Original Paper

Adiponectin Inhibits TNF- α -Activated PAI-1 Expression Via the cAMP-PKA-AMPK-NF- κ B Axis in Human Umbilical Vein Endothelial Cells

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Key Words

Antifibrotic effect • Binding capability • Promoter • Plasminogen activator inhibitor • Adiponectin

Abstract

Background: Tumor necrosis factor (TNF)- α can upregulate the expression of plasminogen activator inhibitor (PAI)-1, an inhibitor of fibrinolysis. Adiponectin (Adp) antagonizes TNF- α by negatively regulating its expression in various tissues. In the present study, the ability of Adp to suppress TNF- α -induced PAI-1 upregulation and the underlying mechanisms were evaluated. **Methods:** Human umbilical vein endothelial cells (HUVECs) were treated with TNF- α in the presence or absence of Adp, and *PAI-1* mRNA and antigen expression, activated signaling pathways, and molecular mechanisms were analyzed by qRT-PCR and ELISA. **Results:** Adp decreased the TNF- α -induced upregulation of *PAI-1* mRNA and protein expression and suppressed TNF- α -induced cAMP-PKA-AMPK inactivation. Adp also suppressed the TNF- α -induced NF- κ B binding capability on the *PAI-1* promoter. Moreover, these Adp-induced effects were further enhanced or prevented by treatment with the cAMP inhibitor Rp-cAMPS or activator forskolin, respectively. **Conclusions:** Our data suggest that Adp abrogates TNF- α -activated PAI-1 expression by activating cAMP-PKA-AMPK signaling to suppress NF- κ B binding to the *PAI-1* promoter in HUVECs. Given the antifibrotic effect of PAI-1 abrogation, Adp may be utilized as a novel agent in the treatment of fibrotic diseases.

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Introduction

Atherosclerosis-associated cardiovascular disease is the leading cause of death in developed countries, and its incidence is increasing rapidly in developing countries. It has been predicted that atherosclerosis will be the primary cause of death worldwide by 2020 [1]. Atherosclerosis is a chronic, systemic disease and multiple factors are involved in its initiation and progression [2], with a growing body of evidence pointing to inflammation and endothelial dysfunction as key factors [3]. Endothelial dysfunction could cause abnormalities of the fibrinolytic system, which plays a significant role in the development of atherosclerotic plaques [4]. Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), and hence is an inhibitor of fibrinolysis [5]. PAI-1 plays a pivotal role in cardiovascular diseases, including arteriosclerosis, since atherosclerotic lesions tend to show increased PAI-1 expression levels [6]. Therefore, studies are needed to determine the regulatory mechanism of PAI-1 expression in order to gain a better understanding of the development of atherosclerosis-associated cardiovascular disease.

Activation of vein endothelial cells by various inflammatory stimuli, including tumor necrosis factor- α (TNF- α), increases the adherence of monocytes, which is considered a crucial step in the development of vascular diseases. Adiponectin (Adp) is an important adipokine involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic, and anti-inflammatory activities. Adp can antagonize TNF- α by negatively regulating its expression in various tissues, such as the liver, as well as in macrophages, and by counteracting its effects. Indeed, low Adp levels are associated with vascular dysfunction, and Adp-based treatment is beneficial for atherosclerosis and endothelial cell dysfunction [7]. In human umbilical vein endothelial cells (HUVECs), TNF- α treatment could increase both *PAI-1* mRNA expression and protein release [8, 9]. Therefore, we hypothesized that Adp might be a key factor involved in inhibiting TNF- α -induced PAI-1 expression.

Adp can stimulate AMP-activated protein kinase (AMPK) phosphorylation and activation in liver cells, thereby enhancing glucose utilization and fatty acid combustion [10]. In addition, Adp can inhibit endothelial nuclear factor-kappa B (NF- κ B) activity stimulated by TNF- α via a cAMP-dependent pathway in human aortic endothelial cells [11]. Furthermore, we previously reported that Adp treatment could inhibit TNF- α -induced NF- κ B p65 activity in HUVECs and increase the AMPK phosphorylation level [12]. Therefore, the aim of the present study was to examine whether Adp regulates TNF- α -induced PAI-1 expression via the cAMP-AMPK-NF- κ B signaling pathway.

