Original Paper

Angiotensin II-Induced Egr-1 Expression is Suppressed by Peroxisome Proliferator-Activated Receptor- γ Ligand 15d-PGJ₂ in Macrophages

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Key WordsEgr-1 • Angiotensin II • Inflammation • Macrophages • PPAR- γ ligand 15d-PGJ₂**Abstract**

Background/Aims: Angiotensin II (Ang II) plays a critical role in regulating vascular inflammatory diseases, such as atherosclerosis and hypertension. Early growth response-1 (Egr-1) is an immediate early gene that acts as a master switch for the inflammatory response. However, the role of Ang II in regulating Egr-1 expression in macrophages, and the effect of peroxisome proliferators-activated receptor- γ (PPAR- γ) ligand 15d-PGJ₂ in this process remain to be investigated. **Methods and Results:** We showed that Ang II significantly up-regulated the expression of Egr-1 in RAW264.7 macrophages, and this effect was markedly attenuated by Eprosartan (an AT1R blocker), SP600125 (a JNK-specific inhibitor) and PD98059 (an ERK-specific inhibitor). Moreover, treatment of macrophages with 15d-PGJ₂, a natural PPAR- γ ligand, significantly reduced Ang II-induced expression of Egr-1 and its inflammatory gene targets (IL-1 β , TNF- α , TGF- β , MCP-1 and ICAM-1) through PPAR- γ activation and ROS formation. In addition, 15d-PGJ₂ treatment markedly inhibited Ang II-induced macrophage migration and proliferation. **Conclusions:** This study for the first time demonstrates that Ang II can induce the expression of Egr-1 via AT1R/JNK and ERK signaling pathways. Activation of PPAR- γ by 15d-PGJ₂ suppresses Egr-1-mediated proinflammatory response, and may be a novel therapeutic strategy for treatment of vascular inflammatory diseases.

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Introduction

Macrophages play critical roles in the development of many vascular inflammatory diseases, including atherosclerosis and hypertension [1]. Angiotensin II (Ang II) is the major bioactive hormone peptide of rennin-angiotensin system, which functions mainly through angiotensin type 1 receptor (AT1R) and its downstream signals, such as mitogen-activated protein kinase (MAPK) pathways [2]. Moreover, Ang II plays an important role in regulating macrophages to induce the production of inflammatory cytokines during various pathological insults [3]. Recently, Li et al found that Ang II activates MAPK pathway in macrophages which promotes the expression of C-reactive protein [4].

Early growth response protein 1 (Egr-1, also known as NGFI-A, zif268, krox-24, and TIS8), is a zinc finger transcription factor, and is rapidly up-regulated in many cell types in response to various stimuli, such as growth factors, cytokines, mechanical stimuli and oxidative stress [5]. Increasing evidence indicates that Egr-1 is a key master regulator to activate the expression of several genes involved in the development of cardiovascular disorders, including atherosclerosis, ischemic pathology, allograft rejection and cardiac hypertrophy [6]. However, the mechanism for Ang II to regulate Egr-1 expression in macrophages remains unclear.

Peroxisome proliferator-activated receptor- γ (PPAR- γ), a ligand-dependent transcriptional factor, is a member of nuclear hormone receptors which regulates target genes by binding PPAR response elements in their promoter regions. It was reported that PPAR- γ plays a critical role in morphogenesis, cell growth and differentiation, lipid and glucose metabolism [7]. PPAR- γ is also involved in many inflammatory diseases, such as hypertension, atherosclerosis, kidney dysfunction and cancer [8, 9]. 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) is a natural peroxisome proliferators-activated receptor- γ (PPAR- γ) ligand [10]. Several studies have shown that 15d-PGJ₂ can modulate the inflammatory response in various diseases, such as diabetes, atherosclerosis and acute pancreatitis [11]. Moreover, 15d-PGJ₂ treatment inhibits the activity of bone marrow-derived monocyte/macrophages in chronic liver injury [12]. However, it is still unknown whether 15d-PGJ₂ affects Ang II-induced Egr-1 expression in macrophages.

