

Thiazolidinediones Reduce Endothelial Expression of Receptors for Advanced Glycation End Products

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Advanced glycation end products (AGEs) are critically involved in atherogenesis in diabetes by binding to receptors for AGE (RAGEs) in vascular cells, thus inducing the expression of proinflammatory mediators. In animal models, interruption of the AGE-RAGE interaction reduces lesion size and plaque development. Therefore, limiting RAGE expression might be an intriguing concept to modulate vascular disease in diabetic patients. The present study investigated whether thiazolidinediones (TZDs), antidiabetic agents clinically used to treat patients with type 2 diabetes, might modulate endothelial RAGE expression. Stimulation of human endothelial cells with rosiglitazone or pioglitazone decreased basal as well as tumor necrosis factor- α -induced RAGE cell surface and total protein expression. In addition, TZDs reduced RAGE mRNA expression in endothelial cells. These effects on RAGE expression were caused by an inhibition of nuclear factor- κ B (NF- κ B) activation at the proximal NF- κ B site of the RAGE promoter. The functional relevance of reduced RAGE expression was demonstrated by showing that pretreatment of endothelial cells with TZDs decreased AGE- as well as β -amyloid-induced monocyte chemoattractant protein-1 expression. In conclusion, TZDs reduce RAGE expression in human endothelial cells, thus limiting the cells' susceptibility toward proinflammatory AGE effects. These data provide new insight on how TZDs, in addition to their metabolic effects, might modulate the development of vascular dysfunction in diabetic patients. *Diabetes* 53:2662–2668, 2004

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AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor- κ B; PPAR, peroxisome proliferator-activated receptor; RAGE, receptor for AGE; TNF- α , tumor necrosis factor- α ; TZD, thiazolidinedione; VCAM-1, vascular cell adhesion molecule-1.

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Patients with diabetes exhibit an increased propensity to develop arteriosclerosis with its sequelae acute myocardial infarction and stroke (1). Advanced glycation end products (AGEs), formed by nonenzymatic glycation of reducing sugars with macromolecules, are crucially involved in atherogenesis in these patients. Binding of AGEs to the receptor for AGEs (RAGE), activates intracellular signaling processes, thus mediating proinflammatory AGE effects (2). Previous work has demonstrated that RAGEs are present on the surface of vascular cells (3). In endothelial cells, tumor necrosis factor- α (TNF- α) as well as AGEs themselves increase RAGE expression, thus rendering these cells more susceptible to proinflammatory AGE effects (4). In addition to AGEs, peptides like S100/calgranulin or β -amyloid have been shown to activate RAGEs (5,6). Ligation of RAGEs in endothelial cells activates the transcription factor nuclear factor- κ B (NF- κ B), subsequently leading to increased expression of proatherogenic mediators such as monocyte chemoattractant protein-1 (MCP-1) or vascular cell adhesion molecule-1 (VCAM-1) (7,8). Given that RAGE expression is increased in the vessel wall in diabetic patients (9), this mechanism might potentially contribute to a vicious circle that promotes lesion development in these patients. In vitro experiments (10,11) as well as animal data (12) suggest that limiting RAGE expression in vascular cells might be an intriguing concept to modulate atherogenesis in the high-risk population of diabetic patients; however, hitherto, nothing is known about such counterbalancing mechanisms.

Thiazolidinediones (TZDs) like pioglitazone and rosiglitazone (13,14) are a group of novel antidiabetic agents that increase insulin sensitivity in peripheral organs, thus lowering blood glucose levels in treated patients. On a molecular level, TZDs activate the transcription factor peroxisome proliferator-activated receptor- γ (PPAR- γ), a nuclear hormone receptor regulating gene expression in response to specific ligands (15). Previous work has established PPAR- γ expression in human endothelial cells, and PPAR- γ -activating TZDs have been shown to modulate gene expression in these cells (16–18). However, to date nothing is known about the effect of TZDs on endothelial RAGE expression.

