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# Adiponectin inhibits macrophage tissue factor, a key trigger of thrombosis in disrupted atherosclerotic plaques

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## Abstract

**Objective**—Adiponectin (APN) is an adipocytokine with anti-atherogenic and anti-inflammatory properties. Hypoadiponectinemia may associate with increased risk for coronary artery disease (CAD) and acute coronary syndrome (ACS). Tissue factor (TF) initiates thrombus formation and facilitates luminal occlusion after plaque rupture, a common cause of fatal ACS. This study tested the hypothesis that APN influences TF expression by macrophages (MΦ), inflammatory cells found in atheromatous plaques.

**Methods**—Human monocyte-derived MΦ or RAW 264.7 cells transfected with TF promoter construct, pretreated with a physiological range of recombinant APN (1–10 μg/ml), received LPS stimulation. TF mRNA and protein levels were quantified by real-time RT-PCR and ELISA. TF pro-coagulant activity was evaluated by one-step clotting assay. TF promoter activity was determined by a dual-luciferase reporter assay. Immunoblot analyses assessed intracellular signaling pathways.

**Results**—APN treatment suppressed TF mRNA expression and protein production in LPS-stimulated human MΦ, compared to vehicle controls. APN treatment also significantly reduced TF pro-coagulant activity in lysates of LPS-stimulated human MΦ, compared to vehicle controls. Moreover, APN suppressed TF promoter activity in LPS-stimulated MΦ compared to controls. APN suppressed phosphorylation and degradation of IκB-α in LPS-stimulated MΦ.

**Conclusions**—APN reduces thrombogenic potential of MΦ by inhibiting TF expression and activity. These observations provide a potential mechanistic link between low APN levels and the thrombotic complications of atherosclerosis.

## Keywords

adiponectin; tissue factor; macrophage; atherothrombosis

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## 1. Introduction

The common cluster of components that comprise the "metabolic syndrome" — including excess visceral fat accumulation, dyslipidemia, impaired glucose tolerance, and hypertension — associate with inflammation, and contribute to an increased risk of developing cardiovascular disease[1]. In patients with coronary artery disease (CAD), atherosclerotic plaque expansion results in progressive luminal obstruction that reduces blood flow and causes tissue ischemia. In contrast to progressive vessel narrowing from stable coronary stenosis, ischemic complications of atherosclerosis frequently occur when non-obstructive atherosclerotic plaques with thin fibrous caps undergo plaque rupture that results in sudden arterial thrombosis.[2] Inflammation contributes to fibrous cap thinning and increases the risk of plaque rupture and ischemic atherothrombotic complications.

Adiponectin (APN), an adipose-specific secretory protein (adipocytokine), has anti-diabetic, anti-atherogenic, and anti-inflammatory properties[3]. Many studies have correlated reduced APN levels in plasma (hypoadiponectinemia) with CAD and with increased risk of cardiovascular events independent of traditional risk factors[4–10]. Some experimental studies, but not all, have shown that APN reduces atherosclerosis by suppressing atherogenic processes within the blood vessel wall[4, 11–16].

Patients with acute coronary syndromes (ACS) have reduced plasma APN compared to patients with stable CAD[17, 18]. In addition, Nakagawa et al. recently reported that nocturnal dysregulation of APN may contribute to ACS in patients with excess visceral fat[19].

Tissue factor (TF; also known as coagulation factor III or tissue thromboplastin) triggers blood coagulation. Upon binding to factor VIIa, the TF/VIIIa complex converts factor X into active proteinase factor Xa — which activates thrombin. Macrophages (MΦ) and other vascular endothelial cells within atheroma express TF[20]. In undisrupted plaques, TF resides within the atheroma, sequestered from luminal blood. Plaque rupture exposes TF within the atheroma to blood, activating the clotting cascade[20]. Even when plaque rupture and thrombosis do not cause arterial occlusion, mural fibrin and platelet deposition likely promote plaque progression and arterial stenosis. We previously demonstrated by microarray screening that APN inhibits the expression of LPS-inducible TF in human MΦ.[15] The current study tested the hypothesis that APN directly regulates MΦ TF expression.

## 2. Materials and Methods

### 2.1. Cell culture

Human monocytes/MΦ were prepared as reported[15]. Differentiated MΦ (Day 10) were incubated in M199 medium containing 1% human serum, with or without recombinant APN, for 24 hours. MΦ then were stimulated with 5 ng/ml of LPS for 6 hours or 15 minutes to analyze TF gene/protein expression (real-time quantitative RT-PCR, ELISA, and one-step clotting assay) or intracellular signaling (immunoblot analysis), respectively. RAW264.7 cells (ATCC, Manassas, VA, USA) were maintained per the supplier's instructions and used for luciferase reporter assay, as described below.