In this study, we aimed to explore the effect of Ang II on Egr-1 expression and investigated whether 15d-PGJ₂ could modulate this process in macrophages. We demonstrated that Ang II markedly up-regulated the expression of Egr-1, and this effect was markedly blocked by the inhibitors of AT1R, JNK or ERK in macrophages. Moreover, 15d-PGJ₂ treatment markedly reduced Ang II-induced expression of Egr-1 and its gene targets and macrophage migration. Thus, the present study for the first time reveals that Ang II is capable of inducing the expression Egr-1 via AT1R/JNK/ERK signal pathway, and this effect was inhibited by 15d-PGJ₂.

Materials and Methods

Antibodies and reagents

Dulbecco modified Eagle medium (DMEM), trypsin and fetal calf serum (FCS) were purchased from Hyclone (South Logan, UT). The anti-Egr-1, anti-AT1R, anti-AT2R and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK, anti-phospho-ERK, anti-ERK and anti-mouse/anti-rabbit conjugated antibodies were from Cell Signaling Technology (Beverly, MA). The 15d-PGJ₂ was purchased from Cayman Chemical (Ann Arbor, MI). The Ang II, Eprosartan (inhibitor of AT1R), PD123319 (inhibitor of AT2 receptor), GW9662 (antagonist of PPAR- γ) and N-acetylcysteine (NAC, inhibitor of ROS production) were obtained from Sigma-Aldrich (St Louis, MO). SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor) and PD98059 (ERK inhibitor) were obtained from Calbiochem (La Jolla, CA). All other chemicals were from Sigma-Aldrich (St Louis, MO), unless specified otherwise.

Cell culture and treatment

Raw 264.7 cell line was purchased from ATCC and cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C under a humidified atmosphere of 95% air and 5% CO₂. To investigate which receptor mediates Ang II-induced expression of Egr-1, cells were pretreated with various inhibitors, including Eprosartan, (1 μ mol/L), PD123319 (1 μ mol/L), SP600125 (10 μ mol/L), PD98059 (10 μ mol/L) and SB203580 (10 μ mol/L) for 0.5 hour and then treated with Ang II (100 nmol/L) for the indicated time points.

Isolation of peritoneal macrophages

8-week-old male mice were injected intraperitoneally with 3% thioglycolate 3 days, peritoneal macrophages were then collected by peritoneal lavage with 10 ml of phosphate-buffered saline and centrifuged at 1,000 rpm for 10 min. Cells were then incubated with RPMI1640 media supplemented 10% fetal bovine serum on the 24-well plate for 1 hour and used for the experiments [13].

Quantitative real-time PCR analysis

Total RNA was extracted from cultured cells with Trizol (Invitrogen). Specific primers used for quantitative real-time PCR (qPCR) assays were 5'-CAGTCCCCTACT CCGCTG-3'(sense) and 5'-TGTGGAACAGATAGTCAGGGAT-3' (anti-sense) for Egr-1, and others as described previously [14, 15]. qPCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) according to the manufacturer's protocol [16].

Western blot

The cells were lysed with extraction buffer (50 mmol/L Tris-HCl pH 7.5, 0.5% Triton X-100, 0.5% NP40, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L PMSF, 1 mmol/L Na₃VO₄) and protease inhibitors. Western blot analysis was performed as previously described. Fifty-sixty micrograms of proteins were separated by SDS-PAGE on a 10% gel and then transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies against Egr-1 (1:1000), AT1R (1:500), AT2R (1:500), JNK (1:500), ERK (1:1000), p38 MAPK (1:500) and β -actin (1:2000) overnight, then incubated with an HRP-conjugated secondary antibody for 1 hour. The bands were scanned, and densitometry analysis was performed with the software Image J 2x (National Institutes of Health, Bethesda, MD) [17-20]. For the phospho-specific protein, we calculated the relative intensity for target proteins through dividing the absolute intensity of phosphorylated proteins by the absolute intensity of total target proteins or β -actin.

