Zhou H / Acta Pharmacol Sin 2004 Oct; 25 (10): 1366-1371

Dilazep and dipyridamole inhibit tissue factor expression on monocytes induced by IgG from patients with antiphospholipid syndrome[1]

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KEY WORDS dilazep; dipyridamole; antiphospholipid syndrome; monocytes; thromboplastin

ABSTRACT

AIM: To investigate whether antiplatelet agents, dilazep and dipyridamole, inhibit tissue factor (TF) expression on monocytes induced by IgG from patients with antiphospholipid syndrome (APS). **METHODS:** Freshly isolated peripheral blood monocytes were allowed to adhere on plastic and then cultured in media containing patient or control antibodies and/or other agonists with or without dilazep or dipyridamole. The TF activity on monocytes was investigated by measuring factor VII_a-dependent generation of factor X_a, using a chromogenic substrate and the TF mRNA expression was examined by real-time PCR (TaqMan PCR). **RESULTS:** The TF activity on monocytes induced by APS IgG (250 mg/L) was inhibited by dilazep (0.15-150 μmol/L) and dipyridamole (0.2-200 μmol/L) in a dose-dependent fashion. But, the TF mRNA expression induced by APS IgG was not inhibited. Theophylline (500 μmol/L), an adenosine receptor antagonist, could counteract the inhibitory effect of dilazep and dipyridamole on TF activity. **CONCLUSION:** Antiplatelet agents, dilazep and dipyridamole, block APS IgG-induced monocytes TF expression at a post-transcriptional level, partly by adenosine receptor pathway. Pharmacological agents that block monocytes TF activity, such as dilazep and dipyridamole, are a novel therapeutic approach in APS.

INTRODUCTION

Tissue factor (TF) is a single chain transmembrane glycoprotein which is a major physiological initiator of blood coagulation *in vivo*. Under normal physiological conditions, TF is not expressed on vascular endothelial cells and monocytes, but it can be induced by stimuli such as lipopolysaccharides (LPS), tumor necrosis factor α (TNFα), interleukin-1 (IL-1), mitogens *et al*[1]. Increased TF expression on endothelial cells and monocytes plays an important role in mediating abnormal coagulation events associated with gram-negative sepsis, atherosclerosis, and arterial or venous thrombosis. The antiphospholipid syndrome (APS) is the association of recurrent venous and arterial thrombosis, and/or repeated fetal loss, as well as thrombocytopenia[2]. Growing evidences suggest that antiphospholipid autoantibodies (aPL), especially anti β_2-glycoprotein I (β_2GPI) antibodies, may stimulate blood cells and vascular endothelium to express TF activity and exert procoagulant effects[3]. Our previous study also showed that certain antiphospholipid antibodies induced monocytes TF activity in APS[4].

[1] Project supported by the Advanced Research Development Program of Jiangsu University (2283000001), the National Natural Science Foundation of China (No 30370602) and National Education Program of China.
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Received 2003-09-15 Accepted 2004-02-20

Dilazep exerts its antiplatelet activity by increasing the adenosine levels in the extracellular space fluid and has cytoprotective and antioxidant effects on endothelial cells as well as antithrombotic effect in experimental animal models[5]. Deguchi reported that dilazep could block TF expression in human umbilical vein endothelial cells (HUVECs) stimulated with TNF, thrombin, PMA and in TNF-stimulated monocytes[6]. Dipyridamole is another vasodilator, often used for coronary diseases and other vascular disorders. It also exerts antiplatelet function, indirectly through a mechanism involving intracellular cyclic 3',5'-adenosine monophosphate, demonstrating a potent antithrombotic effect[7]. Dipyridamole can block LPS-induced TF activity on monocytes.

Although dilazep and dipyridamole have been used in the clinic for many years as vasodilatory and antiplatelet drugs, whether they affect the procoagulant activity of monocytes in APS remains unclear. In the current study, we mainly examined if dilazep and dipyridamole were capable of blocking antibody-induced TF activity on monocytes and tried to find some pharmacological agents available for APS.

MATERIALS AND METHODS

Materials Protein A Sepharose 4B-CL column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Macrophage SFM medium was obtained from Gibco BRL (Grand Island, NY). LPS, dilazep and dipyridamole as well as theophylline were obtained from Sigma (Luis, MO). Human factor VII_a and factor X were provided by Dr Monroe DM. Spectrozyme FX_a was purchased from American Diagnostica Inc (Greenwith, CT). RNA STAT-60™ was from TEL-TEST INC (Friendswood, TX). TaqMan® EZ RT-PCR Kit was obtained from Applied Biosystems (Branchburg, NJ). All other chemicals and reagents used were of the best quality commercially available.