### 2.2. Real-time quantitative RT-PCR

After 6 hours of LPS treatment, total RNA from treated human MΦ were isolated by RNeasy micro kit (QIAGEN, Hilden, Germany). DNase I-treated total RNA was reverse-transcribed, and real-time quantitative PCR with cDNA was performed on an iCycler iQ Real-Time PCR Detection System using SYBR Green I (Bio-Rad, Hercules, CA, USA). GAPDH was used as a reference mRNA to adjust the loading dispersion between samples.

The sequence of sense primers and anti-sense primers was: human tissue factor, 5'-GCCAGGAGAAAGGGGAAT-3' and 5'-CAGTGCAATATAGCATTTGCAGTAGC-3', human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CAATGACCCCTTCATTGACCTC-3' and 5'-AGCATCGCCCCACTTGATT-3'.

### 2.3. Cell Lysates of Monocytes/MΦ and TF ELISA

Monocytes/MΦ were disrupted by repeated freeze–thaw cycles or sonication, and TF was extracted with a buffer of Tris Buffered Saline (50 mM Tris, 100 mM NaCl, pH 7.4) containing 0.1% Triton X-100. Extraction was performed for 18 hours at 2°–8°C, and the lysed cells were centrifuged to remove cell debris. Cell lysates were stored at −70°C until they were assayed. 2 μg of the cell lysate were used to assay tissue factor protein with IMUBIND Tissue Factor ELISA Kit (American Diagnostica Inc, Stamford, CT, USA).

### 2.4. Cell Transfection and Measurement of Luciferase Activity

A human TF promoter fragment (from -278) subcloned into the firefly luciferase reporter vector, pGL2-Basic (TF -278), was obtained from Addgene (Cambridge, MA, USA)[21, 22]. TF-278 was transfected into RAW264.7 cells with lipofectamine LTX (Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. Equivalent transcriptional efficacy was confirmed by co-transfecting the renilla luciferase control vector, pRL-SV40 (Promega, Madison, WI, USA). After transfection, cells were incubated for 24 hours in DMEM supplemented with 10% fetal bovine serum. Then, cells were treated with adiponectin for 24 hours, followed by 6 hours of stimulation with or without LPS (5 ng/ml). Luciferase activity in the cell lysate was measured with a dual luciferase assay kit (Promega) and a luminometer (Promega).

### 2.5. One-Step Clotting Assay for Measuring Tissue Factor Activity

To assess TF activity in MΦ cell lysates, clotting assay was performed as reported previously[23]. Briefly, 50-μl lysates containing 20 ng of MΦ cell protein, after 6 hours of LPS stimulation, were applied to each well in a 96-well plate, after which 50 μl normal pooled plasma (George King Bio-Medical Inc, Overland Park, KS, USA) was added. After incubation for 2 minutes at 37°C, 50 μl of pre-warmed 25-nM calcium chloride (CaCl2) was added. Well absorbance (a surrogate measure for coagulation/fibrin formation) was read by a plate reader at 405 nm with kinetic mode (Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA, USA) every 60 seconds for 180 minutes at 37°C. "Clot initiation time" was determined as the time at which absorbance began to increase continuously. "Half-max time" was determined as the time at which the absorbance reading was half the difference between initial and maximum absorbance.

### 2.6. Immunoblot analysis

Immunoblot analyses with whole-cell lysates of MΦ (20 μg/lane) were performed with a standard method using 10% SDS-PAGE gels and polyvinylidene difluoride membranes (Bio-Rad). The following primary antibodies were used for detection with an ECL prime Western Blotting Detection System (GE Healthcare, Waukesha, WI, USA): anti-phospho-specific IκB-α (Ser32), anti-IκBα, anti-phospho-specific p44/42 MAPK (ERK1/2)(Thr201/Tyr204), anti-phospho-specific SAPK/JNK(Thr183/tyr185), anti-β-Tubulin (all from Cell Signaling Technology, Danvers, MA, USA).

### 2.7. Statistical Analysis

Results are shown as mean±SEM. Two groups were compared using Student's t-test. Between-group comparison of means was performed by ANOVA, followed by t-test. A value of $p<0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. APN suppresses TF expression in human MΦ

In human monocyte-derived MΦ, LPS, but not APN alone (10 μg/ml), increased TF mRNA levels approximately fourfold, compared with controls (Fig. 1A). APN pretreatment, however, significantly inhibited the increased expression of TF in a concentration-dependent manner (76.8% at 10 μg/ml of APN, p< 0.001 vs. LPS alone, n=4; Fig. 1A). Concordant with the suppression of mRNA levels, APN reduced the production of TF protein in a concentration-dependent manner (Fig. 1B).