Given the critical role of RAGEs for atherogenesis in diabetic patients, we examined whether antidiabetic TZDs might modulate endothelial RAGE expression, thus poten-

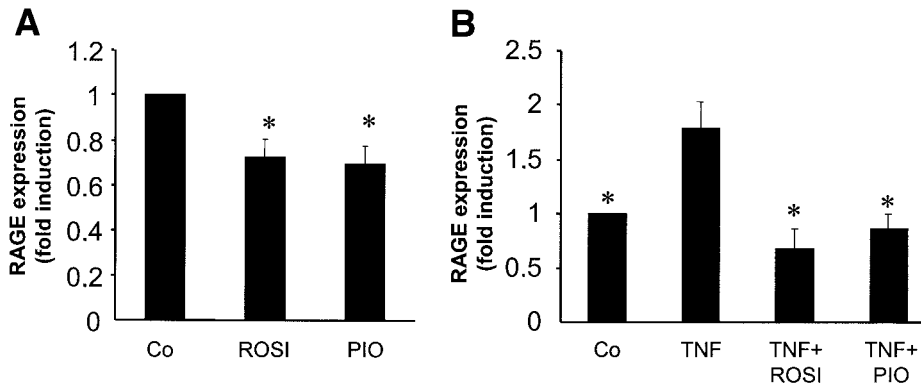


FIG. 1. TZDs inhibit endothelial RAGE cell surface expression. **A:** Human endothelial cells were stimulated with rosiglitazone (ROSI) or pioglitazone (PIO; both at 10 $\mu\text{mol/l}$) for 16 h before mean fluorescence intensity of RAGE expression was measured by flow cytometry. Results are expressed as the fold induction compared with unstimulated cells. Bars represent the means \pm SE ($n = 4$). * $P < 0.05$ compared with unstimulated cells. **B:** Human endothelial cells were stimulated for 16 h with TNF- α (25 ng/ml) in the absence or presence of rosiglitazone or pioglitazone (both at 10 $\mu\text{mol/l}$) before flow cytometry analysis was performed. Results (mean fluorescence intensity) are expressed as the fold induction compared with unstimulated cells. Bars represent the means \pm SE ($n = 4$). * $P < 0.05$ compared with unstimulated cells. Co, control.

tially decreasing the cells' susceptibility toward proinflammatory AGE effects.

RESEARCH DESIGN AND METHODS

Cell culture. Human umbilical vein endothelial cells were purchased from Biochrom and cultured according to the manufacturer's protocol. Endothelial cells were >99% von Willebrand factor positive as determined by flow cytometry, and they were used at passages 2 to 5 for all experiments. Bovine endothelial cells were isolated and cultured as described before (18).

Preparation of AGE-BSA. AGE-BSA was prepared as previously described (7).

Flow cytometry analysis. Flow cytometry analysis was performed as previously described (19). To assess the effect of TZDs on basal RAGE

expression, human endothelial cells were treated for 16 h with rosiglitazone or pioglitazone before staining was performed as described below. In other experiments, endothelial cells were treated with TNF- α (25 ng/ml) in the presence or absence of TZDs. After 16 h cells were harvested and stained with a polyclonal chicken anti-human RAGE antibody. After washing, endothelial cells were stained with fluorescein isothiocyanate-conjugated anti-chicken antibodies. Controls used isomatched IgG. Finally, endothelial cells were washed three times and stored in 1% paraformaldehyde (Sigma) at 4°C until flow cytometry analysis was performed within 24 h.

Western blot analysis. For Western blot analysis of RAGE expression, human endothelial cells were stimulated as described above. Standard Western blot analysis on total cell lysates was performed using mouse anti-RAGE antibodies (mAbs; Santa Cruz). To ensure equal loading of intact protein, membranes were stripped and restained with antibodies against α -tubulin.

