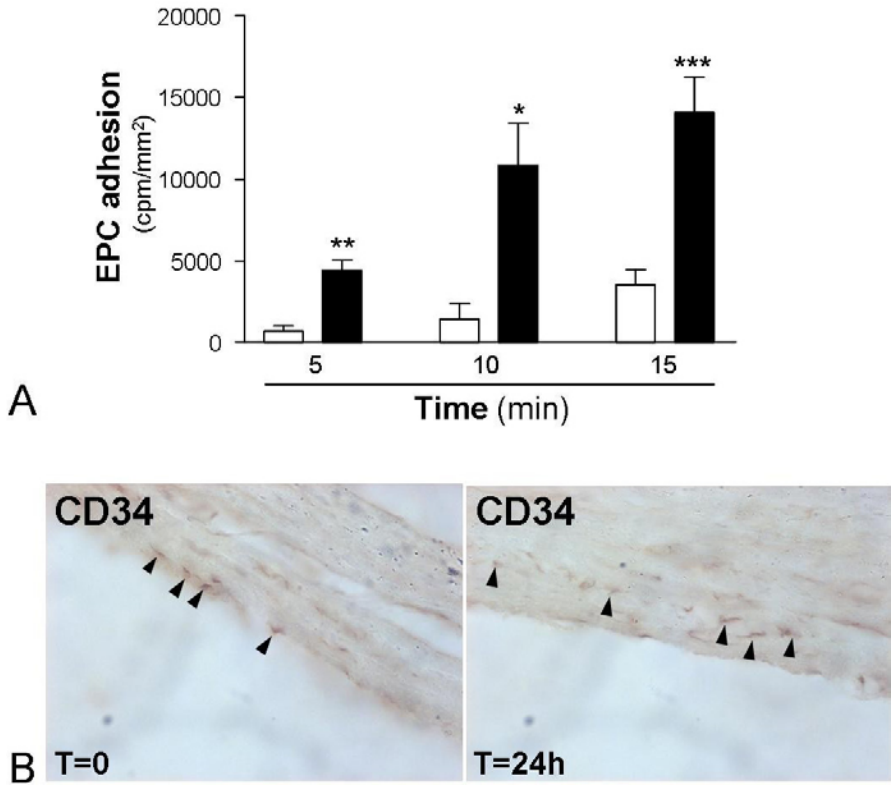

Figure 1. Adhesion of EPC to human internal mammary arteries (HIMA). Panel A: Sections of 0.8 cm^2 of HIMA from non-diabetic (white bars) and diabetic patients (black bars) were exposed to a constant flow (0.3 ml/min) of EPC labelled with ^{111}In-oxine in a perfusion chamber. Adhesion of EPC was measured as the cpm/mm^2 in a gamma counter. Bar graphs show the mean±SD of 6 different arteries. **P*< 0.05, ***P*< 0.01, ****P*< 0.001. Panel B: Immunohistochemical analysis showing the presence of $CD34^+$ cells in a diabetic HIMA perfused with EPC.

an experiment in which radiolabelled EPC were passed through a human internal mammary artery mounted in a perfusion chamber using a constant flow of 0.3 ml/min of EPC. We observed that the EPC attached to the arteries in a time-dependent manner (5-15 min). Interestingly, we found that EPC attached more to arteries isolated from diabetic patient (Figure 1, panel A). This difference was statistically significant.

To visualize the adhesion of EPC to the arteries, we fixed a group of arteries immediately after the flow experiment (t=0) or after 24 h incubation at 37°C/5% CO_2/95% humidity (t=24 h) and then the arteries (all of them were endothelium-denuded) were proceeded for the microscopic analysis of $CD34^+$ cells. As shown in Figure 1 (panel B), $CD34^+$ positive cells were located initially in the luminal side. After 24 h incubation, however, the cells were also located further inside the artery.