Cell migration assay

Cell migration was determined in Boyden chambers as described [21]. Briefly, cells were serum-starved for 8 hours and then exposed to 15d-PGJ₂ or vehicle (negative control) for 0.5 hour. Then 4×10^4 cells were seeded to the upper chamber. Cell migration was allowed to proceed for 16 hours at 37°C in 5% CO₂. Cells migrated to the lower surface of the filter were stained 1% crystal violet in 2% ethanol for 20 min, quantified by cell counting under a microscope by randomly choosing different views and taking average counting.

Statistical analysis

All data were expressed as mean $\pm$ SEM. Differences between groups were evaluated for statistical significance using a Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Ang II enhances Egr-1 expression in macrophages

To determine the effect of Ang II on Egr-1 expression, RAW 264.7 cells were treated with Ang II for 0.5, 1, 2 and 4 hours after overnight starvation. Western bolt analysis showed that the expression of Egr-1 was significantly increased after Ang II treatment and peaked at 2 hours (Fig. 1A). Moreover, this increased expression of Egr-1 was in a dose-dependent manner (Fig. 1B). These results indicate that Ang II can induce Egr-1 expression in microphages.

Fig. 1. Ang II increases Egr-1 expression in macrophages. (A) RAW264.7 cells were treated with Ang II (100 nmol/L) for 0, 0.5, 1, 2 and 4 hours after overnight starvation. The expression of Egr-1 was determined by Western blot analysis. Representative Western blots were shown (top). The intensity of protein bands was quantified and shown as the ratio to control (0 h) after normalization by β -actin (bottom). (B) RAW264.7 cells were treated with different doses of Ang II (0, 10, 100 and 1000 nmol/L) for 2 hours after overnight starvation. The expression of Egr-1 was determined by Western blot analysis (top). The intensity of protein bands was quantified as in A (bottom). Results are expressed as mean $\pm$ SEM (n=3). * P < 0.05 versus control (0 hour).

Ang II upregulates Egr-1 expression through AT1R

Ang II is known to regulate cells through the activation of angiotensin II type 1 and type 2 receptors (AT1R and AT2R) [22]. We then determined the effect of Ang II on the expression of AT1R and AT2R in both Raw264.7 cells and peritoneal macrophages. We found that Ang II treatment significantly increased the expression of AT1R but not AT2R in both cell types (Fig. 2A). To determine which receptor is involved in the regulation of Egr-1 by Ang II, peritoneal cells were pretreated with Eprosartan (inhibitor of AT1R) and PD123319 (inhibitor of AT2R) for 0.5 hour and then stimulated with Ang II for additional 2 hours. As shown in Fig. 2B and C, Ang II-induced upregulation of Egr-1 mRNA and protein was significantly abolished by Eprosartan in macrophages, but not by PD123319. Moreover, the expression pattern of Egr-1 was confirmed in the RAW 264.7 (Fig. 2D). These results suggest that Ang II induces Egr-1 expression through the activation of AT1R.

Ang II induces Egr-1 expression via the JNK and ERK pathways

Since MAPKs (including JNK, p38 and ERK) are the major downstream effectors of AT1R, we then examined the levels of JNK, p38 and ERK phosphorylation in RAW 264.7 cells after Ang II treatment at various time points. Consistent with previous reports [23], we found that Ang II treatment markedly increased the levels of JNK, p38 and ERK phosphorylation, which peaked at 15 min (p38 MAPK and ERK) or 30 min (JNK) (Fig. 3A), indicating that Ang II can induce the activation of JNK, p38 and ERK signaling pathways in macrophages.

To further determine which signaling pathway contributes to Ang II-induced Egr-1 expression, RAW 264.7 cells were pretreated with specific MAPK inhibitors, including SP600125 (a JNK inhibitor), SB203580 (a p38 MAPK inhibitor) and PD98059 (a ERK inhibitor) for 0.5 hour and then stimulated with Ang II for additional 2 hours. As shown in Fig. 3B, SP600125 and PD98059 significantly abolished Ang II-induced expression of Egr-1, whereas SB203580 had the opposite effect.

