Asymmetric dimethylarginine induces TNF-α production via ROS/NF-κB dependent pathway in human monocyctic cells and the inhibitory effect of reinioside C

Guo-Gang Zhang a, Yong-Ping Bai a, Mei-Fang Chen a, Rui-Zhen Shi a, De-Jian Jiang b, Qiong-Mei Fu a, Gui-Shan Tan b, Yuan-Jian Li a,b,*

a Department of Cardiovascular Medicine, Xiangya Hospital of Central South University, Changsha 410008, China
b Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha 410078, China

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Abstract

Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, has been implicated in vascular inflammation through induction of reactive oxygen species (ROS) and proinflammatory genes in endothelial cells. However, relatively few attentions have been paid to the effect of ADMA on monocytes, one of the important cells throughout all stages of atherosclerosis. In the present study, we found that reinioside C, the main component extracted from Polygala fallax Hemsl., dose-dependently inhibited tumor necrosis factor-α (TNF-α) production induced by ADMA in monocytes. Furthermore, reinioside C attenuated ADMA-induced generation of reactive oxygen species and activation of nuclear factor-κB (NF-κB) activity in monocytes in a dose-dependent manner, this effect was inhibited by L-arginine (NOS substrate) and PDTC (inhibitor of NF-κB). These data suggest that reinioside C could attenuate the increase of TNF-α induced by exogenous ADMA through inhibition of ROS/NF-κB pathway in monocytes.

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1. Introduction

It is widely accepted that ADMA, an endogenous NOS inhibitor, is a risk factor of atherosclerosis (Stühlinger, 2007). Previous studies have demonstrated that plasma ADMA level is significantly elevated in hypercholesterolemia in animals and humans (Lu et al., 2004; Boger et al., 2000). Recently, it has been reported that the plasma level of ADMA is higher in patients with acute coronary syndrome (ACS) than those in patients with stable coronary artery disease (CAD) (Krempl et al., 2005). The ADMA level is positively correlated with carotid artery intima-media thickness (IMT) and major adverse cardiovascular events in CAD patients after Percutaneous Transluminal Coronary Angioplasty (PTCA) (Lu et al., 2003; Miyazaki et al., 1999; Siroen et al., 2005). These data suggest that ADMA may be involved in the development of ACS, while the mechanism is not so clear. Many studies have demonstrated that exogenous ADMA can induce apoptosis and inflammatory responses in endothelial cells (Yuan et al., 2007; Jiang et al., 2006). However, relatively few attentions have been paid to the effect of ADMA on monocytes, one of the important cells in atherogenesis. Until recently Smirnova IV et al. (2004) reported that ADMA can promote formation of foam cells by up-regulating lectin-liked oxidized low-density lipoprotein receptor-1 (LOX-1) expression in monocytes.

TNF-α, an important cytokine involved in the development of atherosclerosis, can significantly up-regulate the expression of adhesion molecules including soluble intercellular adhesion
molecule-1 (sICAM-1), vascular cell adhesion molecule-1 (VCAM-1) or monocyte chemoattractant protein-1 (MCP-1) in hypercholesterolemic humans or in cultured endothelial cells (Tan et al., 2007; El Messal et al., 2006). In hypercholesterolemic mice, the atherosclerosis lesions were markedly decreased by blocking TNF-α action using specific antibodies (Ogiwara et al., 2004; Rus et al., 1991). These data suggested a key role of TNF-α on atherogenesis and occurrence of ACS. TNF-α is secreted from various cells including monocytes. Some kinds of stimuli such as oxidized low-density lipoprotein (ox-LDL), high glucose and Ang II can up-regulate TNF-α production at transcriptional level (Tan et al., 2007; Chen et al., 2007; Wang et al., 2007). Further, the ROS sensitive-activation of NF-κB serves as a critical signal in the activation of TNF-α transcription (Bai et al., 2007). Based on the previous reports that ADMA increases intracellular ROS and activates NF-κB in endothelial cells, we speculate that ADMA can increase TNF-α production via activating ROS/NF-κB dependent pathway in monocytes.