Purification of serum IgG Serum samples were obtained from the patients diagnosed APS and healthy control. All patients had the history of thrombosis. None of the controls had the history of autoimmune diseases, bleeding disorders, thrombosis. Serum IgG was purified using Protein A Sepharose 4B-CL column according to the manuals[8]. Purity of IgG fractions was >95 % assessed by sodium dodecyl sulphate-polyacylamide gel electrophorosis (SDS-PAGE) and also by ELISA for the detection of anti-β_2GPI [25-28 RU (relative unit), normal IgG<2 RU].

Isolation of peripheral blood monocytes Peripheral blood cells were obtained from healthy donors by vein puncture using 0.129 mol/L sodium citrate (1/9, v/v) as anticoagulant. Peripheral blood mononuclear cells (PBMC) were isolated using Accu-prep™ (Accurate Chemical & Scientific Corp, Westbury, NY) gradient centrifugation as described previously[4]. The cells were washed and resuspended in macrophage SFM medium and seeded on 96-well plate. The cells were allowed to adhere to plastic cultural plate for 1 h at 37 ºC in humidified atmosphere of 5 % CO_2. The non-adherent cells were removed by washing 3 times with medium. Monocytes (adherent cells) were further cultured in the conditioned medium for determination of TF expression. The viability of the cells assessed by trypan blue exclusion was greater than 95 % after 6 h of incubation.

Determination of TF activity on monocytes TF activity on monocytes surface was determined as factor X activation by factor VII_a/TF complex[9]. The cells (5×10^4/well) were incubated in the medium with or without dilazep or dipyridamole for 15 min and stimulated with APS IgG or control IgG or other agonists for 6 h. The concentrations used in assays were: dilazep 0.15-150 μmol/L; dipyridamole 0.2-200 μmol/L; LPS 500 μg/L; IgG 250 mg/L; theophylline, 500 μmol/L. After incubation with each stimulant or inhibitor, the cells were washed twice using HEPES-buffered saline. Factor VII_a and X were added to the cells with final concentration of 100 pmol/L and 135 nmol/L respectively in the buffer. The generated factor X_a was determined using a chromogenic substrate Spectozyme FX_a.

Analysis of TF mRNA by TaqMan PCR[10] The total RNA of monocytes was prepared using RNA STAT-60 after treating the cells with dilazep (150 μmol/L), dipyridamole (200 μmol/L), LPS (500 μg/L), IgG (250 mg/L) or other agonists. Quantitative RT and PCR assays were performed in triplicate on a Perkin-Elmer 7700 TaqMan® PCR machine using a TaqMan EZ-RT PCR kit (Perkin-Elmer). Primers were designed using the Perkin-Elmer computer program Primer Express. The forward primer for human TF was 5'-TCAGGTGATCCACCCACCTT-3' corresponding to 1561-1580 residues, and the reverse primer was 5'-GCACCCAATTTCCTTCCATTT-3' corresponding to 1672-1692. The control housekeeping gene was human cyclophilin (huCYC) (Pre-Developed TaqMan® Assay Reagents, Biosystems, Foster, CA). Standard RT extension, PCR

annealing, and amplification temperatures were used as detailed by the Perkin-Elmer TaqMan manual. Relative quantitation was determined by standard $2^{(-\Delta\Delta CT)}$ calculations.

Statistical analysis Data were expressed as mean±SEM. The difference between the TF activity induced by agonists and antagonists was calculated by analysis of variance with *t*-test using the StatView 4.1 package software (ABACUS CONCEPTS, Berkeley, CA, USA) for the Macintosh. $P<0.05$ was considered as statistically significant.

RESULTS

APS IgG enhanced TF activity on monocytes As shown in Fig 1, the TF mRNA expression and the activity on cell surface (shown by factor X_a generation) could reach to 2.82±0.50 [$2^{(-\Delta\Delta CT)}$] fold change and 3.20±0.57 nmol/L, respectively stimulated by patient IgG (250 mg/L), significantly higher than that by similar concentration of normal IgG shown 0.52±0.20 [$2^{(-\Delta\Delta CT)}$] and 0.60±0.18 nmol/L, respectively ($P<0.01$). The effects of patient IgG were even stronger than that of LPS (500 μg/L) with 2.15±0.54 [$2^{(-\Delta\Delta CT)}$] and 1.94±0.52 nmol/L, which is known to cause TF expression on endothelial cells and monocytes. The procoagulant activity (PCA) was undetectable when VII_a was omitted from the assay (data not shown), indicating that PCA is indeed TF-mediated.