4.2. Relationship between the expression of COX-2 and SDF-1 in human internal mammary arteries

To explain the increase in the adhesion of EPC to human arteries isolated from diabetic patients we analyzed the expression of COX-2 and SDF-1 in cross-sections of HIMA isolated from diabetic and non-diabetic patients by immunohistochemical staining and confocal microscopy. Figure 2 (panel A) shows immunostaining of COX-2 and SDF-1 in the media layer of human arteries. We found a positive Pearson correlation in the expression of both proteins (r^2=0.7, *P*= 0.0006) as shown in panel B. The expression of both COX-2 and SDF-1 in HIMA from diabetic patients was increased with respect to non-diabetic patients, as shown in panel C.

4.3. Effect of pioglitazone on the adhesion of EPC to human internal mammary arteries

We have previously shown that treatment of EPC in culture with pioglitazone (1 μM) increased the adhesion of these cells to fibronectin (17). In this work we analyzed the effect of pioglitazone on EPC adhesion on HIMA from diabetic and non-diabetic patients using a dynamic system (perfusion chamber). The incubation of EPC with pioglitazone (1 μM) increased their subsequent adhesion to HIMA from non-diabetic patients (Figure 3, left bars). Pioglitazone, however, did not significantly increase the adhesion of EPC to HIMA from diabetic patients (Figure 3, right bars).

It has been described that EPC isolated from diabetic patients show impairment in proliferation and cellular adhesion (26). Thus, we performed a set of experiments where the EPC were treated with a high concentration of glucose (15 mM) or mannitol (15 mM) for