Antiatherosclerotic effects of some drugs such as simvastatin and xathones were closely related to the decrease of TNF-α in ACS patients is higher than the level in CAD patients (Pasqui et al., 2006). In hypercholesterolemic mice, the atherosclerosis lesions were markedly decreased by blocking TNF-α action using specific antibodies (Ogiwara et al., 2004; Rus et al., 1991). These data suggested a key role of TNF-α on atherogenesis and occurrence of ACS. TNF-α is secreted from various cells including monocytes. Some kinds of stimuli such as oxidized low-density lipoprotein (ox-LDL), high glucose and Ang II can up-regulate TNF-α production at transcriptional level (Tan et al., 2007; Chen et al., 2007; Wang et al., 2007). Further, the ROS sensitive-activation of NF-κB serves as a critical signal in the activation of TNF-α transcription (Bai et al., 2007). Based on the previous reports that ADMA increases intracellular ROS and activates NF-κB in endothelial cells, we speculate that ADMA can increase TNF-α production via activating ROS/NF-κB dependent pathway in monocytes.

Antithrombotic effects of some drugs such as aspirin and xanthones were closely related to the decrease of TNF-α production (Jiang et al., 2007, 2004). Polygala fallax Hemsl., a commonly used Chinese medicinal herb, has been used to treat some diseases such as infective inflammation and hypercholesterolemia. Reinioside C (Fig. 1) is the main component extracted from P. fallax Hemsl. (Xu et al., 2006). Previous study has shown that reinioside C could inhibit the elevated expression of LOX-1 mRNA and protein induced by ox-LDL in the human umbilical vein endothelial cells (HUVECs) (Bai et al., 2006). In the present study, we investigated the TNF-α production induced by ADMA and the possible inhibitory effect of reinioside C in human monocytic cell.

2. Materials and methods

2.1. Reagents

RPMI 1640, benzylpenicillin and streptomycin were obtained from Gibco-BRL. Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). Asymmetric dimethylarginine standard, pyrollidine dithiocarbamate (PDTC), and L-arginine were purchased from Sigma. Reionside C (purity was 99.0% by HPLC and the structure was elucidated on the basis of spectral evidences) was extracted from P. fallax Hemsl. (School of Pharmaceutical Sciences, Central South University, China). [γ-32P] ATP was obtained from Funui Biological Engineering Institute (Beijing, China). ROS detection kits and BCA protein kits were purchased from Beyotime Company (Jiangsu, China). Elisa kits for measurement of TNF-α were obtained from Senxiong Biological Limited Corporation (Shanghai, China). Gel shift assay system for the determination of NF-κB activity, the probe of NF-κB and anti-IκB-α were obtained from Promega.

2.2. Cell culture and treatment

Human monocytoid cells (THP-1, ATCC) were purchased from Cell Culture Center of Xiang-Ya Medical School (Changsha, China). THP-1 cells were cultured in RPMI medium 1640 containing 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. The viability of monocytic cells was assessed before the experiment using trypan blue exclusion and was always >95%. When the cells had reached subconfluence, they were passaged into six-well culture dishes at an optimal density of 105 cells/ml. In the first part, the cells were exposed to ADMA for different time periods (0, 6, 12, 24 and 48 h) and of different concentrations (3, 10 or 30 μM). In the second part, the cells were exposed to ADMA (30 μM) and different concentrations of reinioside C (1, 3 or 10 μM).

2.3. Determination of TNF-α concentration

TNF-α level in culture medium was assayed by enzyme linked immunosorbent assay (ELISA). The values were measured at 405 nm by a microplate reader (Biotek). The standard curve for TNF-α measured by this ELISA kit was linear from 0 to 1000 pg/ml; the detection limit was 16 pg/ml. The cellular protein concentration in cell lysates was determined with a protein assay kit with bovine serum albumin used as a standard. Protein amounts are expressed in picograms per milligram of total cellular protein assayed in corresponding cellular lysates.