### 3.2. APN suppresses pro-coagulant activity in LPS-stimulated MΦ

Evaluation of the effect of APN on clot formation through TF used a one-step clotting assay with MΦ cell lysates. LPS stimulation shortened coagulation half-max time, which represents TF activity on clot formation (no stimulation: 42.4±0.5 minutes vs. LPS: 27.9±0.7 minutes, n=4, p<0.0001; Fig. 2). APN pre-treatment, however, significantly extended the half-max time up to 37.3±0.4 minutes at 10 μg/ml of APN (vs. LPS alone, n=4, p< 0.0001), indicating APN suppression of pro-coagulant TF activity (Fig. 2).

### 3.3. APN inhibits LPS-induced TF promoter activity

To elucidate the mechanism by which APN exposure regulates the TF gene, we evaluated the effect of APN on TF transcription. We used a mouse MΦ cell line (RAW 264.7) to optimize transfection efficiency with the TF promoter reporter construct (Fig. 3A). In these construct-transfected cells, LPS increased luciferase activity by 4.94±0.27 times, compared with vehicle control (Fig. 3B). Adiponectin pre-incubation reduced LPS-induced increase in TF promoter activity by 76% (10 μg/ml of APN, 1.94±0.19 times, n=4, <0.01, Fig. 3B).

### 3.4. APN suppresses the phosphorylation and degradation of IkB-α in MΦ

To evaluate the intracellular mechanism of APN on TF transcription, we performed immunoblot analysis with human MΦ. In monocytes/MΦ, LPS stimulation activates several signaling pathways distal to TLR4 — NF-κB, JNK, and ERK1/2 — that facilitate the transcription of TF[21, 24]. APN inhibits activation of NF-κB, but not of JNK or ERK1/2 signaling pathways, as shown by Western blot analysis. APN inhibited LPS-induced phosphorylation and degradation of IκB-α that inhibits NF-κB function (Fig. 4). APN-induced stabilization of IκB-α points to an important molecular mechanism for APN inhibition of NF-κB-regulated TF expression.

## 4. Discussion

Many mechanisms may contribute to heightened cardiovascular risk associated in some studies with hypoadiponectinemia. APN appears to regulate plaque inflammation and stability in particular, by functioning as an anti-inflammatory adipocytokine that reduces the production of inflammatory cytokines and chemokines, such as tumor necrosis factor alpha (TNFα) and interferon-inducible protein 10 (IP-10)[15, 25]. APN also increases expression of interleukin-10 (IL-10), a cytokine with anti-inflammatory properties, and of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1)[26]. Moreover, APN increases high-density lipoprotein assembly in the liver that may subsequently remove cholesterol from atheromata[27]. These actions may stabilize plaques and reduce their size and risk of rupture.

TF produced by MΦ, the most abundant inflammatory cells in atheromatous plaques, likely triggers many thrombotic complications of atherosclerosis. The present study shows that APN reduces LPS-stimulated expression of TF mRNA and protein by qRT-PCR, TF luciferase promoter, and ELISA experiments. In addition, one-step clotting assay

experiments demonstrated that APN treatment significantly inhibits TF-dependent MΦ pro-coagulant activity.

Notably, an NF-κB binding site exists in the promoter region of TF, and LPS directs NF-κB activation by initiating Toll-like receptor 4–dependent phosphorylation/degradation of the NF-κB inhibitor IκB[21]. Mechanistic studies that examined APN regulation of LPS-stimulated NF-κB, JNK, and ERK1/2 intracellular signaling in MΦ showed that APN attenuates NF-κB signaling by reducing the phosphorylation of IκB-α. APN can also suppress the phosphorylation and subsequent degradation of IκB-α in vascular endothelial cells[28]. Our immunoblot analyses with MΦ showed that APN treatment reduces the phosphorylation and degradation of IκB-α without affecting the activation of JNK and ERK1/2 signaling cascades. Thus, APN presumably suppresses NF-κB binding to the TF promoter region through the limitation of inflammatory signaling downstream of TLR4 in MΦ.

Kato et al. reported that APN deficiency accelerates thrombus formation through the enhancement of platelet aggregation in mice[29]. Although their studies focused on platelet factors rather than coagulation factors, APN inhibition of TF might contribute to enhanced thrombosis in APN-deficient mice. Previous studies have shown that APN inhibits TF expression and enhances TF pathway inhibitor expression in human endothelial cells[30]. During atherogenesis, monocytes adhere to injured endothelial cells and invade subendothelial space, then accumulate lipids to become foam cells in fatty streaks and vulnerable plaques. The dominant source of TF in the arterial wall during this process, as a determinant of plaque thrombogenicity, gradually shifts from endothelial cells to inflammatory MΦ, an abundant cell type in advanced atheroma[20]. Indeed, LPS, used as a model pro-inflammatory stimulus in the present study, may not itself trigger thrombotic complications in most ACS. Thus, future studies in vitro and in vivo with this regard should address APN's modulatory effects on other putative atherogenic stimuli. Our finding that APN attenuates MΦ TF expression, however, may provide in part a mechanistic basis for increased risk of atherothrombotic events in individuals with hypoadiponectinemia[17, 19].