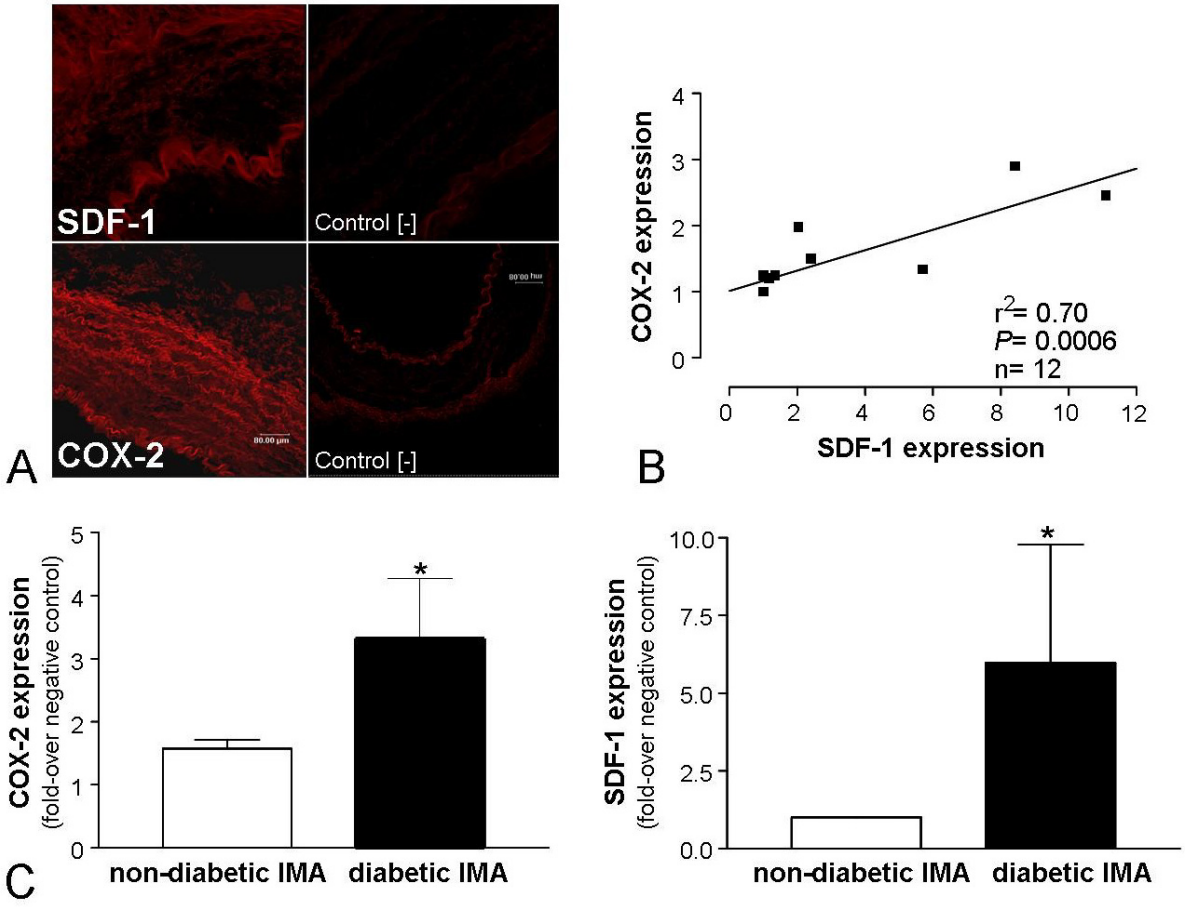

Figure 2. Relationship between the expression of COX-2 and SDF-1 in human internal mammary arteries (HIMA). Panel A: Presence of COX-2 and SDF-1 in HIMA assessed by immunohistochemical analysis. Right, negative controls (Control [-]) using irrelevant-isotype matched IgG. Panel B: Graph shows the Pearson correlation of the immunostaining corresponding to COX-2 and SDF-1. Panel C: Differential expression of COX-2 (left panel) and SDF-1 (right panel) in non-diabetic and diabetic IMA. (Depending on the graph scale low deviations of the mean do not appear in the computer originated graphs).

72 h, in the presence or absence of pioglitazone (1 μM), and then the adhesion to HIMA from diabetic patients was analyzed. Glucose treatment diminished the adhesion of EPC to human arteries in approximately 50% (Figure 4). Interestingly, pioglitazone treatment significantly reversed the effect of high glucose.

4.4. Mechanism of action of pioglitazone

We analyzed the involvement of the PPARγ receptor in the effect of pioglitazone on EPC adhesion. EPC were pre-treated with the PPARγ receptor antagonist GW9662 (2 μM, for 30 min) prior to pioglitazone treatment. As shown in Figure 5 (panel A) the effect of pioglitazone on the adhesion of EPC to human non-diabetic internal mammary arteries was abolished in the presence of GW9662. In another set of experiments, we studied the effect of the endogenous agonist of PPARγ receptor, 15d-PGJ_2 on the adhesion of EPC to human non-diabetic internal mammary arteries in order to test a non-thiazolidinedione PPARγ agent and thus reinforce the idea of a significant PPARγ-dependence. 15d-PGJ_2 (5 μM) increased the adhesion of EPC to human arteries as shown in Figure 5 (panel B).

On the other hand, it is known that CXCR-4 is a membrane receptor of EPC involved in EPC adhesion (22, 25, 28). Thus, we also analyzed whether treatment with pioglitazone increases the expression of CXCR-4 analyzed by flow cytometry. Figure 6 shows that pioglitazone (1 μM) increased the expression of CXCR-4 in EPC maintained in exogenously added glucose (5 mM) and that this effect was reversed by the PPARγ antagonist GW9662. Treatment with pioglitazone (1 μM) did not modify the effect of high glucose concentrations on CXCR-4 expression in a significant manner. We also found that the expression of CXCR-4 decreased in EPC treated with glucose (15 mM, for 72 h) (Figure 6, panel B).