Dilazep and dipyridamole inhibited the TF activity induced by APS IgG Dilazep and dipyridamole strongly inhibited the effects of APS IgG on monocytes TF activity in a dose-dependent manner (Fig 2). Dilazep 150 μmol/L or dipyridamole 200 μmol/L blocked around 80 % and 75 % of effects of LPS (500 μg/L) and APS IgG (250 mg/L), respectively. The 50 % of inhibitory effects of dilazep or dipyridamol on LPS and APS IgG were 18 μmol/L, 20.25 μmol/L, and 20 μmol/L, 24 μmol/L, respectively.

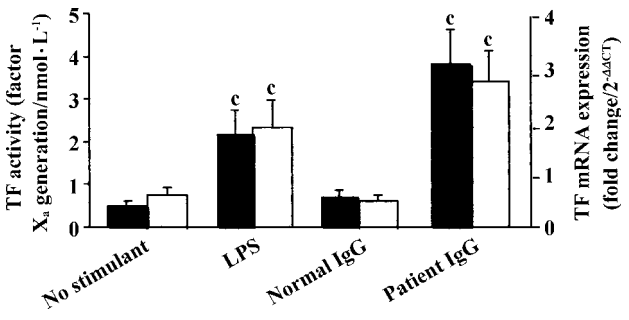

Fig 1. TF expression on monocytes stimulated with LPS and patient IgG. Monocytes (5×10^4/well) was stimulated with 500 μg/L of LPS and 250 mg/L of patient IgG. The TF activity on monocytes surface was determined as factor X_a generation using Spectrozyme FX_a substrate (closed columns) and TF mRNA was investigated by TaqMan PCR (open columns). *n*=3. Mean±SEM. $^cP<0.01$ *vs* no stimulant.

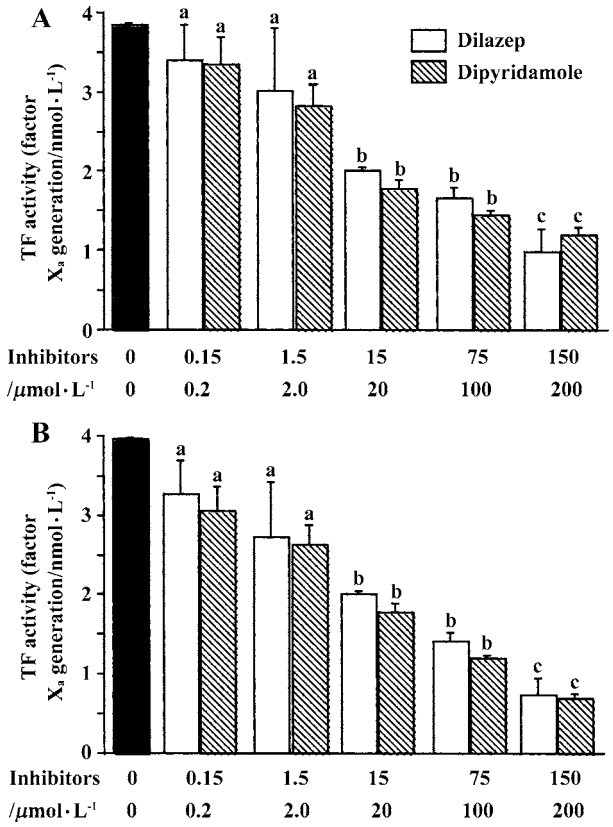

Fig 2. Inhibitory effects of dilazep and dipyridamole on LPS- and APS IgG-induced TF activity on monocytes. Monocytes (5×10^4/well) was stimulated with 250 mg/L of patient IgG (A) or 500 μg/L of LPS (B) without or with 0.15-150 μmol/L of dilazep or 0.2-200 μmol/L of dipyridamole. The TF activity on monocytes surface was determined as factor X_a generation using Spectrozyme FX_a substrate. *n*=3. Mean±SEM. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* no inhibitors.