2.4. Determination of ROS

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in fluorospectro-photometer (F4000, Japan). Cells in 6-well culture dishes were incubated with ADMA (30 μM)
for various times in the absence or presence of reinioside C (1, 3 or 10 μM), PDTC (100 μM) or L-arginine (0.5 mM). The cells were washed with RPMI medium 1640 and incubated with DCFH-DA at 37 °C for 20 min. DCF fluorescence distribution of 20,000 cells was detected by fluorospectro-photometer analysis at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

2.5. Preparation of nuclear extracts

Monocyte cells were seeded into 25 cm² culture flasks at an optimal density of 10⁵ cells/ml. After incubation for about 2–3 d, cells were collected and incubated with 400 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice. After vortexing, cell lysates were centrifuged by 12,000 g for 3 min at 4 °C and nuclei were resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 04 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerin 1 mM DTT, 1 mM PMSF) and vigorously vortexed at 4 °C for 15 min. Nuclear lysates were next centrifuged at 12,000 g for 5 min and the supernatant containing the nuclear proteins was carefully removed. Protein aliquots were either frozen at −70 °C or immediately used for EMSA.

2.6. Electrophoretic mobility shift assay (EMSA)

The EMSA for determining the NF-κB DNA-binding activity was performed by incubating aliquots of nuclear extracts containing 15 μg total protein with γ-32P-labelled double-stranded NF-κB specific oligonucleotide probe (sense: 3′-TCAACTCCCTGAAAGGGTCCG-5′; 5′-AGTTGAGGG-GACTTTCCCAGGC-3′) by T4 polynucleotide kinase. The labelled probe was purified through Sephadex G-25. After 10 min of incubation at room temperature, the mixture was run on a 4% nondenaturing polyacrylamide gel in 0.5×TBE buffer. After electrophoresis, the gels were dried and the DNA-protein complexes were detected by autoradiography.

2.7. Western immunoblotting analysis of IκB-α protein

The cellular levels of IκB-α protein were determined by Western immunoblotting analysis. Briefly, cell proteins were separated by SDS/12.5%(w/v)-PAGE, followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκB-α antibody. Bands corresponding to IκB-α were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyt2 version 1.1).

2.8. Statistical analysis

Results are expressed as means±SEM. The data were analyzed by ANOVA followed by Newmann–Keuls–Student test for multiple comparisons. The statistical significance was considered if P<0.05.

3. Results

3.1. Effect of ADMA on TNF-α production in cultured THP-1

We examined the time course and concentration dependence of ADMA-induced TNF-α production in cultured THP-1.
cells were exposed to ADMA for different time periods (0, 6, 12, 24 and 48 h) and of different concentrations (3, 10 or 30 μM). Time and concentration course experiment revealed that ADMA time- and concentration-dependently increased TNF-α production and treatment with ADMA at a higher concentration of 30 μM for 24 h caused a significant increase of TNF-α (p < 0.01) (Fig. 2).

3.2. ROS–NF–κB pathway mediated TNF-α production induced by ADMA

We examined the time course of ADMA-induced intracellular ROS production and NF–κB activity in cultured THP-1. The cells were exposed to ADMA (30 μM) for different time periods (0, 1, 2 and 4 h). Time course experiment revealed that ADMA (30 μM) time-dependently increased intracellular ROS production and activated NF–κB activity, both reached the peak at 2 to 4 h in cultured THP-1 (Figs. 3 and 4A).

The activation of NF–κB might be caused by enhanced phosphorylation of the inhibitor protein IκB–α. Therefore the effect of ADMA on the phosphorylation status of IκB–α was investigated. As shown in Fig. 4B, time course experiment revealed that ADMA (30 μM) time-dependently decreased the level of IκB–α protein in cultured THP-1.

Fig. 4. Time course of ADMA-induced NF–κB/DNA-binding activity in cultured THP-1 (A). Cells were exposed to ADMA (30 μM) for 0, 1, 2 and 4 h. Competition experiment where unlabeled NF–κB probe was added at 100-fold excess concentrations to the 32P-labeled probe. 1: unlabeled probe 2: 0 h, 3: 1 h, 4: 2 h, 5: 4 h; Time course of ADMA induced the phosphorylation status of IκB–α in cultured THP-1 (B). Cells were exposed to ADMA (30 μM) for 0, 1, 2 and 4 h. 1: 0 h, 2: 1 h, 3: 2 h, 4: 4 h.