5. DISCUSSION

The most important results of the current study may be summarized as follows: 1) EPC obtained from healthy donors show a higher adhesion capability to

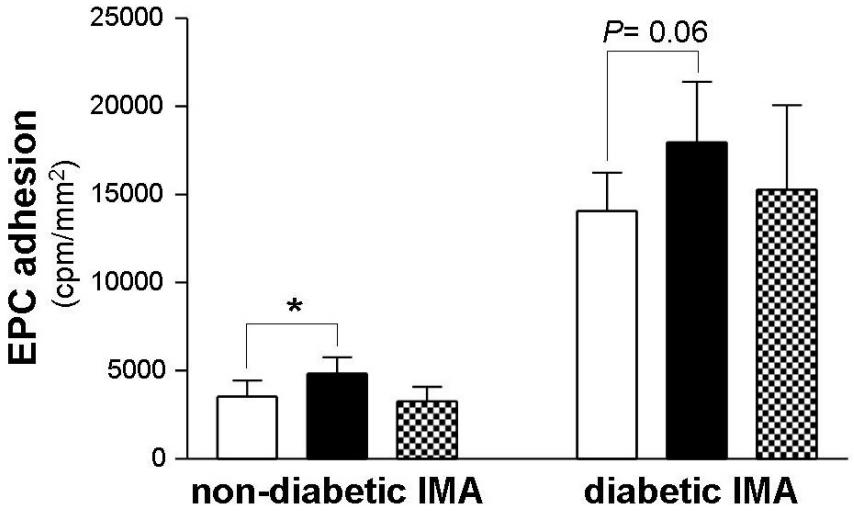

Figure 3. Pioglitazone increases adhesion of EPC to human internal mammary arteries (HIMA). Sections of HIMA were exposed to a constant flow (0.3 ml/min, 15 min) of EPC labelled with ^{111}In-oxine. Adhesion of control EPC (white bars) and EPC pre-treated with 1 μM pioglitazone (black bars) or 10 μM pioglitazone (squared bars) to non-diabetic and diabetic arteries was measured as cpm/mm^2 in a gamma counter. Bar graphs show the mean±SD of 6 different arteries. *P< 0.05.

diabetic internal mammary arteries; 2) This increased adhesion might be related to an increased expression of COX-2 and SDF-1 in diabetic vessels, which correlated with each other; 3) Incubation EPC with high glucose concentration decreases its adhesion to diabetic vessels; 4) This decreased adhesion is prevented by incubation with pioglitazone (1 μM) in a PPARγ-dependent manner.

One of the most surprising results of the current study is the increasing adhesion of EPC to diabetic IMA (Figure 1, panel A). This would be in contrast to the decreased adhesion of EPC from diabetic patients described previously (5, 27), and might be regarded as a contradiction compared to the decreased endothelial quality in diabetic patients. However, as shown in the current study (Figure 2), this fact could be related to an increased expression of the inflammation-related proteins COX-2 and SDF-1 observed in diabetic vessels compared to non-diabetic controls (Figure 2, panel B). Indeed, the relationship between COX-2 expression and cell adhesion and angiogenesis has been documented in other models, and it has been reported that inhibition of COX-2 suppressed integrin $\alpha_v\beta_3$-dependent angiogenesis (28). Interestingly, and in a close relationship to the underlying mechanism described in the present study, it has been shown that elevation of SDF-1 expression in the subacromial bursa of patients can be down-regulated by COX-2 inhibitors (29).