Taken together, the results of our study suggest a mechanistic link between APN and the thrombotic complications of atherosclerosis (Fig. 5). Therapeutic interventions that elevate APN in CAD patients may not only prevent the progression of atheromatous plaques, but also may reduce atherothrombotic events by suppressing MΦ TF expression.

## Acknowledgments

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## Highlights

Adiponectin suppresses tissue factor expression in LPS-stimulated macrophages (MΦ).

Adiponectin reduces pro-coagulant activity in LPS-stimulated MΦ.

The inhibitory mechanisms of tissue factor by adiponectin were studied.

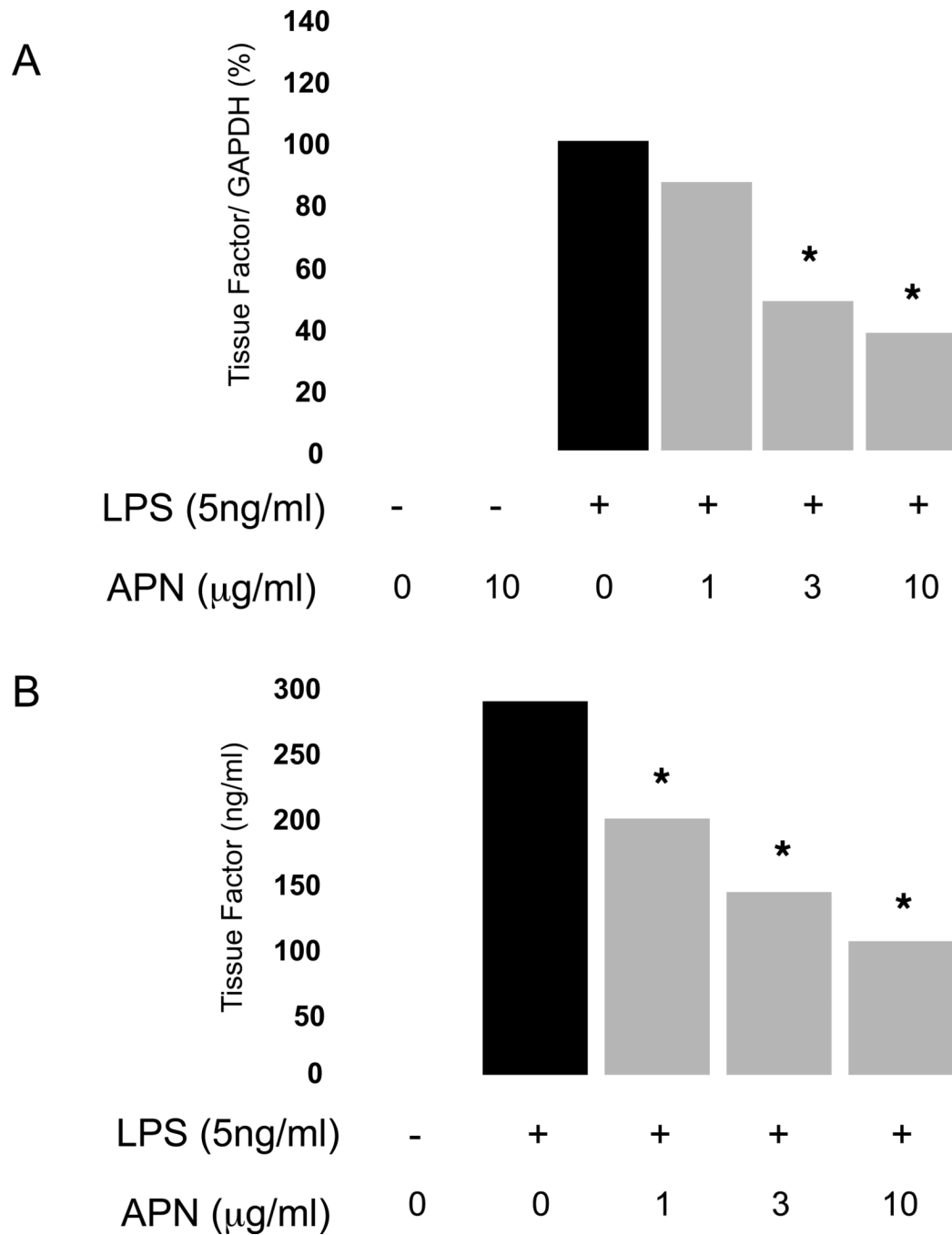The increment of adiponectin may prevent atherothromosis in coronay artery disease.