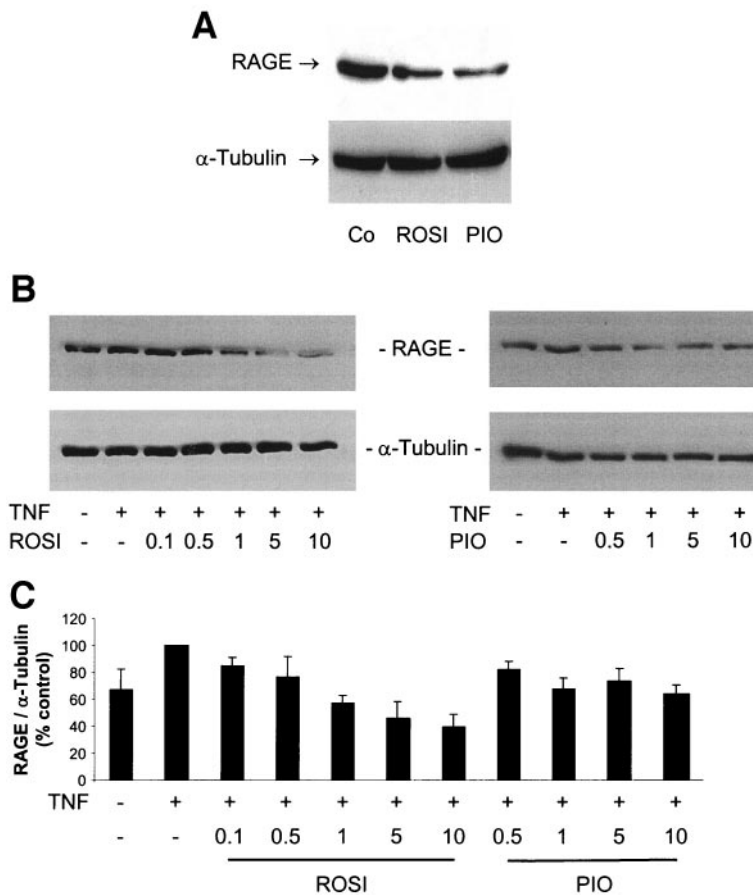


FIG. 2. TZDs inhibit endothelial RAGE protein expression. **A:** Human endothelial cells were stimulated with rosiglitazone (ROSI) or pioglitazone (PIO; both at 10 $\mu\text{mol/l}$) for 16 h before Western blot analysis was performed. Equal loading of intact protein was assured by staining for α -tubulin. Three independent experiments yielded similar results. **B:** Human endothelial cells were stimulated for 16 h with TNF- α (25 ng/ml) in the absence or presence of rosiglitazone or pioglitazone at the concentrations indicated (μM) before Western blotting was performed. Equal loading of intact protein was confirmed by staining for α -tubulin. Blots demonstrate one of four similar experiments. **C:** Densitometric analysis of four independent Western blot experiments showing the effect of TZDs on TNF- α -induced endothelial RAGE protein expression normalized to α -tubulin. Bars represent the means \pm SE. Co, control.

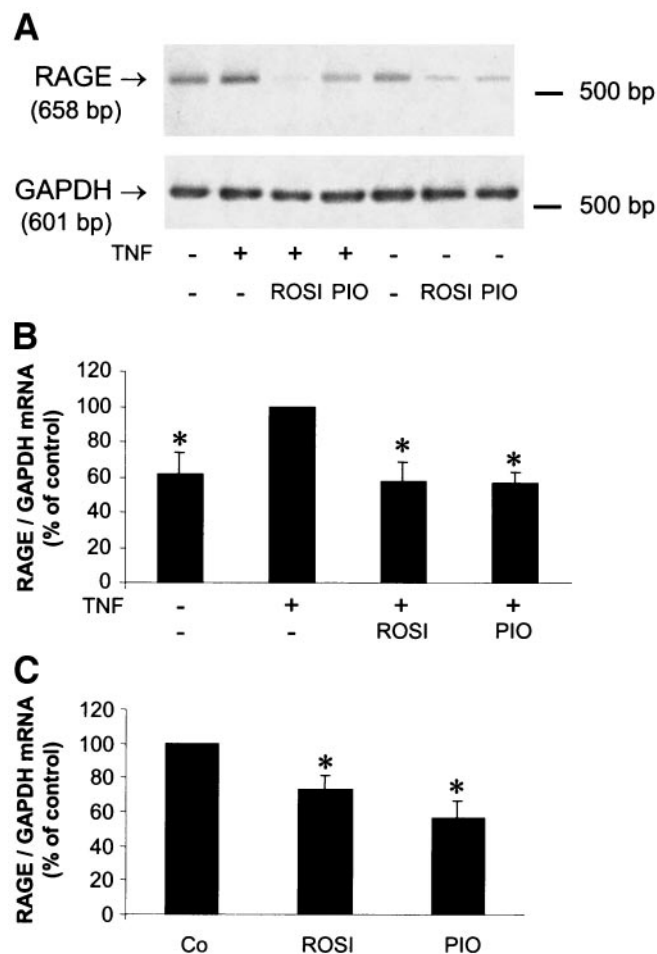


FIG. 3. TZDs reduce endothelial RAGE mRNA expression. **A:** Unstimulated as well as TNF- α -stimulated human endothelial cells were treated with rosiglitazone (ROSI) or pioglitazone (PIO; both at 10 μ M), and RT-PCR was performed after 6 h. mRNA expression of the housekeeping gene GAPDH is also shown. **B:** Densitometric analysis of three independent experiments on the effect of TZDs on TNF- α -induced RAGE mRNA expression normalized to GAPDH mRNA. Bars represent the means \pm SE. * P < 0.05 compared with TNF- α -stimulated cells. **C:** Densitometric analysis of six independent experiments on the effect of TZDs on basal RAGE mRNA expression normalized to GAPDH mRNA. Bars represent the means \pm SE. * P < 0.05 compared with unstimulated cells. Co, control.

