Original Article

Pioglitazone Inhibits Angiotensin II–Induced Senescence of Endothelial Progenitor Cell

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We investigated whether a peroxisome proliferator–activated receptor (PPAR) agonist would effect the angiotensin II (Ang II)–induced senescence of endothelial progenitor cells (EPCs). EPCs were isolated from peripheral blood and characterized. Both reverse transcription–polymerase chain reaction (RT-PCR) and Western blotting were used to assess gp91phox expression and angiotensin type 1 receptor (AT_1R) levels. Immunofluorescence of nitrotyrosine provided evidence of peroxynitrite formation. Our data indicate that Ang II increased the expression of gp91phox mRNA, which was significantly diminished by pioglitazone, a PPARγ agonist. Western blotting revealed that Ang II stimulated an increase in the gp91phox protein, whereas co-treatment with pioglitazone significantly reduced this increase. In addition, pioglitazone also inhibited Ang II–induced peroxynitrite formation. Interestingly, pioglitazone decreased the expressions of AT_1R mRNA and protein. The exposure of cultured EPCs to Ang II (100 nmol/L) significantly accelerated the rate of senescence compared to that of the control cells during 14 d in culture, as determined by acidic β-galactosidase staining. Ang II–induced EPC senescence was significantly inhibited by co-treatment with pioglitazone. Because cellular senescence is critically influenced by telomerase, which elongates telomeres, we also measured telomerase activity by means of PCR-ELISA–based assay. The results showed that Ang II significantly diminished telomerase activity, and this effect was significantly abolished by co-treatment with pioglitazone. In conclusion, pioglitazone inhibited Ang II–induced senescence of EPCs *via* down-regulation of the expression of AT_1R. (*Hypertens Res* 2008; 31: 757–765)

***Key Words*: endothelial progenitor cells, angiotensin II, senescence, oxidative stress**

Introduction

There is a wide range of experimental evidence that angiotensin II (Ang II) promotes the production of free radicals in the pathogenesis of cardiovascular disease. Studies have also shown that expression of NADPH oxidase subunits plays a role in the formation of human atherosclerotic lesions as well as animal models of hypertension and atherosclerosis (*1*–*3*). In vascular endothelial cells, Ang II also induces superoxide anion generation *via* the activation of NADPH oxidase (*4*), which mediates endothelial dysfunction and peroxynitrite formation (*5*). Recently, some studies have indicated that, after exposure to Ang II, an elevation of superoxide production occurs through the Ang II type 1 (AT_1) receptor (AT_1R) activation of NADPH oxidase in the coronary arterioles and human umbilical vein endothelial cells (*6*, *7*). In previous studies, we have demonstrated that Ang II promotes the senescence of human endothelial progenitor cells (EPCs) through enhanced oxidative stress (*8*). Given the important role of EPCs for neovascularization of ischemic tissue, the functional impairment of EPCs may contribute to an insufficient regeneration of the endothelium, which could lead to endothelial dysfunction.

Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear receptor family.

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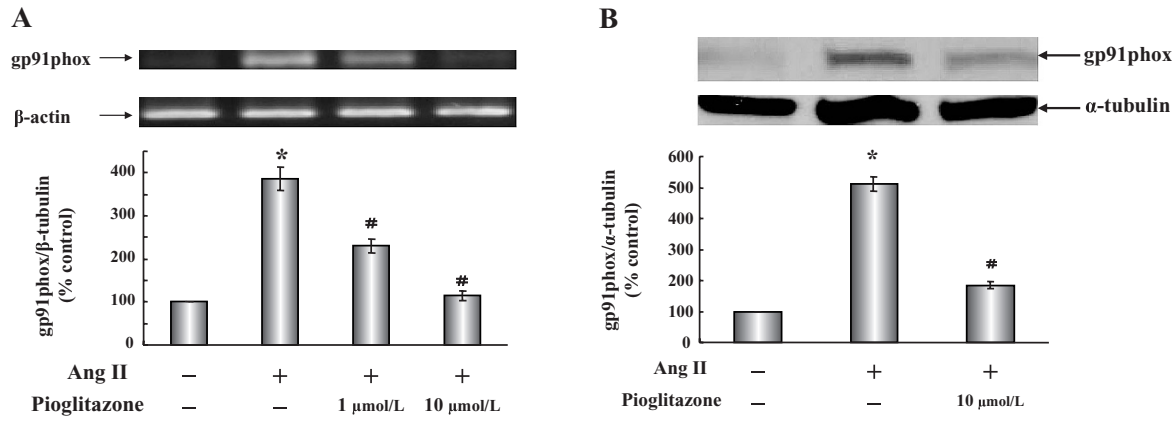