A

Tissue Factor/ GAPDH (%)

| | | | | | |
|---|---|---|---|---|---|
| LPS (5ng/ml) | - | - | + | + | + | + |
| APN (μg/ml) | 0 | 10 | 0 | 1 | 3 | 10 |

B

Tissue Factor (ng/ml)

| | | | | | |
|---|---|---|---|---|---|
| LPS (5ng/ml) | - | + | + | + | + |
| APN (μg/ml) | 0 | 0 | 1 | 3 | 10 |

**Fig. 1. APN suppresses TF gene expression and protein production in LPS-stimulated human MΦ**

Human monocyte-derived MΦ (day 10) were treated with the indicated concentration of recombinant human APN in starvation media for 24 hours, and then stimulated with LPS (5 ng/ml) for 6 hours. (A) Gene expression of TF analyzed by real-time quantitative RT-PCR. mRNA levels of GAPDH served as an internal control for adjustment between samples. (B) TF protein in TF cell lysates assayed by ELISA. Data are expressed relative to the values of LPS-alone treatment (100%) in mean±SEM (n=4). *$p$<0.001 vs. LPS.
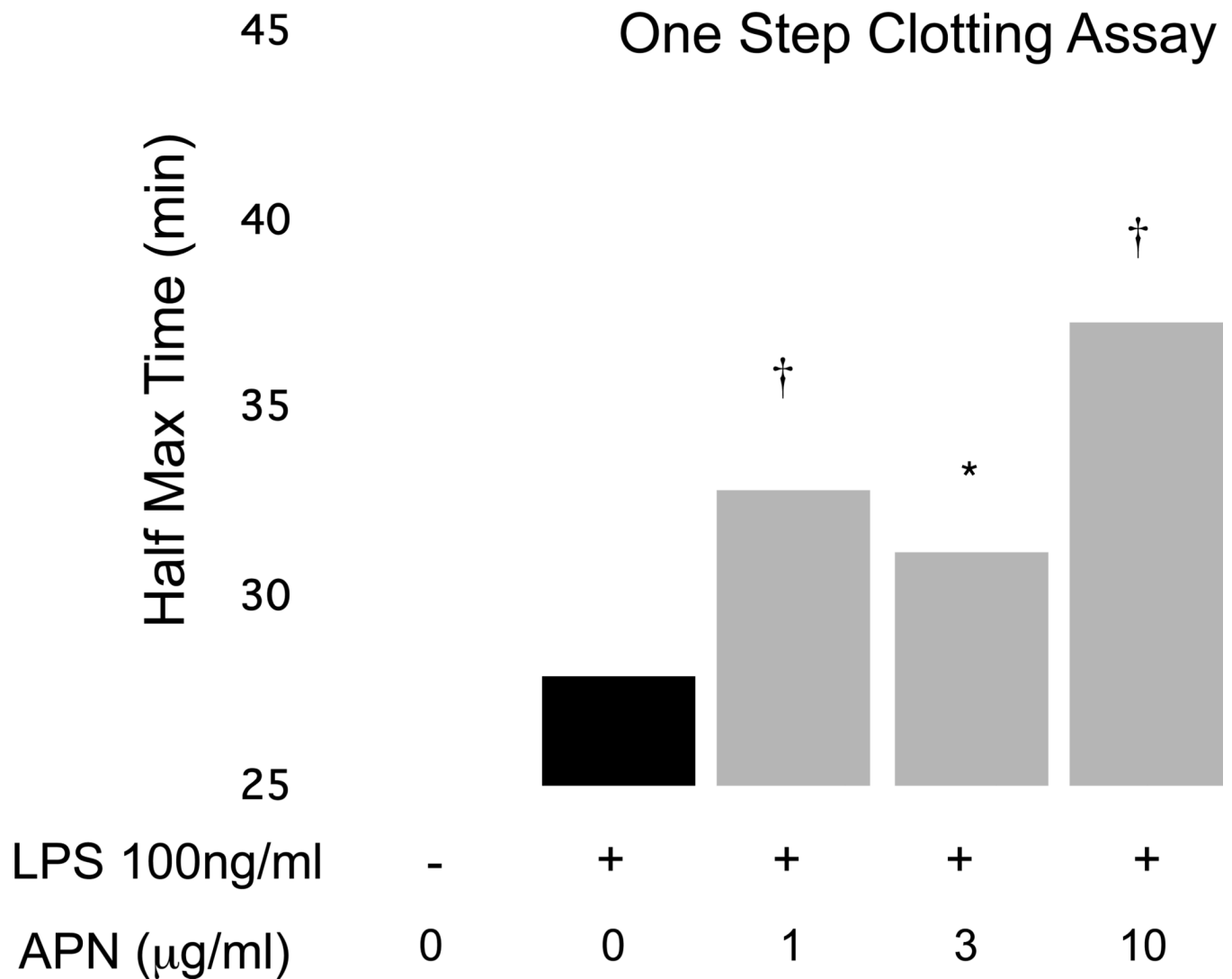
**Fig. 2. APN reduces pro-coagulant activity initiated by TF in LPS-stimulated human MΦ**
A one-step clotting assay was performed with human MΦ lysates, treated as indicated. "Clot initiation time" was the time at which absorbance began to increase continuously. "Half-max time" was the time at which the absorbance reading reached half of the difference between initial and maximum absorbance. Data are expressed in mean "half-max time" ±SEM (n=4). *$p$<0.001, †$p$<0.0001 vs. LPS.