Densitometric analysis was performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

RT-PCR. Total RNA from human endothelial cells was isolated using a column-based RNA isolation kit (Qiagen). We reverse-transcribed 2 μ g of total RNA into cDNA with 1 unit/ml reverse transcriptase (Superscript; Gibco) at 37°C for 1 h. Amplification of RAGE cDNA used the following primers: forward: 5'-GGGCTCTTCACACTGC-3'; reverse: 5'-CTCCAGTACTACTCTCGCTGTC-3'. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out with the following primers: forward: 5'-CCACCATGGCAAATTCATGGCA-3'; reverse: 5'-TCTAGACGGCAGGTCAGTCCACC-3'.

Electrophoretic mobility shift assay. Nuclear extracts of human endothelial cells were prepared as described before (20). Endothelial cells were stimulated for 2 h with TNF- α (25 ng/ml) with or without rosiglitazone or pioglitazone (10 μ M/l) before the preparation of nuclear extracts. Oligonucleotides for the proximal (nucleotide number -686 to -678; 5'-AGAGTGGGGAACCCCTCCCA-3') and distal NF- κ B site (nucleotide number -1,533 to -1,525; 5'-CTCGGAGGGAGTTTCTGCTA-3') of the human RAGE promoter were annealed with a complementary primer and radiolabeled using [α -³²P]d eoxycytidine triphosphate (ICN). Protein-DNA complexes were separated from free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels in 0.5 \times Tris-borate-EDTA, and bands were visualized by autoradiography. Supershift analysis used a mouse anti-human p50 antibody (Santa Cruz) added 30 min before the addition of radiolabeled NF- κ B probes.

Transient transfection assays. Bovine endothelial cells were transiently transfected with RAGE promoter-luciferase constructs and a pCMV- β -galactosidase construct, using SuperFect according to the manufacturer's protocol. For PCR, the following oligonucleotide was used to amplify the proximal 750-bp fragment of the human RAGE promoter including the proximal NF- κ B site: sense 5'-GGGCTCGAGCCGACTAGCTACCATCTGATC-3'. To amplify the proximal 675-bp promoter region lacking the proximal NF- κ B site the following sense oligonucleotide was used: 5'-GGGCTCGAGCCATTAAGATCCGGCAGGACTG-3'. For both PCRs we used the antisense oligonucleotide: 5'-ATTCAGCGGATCCCTGCTCCTCCAGGTCC-3'. The resulting PCR fragments were cloned into the *Xho*I and *Bam*HI sites of the pGL3 luciferase reporter plasmids to achieve two constructs: pGL3/RAGE750 (including the proximal NF- κ B site) and pGL3/RAGE675 (lacking the proximal NF- κ B site). **MCP-1 enzyme-linked immunosorbent assay.** To examine the effect of reduced RAGE expression on the proinflammatory AGE and RAGE activator's effect, human endothelial cells were pretreated with TZDs for 12 h before 16 h of stimulation with AGE-albumin (500 μ g/ml), β -amyloid (125 nmol/l), or TNF- α (25 ng/ml). Enzyme-linked immunosorbent assay (ELISA) for MCP-1 (R&D Systems) was performed on cell-free supernatants according to the manufacturer's protocol.

Northern blot analysis. For Northern blot experiments, human endothelial cells were pretreated with rosiglitazone for 12 h before 6 h of stimulation with β -amyloid (125 nmol/l) or TNF- α (25 ng/ml). We used 5 μ g of total RNA in standard Northern blot analysis, using cDNA probes against MCP-1 or GAPDH.

Statistical analysis. Results of the experimental studies are reported as the means \pm SE. Differences were analyzed by 1-way ANOVA followed by the appropriate post hoc test. A P value < 0.05 was regarded as significant.

RESULTS

TZDs reduce endothelial RAGE protein expression.