Fig. 1. *Effect of pioglitazone on Ang II–stimulated gp91phox mRNA and protein of NAD(P)H oxidase in EPCs. EPCs were pre-treated with the indicated doses of pioglitazone for 24 h before Ang II stimulation (100 nmol/L). Pioglitazone significantly diminished the Ang II–induced increase in gp91phox mRNA (A) and protein (B) expression. Representative images from five independent experiments are shown (top). Bar graphs show gp91phox mRNA and protein normalized by β-actin and α-tubulin, respectively (bottom). Data are expressed as a ratio of the test value to the value for unstimulated cells (set at 100%). Data represent the means±SEM of five independent experiments. *$p<0.05$ vs. the control cells. #$p<0.05$ vs. EPCs treated with Ang II alone.*

Emerging evidence indicates that the PPAR-signaling pathways play critical roles in the regulation of a variety of biological processes within the cardiovascular system (*9*). Considerable evidence indicates that treatment with PPARγ agonists improves endothelial function in diabetic animal models and diabetic patients (*10*). However, it remains unclear whether this endothelial protective effect is secondary to the improved glucose metabolism achieved by the PPARγ agonists, or whether these drugs confer the endothelial protection directly.

To our knowledge, the interaction among Ang II, pioglitazone, and oxidative stress in EPCs has not been investigated. In view of our previous findings that Ang II promotes senescence of EPCs through enhanced oxidative stress (*8*), we further investigated the potential effect of pioglitazone on Ang II–induced EPCs senescence. Our data demonstrated that pioglitazone reduced Ang II–induced oxidative stress and senescence in EPCs, and that this effect was partially achieved through down-regulation of AT_1R expression.

Methods

Isolation of Mononuclear Cells and Cell Culture

EPCs were cultured according to a previously described technique (*11*, *12*). Briefly, peripheral blood mononuclear cells (PB-MNCs) were isolated from healthy volunteers by density gradient centrifugation with Histopaque 1077 (Sigma Chemical Co., St. Louis, USA). After purification with 3 washing steps, 10×10^6 PB-MNCs were plated on fibronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2) (Clonetics, Walkersville, USA) supplemented with 3 μg/mL bovine brain extract, 30 μg/mL gentamycin, 50 μg/mL amphotericin B, 10 μg/mL human epidermal growth factor, and 5% fetal bovine serum (FBS). The 6-well plates were replated at day 4. At day 8 of the culture, the PB-MNCs were pre-treated for 24 h with one of the several concentrations of pioglitazone. Thereafter, Ang II (100 nmol/L) was added to the PB-MNCs.

Colony-Forming Assay

The colony-forming assay was performed according to a previously described technique (*13*, *14*). We cultured isolated PB-MNCs in EBM-2 with supplements and then treated the PB-MNCs at day 0 of the culture with Ang II (100 nmol/L) for 48 h after no pre-treatment or pre-treatment with pioglitazone (10 μmol/L) for 24 h before colony assay. After 48 h, we seeded the nonadherent cells (1×10^6) in methylcellulose plates (MethocultGF H4434; CellSystems, Kirkland, USA) for a final assessment of the number of colonies. We used nonadherent rather than adherent cells for the colony assay to avoid contamination with mature endothelial cells and non-progenitor cells, because these cells could affect the colony formation of EPCs (*14*). The number of colonies was counted 7 d after plating. A colony of EPCs consisted of multiple thin, flat cells emanating from a central cluster of rounded cells. A central cluster alone without associated emanating cells was not counted as a colony. Colonies were counted in a minimum of four wells by observers who were unaware of the research protocol.