A

-227 AP-1 NF-κB Egr-1 >

TF

B

Fold Induction
(Luciferase Activity/ SV40)

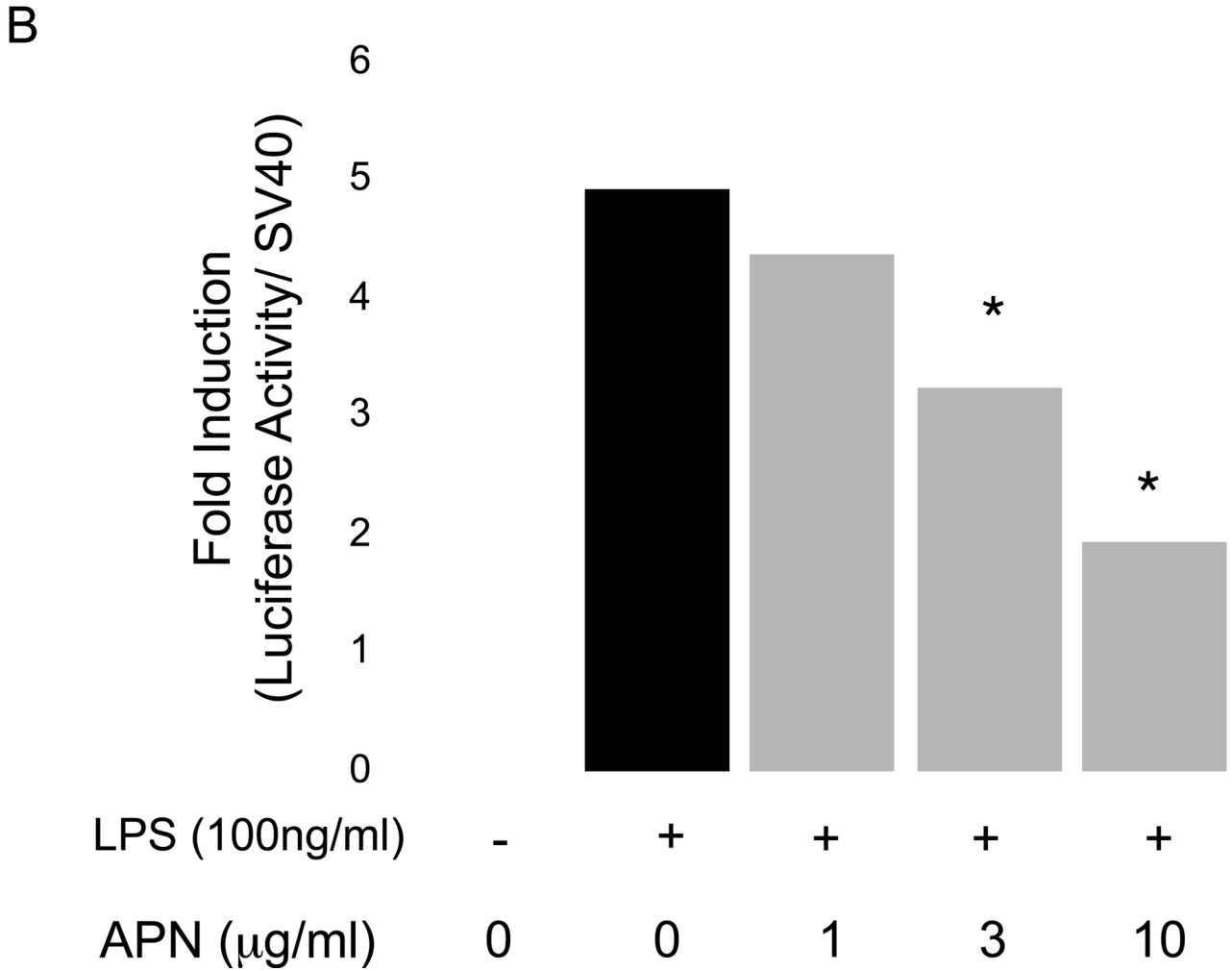| | | | * | * |
|---|---|---|---|---|
| LPS (100ng/ml) | - | + | + | + | + |
| APN (μg/ml) | 0 | 0 | 1 | 3 | 10 |