The effect of rosiglitazone and pioglitazone on endothelial cell surface RAGE expression was examined by flow cytometry analysis. Treatment of human endothelial cells with rosiglitazone or pioglitazone decreased basal RAGE expression to 73 \pm 9 or 70 \pm 9%, respectively (P < 0.05 for both, compared with unstimulated cells; n = 5) (Fig. 1A). Because TNF- α is known to increase endothelial RAGE expression, we next examined the effect of TZDs on TNF- α -induced RAGE cell surface expression. Stimulation of endothelial cells with TNF- α (25 ng/ml) for 16 h led to a 1.8 \pm 0.3-fold increase in cell surface RAGE expression (P < 0.05, n = 4), whereas concomitant treatment with rosiglitazone or pioglitazone significantly reduced this increase to the baseline level of unstimulated cells (P < 0.05 for both, compared with TNF- α -treated cells; n = 4) (Fig. 1B).

Both rosiglitazone as well as pioglitazone reduced basal RAGE protein expression assessed by Western blot analysis (Fig. 2A). In addition, TZDs reduced TNF- α -induced total RAGE protein expression in a concentration-dependent manner with a maximal reduction to 40 \pm 9 and 63 \pm 9% at 10 μ M/l rosiglitazone and pioglitazone, respectively (Fig. 2B and C). Two different PPAR- α activators, fenofibrate or WY14643, did not significantly alter basal or TNF- α -induced RAGE expression (data not shown).

TZDs reduce RAGE mRNA expression in human endothelial cells.

To examine whether the decrease in endothelial RAGE protein expression by TZDs resulted from reduced mRNA expression, we treated unstimulated as well as TNF- α -stimulated human endothelial cells with rosiglitazone and pioglitazone and performed RT-PCR after 6 h. TZD treatment of cells decreased TNF- α -induced RAGE mRNA expression to 56 \pm 12 and 59 \pm 11% at 10 μ M/l rosiglitazone and pioglitazone, respectively (P < 0.05 compared with TNF- α -stimulated cells, n = 3) (Fig. 3A and B). In addition, rosiglitazone and pioglitazone