Prompt endothelial repair after a vascular injury seems to be highly dependent on adequate release of bone marrow and inflammation-related precursors which may mediate an early angiogenic stimulus directly and by stimulation of local endothelial cells (15, 16). Therefore, our *in vitro* model consisted on early outgrowth EPC obtain from peripheral blood mononuclear cells and cultured for 7 days. Adhesion of early outgrowth EPC to denuded vessels is highly dependent on the interaction of membrane chemokine family receptor CXCR-4 in the membrane of EPC with the interstitial SDF-1, expressed in the ischemic tissue. Expression of SDF-1 is highly induced by local hypoxia and cellular stress (hypoxia inducible factor) (16). Therefore, an increased inflammatory stress in diabetic vessels might be regarded as the responsible factor of increased adhesion of donor EPC ("healthy seed") to the diabetic vessels ("diseased soil"). In an animal experimental model, however, decreased plasma levels of SDF-1 have been related to impaired mobilization of EPC after hind-limb ischemia (30). Thus, a close SDF-1/CXCR-4 regulation from the bone marrow to plasma and ischemic tissues seems to regulate EPC release and homing. According to our results, an important factor to dampen the EPC-binding capacity is the decreased adhesive capacity of EPC after incubation with high glucose (Figure 4). The mechanism responsible for this glucose-induced decrease of EPC adhesion could also be related to glycation following pre-incubation with glucose. In fact, glycation seems to impair EPC-mediated vascular repair (31) and receptors of advanced glycation end products (RAGE) have been involved in EPC homing (32). Moreover, previous studies have shown that high glucose is able to alter EPC metabolism (33), increase EPC senescence trough a p38 MAPK dependent pathway (34), impair EPC differentiation, process can be restored modulating Akt/FoxO1 activity (35, 36), prevent the proliferation and function of early and late EPC, effect that is prevented by nitric oxide donors (37) and significantly reduce the protein expression and activity of cathepsin L, which is involved in matrix degradation and required for invasion of EPC into the ischemic tissue (38). Therefore, high glucose may limit the functional capacity of EPC to improve neovascularization and vascular repair in diabetic patients through multiple mechanisms.

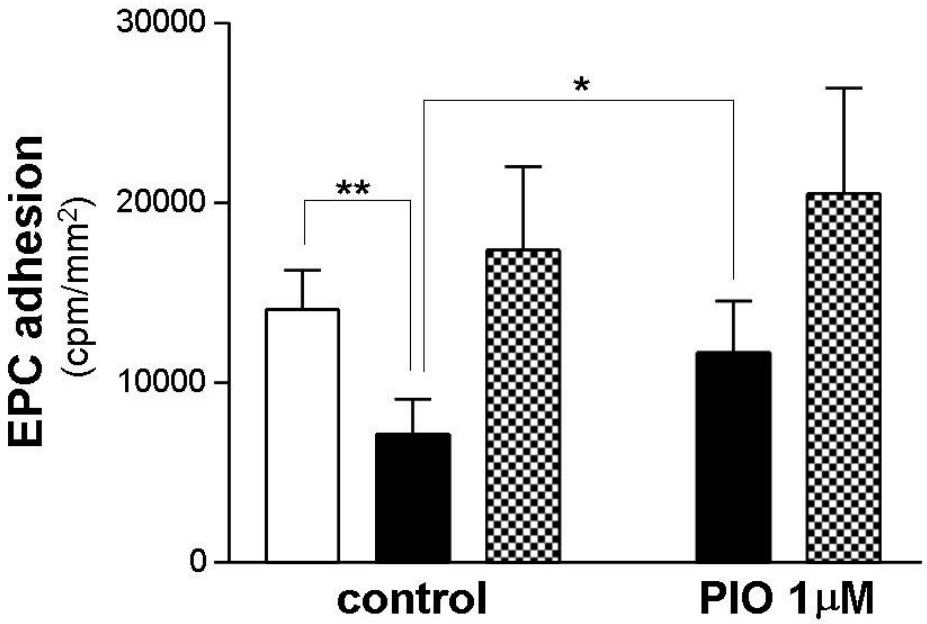

Figure 4. Effect of pioglitazone on the adhesion of EPC treated with high glucose to human internal mammary arteries (HIMA) from diabetic patients. EPC were cultured in basal MV-2 medium containing 5 mM glucose (white bars) or in media supplemented with 15 mM glucose (black bars) or 15 mM mannitol (squared bars) for 72 h in the absence (control) or presence of 1 μM pioglitazone (PIO 1 μM). Sections of diabetic HIMA were exposed to a constant flow (0.3 ml/min) of EPC loaded with ^{111}In-oxine in a perfusion chamber. Adhesion of EPC was measured as the cpm/mm^2 in a gamma counter. Bar graphs show the mean±SD of 6 different arteries. In these experiments exogenous glucose was added to increase its concentration from 5 mM (glucose concentration in basal MV-2 medium) to 15 mM. *P< 0.05.