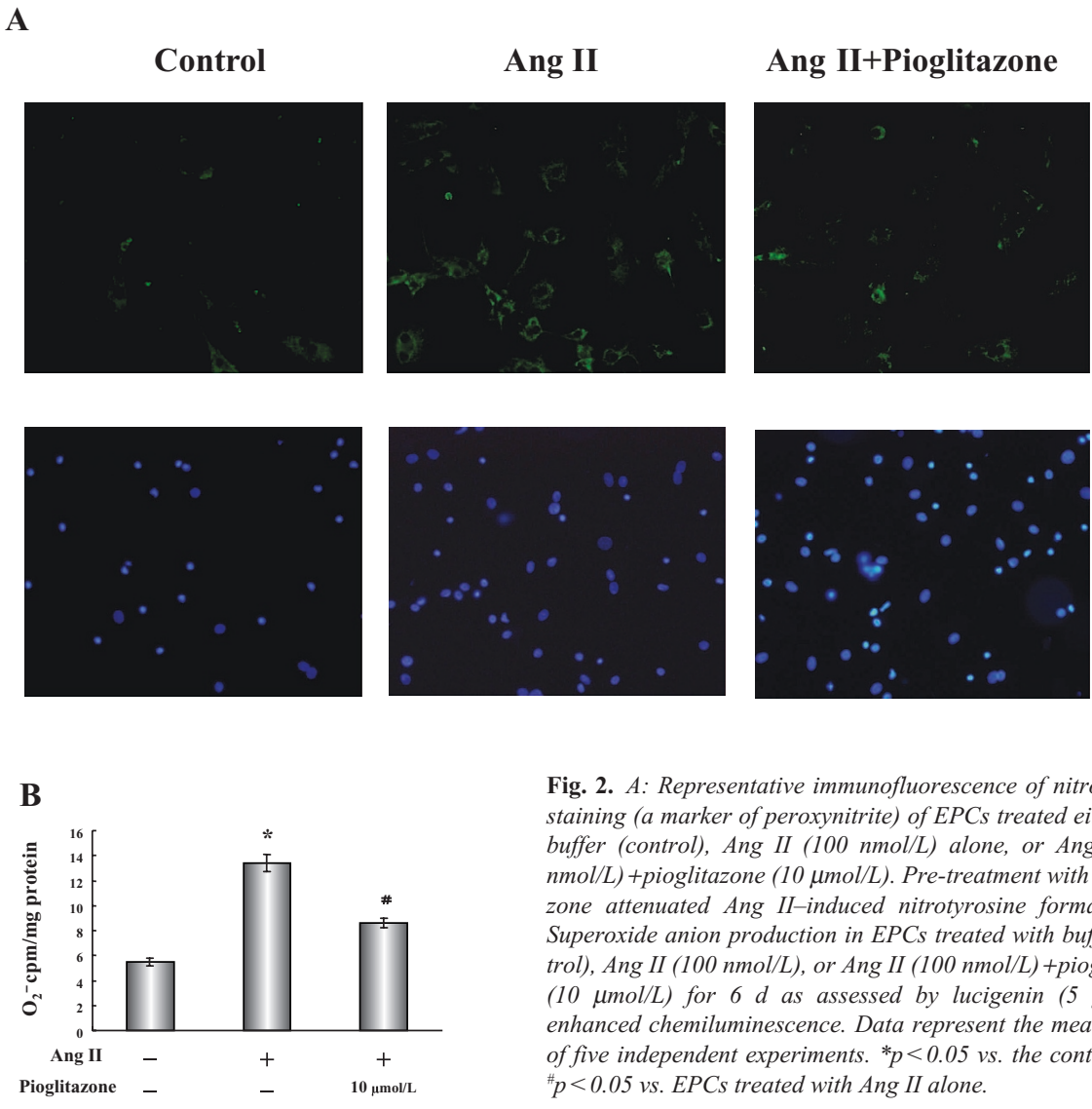

Fig. 2. *A: Representative immunofluorescence of nitrotyrosine staining (a marker of peroxynitrite) of EPCs treated either with buffer (control), Ang II (100 nmol/L) alone, or Ang II (100 nmol/L) +pioglitazone (10 μmol/L). Pre-treatment with pioglitazone attenuated Ang II–induced nitrotyrosine formation. B: Superoxide anion production in EPCs treated with buffer (control), Ang II (100 nmol/L), or Ang II (100 nmol/L) +pioglitazone (10 μmol/L) for 6 d as assessed by lucigenin (5 μmol/L)-enhanced chemiluminescence. Data represent the means±SEM of five independent experiments. *$p < 0.05$ vs. the control cells. #$p < 0.05$ vs. EPCs treated with Ang II alone.*

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

To investigate the effect of pioglitazone on Ang II–stimulated gp91phox and AT_1R expression, EPCs were pre-treated with one of several concentrations of pioglitazone for 24 h before Ang II–stimulation (100 nmol/L) for 1 h. Total RNA was extracted by using an RNeasy RNA extraction kit (Qiagen, Chatsworth, USA). Briefly, cells were lysed in guanadinium isothiocyanate buffer, and RNA was purified following the manufacturer's instructions. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)–treated H_2O. To generate cDNA, 1 μg total RNA was treated with DNaseI (Ambion, Austin, USA) to remove any contaminating genomic cDNA. The DNase-treated RNA (100 ng) was then converted into cDNA by using murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Bethesda, USA). The transcribed cDNA was then used for polymerase chain reaction (PCR) amplification to estimate the expression of gp91phox. Two specific primers matching the published sequences were used to identify and amplify gp91phox (sense primer, 5′-CAACAAGAGTTCGAAGACAA-3′; antisense primer, 5′-CCCCTTCTTCTTCATCTGTA-3′). The PCR products were 689 bp in length. The amplification consisted of 44 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. The PCR products were then visualized in 1.5% ethidium bromide–stained agarose gels. β-Actin was amplified as a reference. The effect of pioglitazone on Ang II–stimulated AT_1R mRNA expression was also evaluated. For this purpose, EPCs that had been pre-treated with pioglitazone at either 1 or 10 μmol/L for 24 h, as well as EPCs that were not pre-treated, were incubated with Ang II at 100 nmol/L for 1 h, and then AT_1R mRNA expression was examined. For amplification, a primer pair specific for human AT_1R (sense, 5′-GTCATG