Dilazep and dipyridamole did not affect the TF mRNA expression The effects of dilazep and dipyridamole on TF mRNA expression of monocytes stimulated with LPS (500 μg/L) and APS patient IgG (250 mg/L) were also evaluated. As seen in Fig 3, Dilazep 150 μmol/L and dipyridamole 200 μmol/L were used in the assays according to their inhibitory effects on TF activity. Interestingly, dilazep and dipyridamole did not change monocytes TF mRNA level induced by both of

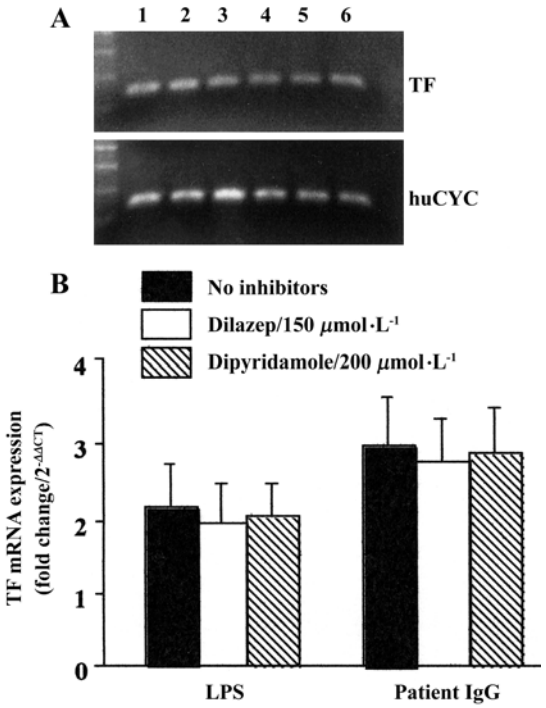

Fig 3. Dilazep and dipyridamole did not affect TF mRNA expression on monocytes induced by LPS or APS IgG. Monocytes (5×10^6/well) was stimulated with 500 μg/L of LPS and 250 mg/L of patient IgG without or with 150 μmol/L of dilazep or 200 μmol/L of dipyridamole. The TF mRNA was investigated by TaqMan PCR using TF primers. (A) The bands of PCR products on gel analysis. Lane 1, treated by LPS; lane 2, by LPS and dilazep; lane 3, by LPS and dipyridamole; lane 4, treated by APS IgG; lane 5, by APS IgG and dilazep; lane 6, by APS IgG and dipyridamole. (B) The relative quantitation was determined by standard $2^{(-\Delta\Delta CT)}$ calculations. *n*=3. Mean±SEM.

LPS and patient IgG, although the similar concentration of them strongly inhibited the TF activity on monocytes (Fig 2).

Theophylline counteracted the inhibitory effects of dilazep and dipyridamole To clarify whether the inhibitory effects of dilazep and dipyridamole on monocytes TF activity is mediated by adenosine receptor, the effect of an adenosine receptor antagonist, theophylline, on the inhibitory effects of dilazep and dipyridamole was investigated. The data showed that 500 μmol/L of theophylline counteracted about 75 % of the inhibitory effect of dilazep (150 μmol/L) and dipyridamole (200 μmol/L) on monocytes TF activity stimulated with LPS or patient IgG (Fig 4).

DISCUSSION

Monocytes, like endothelial cells and other cells in contact with blood, do not constitutively express TF activity. They synthesize and express TF following stimulation with LPS, inflammatory cytokines, and certain other stimuli. Several studies demonstrate that increased TF expression was associated with IgG anticardiolipin antibodies, which is responsible of hypercoagulability in APS[11]. Anti-β_2GPI human monoclonal antibodies derived from peripheral B cells of APS patients were shown to enhance monocytes TF activity and TF mRNA level in a β_2GPI-dependent fashion, as compared to appropriate controls[12]. In our current study, all selected APS patients with thrombosis showed higher titer of anti-β_2GPI in their serum as well as in their IgG fractions than that of normal controls. The selected APS patients IgG significantly enhanced TF expression on blood monocytes with both of activity and mRNA levels (Fig 1). Thus, that certain autoantibodies to β_2GPI are capable of inducing TF activity on monocytes contributes to the thrombotic diathesis in APS.

Antiplatelet agents, dilazep and dipyridamole, are commonly used in patients at risk of developing thrombotic diseases. Dilazep is also known to dilate coronary, cerebral, and renal vessels by blocking the calcium influx and to prevent the occurrence of thrombus formation in experimental animals[13]. Dipyridamole is often used in coronary diseases not only as a vasodilator but also as an inhibitor of platelet aggregation for over 20 years. It has been already found that dipyridamole blocks the LPS-induced increase in monocyte-associated TF activity and PMA stimulated O_2-release from monocytes and polymorphonuclear leukocytes[14]. These findings prompted us to investigate the effects of dilazep and dipyridamole on the procoagulant activity of monocytes induced by APS IgG. The results of our present study showed that both of them significantly inhibited the APS-induced TF activity on monocytes, in the fashion of dose-dependent (Fig 2), suggesting some pharmacological agents that block monocytes TF activity are a novel therapeutic approach in APS.