In this context, the search of a compound to reverse glucose-induced decreased EPC adhesion may be considered as a desirable objective in the state-of-the-art experimental approach. According to previous reports which described the increased adhesion of EPC to a fibronectin matrix (17) we tried to assess whether these effects may take place in the model of *ex vivo* internal mammary arteries obtained from cardiac surgery of atherosclerotic patients (diabetics and non-diabetics). Incubation of EPC with 1 μM pioglitazone increased the adhesion of EPC to non-diabetic internal mammary arteries (Figure 3). At the same time, we found that pioglitazone at 10 μM did not possess any significant effect. This biphasic effect has previously found when the pioglitazone-mediated EPC adhesion was tested on an inert fibronectin matrix, and was found to be due to TGF-β1 secretion after incubation with pioglitazone at 10 μM (17).

Notably, the effect of pioglitazone was reversed with preincubation with the PPARγ blocker GW9662 (Figure 5), and this is a widely accepted pharmacological approach for PPARγ inhibition (39). We equally found a pioglitazone-mediated increased expression of the CXCR-4, a receptor involved in the adhesion of EPC (15, 16), which might be important in the mechanism of action of pioglitazone on EPC adhesion. In terms of adhesion of EPC to arteries from diabetic patients, we found a tendency to increase the adhesion in the presence of pioglitazone, although this effect was not statistically significant, as observed in non-diabetic arteries. Previous studies have shown that pioglitazone increases the numbers and improves the functional capacity of EPC in patients with Diabetes mellitus (40) and also in patients with CAD (20). In addition, pioglitazone prevents apoptosis of EPC, increase SDF-1-induced migratory capacity and neoangiogenesis *in vivo* (14).

A limitation of the present study is the exclusive usage of EPC from healthy donors. Indeed, due to the clinical instability of the diabetic patients included in our study (cardiac surgery patients in the clinical care setting), and the large amount of blood required to isolated EPC it was not possible to obtained EPC from these patients. As an alternative, we induced EPC dysfunction exposing these cells to high glucose concentrations. However, we should bear in mind that not only high glucose concentrations but other mechanisms including lipids, oxidative stress and inflammation are likely to be involved in diabetes-induced EPC impairment (5).

In conclusion, the present study shows that, in type 2 diabetes, internal mammary arteries possess an increased EPC adhesion capability related to increased COX-2 and SDF-1 expression. However, under this pathophysiologic condition the intrinsic adhesive properties of EPC are impaired, associated to a down-regulation of CXCR-4. Interestingly, pioglitazone is able to enhance

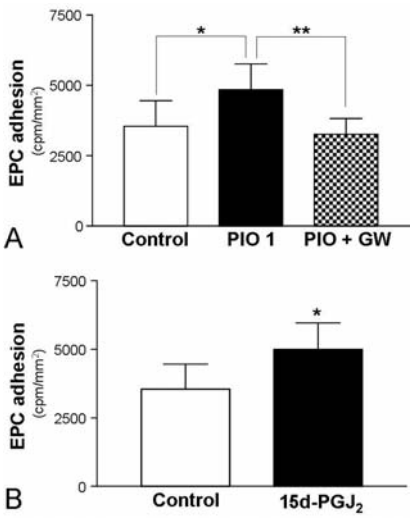

Figure 5. The effect of pioglitazone on EPC adhesion to human internal mammary arteries (HIMA) is mediated by PPARγ. Panel A: Effect of the PPARγ antagonist GW9662 on the effect of pioglitazone on the adhesion of EPC to HIMA. Adhesion of control EPC (control) and EPC incubated with 1 μM pioglitazone for 24 h in the absence (PIO 1) or presence of 2 μM GW9662 (added 30 min prior to pioglitazone; PIO + GW) is shown. Panel B: Effect of the PPARγ agonist 15d-PGJ_2 on the adhesion of EPC to HIMA. Adhesion of control EPC (control) and EPC treated with 5 μM 15d-PGJ_2 for 24 h (15d-PGJ_2) is shown. HIMA were exposed to a constant flow (0.3 ml/min for 15 min) of EPC labelled with ^{111}In-oxine in a perfusion chamber. Adhesion of EPC was measured as the cpm/mm^2 in a gamma counter. Bar graphs show the mean±SD of 6 different arteries. **P*< 0.05, ***P*< 0.01.