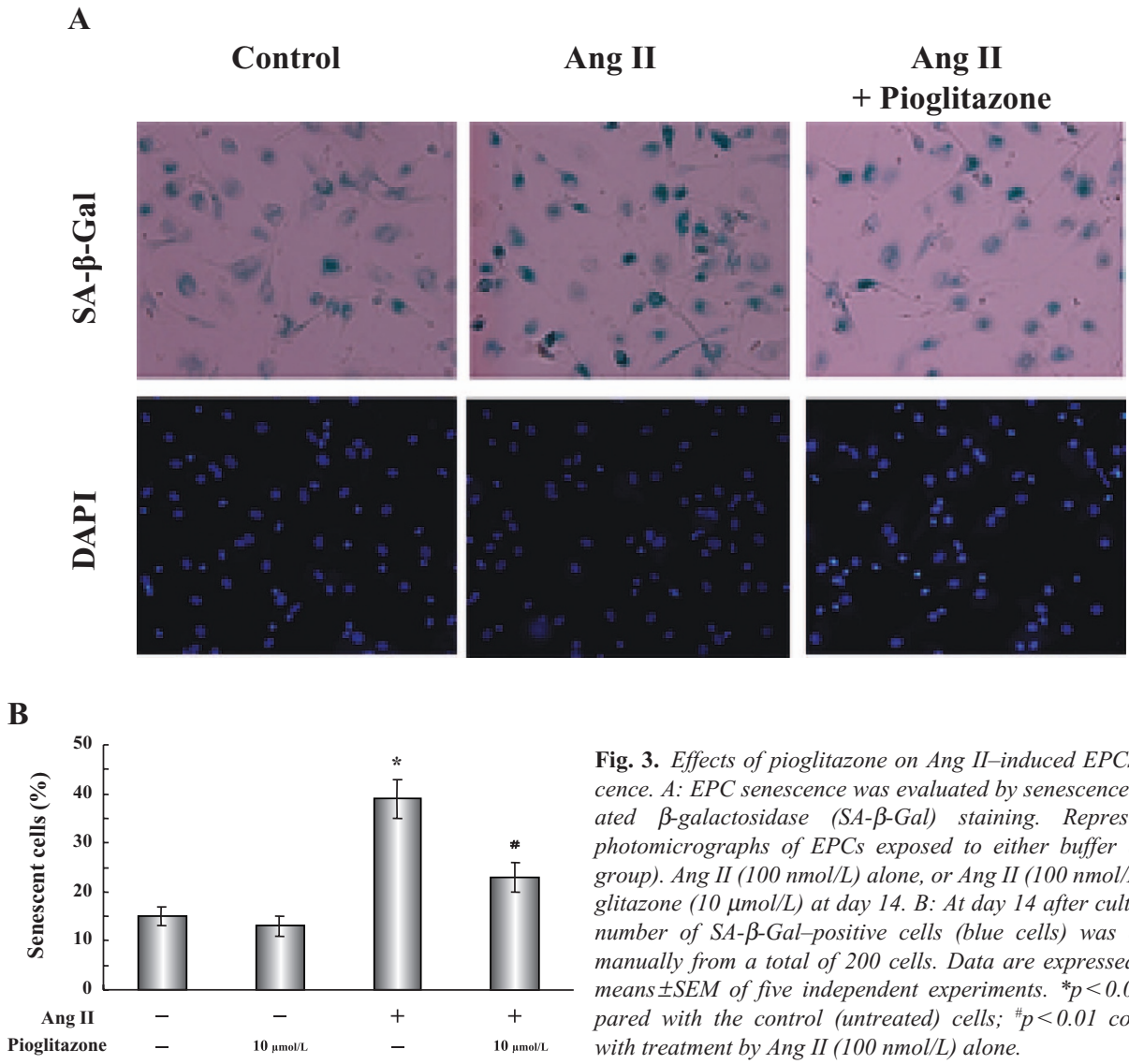

Fig. 3. *Effects of pioglitazone on Ang II–induced EPCs senescence. A: EPC senescence was evaluated by senescence-associated β-galactosidase (SA-β-Gal) staining. Representative photomicrographs of EPCs exposed to either buffer (control group). Ang II (100 nmol/L) alone, or Ang II (100 nmol/L) + pioglitazone (10 μmol/L) at day 14. B: At day 14 after culture, the number of SA-β-Gal–positive cells (blue cells) was counted manually from a total of 200 cells. Data are expressed as the means ± SEM of five independent experiments. *$p < 0.01$ compared with the control (untreated) cells; #$p < 0.01$ compared with treatment by Ang II (100 nmol/L) alone.*

ATTCCTACTTTATACAGTATC-3′; antisense pair, 5′-AGCCAGGTATCGATCAATGCTGAGACA-3′) was used. The PCR product was 304 bp in length. The amplification consisted of 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR products were visualized on 1.5% ethidium bromide–stained agarose gels. β-Actin was amplified as a reference.