**Fig. 3. APN inhibits TF promoter activity in LPS-stimulated RAW 264.7 cells**
TF promoter reporter construct-transfected RAW 264.7 cells were pre-treated with APN for 24 hours, and then stimulated with LPS (100 ng/ml) for 6 hours. The promoter activity in the harvested cells was determined by dual luciferase assay. SV40 indicates control vector (pRL-SV40) (n=4, *p<0.0001 vs. LPS).
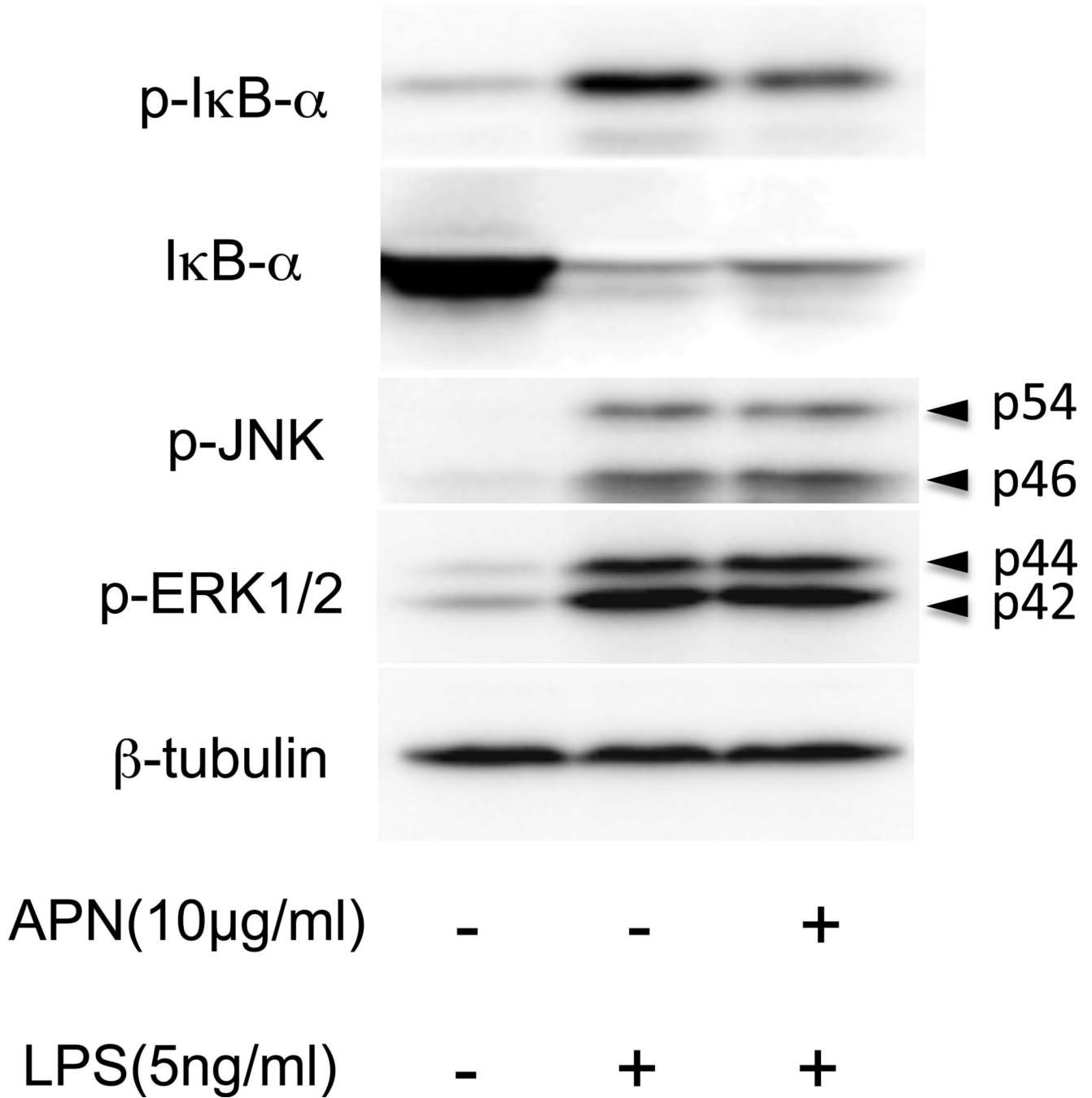
**Fig. 4. APN attenuates LPS-triggered intracellular activation of IκB-α responsible for TF transcription in human MΦ**