To test this hypothesis, HUVECs were treated with TNF- α in the absence and presence of Adp, and the effects on PAI-1 mRNA and protein expression were evaluated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. In addition, the binding capacity of NF- κ B on the *PAI-1* promoter was assessed under various treatments with chromatin immunoprecipitation (ChIP) assays using mutant promoter constructs. The role of the cAMP/protein kinase A (PKA) signaling pathway was evaluated using specific inhibitors to determine the underlying regulatory mechanism. These results are expected to provide new insights into the molecular pathological mechanisms contributing to the development and progression of atherosclerosis and suggest new treatment targets.

Materials and Methods

HUVEC isolation and culture

HUVECs were isolated from human umbilical cords collected from normal deliveries at the First Affiliated Hospital of Gannan Medical University. Signed consent was obtained from all donors, and the study was approved by the Institutional Review Board of the First Affiliated Hospital of Gannan Medical

University. The vein in the cord was washed with pre-warmed phosphate-buffered saline (PBS) using a 50-ml syringe and then filled with Hanks' balanced salt solution (HBSS) containing 0.2% collagenase type II. After 15 min, HBSS was collected in a tube and centrifuged at $300 \times g$ for 5 min. Isolated cells were plated in 100-mm-diameter culture dishes coated with 0.1% gelatin (Sigma Aldrich, St. Louis, MO, USA) and cultured in M199 medium containing 10% fetal bovine serum and 1% antibiotics. The cells were grown at 37°C in a humidified atmosphere with 5% CO₂. The cells plated on the culture dish were regarded as passage 0, and those obtained from passages 3 to 4 were used in the experiments.

Cell treatments

To detect the effect of TNF- α on PAI-1 expression, HUVECs were treated with various concentrations of recombinant TNF- α protein (1, 10, 20, 50, or 100 ng/ml) for 12 h or with 10 ng/ml TNF- α for 1, 3, 6, 12, or 24 h. To detect the suppressive effect of Adp on TNF- α -induced effects, HUVECs were treated with 10 ng/ml TNF- α plus 0 (control), 10 μ g/ml, 20 μ g/ml, or 30 μ g/ml Adp. To detect the role of the cAMP/PKA pathway in the regulation of Adp and TNF- α -induced effects, HUVECs were treated with 10 ng/ml TNF- α plus 30 μ g/ml Adp in the presence or absence of a cAMP/PKA pathway inhibitor (10 μ M RP-cAMPs) or activator (10 μ M forskolin).

RNA extraction, reverse transcription, and PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in a 25- μ l volume. Expression levels were quantitated by real-time PCR using the ABI PRISM 7500 sequence detection system with SYBR Green qPCR SuperMix (Invitrogen). The expression level of each mRNA was normalized to that of β -actin (endogenous control) and calculated using the 2^{- $\Delta\Delta$ Ct} method. The primers were as follows: PAI-1-F: 5'-TGCCCTCTACTTCAACGG-3', PAI-1-R: 5'-GTCGGTCATTCCCAGGTT-3'; β -actin-F: 5'-ATCTGGCACCACCTTCTA-3', β -actin-R: 5'-CTCGGTGAGGTCTCATGA-3'.

ELISA for PAI-1, NF- κ B, and PKA

ELISA kits were used to detect the expression of PAI-1 (Human PAI-1 Total Antigen Assay; Molecular Innovations, Novi, MI, USA), NF- κ B (P65) activity (KAA065; Rockland, Limerick, PA, USA), and PKA activity (K027-H1; Arbor Assays, Ann Arbor, MI, USA), according to the manufacturers' protocols.

Western blotting

HUVECs treated under the various conditions described in the Cell treatments section above were washed twice with ice-cold PBS and resuspended in ice-cold RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride and a cocktail of protease inhibitors (1:100 dilution; Beyotime, Nantong, China). Samples were centrifuged at 4°C for 15 min at 14,000 rpm. Supernatants were recovered and total proteins were quantified using a BCA Protein Assay kit (Thermo Scientific Pierce, Rockford, IL, USA). The western blot analysis was carried out according to our previous description [13]. Briefly, equal amounts of protein were loaded and separated on 8–12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h at room temperature with 5% milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). They were then incubated for 1 h with the primary antibodies (anti-AMPK, 1:2,000; anti-p-AMPK, 1:2,000; Abcam, Cambridge, MA, USA), washed three times with TBST, incubated with the secondary antibody for 40 min, washed three more times with TBST, and finally visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

cAMP assays

The cells (2×10^5 cells/well) were seeded in wells of a 24-well plate. After experimental treatment, the incubation medium was aspirated. The cells were lysed with 200 μ l of 1 M HCl containing 0.1% Triton X-100, and the lysis solution was then transferred to microcentrifuge tubes. After centrifugation at $1300 \times g$ for 5 min at room temperature, the cellular cAMP concentrations in the supernatants were determined using a direct enzyme immunoassay kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Promoter constructs, mutagenesis, and luciferase assay