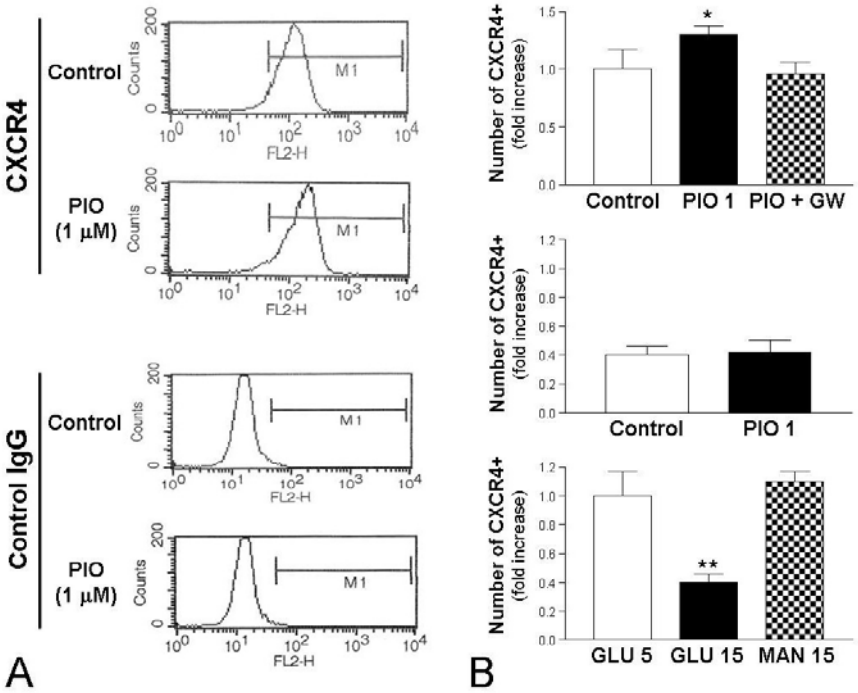

Figure 6. Expression of CXCR-4 in EPC and modulation by pioglitazone. Panel A: typical experiment of flow cytometry showing the expression of CXCR-4 in control EPC (control) and in EPC treated with pioglitazone (PIO, 1 μM). Control experiments using irrelevant-isotype matched IgG are also shown. Panel B: (Upper) expression of CXCR-4 analyzed by flow cytometry (mean±SD of 5 experiments) in EPC cultured in MV-2 medium (5 mM glucose) under control conditions (control), in cells treated with 1 μM pioglitazone (PIO 1) and in cells pre-incubated with μM GW9662 (added 30 min prior to pioglitazone) and treated with 1 μM pioglitazone (PIO + GW); (Middle), expression of CXCR-4 analyzed by flow cytometry (mean±SD of 5 experiments) in EPC cultured in media containing 15 mM glucose in the absence (control) or presence of 1 μM pioglitazone (PIO 1); (Bottom), effect of 5 mM glucose (GLU 5), 15 mM glucose (GLU 15) and 15 mM mannitol (MAN 15) on EPC expression of CXCR-4 analyzed by flow cytometry (mean±SD of 5 experiments). In these experiments exogenous glucose was added to increase its concentration from 5 mM (glucose concentration in basal MV-2 medium) to 15 mM. **P*< 0.05; ***P*< 0.01.

EPC adhesiveness in a PPARγ-dependent manner. Further experiments using EPC from disease patients should be needed to determine the potential clinical implications of the presented results in the setting of therapy with autologous EPC.

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