TF expression is known to be regulated at both transcriptional and posttranscriptional levels. In the current study, APS IgG induced TF expression on monocytes by increasing TF gene levels and its functional activity. Interestingly, both of dilazep and dipyridamole inhibited APS IgG-induced monocytes surface TF activity, but they did not inhibit the TF mRNA expression (Fig 3). Deguchi[6] reported that dilazep inhibited TF expression in HUVECs and monocytes stimulated by PMA and thrombin at both transcriptional and post-

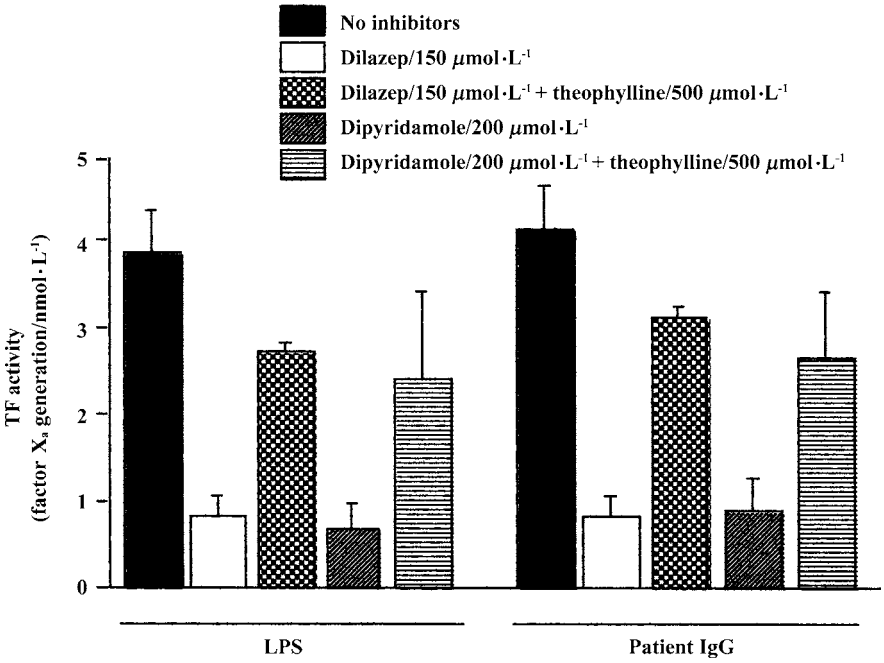

Fig 4. Theophylline counteracted the inhibitory effects of dilazep and dipyridamole. Monocytes (5×10^4/well) were stimulated with 500 μg/L of LPS or 250 mg/L of patient IgG in the presence of 150 μmol/L dilazep or 200 μmol/L dipyridamole with or without 500 μmol/L of theophylline. The TF activity on monocytes surface was determined as factor X_a generation using Spectrozyme FX_a substrate. *n*=3. Mean±SEM.

transcriptional levels, but dilazep did not inhibit TF mRNA expression induced by TNF in HUVECs. It can be explained that the signaling pathway of TNF-induced TF expression is different from that of PMA- or thrombin-induced TF expression. Our results demonstrated that dilazep and dipyridamole regulate APS IgG-stimulated TF surface expression at the posttranscriptional level. The signaling pathway of APS IgG-stimulated TF expression may be also different from that of PMA or thrombin. It has been known that adenosine down-regulate TF expression on endothelial cells via its A2a and A3 receptors[15,16]. Moreover, dilazep and dipyridamole were previously reported to decrease the rate of adenosine metabolism, leading to increased plasma adenosine levels. Our data showed that the inhibitory effects of dilazep and dipyridamole on monocytes TF activity were counteracted by adenosine receptor antagonist, theophylline (Fig 4), indicating that the inhibitory effects of dilazep and dipyridamole were mediated, at least in part, by adenosine receptors. All together, these findings suggest that adenosine and its receptors play important roles in the regulation of TF expression.

In summary, this study showed that some autoantibodies, especially to β_2GPI antibodies, induced TF activity on monocytes contribute to the thrombotic diathesis in APS. Antiplatelet agents, dilazep and dipyridamole, inhibit APS IgG-induced TF activity on monocytes at post-transcriptional levels and their inhibitory effects is mediated, in part, by adenosine receptors pathway. Besides their well-known vasodilatory and antiplatelet effects, dilazep and dipyridamole exert potent inhibitory activity on the procoagulant activity of monocytes and maybe a novel therapeutic approach in APS.

ACKNOWLEDGEMENTS We thank Dr Roubey RAS for expert supervision and Dr Monroe DM for providing factor VII_a and X.

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