The upstream promoter fragments located at 1000 bp from the transcriptional start site of the human *PAI-1* promoter were generated by PCR amplification from human genomic DNA and inserted into the pGL3 promoter vector carrying the firefly luciferase reporter gene (Promega). The NF- κ B-binding sites from -288 bp to -297 bp of the full-length *PAI-1* promoter region were identified using ConSite (<http://consite.genereg.net/>). Site-directed mutagenesis was then employed to mutate each predicted binding site using QuikChange II Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA, USA). All of the *PAI-1* promoter constructs generated were confirmed by DNA sequencing. The plasmid DNA constructs were transfected into HUVECs for 24 h and then the cells were disrupted using a lysis buffer (Promega). The resultant cell lysate (20 μ l) was mixed with luciferase reagent (50 μ l), and the luminescent value was measured. All assays were carried out in triplicate.

ChIP assays

Each group of cells was cross-linked with 1% formaldehyde for 10 min at room temperature and subsequently quenched with glycine. The cells were lysed and the nuclei were isolated from the transfected cells. The resultant chromatin was resuspended in Lysis Buffer 3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.5 mM ethylene glycol tetraacetic acid, 0.1% Na-deoxycholate, 0.5% *N*-lauroylsarcosine, and a cocktail of protease inhibitors) and sonicated to obtain DNA fragments of 100–1000 bp. The samples were treated with 1% Triton X-100 (final concentration) and subjected to centrifugation at 20,000 $\times g$ at 4°C for 10 min. The supernatants of the samples were incubated overnight at 4°C on a rotator with either 4 μ g of NF- κ B antibody or IgG antibody, which served as the sham control. Following incubation, 25 μ l of magnetic A/G beads (Thermo Scientific) was added to each reaction, and the suspension was incubated for 3 h at 4°C with rotation. The beads were washed four times with RIPA buffer (10 mM Tris-HCl, 0.25 M LiCl, 0.5% NP-40, and 0.5% sodium deoxycholate, pH 7.5) and two times with Tris-EDTA buffer supplemented with 50 mM NaCl. The cross-linking was disrupted by treatment with 10% Chelex-100 by boiling. The samples were treated with RNase A and Proteinase K. The resultant DNA was used as a template to analyze the NF- κ B-binding region within the *PAI-1* promoter. The abundance of promoter sequences that were bound by NF- κ B was analyzed by real-time PCR using 2 μ l of the DNA as a template. The experiments were repeated at least three times in triplicate, and the qRT-PCR results are expressed as the percentage of the input [14].

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Chicago, IL, USA). The results are shown as means $\pm$ standard deviation and analyzed using one-way ANOVA followed by post-hoc LSD test. A value of $P < 0.05$ was considered statistically significant.

Results

TNF- α upregulates *PAI-1* expression

The qRT-PCR and ELISA results showed that the treatment of HUVECs with TNF- α for 5 h could upregulate *PAI-1* mRNA expression and PAI-1 antigen levels at all concentrations tested from 1 ng/ml to 100 ng/ml (Fig. 1A, B), with both levels showing a peak in response to 10 ng/ml TNF- α . We further detected PAI-1 mRNA and antigen levels after 10 ng/ml TNF- α treatment at different time points. Both the mRNA and antigen levels increased dramatically after 1 h of treatment, reached a peak at 6 h, and then were maintained at this high level thereafter (Fig. 1C, D). Therefore, treatment with 10 ng/ml TNF- α for 6 h was used for subsequent assays.