15d-PGJ₂ suppresses Ang II-induced Egr-1 expression via PPAR- γ activation and ROS formation

It is known that PPAR- γ is expressed in macrophages in response to Egr-1-dependent inflammation [12]. We then determined whether the activation of PPAR- γ can regulate

Fig. 4. 15d-PGJ₂ suppresses Ang II-induced Egr-1 expression. RAW264.7 cells were pretreated with GW9662 (1 μ mol/L) or NAC (2.5 mmol/L) for 0.5 h and then treated with 15d-PGJ₂ (5 μ mol/L) for additional 2 hours. The expression of Egr-1 was determined by qPCR analysis (A) and Western blot analysis (B). The intensity of protein bands was quantified (bottom). Data are expressed as mean $\pm$ SEM (n=3). * P < 0.05 versus control. # P < 0.05 versus Ang II alone.

Fig. 5. 15d-PGJ₂ inhibits inflammatory cytokine expression in macrophages after Ang II stimulation. RAW264.7 cells were pretreated with 15d-PGJ₂ (5 μ mol/L) for 0.5 h and then stimulated by Ang II for additional 2 hours. The mRNA levels of IL-1, TNF- α , MCP-1, TGF- β 1 and ICAM were measured by qPCR analysis. Data are expressed as mean $\pm$ SEM (n=3). * P < 0.05 versus control, # P < 0.05 versus Ang II alone.

Ang II-induced Egr-1 expression. RAW 264.7 cells were pretreated with 15d-PGJ₂ for 0.5 hour and then stimulated with Ang II for additional 2 hours. Ang II treatment markedly upregulated Egr-1 expression, whereas 15d-PGJ₂ significantly attenuated this effect (Fig. 4A and B). Moreover, the inhibition of Egr-1 by 15d-PGJ₂ was partially restored in the presence of PPAR- γ inhibitor GW9662 or ROS inhibitor NAC (Fig. 4A and B), indicating that 15d-PGJ₂ reduces the expression of Egr-1 at least partially through activation of PPAR- γ and ROS pathways.

Fig. 6. 15d-PGJ₂ reduces macrophage migration and proliferation after Ang II stimulation. RAW264.7 cells were pretreated with 15d-PGJ₂ (5 μ mol/L) for 0.5 h and then stimulated by Ang II for additional 16 hours. (A) Cell migration was measured by stained with 1% crystal violet in 2% ethanol. A representative field was shown for each condition (top). Quantitative analysis of cell migration was shown (bottom). Bar: 50 μ m. (B) MTT analysis of cell proliferation was shown (right). Data are expressed as mean $\pm$ SEM (n=3). * P < 0.05 versus control. # P < 0.05 versus Ang II alone.

15d-PGJ₂ inhibits the expression of Egr-1-mediated inflammatory cytokines in macrophage after Ang II stimulation

Egr-1 is an initial factor of inflammation [24]. Ang II can increase the expression of inflammatory gene targets of Egr-1 in macrophages. We next examined whether the activation of PPAR- γ can affect the expression of Egr-1-mediated inflammatory genes. RAW 264.7 cells were pretreated with 15d-PGJ₂ for 0.5 hour and then stimulated with Ang II for additional 2 hours. 15d-PGJ₂ treatment significantly inhibited the Ang II-induced up-regulation of IL-1 β , TNF- α , TGF- β , MCP-1 and ICAM mRNA expression (Fig. 5). Together, these data indicate that 15d-PGJ₂ can inhibit Egr-1-mediated inflammatory response in macrophages.