Human MΦ were incubated with or without (left lanes) 10 μg/ml of APN for 24 hours, and subsequently stimulated with 5 ng/ml of LPS for 15 minutes. Whole-cell lysates were fractionated by SDS-PAGE and immunoblotted with indicated antibodies. β-tubulin served as a loading control.
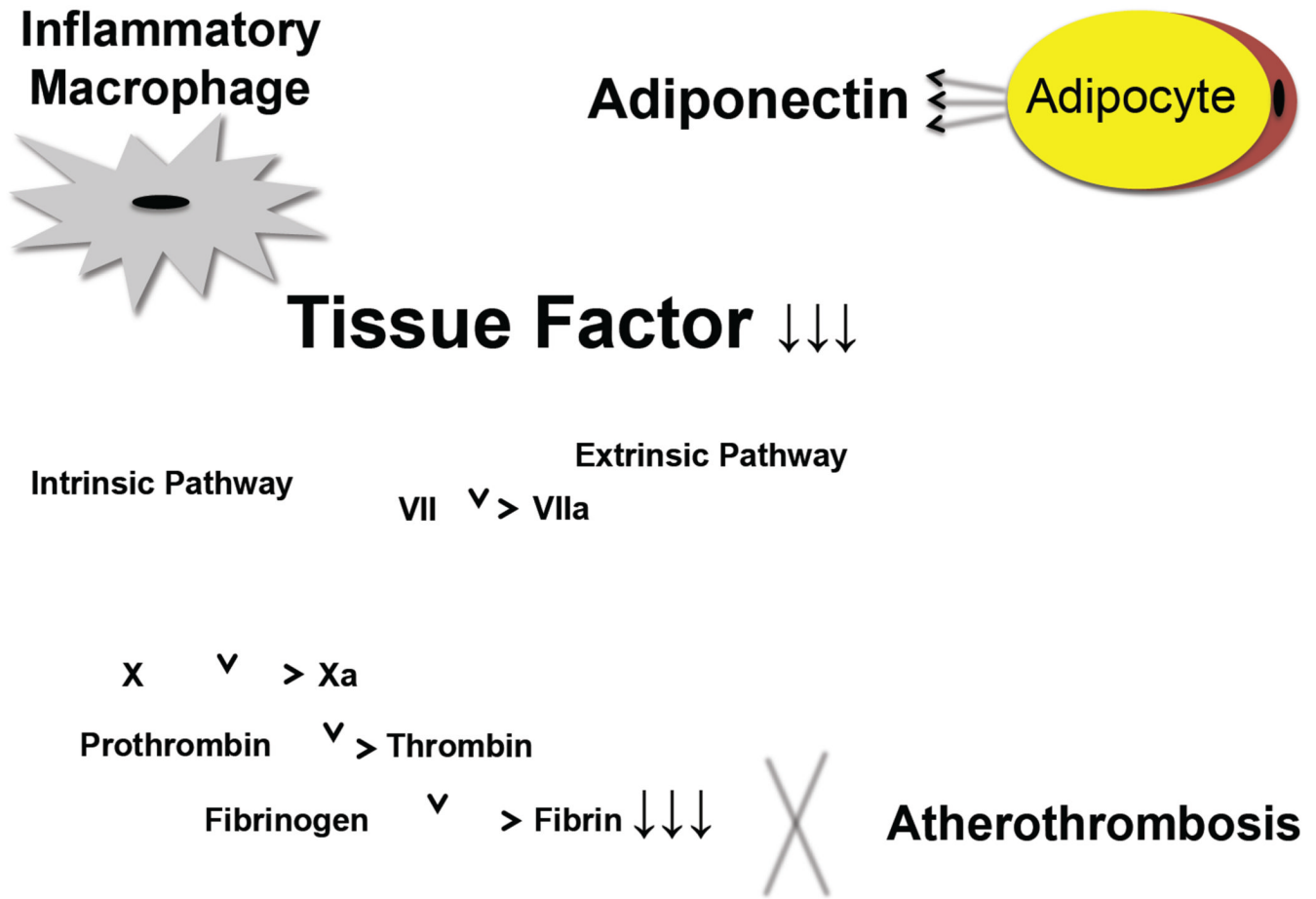
**Fig. 5. APN inhibits MΦ TF in coagulation cascade, a potential mechanism in the prevention of atherothrombosis**

APN secreted from adipocytes interacts with MΦ in disrupted atheromatous plaques and inhibits TF production. It subsequently attenuates the activation of the coagulation cascade and arterial thrombus formation — a key pathology in atherothrombosis.