Adp prevents TNF- α -induced *PAI-1* upregulation

To investigate the role of Adp in regulating the TNF- α -induced *PAI-1* upregulation, HUVECs were treated with 10 ng/ml TNF- α with or without various concentrations of Adp (10 μ g/ml, 20 μ g/ml, 30 μ g/ml) for 6 h. The qRT-PCR and ELISA results showed that Adp co-treatment could decrease the TNF- α -induced PAI-1 mRNA and antigen levels at all

Fig. 1. The effect of TNF- α on PAI-1 mRNA and antigen levels in HUVECs. A) PAI-1 mRNA levels after treatment with various concentrations of TNF- α (1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml). B) PAI-1 antigen levels after treatment with various concentrations of TNF- α (1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml). C) PAI-1 mRNA levels after treatment with 10 ng/ml TNF- α at different time points. D) PAI-1 antigen levels after treatment with 10 ng/ml TNF- α at different time points. * $P < 0.05$.

Fig. 2. The effect of adiponectin (Adp) on TNF- α -induced PAI-1 upregulation in HUVECs. A) PAI-1 mRNA levels after treatment with various concentrations of Adp (10 μ g/ml, 20 μ g/ml, 30 μ g/ml) plus 10 ng/ml TNF- α . B) PAI-1 antigen levels after treatment with various concentrations of Adp (10 μ g/ml, 20 μ g/ml, 30 μ g/ml) plus 10 ng/ml TNF- α . * $P < 0.05$.

concentrations (Fig. 2A, B) in a dose-dependent manner. These results indicated that Adp could inhibit TNF- α -activated PAI-1 expression.

Adp inhibits TNF- α -induced PAI-1 by activating the cAMP-PKA-AMPK signaling pathway

To investigate the mechanism by which Adp inhibits TNF- α -activated PAI-1 expression, we evaluated the potential role of the cAMP-PKA-AMPK signaling pathway. As shown in Fig. 3 A-C, TNF- α treatment inactivated the cAMP-PKA-AMPK signaling pathway, and Adp co-treatment weakened this effect. Furthermore, co-treatment with the cAMP inhibitor

Fig. 3. Adiponectin (Adp) inhibits TNF- α -induced PAI-1 upregulation by activating the cAMP-PKA-AMPK signaling pathway. A) Effect of 30 μ g/ml Adp and/or 10 ng/ml TNF- α treatment on cAMP levels. B) Effect of 30 μ g/ml Adp and/or 10 ng/ml TNF- α treatment on PKA activity. C) Representative gel (left) and quantification (right) of the effect of 30 μ g/ml Adp and/or 10 ng/ml TNF- α treatment on the AMPK phosphorylation level. D) Effect of co-treatment with the cAMP inhibitor Rp-cAMPs or cAMP activator forskolin plus 30 μ g/ml Adp and 10 ng/ml TNF- α on the PAI-1 mRNA level. E) Effect of co-treatment with the cAMP inhibitor Rp-cAMPs or the cAMP activator forskolin plus 30 μ g/ml Adp and 10 ng/ml TNF- α on the PAI-1 antigen level.

Rp-cAMP or activator forskolin increased or decreased the PAI-1 mRNA and antigen levels, respectively, compared to the levels observed using 30 μ g/ml Adp and 10 ng/ml TNF- α alone (Fig. 3D, E). These results revealed that Adp inhibits TNF- α -induced PAI-1 by activating the cAMP-PKA-AMPK signaling pathway.

Adp inhibits TNF- α -induced PAI-1 by suppressing the binding capacity of NF- κ B on the PAI-1 promoter

A binding site of NF- κ B was found in the PAI-1 promoter from -288 bp to -297 bp (GGTATTCCCC). Therefore, we investigated the role of NF- κ B in regulating PAI-1 transcription. As shown in Fig. 4A, 10 ng/ml TNF- α treatment increased NF- κ B activity, which was significantly decreased by treatment with 30 μ g/ml Adp plus 10 ng/ml TNF- α . In addition, the ChIP assays showed that TNF- α treatment increased the luciferase activity of the wild-type full-length PAI-1 promoter construct, which was decreased by 30 μ g/ml Adp plus 10 ng/ml TNF- α ; however, this co-treatment had no obvious effect on the luciferase activity of the mutant-type full-length PAI-1 promoter construct. As shown in Fig. 4C, TNF- α