Fig. 5. Effect of reinioside C on elevated level of TNF-α induced by ADMA in medium of THP-1 cells. Cells were preincubated with different concentrations of reinioside C (1, 3 or 10 μM), t-arginine (0.5 mM) or PDTC (100 μM) for 1 h, and then 30 μM ADMA was added and incubated for an additional 24-hour period. Data are expressed as means±SEM, n = 6. Compared with control, **p < 0.01; Compared with ADMA group, *p < 0.05, **p < 0.01. +reinioside C=ADMA + reinioside C; +t-arginine=ADMA + t-arginine; +PDTC=ADMA + PDTC.

Fig. 6. Effect of reinioside C on elevated level of intracellular ROS induced by ADMA in medium of THP-1 cells. Cells were preincubated with different concentrations of reinioside C (1, 3 or 10 μM), t-arginine (0.5 mM) or PDTC (100 μM) for 1 h, and then 30 μM ADMA was added and incubated for an additional 2 h period. Data are expressed as means±SEM, n = 6. Compared with control, **p < 0.01; Compared with ADMA group, *p < 0.05, **p < 0.01. +reinioside C=ADMA + reinioside C; +t-arginine=ADMA + t-arginine; +PDTC=ADMA + PDTC.
The effect of reinioside C on ADMA induced the phosphorylation status of IκB, which is an important role in the pathogenesis of endothelial dysfunction, and ADMA was added and incubated for an additional 2 h period. Further, pretreatment with l-arginine (0.5 mM), PDTC (100 μM) prevented ADMA-induced intracellular ROS production, and that reinioside C (10 μM) itself had no effect on the intracellular ROS level in THP-1 (Figs. 6 and 7A).

We also investigated the effect of reinioside C (1, 10, 30 μM) on the phosphorylation status of IκB-α induced by ADMA. As shown in Fig. 7B, reinioside C concentration-dependently inhibited the decrease of IκB-α level induced by ADMA (30 μM), the same effects were also observed when the cells were pre-treated with l-arginine (0.5 mM) and PDTC (100 μM).

4. Discussion

The development of ACS is often associated with inflammation. TNF-α, an inflammatory cytokine, plays an important role in the stability of plaque which induces the occurrence of ACS (Wang et al., 2004). Monocytes, participating in inflammatory responses, can take up oxidized lipoproteins to form foam cells and secrete some cytokines such as TNF-α and MCP-1 in the atherosclerotic lesions. Circulating monocytes from patients of ACS showed high level of proinflammatory cytokines including TNF-α, and monocyte activation is believed to play an important role in the pathogenesis of ACS (Del Fresno et al., 2007). Many studies suggest that ADMA may be a potential atherogenic molecule contributing to the initiation and progress of atherosclerotic diseases, and interactions of ADMA with cytokines are important steps in the inflammatory processes of atherosclerosis. Recently, it was reported that ADMA, an endogenous inhibitor of NOS, can promote formation of foam cells by up-regulating LOX-1 expression in monocytes (Smirnova et al., 2004). Therefore, we speculate that ADMA may have direct proinflammatory effect on cytokines production in monocytes. Here we found that exogenous ADMA increased the expression of TNF-α in monocyte cell line THP-1 in a concentration- and time-dependent manner, and this effect was markedly attenuated by reinioside C, l-arginine (NOS substrate) and PDTC (inhibitor of NF-κB).

The expression of proinflammatory cytokines, including TNF-α and MCP-1, is regulated principally at the level of transcription. In monocytes, there are several transcriptional factor-binding sites in cytokines promoter including NF-κB (Guha et al., 2000). Activated NF-κB can bind to cytokines promoter, which is critically involved in cytokines gene regulation by various stimuli including ox-LDL, homocysteine (Hcy) and so on (Wilson et al., 2000). In the present study we found that exogenous ADMA increased the level of TNF-α, concomitantly with activation of NF-κB activity and intracellular ROS elevation. These effects were inhibited by pretreatment with reinioside C or PDTC.