15d-PGJ₂ reduces macrophage migration and proliferation after Ang II stimulation

15d-PGJ₂ has also been reported to play an important role in regulating the migration of inflammatory cells [21]. Therefore, we examined the effect of 15d-PGJ₂ on Ang II-induced macrophage migration and proliferation. A trans-well migration assay showed that treatment of macrophages with 15d-PGJ₂ markedly decreased Ang II-induced migration of macrophages (Fig. 6A). MTT analysis further indicated that 15d-PGJ₂ significantly reduced macrophage proliferation induced by Ang II (Fig. 6B).

Discussion

In this study, we for the first time demonstrated that Ang II stimulation drastically increased Egr-1 expression in macrophages. This effect was mainly mediated via AT1R/JNK/ERK signaling pathways. Moreover, 15d-PGJ₂, a natural PPAR- γ ligand, markedly reduced Ang II-induced expression of Egr-1 and its inflammatory gene targets as well as macrophage migration.

Egr-1 is known as the master transcription factor that plays a critical role in regulating cell proliferation, differentiation and apoptosis. It is rapidly and transiently expressed in many cell types in response to various stimuli [25]. For example, hypoxia rapidly induces Egr-1 expression in mononuclear phagocytes [26]. Importantly, Egr-1 has been implicated in the pathophysiology of several vascular diseases. Inhibition of its activity by siRNA or

small molecules markedly attenuates the proliferation of vascular smooth muscle cells and neointimal growth [27]. In addition, knockout of Egr-1 markedly reduces the development of atherogenesis [28]. However, how Ang II regulates Egr-1 in macrophages is still unknown. In this study, our data showed that Ang II dramatically increased Egr-1 expression (Fig. 1), and this effect was abolished by the inhibitors of AT1R, JNK and ERK (Fig. 2 and 3). Collectively, these data suggest that the activation of AT1R/JNK/ERK signaling pathways is required for Ang II-induced expression of Egr-1 in macrophages.

PPAR- γ , a member of the nuclear receptor superfamily, is mainly found in adipocytes and functions in regulating adipocyte differentiation and glucose homeostasis [29]. Several studies demonstrated that PPAR- γ can be dramatically induced in monocytes/macrophages and T cells by inflammatory cytokines [29]. Increasing evidence suggests that a natural PPAR γ agonist 15d-PGJ₂ is a key anti-inflammatory mediator [30], and the anti-inflammatory action is mediated through either PPAR- γ -dependent or independent pathways [31]. For example, 15d-PGJ₂ regulates the expression of TNF- α and MCP-1 in bone marrow-derived monocytes/macrophages via a PPAR- γ -dependent manner [21]. Furthermore, NF- κ B is implicated in the inhibitory effects of 15d-PGJ₂ on the inflammatory response [32]. However, little is known about the effect of 15d-PGJ₂ on the expression of Egr-1 and its target genes. Accumulating evidence indicates that Egr-1 is a zinc-finger transcription factor that can bind to the consensus DNA sequence GCG[T/G]GGGCG in the promoter and enhancer regions of many genes. It induces the expression of many inflammatory genes, such as IL-1 β , TNF- α , MCP-1 and ICAM as well as procoagulant genes [26, 33]. Our results here showed that 15d-PGJ₂ treatment markedly reduced Ang II-induced expression of Egr-1 as well as the expression of IL-1 β , TNF- α , TGF- β , MCP-1 and ICAM in macrophages, and PPAR- γ inhibitor GW9662 or ROS inhibitor NAC markedly abolished these effects (Fig. 4). Thus these results suggest that the inhibitory effect of 15d-PGJ₂ on Egr-1 is partially via PPAR- γ and ROS production.

In conclusion, our results demonstrated that Ang II markedly enhanced Egr-1 expression in macrophages via AT1R/JNK/ERK signaling pathways. 15d-PGJ₂ significantly inhibits the expression of Egr-1 and its downstream targets as well as macrophage migration partially through activation of PPAR- γ and ROS pathways. Thus, the present study has identified a novel mechanism of Ang II-regulated Egr1 expression, which may provide a new therapeutic target for vascular inflammatory diseases.

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Disclosure Statement

The authors declared no conflict of interest.

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