Fig. 4. Adiponectin (Adp) inhibits TNF- α -induced PAI-1 upregulation by suppressing the binding capacity of NF- κ B on the PAI-1 promoter. HUVECs were treated with 30 μ g/ml Adp and/or 10 ng/ml TNF- α , and cells were harvested for A) NF- κ B activity detection, B) luciferase assays, and C) ChIP assays to detect the binding capacity of NF- κ B on the PAI-1 promoter. * $P < 0.05$

Fig. 5. Adiponectin (Adp) suppresses NF- κ B activity and binding capacity by activating the cAMP-PKA-AMPK signaling pathway. HUVECs were treated with the cAMP inhibitor Rp-cAMPs or activator forskolin plus 30 μ g/ml Adp and 10 ng/ml TNF- α cells were harvested for A) NF- κ B activity detection, B) luciferase assays, and C) ChIP assays to detect the binding capacity of NF- κ B on the PAI-1 promoter. * $P < 0.05$.

treatment increased NF- κ B binding on the PAI-1 promoter, which was decreased by 30 μ g/ml Adp plus 10 ng/ml TNF- α .

Adp decreases NF- κ B activity and binding capacity by activating the cAMP-PKA-AMPK signaling pathway

As shown in Fig. 5A, co-treatment with the cAMP inhibitor Rp-cAMPs or activator forskolin increased or decreased NF- κ B activity, respectively, compared to that of the treatment group with 30 μ g/ml Adp and 10 ng/ml TNF- α alone. In addition, Rp-cAMPs or forskolin co-treatment increased or decreased the luciferase activity of the wild-type full-length PAI-1 promoter construct, respectively, compared to that of the 30 μ g/ml Adp and 10 ng/ml TNF- α treatment (Fig. 5B). However, there was no obvious effect of either the cAMP inhibitor or activator on luciferase activity of the mutant-type full-length PAI-1 promoter construct in 30 μ g/ml Adp and 10 ng/ml TNF- α -treated HUVECs. As shown in Fig. 5C, Rp-

cAMPs or forskolin co-treatment, respectively, increased or decreased NF- κ B binding on the *PAI-1* promoter compared to that of the 30 μ g/ml Adp and 10 ng/ml TNF- α treatment. These results further indicated that Adp suppresses NF- κ B activity and binding by activating the cAMP-PKA-AMPK signaling pathway.

Discussion

The abnormal function of the fibrinolytic system is a causal pathophysiological factor in atherosclerosis [4, 15]. Several studies have shown that PAI-1 deficiency is associated with a reduced tissue fibrosis. PAI-1 is a plausible target for controlling the pathogenesis of fibrosis [5]; accordingly, it is critical to find new methods to decrease PAI-1 expression for the treatment and prevention of fibrosis-related diseases, such as atherosclerosis-associated cardiovascular disease. As one of the key regulatory factors in the fibrinolysis process of plasmin, TGF- α positively regulates *PAI-1* expression in many cell types, such as granulosa cells, the human hepatoma cell line HepG2, and HUVECs [8, 16, 17]. Therefore, we aimed to identify regulators of TGF- α -induced PAI-1 upregulation. We demonstrated that Adp could decrease the TNF- α -induced elevation of PAI-1, a major regulator of the fibrinolytic system. In addition, we found that Adp exerts these regulatory effects via the cAMP-PKA-AMPK-NF- κ B axis. Our results provide new insight into the molecular mechanisms underlying the development of atherosclerosis-associated cardiovascular disease and suggest therapeutic strategies.

Adp appears to regulate plaque inflammation and stability by functioning as an anti-inflammatory adipocytokine that reduces the production of inflammatory cytokines and chemokines, such as TNF- α [18, 19]. In addition, TNF- α upregulates PAI-1 expression in pleural mesothelial cells [20]. However, this is the first study to show that Adp plays a role in TNF- α -induced PAI-1 expression at both the mRNA and protein levels in a dose-dependent manner in HUVECs. Collectively, these results reveal that Adp might play an important role in regulating the fibrinolytic system. In addition, elevated PAI-1 levels may be involved in obesity, metabolic syndrome, diabetes mellitus, thrombosis, endometriosis, atherosclerosis, cancer, and multiple organ fibrosis [21-24]; we predicated that Adp may be utilized as a novel agent in the treatment of these human diseases. However, to assess the effects of Adp on fibrinolytic balance, the expression levels of t-PA, u-PA, and u-PA receptor (u-PAR) need to be examined because fibrinolysis is determined by a balance of inhibitors and activators.