Western Blotting

The effect of pioglitazone on Ang II–stimulated AT_1R protein and gp91phox protein was investigated. For this purpose, EPCs that had been pre-treated with pioglitazone at 10 μmol/L for 24 h, as well as EPCs that were not pre-treated, were incubated with Ang II at 100 nmol/L for 24 h, and then AT_1R protein and gp91phox protein expressions were examined. The general protocol for Western blotting was as described previously (*15*). Briefly, EPCs proteins were size-separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred to a PVDF membrane (Millipore, Bradford, USA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST) and incubated with rabbit anti-human AT_1R (N-10; Santa Cruz Biotechnology Inc., Santa Cruz, USA) anti-serum at a dilution of 1:300 for 12 h at 4°C. Goat polyclonal antibody against human gp91phox (Santa Cruz Biotechnology Inc.) was also used at a dilution of 1:100. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK). The profile of each band was plotted by using NIH Image software, and the densitometric intensity corresponding to each band was measured as an intensity value. Both AT_1R and gp91phox protein intensity were normalized by using α-tubulin expression.

Immunofluorescence

Confirmation of the endothelial-cell lineage was performed as previously described (*11*, *12*). Briefly, indirect immunostaining was performed by using endothelial-specific antibodies against vascular endothelial growth factor 2 and CD31, by measuring the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low density lipoprotein (acLDL), or by measuring the binding of fluorescein isothiocyanate (FITC)–labeled Ulex europaeus agglutinin 1 (UEA-1) (data not shown). EPCs at day 14 after culture, that is, EPCs exposed to Ang II after no pre-treatment or pre-treatment with pioglitazone for 6 d, were immunostained using polyclonal antibody against nitrotyrosine (1:100; Upstate Biotechnology, Lake Placid, USA), a marker for peroxynitrite. Incubation with the FITC-conjugated goat anti-rabbit IgG (1:200) took place for 90 min in the dark. Coverslips were placed over the cells and sealed with nail polish. A Vectorshield H-1200 Mounting Kit (Vector Laboratories, Burlingame, USA) was used for immunofluorescence. Cells were visualized by fluorescence microscopy with standard FITC excitation/emission filter combinations.

Determination of Superoxide Anion Formation by Lucigenin-Enhanced Chemiluminescence

Basal superoxide anion formation was measured by lucigenin-enhanced chemiluminescence as previously described (*16*). In brief, EPCs at day 14 after culture, that is, EPCs exposed to Ang II after no pre-treatment or pre-treatment with pioglitazone for 6 d, were transferred into a scintillation vial containing lucigenin and Krebs/HEPES buffer (final composition in nmol/L: lucigenin 0.005, NaCl 99.01, KCl 4.69, $CaCL_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.03, $NaHCO_3$ 25, Na-HEPES 20, glucose 5.6; pH 7.4). Signals were assessed over 20 min at 37°C in a luminometer at 30 s intervals under basal conditions in the absence of additional NADPH. The chemiluminescence signal was adjusted for the amount of protein of EPCs extract.

Senescence-Associated β-Galactosidase Activity Assay

Senescence-associated β-galactosidase (SA-β-Gal) activity was measured as previously described (*17*). Briefly, EPCs at day 14 after culture, that is, EPCs exposed to Ang II after no pre-treatment or pre-treatment with pioglitazone for 6 d, were washed in PBS, fixed for 3 min (room temperature) in 2% paraformaldehyde, washed, and incubated for 24 h at 37°C (no CO_2) with fresh SA-β-Gal stain solution: 1 mg/mL 5-bromo-4-chloro-3-indyl β-D-galactopylanoside (X-Gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium fericyanide, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet-40. EPCs were counterstained with 4′,6-diamino-phenylindole (DAPI) (0.2 μg/mL in 10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L EDTA, 100 mmol/L NaCl) for 10 min and then the total cell number was counted.

Telomeric Repeat Amplification Protocol Assay

For quantitative analyses of telomerase activity in EPCs at day 14 after culture, that is, EPCs exposed to Ang II after no pre-treatment or pre-treatment with pioglitazone for 6 d, the Telomeric Repeat Amplification Protocol (TRAP) assay, in which the telomerase reaction product is amplified by PCR, was performed using a *TeloTAGGG* PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol as previously described (*8*).

Statistical Analysis

Data are expressed as the means±SEM from at least 5 independent experiments. Statistical analysis was performed by one-way ANOVA (LSD test) for multiple testing. Probability values of $p<0.05$ were considered statistically significant.