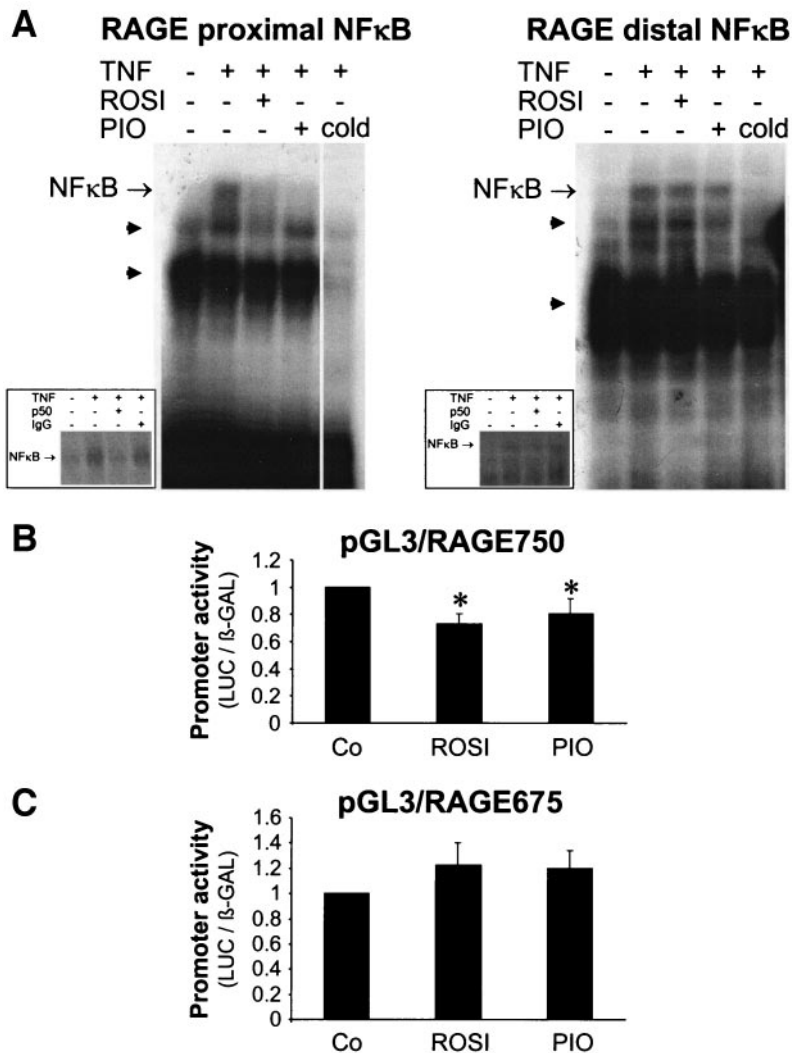


FIG. 4. TZDs inhibit NF-κB activation in the RAGE promoter. **A:** Representative EMSA of human endothelial cells treated with TNF-α (25 ng/ml) for 2 h in the absence or presence of rosiglitazone (ROSI) or pioglitazone (PIO; both at 10 μmol/l). EMSA used oligonucleotides corresponding to the proximal (nucleotide number -692 to -673) and distal (nucleotide number -1,539 to -1,520) NF-κB sites of the RAGE promoter. Three independent experiments yielded similar results. Supershift analysis using a mouse anti-human p50 antibody added 30 min before the addition of radiolabeled NF-κB probes confirmed the specificity of the NF-κB bands (inserts). **B** and **C:** Bovine endothelial cells were transiently transfected with a reporter construct containing the proximal part of the RAGE promoter including (-750 to 0 bp; pGL3/RAGE750) (**B**) or lacking (-675 to 0 bp; pGL3/RAGE675) (**C**) the proximal NF-κB site and stimulated for 12 h. Results of luciferase activity (LUC) were normalized to β-galactosidase (β-GAL). Bars represent the means ± SE. * $P < 0.05$ compared with unstimulated cells ($n = 9$ in **B**; $n = 5$ in **C**). Co, control.

reduced basal RAGE mRNA expression to 73 ± 8 and $56 \pm 10\%$, respectively ($P < 0.05$ compared with unstimulated cells, $n = 6$) (Fig. 3A and C).

TZDs inhibit NF-κB activation in the RAGE-promoter. TNF-α-induced endothelial RAGE expression is regulated by activation of the proximal NF-κB site (nucleotide number -686 to -678) in the RAGE promoter (4). To examine whether TZDs might interact with this pathway, electrophoretic mobility shift assay (EMSA) was performed using oligonucleotides corresponding to the respective site within the RAGE promoter. As shown in Fig. 4A, rosiglitazone as well as pioglitazone inhibited NF-κB protein binding to the proximal NF-κB site, but they had no effect on the distal NF-κB site (nucleotide number -1,533 to -1,525), which is activated by TNF-α but not involved in TNF-α-induced endothelial RAGE expression (4).

Next we examined the effect of TZDs on baseline RAGE promoter activity, performing transient transfection assays using a RAGE promoter reporter construct containing the proximal part of the RAGE promoter (-750 to 0 bp) including the proximal NF-κB site. TZD treatment of cells transfected with this construct significantly reduced baseline promoter activity (Fig. 4B). In contrast, TZDs had no effect on baseline promoter activity after transfection of a

promoter reporter construct that was lacking this proximal NF-κB site (Fig. 4C). Previous data have shown that baseline RAGE expression is also regulated via this NF-κB site (4,21). Therefore, our data suggest that the interaction of TZDs with the proximal NF-κB site may explain the reduction of both TNF-α-induced as well as baseline RAGE expression.

TZD-mediated reduction of endothelial RAGE expression decreases proinflammatory AGE effects in human endothelial cells. In human endothelial cells, activation of RAGEs has previously been shown to upregulate the expression of proinflammatory mediators such as MCP-1. To assess the functional relevance of reduced endothelial RAGE expression, human endothelial cells were pretreated with rosiglitazone or pioglitazone for 12 h and then stimulated with AGE-BSA (500 μg/ml) or the RAGE activator β-amyloid before investigating MCP-1 protein release. Stimulation of endothelial cells with AGE-BSA for 16 h induced MCP-1 protein secretion from 13.3 ± 9.3 to 1181.8 ± 309 ng/ml, as determined by ELISA on cell-free supernatants ($P < 0.05$, $n = 5$). A 12-h pretreatment of cells with rosiglitazone or pioglitazone significantly reduced AGE-mediated MCP-1 release to 192.5 ± 108.8 and 130.8 ± 60 ng/ml, respectively ($P < 0.05$ for both, compared with AGE-treated cells; $n = 5$) (Fig. 5A). Similar