PAI is clearly induced by TNF- α . However, the exact mechanisms of PAI-1 expression are complex and remain to be elucidated. Various transcription factors, such as CREB-binding protein, upstream stimulatory factors 1 and 2, Smad2, Smad3, CCAAT box-binding transcription factor, specificity protein 1, and p53, have been implicated in the regulation of PAI-1 expression [25]. However, the regulatory mechanism of *PAI-1* expression is cell type-dependent [26]. TNF- α elicits an inflammatory response via several signal transduction pathways, including the NF- κ B pathway [27]. Given that the *PAI-1* promoter contains a binding site for NF- κ B, we hypothesized that Adp regulates TNF- α -induced PAI-1 expression by altering the binding ability of NF- κ B. This hypothesis was supported by the results of luciferase and ChIP assays, clearly showing that Adp inhibits TNF- α -induced increases in NF- κ B binding to the *PAI-1* promoter, but not to a mutated promoter construct. Therefore, Adp appears to regulate TNF- α -induced PAI-1 antigen expression by transcriptional regulation by affecting the binding activity of NF- κ B. However, other transcription factor-binding sites have also been identified on the *PAI-1* promoter, such as binding sites for AP-1 and SP-1 [20, 28]. For example, glutathione suppresses transforming growth factor- β -induced PAI-1 expression by inhibiting the binding of AP-1 or SP-1 to the *PAI-1* promoter [28]. Therefore, further studies are needed to identify the relationships between these transcription factors with respect to the inhibitory mechanism of Adp on TNF- α -induced *PAI-1* mRNA upregulation.

Extensive evidence indicates that canonical and non-canonical pathways, such as epidermal growth factor receptor, Src kinase, and mitogen-activated protein kinase

pathways, are involved in the regulation of PAI-1 expression [25]. However, the mechanisms of PAI regulation are complex, and other signaling pathways might be involved. Our results indicated that TNF- α treatment could reduce cAMP levels, PKA activity, and AMPK phosphorylation levels, indicating that TNF- α might induce PAI-1 expression via the cAMP/PKA/AMPK signaling pathway. Furthermore, we found that Adp could reduce the inhibitory effects of TNF- α on cAMP levels, PKA activity, and AMPK phosphorylation levels. cAMP/PKA pathways are essential upstream regulators of AMPK activity [29, 30]. Accordingly, we predicted that Adp exerts its effects on TNF- α -induced PAI-1 upregulation via the cAMP/PKA/AMPK signaling pathway. The abrogation or enhancement of the observed effects of Adp on PAI-1 expression and NF- κ B binding capacity in the presence of the cAMP inhibitor Rp-cAMPs or cAMP activator forskolin, respectively, supported this hypothesis. However, histone deacetylase inhibitor impairs plasminogen activator inhibitor-1 expression by inhibiting the TNF- α -activated MAPK/AP-1 signaling cascade [20], and notoginsenoside R1 inhibits TNF- α -induced PAI-1 overexpression in human aortic smooth muscle cells by suppressing ERK and PKB signaling pathways [31]. These results indicate that the exact mechanisms of PAI-1 expression are far more complex than already outlined.

Our study had some limitations, mentioned briefly above. Firstly, the effects of Adp on the expression levels of t-PA, u-PA, and u-PAR were not analyzed and therefore the effects of Adp on fibrinolytic balance are unclear and require further study. Secondly, further studies are needed to identify the roles of relationships between transcription factors in the inhibitory effects of Adp on TNF- α -induced *PAI-1* mRNA upregulation. Thirdly, given the large number of factors involved, genomics and proteomics approaches are needed to analyze the exact mechanism of PAI-1 expression.

Conclusion

In conclusion, the present study demonstrated that Adp could decrease TNF- α -induced PAI-1 expression by suppressing NF- κ B binding to the *PAI-1* promoter in HUVECs via the cAMP/PKA/AMPK signaling pathway. Given that PAI-1 is an inhibitor of fibrinolysis, we expect that Adp may be utilized as a novel agent in the treatment of fibrotic diseases. However, more detailed investigations, especially *in vivo* studies, are needed to confirm these findings and to determine the clinical potential and safety of Adp treatment.

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Disclosure Statement

The authors declare no conflict of interest.

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