Results

Pioglitazone Reduces Ang II–Induced gp91phox Expression in Human EPCs

We previously demonstrated that Ang II–induced gp91phox mRNA expression occurred dose-dependently with a maximal effect achieved at 100 nmol/L (*8*). Therefore, in this study, we examined whether pioglitazone could modulate Ang II–induced gp91phox mRNA expression in human EPCs. Our results showed that pioglitazone markedly diminished the Ang II–induced increase in gp91phox mRNA expression (Fig. 1A). Similarly, pioglitazone markedly diminished the Ang II–induced increase in gp91phox protein expression (Fig. 1B), although we did not observe any effects of pioglitazone itself on the basal level of gp91phox protein (data not shown).

Pioglitazone Inhibits Ang II–Induced Peroxynitrite Formation and Superoxide Production in Human EPCs

As shown in Fig. 2A, Ang II markedly increased nitrotyrosine staining, suggesting an increase in peroxynitrite formation. The Ang II–induced peroxynitrite production was prevented by pioglitazone treatment (Fig. 2A). In addition, we assessed O_2^- formation in EPCs after treatments. Ang II markedly increased O_2^- formation, while pretreatment with pioglitazone significantly inhibited Ang II–induced up-regulation of O_2^- formation (Fig. 2B).

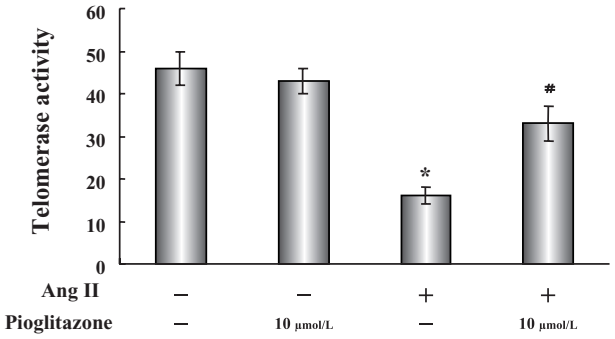

Fig. 4. *Effect of pioglitazone on Ang II–induced telomerase activity. EPCs at day 8 were pre-treated with or without pioglitazone for 24 h before Ang II stimulation (100 nmol/L and telomerase activity was measured at day 14 of culture. Data are expressed as the means±SEM of five independent experiments. *$p < 0.01$ compared with the control (untreated) cells; #$p < 0.01$ compared with treatment by Ang II (100 nmol/L) alone.*

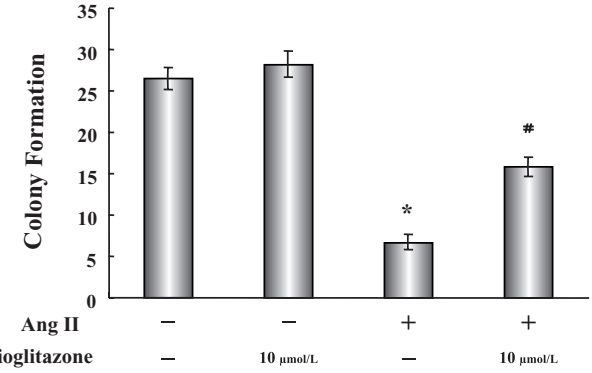

Fig. 5. *Effect of pioglitazone on the impairment of Ang II–induced colony formaing unit. EPCs were seeded in methylcellulose plates, and the number of colony-forming units was examined after an additional 7 d of cultivation. Data are expressed as the means±SEM of five independent experiments.*

Pioglitazone Reduces Ang II–Induced EPCs Senescence

To assess the onset of senescence, acidic β-galactosidase was detected as a biochemical marker of the acidification that occurs at the onset of cellular senescence. Prolonged cultivation of EPCs resulted in an increase in SA-β-Gal–positive cells. Coincubation with Ang II accelerated the increase in SA-β-Gal–positive cells in 100 nmol/L Ang II–treated EPCs was markedly increased compared to that in the control cells (Fig. 3A). As shown in Fig. 3, the Ang II–induced increase in SA-β-Gal–positive cells was significantly attenuated by the pre-treatment with pioglitazone.

Effects of Pioglitazone on Ang II–Induced Impairment of Telomerase Activity in EPCs

Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the telomere length reduction induced by each cell division. Therefore, we measured telomerase activity by a TeloTAGGG Telomerase PCR ELISA. As demonstrated in Fig. 4, 100 nmol/L Ang II significantly diminished telomerase activity, and this effect was significantly abolished by pre-treatment with pioglitazone.