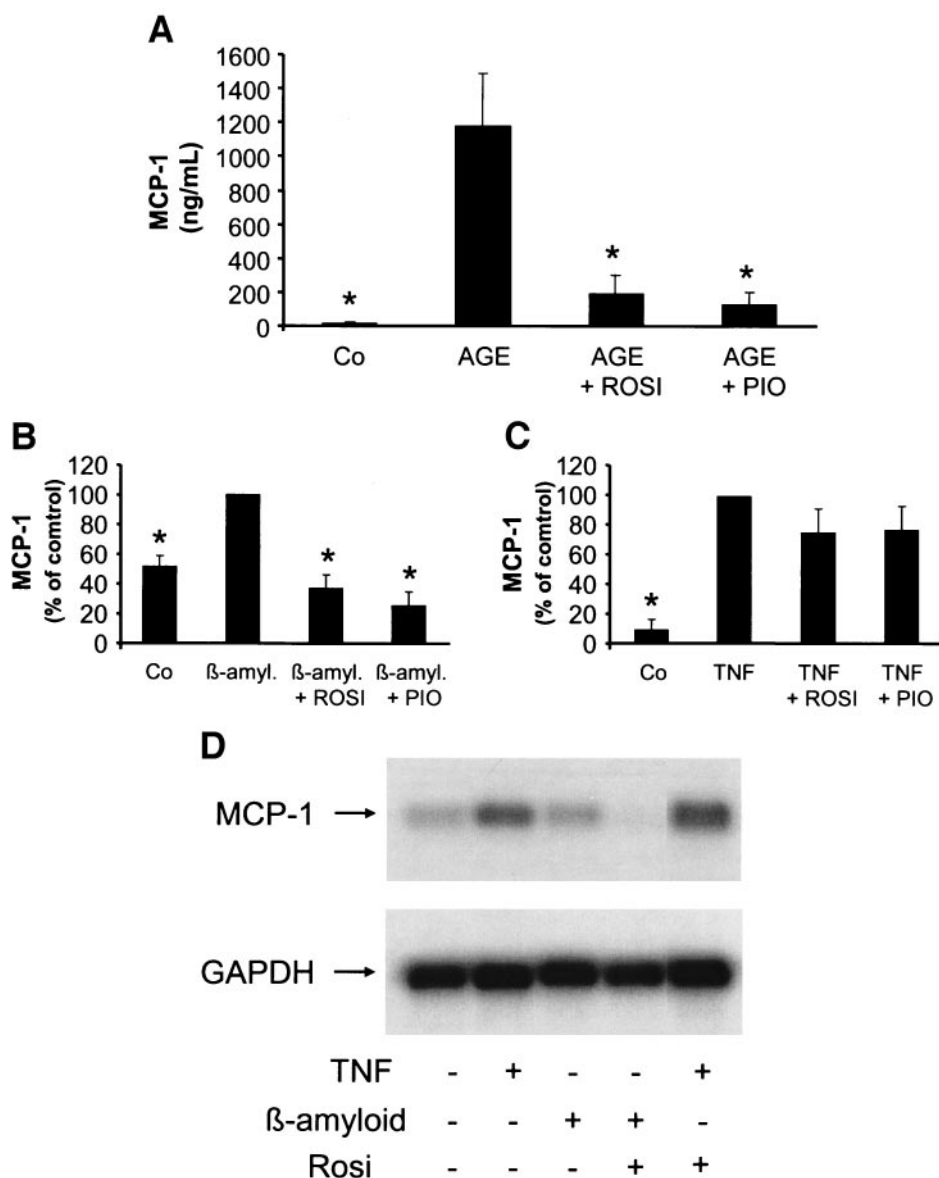


FIG. 5. TZD-mediated reduction of endothelial RAGE expression decreases proinflammatory AGE effects in human endothelial cells. **A:** Human endothelial cells were pretreated with rosiglitazone (ROSI) or pioglitazone (PIO) for 12 h before stimulation with AGE-BSA (AGE) (500 μg/ml). After 16 h, MCP-1 ELISA was performed on cell-free supernatants. Bars represent the means ± SE. **P* < 0.05 compared with AGE-stimulated cells (*n* = 5). **B:** Human endothelial cells were pretreated with rosiglitazone or pioglitazone for 12 h before stimulation with β-amyloid (125 nmol/l). After 16 h, MCP-1 ELISA was performed on cell-free supernatants. Results are expressed as percent of β-amyloid-stimulated cells (% of control). **P* < 0.05 compared with AGE-stimulated cells (*n* = 5). **C:** Human endothelial cells were pretreated with rosiglitazone or pioglitazone for 12 h before stimulation with TNF-α (25 ng/ml). After 16 h, MCP-1 ELISA was performed on cell-free supernatants. Results are expressed as percent of TNF-α-stimulated cells (% of control). **P* < 0.05 compared with TNF-α-stimulated cells (*n* = 5). **D:** Human endothelial cells were pretreated with rosiglitazone for 12 h before 6-h stimulation with TNF-α or β-amyloid. Representative Northern blot is shown. Three independent experiments demonstrated similar results. Co, control.

