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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.

1. Introduction

The common cluster of components that comprise the "metabolic syndrome"—including excess visceral fat accumulation, dyslipidemia, impaired glucose tolerance, and hypertension—associates with inflammation, and contributes 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 (ACSs) 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].

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Tissue factor (TF; also known as coagulation factor III or tissue thromboplastin) triggers blood coagulation. Upon binding to factor VIIa, the TF/VIIa 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 h. MΦ then were stimulated with 5 ng/ml of LPS for 6 h or 15 min to analyze TF gene/protein expression (real-time quantitative RT-PCR, ELISA, and one-step clotting assay) or intracellular signaling (immunoblot analysis), respectively. RAW 264.7 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 h 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 h at 2–8 °C, and the lysed cells were centrifuged to remove cell debris. Cell lysates were stored at −80 °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 RAW 264.7 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 h in DMEM supplemented with 10% fetal bovine serum. Then, cells were treated with adiponectin for 24 h, followed by 6 h of stimulation with or without LPS (100 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 h 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 min 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 s for 180 min 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 min vs. LPS: 27.9 ± 0.7 min, $n = 4$, $p < 0.0001$; Fig. 2). APN pre-treatment, however, significantly extended the half-max time up to 37.3 ± 0.4 min at 10 μg/ml of APN (vs. LPS alone, $n = 4$, $p < 0.0001$), indicating APN suppression of pro-coagulant TF activity (Fig. 2).