Pioglitazone Inhibits the Impairment of Ang II–Induced Colony Formation

Having demonstrated that pioglitazone inhibited the onset of Ang II–induced senescence, we performed an outgrowth assay to examine whether the pioglitazone-induced delay in senescence would translate into an increase in the clonal expansion potential of the cultivated EPCs. As shown in Fig. 5, the number of colonies was significantly lower in EPCs treated with Ang II than in the control culture. The Ang II–induced impairment of colony formation was significantly abolished by pre-treatment with pioglitazone.

Pioglitazone Inhibits Ang II–Induced AT_1R Expression

We demonstrated in our previous study (*18*) that the Ang II–induced increases in the expressions of AT_1R mRNA and protein are time-dependent, with a maximal increase at 1 h and 24 h, respectively. Therefore, in the present study we examined the effects of pioglitazone on AT_1R mRNA and protein expression at 1 h and 24 h, respectively. Ang II stimulated a significant increase in AT_1R mRNA and protein expression, and pre-treatment with pioglitazone prevented these Ang II–induced effects (Fig. 6).

Discussion

Recent studies have demonstrated that atherosclerotic risk factors inversely correlate with the number of EPCs (*14*, *19*). Moreover, angiotensin-converting enzyme inhibitor therapy with ramipril was reported to augment circulating EPCs with enhanced functional activity in patients with stable coronary artery disease (*20*). Given the concept of postnatal neovascularization contributed by EPCs, a further understanding of the mechanisms that regulates EPC biological activity could provide new insights into the pathogenesis of vasculogenesis. Here we have shown for the first time that pioglitazone inhibits Ang II–stimulated gp91phox expression in EPCs, which may contribute to the inhibitory effect of pioglitazone on Ang II–induced oxidative stress, as evidenced by peroxynitrite for-

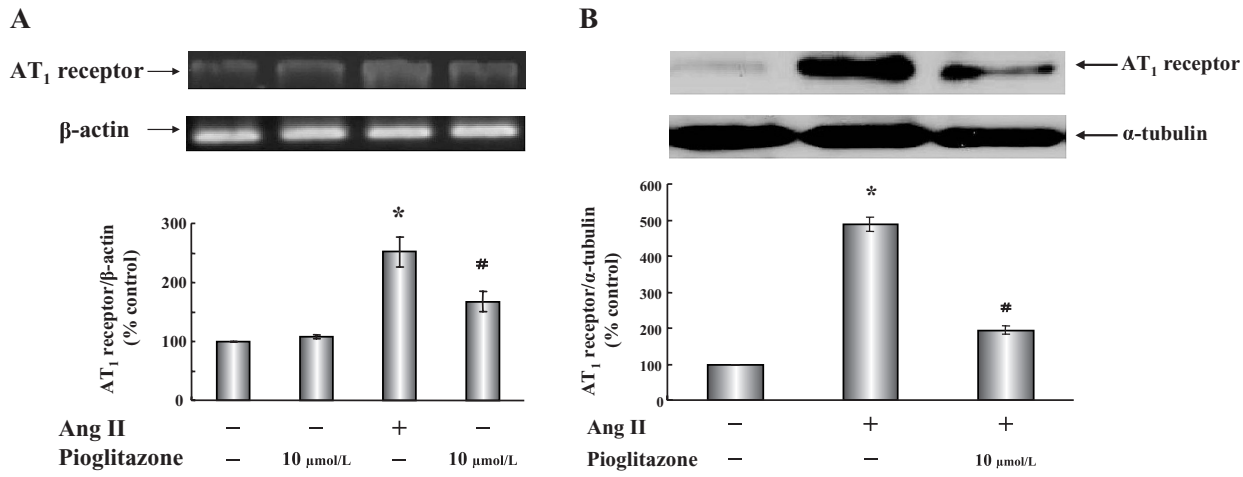

Fig. 6. *Effect of pioglitazone on AT_1 receptor expression. Ang II (100 nmol/L) stimulated a significant increase in AT_1R mRNA (A) and protein (B) expression, whereas pre-treatment with pioglitazone (10 μmol/L) reduced these increases. Data are expressed as the mean ± SEM of five independent experiments. *$p < 0.01$ compared with the control (untreated) cells; #$p < 0.01$ compared with treatment by Ang II (100 nmol/L) alone.*

mation. Secondarily, we have demonstrated for the first time that pioglitazone reduces Ang II–induced acceleration of senescence in EPCs, at least in part through the down-regulation of AT_1R.

The present study has shown that pioglitazone significantly prevented Ang II–induced AT_1R mRNA and protein expression. It appears that the inhibitory effect of pioglitazone on Ang II–induced senescence may be partially due to the AT_1R down-regulation induced by pioglitazone. However, the prevention of Ang II–mediated responses by pioglitazone may involve other effects, such as direct effects on the senescence process. Our study provides the foundation for future studies to address the mechanisms of Ang II–induced senescence in EPCs.