results were obtained upon stimulation with the RAGE activator β-amyloid: we found that β-amyloid was a less potent inducer of MCP-1 release, but pretreatment of endothelial cells with TZDs significantly inhibited this increase (Fig. 5B). In addition, rosiglitazone reduced β-amyloid-induced MCP-1 mRNA expression, as determined by Northern blot analysis (Fig. 5D). In contrast, TZD pretreatment did not affect TNF-α-induced MCP-1 protein or mRNA expression, ruling out a direct effect of TZDs on MCP-1, as previously described (17) (Fig. 5C and D). These data suggest that TZD-mediated reduction of RAGE expression limits the cells susceptibility toward proinflammatory AGE or RAGE activators' effects.

DISCUSSION

The present study reports a reduction of RAGE expression in human endothelial cells by antidiabetic TZDs, with subsequently reduced endothelial susceptibility toward proinflammatory AGE effects.

TZDs, originally described to control gene expression in glucose homeostasis and adipogenesis, have recently been

identified as regulators of gene expression in human endothelial cells. In these cells, TZDs exhibit anti-inflammatory effects on various mediators of atherosclerosis (reviewed in 22). Our study extends the understanding of TZDs' action in vascular cells by demonstrating a reduction of basal as well as TNF-α-induced RAGE expression in human endothelial cells. Our Western blot data suggest that rosiglitazone may be a stronger inhibitor of TNF-α-induced endothelial RAGE expression. Still, there is no difference in the effects of rosiglitazone and pioglitazone on RAGE mRNA and cell surface protein expression, suggesting that the difference observed in Western blot analyses may be caused by the variability of the method and may thus not reflect a true difference between the two TZDs.

Previous work has established that TNF-α activates both NF-κB sites in the RAGE promoter, with only the proximal site being of importance to induce endothelial RAGE expression (4). We demonstrate that TZDs' reduction of TNF-α-induced RAGE expression is mediated by an inhibition of NF-κB activation at this respective site. In

addition, our data suggest that TZDs' interaction with this NF- κ B site may also explain the reduction in baseline RAGE expression, because baseline promoter activity is also regulated via this NF- κ B site (4,21). These findings are in accordance with previous observations that PPAR activators can interfere with NF- κ B activation in human endothelial cells (20,23). PPARs have been shown to alter cofactor recruitment as well as cofactor binding to transcription factors like NF- κ B (24), and such mechanisms may explain the inhibitory effect of TZDs on the proximal NF- κ B site. Interestingly, TZDs did not affect binding of NF- κ B proteins to the distal NF- κ B site, and this difference may be caused by different cofactors involved in NF- κ B activation of this site. Alternatively, the lack of an effect on activation of the distal NF- κ B site may stem from differences in the upstream signaling pathways that lead to activation of these two sites.

The reduction of endothelial RAGE expression by TZDs decreases the cells' susceptibility toward proinflammatory AGE effects, as shown by impaired MCP-1 release after TZD pretreatment. In contrast, TZDs did not affect TNF- α -induced MCP-1 expression, ruling out a direct effect of PPAR- γ activators. These data are consistent with previous reports showing no effect of TZDs on endothelial MCP-1 expression (17).

Our findings might have important pathophysiological and clinical implications for the high-risk population of diabetic patients: these patients do exhibit increased AGE levels on one hand as well as enhanced RAGE expression in the vasculature on the other hand. In vascular cells like endothelial cells, AGE-RAGE interaction leads to a long-lasting and extensive expression of proatherogenic mediators, such as MCP-1 or VCAM-1. These mediators furnish lesion development by facilitating leukocyte recruitment with subsequent activation of these cells in the vessel wall (25). TNF- α , one of the cytokines released from these inflammatory cells in the plaque, may then in turn increase endothelial RAGE expression, thus creating a vicious circle that perpetuates the atherogenic process in diabetic patients. Animal data demonstrating that interruption of the AGE-RAGE interaction decreases lesion size in a mouse model of arteriosclerosis (12) have led to the consideration that limiting RAGE activation might be a therapeutic tool to influence vascular disease in diabetic patients (3). The reduction of RAGE expression by antidiabetic TZDs, as shown here, might represent such a novel way to limit RAGE-mediated cell activation in the vessel wall, thus potentially modulating atherogenesis in patients with diabetes. Clinical studies suggest beneficial effects of TZD treatment on markers of arteriosclerosis in diabetic patients (26), and further studies have to evaluate to what extent a reduction of endothelial RAGE expression may contribute to these findings.

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