Telomerase is capable of counteracting the onset of senescence (*21–23*). In a previous study (*8*), we have shown that Ang II inhibits telomerase activity *via* inhibition of the accumulation of reactive oxygen species. A variety of studies have investigated the role of telomeres and telomerase in cellular senescence. Recent studies have demonstrated that introduction of *h*TERT into human vascular cells can extend their life span and preserve a younger phenotype, underlying the important role of telomerase and of telomere stabilization in the longevity and functional activity of endothelial cells (*23*). Moreover, Murasawa *et al*. (*24*) have revealed that overexpression of *h*TERT by adenovirus-mediated gene delivery could result in a delay in senescence and recovery or enhancement of the regenerative properties of EPCs. Keeping these findings in mind, we speculated that Ang II accelerates the onset of senescence through telomerase inactivation, while pioglitazone inhibits Ang II–stimulated senescence in EPCs through inhibition of Ang II–induced telomerase inactivation.

It is conceivable that aging or senescence is associated with dysfunctional and defective vasculogenesis. Recent results from animal studies have indicated that transplantation of bone marrow (including EPCs) from old mice into young mice led to minimal neovascularization of young bone marrow (*25*). More importantly, Heiss *et al*. (*26*) demonstrated that age-related alterations in the number and function of EPCs correlate directly with the degree of senescent endothelial dysfunction. These studies provide evidence to support an age–dependent impairment in neovascularization that is heavily influenced by the EPCs phenotype. The endothelial protective effects of PPARγ agonists are of clinical significance. Endothelial dysfunction causes up-regulation of adhesion molecules, enhances migration of monocytes into the vessel wall, and increases proliferation of smooth muscle cells (*27*, *28*). Therefore, therapeutic interventions that improve vascular activity in EPCs, such as PPARγ agonists, may have tremendous potential for the treatment of cardiovascular diseases.

We used two different culture methods in the present study, because there is no established culture assay for EPCs. That is, we used nonadherent cells for the colony assay (*14*), and used adherent cells for the rest of the study (*29*). We used nonadherent cells, but not adherent cells, for the colony assay to avoid contamination with mature endothelial cells and nonprogenitor cells, because a previous report indicates that these cells can affect EPC colony formation (*14*). On the other hand, our EPCs culture method was mostly based on the original paper by Asahara *et al*. (*29*). In the future, it will be necessary to establish a standard method for the culture of EPC. In recent studies, EPCs have generally benn defined by DiLDL/lectin double-positive cells (*30*, *31*). The molecular and phenotypic determinants of EPCs and their precursors remain largely unknown. Attempts to accurately characterize

these cells have been hampered by several hurdles (*32*). Indeed, a considerable overlap exists among proteins expressed on the surface of angioblast-like EPCs, mature ECs sloughed from the vessel wall, and hematopoietic cells. Specifically, both putative EPCs with angioblastic potential and vessel wall–derived mature ECs may express similar endothelial-specific markers, including vascular endothelial growth factor receptor-2 (VEGFR2), Tie-1, Tie-2, vascular endothelial cadherin, and CD34 (*33*, *34*). Consequently, an array of different methodologies has been used to determine the number of circulating EPCs, including CD34+. VEGFR2+, CD133+, CD34+/VEGFR2+, CD34+/CD133+/VEGFR2, CD34+/CD117+/VEGFR2+, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine–labeled acLDL uptake/lectin binding, and early- and late-outgrowth EPCs colonies (*35*). As a result, these variations in method have created confusion regarding data interpretation and may explain the inconsistent results obtained in different studies. As noted, it remains to be determined whether or not changes in CD34+/KDR+ cells fully reflect changes in EPCs capable of arterial repair and angiogenic activity (*35*). Clearly, further studies will be needed to identify a specific marker of EPCs. In addition, we have shown that Ang II decreased the number of EPC colonies, and that this effect of Ang II was significantly abolished by the pre-treatment with pioglitazone. The EPC colony has been used as a quantitative variable for EPCs in peripheral blood and has been regarded as a cardiovascular prognostic factor (*14*, *36*). Considering the growing importance of the EPC colonies in cardiovascular research, it is crucial to investigate the characteristics of the EPC colony in detail. Very recently, Hur *et al*. (*37*) reported for the first time that a specific subset of T cells (CD3+CD31+CXCR4+) make up the central cluster of EPC colonies. They also found that this subset of T cells could enhance EPC differentiation and angiogenesis, resulting in neovascuclarization *in vivo*. Finally, although we have shown that pioglitazone may attenuate Ang II–induced senescence and oxidative stress *in vitro*, it remains unknown whether these protective effects of pioglitazone on EPCs could extend to an *in vivo* setting.

In conclusion, given that EPCs may contribute to postnatal neovascularization, the protective effects of pioglitazone on Ang II–induced cellular senescence in EPCs may affect the process of vascular remodeling.

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