Accumulating evidences indicate that oxidative stress plays an important role in the pathogenesis of endothelial dysfunction and atherosclerosis (Jacobi et al., 2005). Recently, ROS has been considered as second messengers leading to NF-κB activation in response to extracellular stimuli, and then up-

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Fig. 7. The effect of reinioside C and PDTC on ADMA-induced NF-κB/DNA-binding activity in cultured THP-1 (A). Cells were preincubated with different concentrations of reinioside C (1, 3 or 10 μM) or PDTC (100 μM) for 1 h, and then 30 μM ADMA was added and incubated for an additional 2 h period. Competition experiment where unlabeled NF-κB probe was added at 100-fold excess concentrations to the 32P-labeled probe. 1: unlabeled probe, 2: control, 3: ADMA (30 μM), 4: reinioside C (1 μM)+ADMA (30 μM), 5: reinioside C (3 μM)+ADMA (30 μM), 6: reinioside C (10 μM)+ADMA (30 μM), 7: l-arginine (0.5 mM)+ADMA (30 μM), 8: PDTC (100 μM)+ADMA (30 μM).

The effect of reinioside C on ADMA induced the phosphorylation status of IκB-α in cultured THP-1 (B). Cells were preincubated with different concentrations of reinioside C (1, 3 or 10 μM) or PDTC (100 μM) for 1 h, and then 30 μM ADMA was added and incubated for an additional 2 h period. 1: control, 2: ADMA (30 μM), 3: reinioside C (1 μM)+ADMA (30 μM), 4: reinioside C (3 μM)+ADMA (30 μM), 5: reinioside C (10 μM)+ADMA (30 μM), 6: l-arginine (0.5 mM)+ADMA (30 μM), 7: PDTC (100 μM)+ADMA (30 μM).

3.3. Effect of reinioside C on ADMA-induced TNF-α expression

Firstly, we determined the effects of reinioside C (1, 10, 30 μM) on ADMA-induced TNF-α elevation, l-arginine (0.5 mM) or PDTC (100 μM) was also added. The results showed that reinioside C concentration-dependently reduced intracellular ROS elevation and NF-κB activation induced by ADMA (30 μM, 2 h). Further, pretreatment with l-arginine (0.5 mM), PDTC (100 μM) prevented ADMA-induced intracellular ROS production, and that reinioside C (10 μM) itself had no effect on the intracellular ROS level in the medium of THP-1 (Fig. 5).

Furthermore, we investigated whether ROS–NF-κB pathway was involved in the effect of reinioside C (1, 10, 30 μM) on ADMA-induced TNF-α elevation. The results showed that reinioside C concentration-dependently reduced intracellular ROS elevation and NF-κB activation induced by ADMA (30 μM, 2 h). Further, pretreatment with l-arginine (0.5 mM), PDTC (100 μM) prevented ADMA-induced TNF-α production, and that reinioside C (10 μM) itself had no effect on TNF-α level in the medium of THP-1 (Fig. 5).

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regulating the gene expression of proinflammatory cytokines including TNF-α, OX-LDL, high glucose and ADMA increased the production of intracellular ROS and induced TNF-α expression in endothelial cells, which were suppressed by anti-oxidant agents (Bai et al., 2007; Yang et al., 2007; Jiang et al., 2003). Our study demonstrated that ADMA significantly increased intracellular ROS production as shown by DCF fluorescence intensity and induced expression of TNF-α in THP-1, which were abolished by pretreatment with reinside C and PDTC. Although PDTC is a well-known inhibitor of NF-κB, there was evidence supporting that PDTC suppresses NF-κB activation through its antioxidant property (Yokoo and Kitamura, 1996), which possibly accounts for the reduced ROS formation by PDTC in our experiment. These results suggest that ADMA-induced increase of TNF-α was related to the elevation of intracellular ROS level, which resulted in the subsequent activation of NF-κB in THP-1. Reinside C attenuates the increasing levels of TNF-α induced by ADMA in THP-1 through inhibiting ROS/NF-κB signaling pathway, more precisely, through inhibiting the ROS rise at the cellular level.

It is worthy of being mentioned that the effective concentrations of ADMA used in our study were much higher than plasma level of ADMA achieved in patients with ACS. In fact, the intracellular ADMA level has been reported to be much more concentrated, even 10 folds higher than the reported range for plasma values. Thus, higher concentrations of exogenous ADMA should be used to elevate the intracellular ADMA level in the in vitro experimental system. The concentrations of exogenous ADMA used in our study were based on our previous studies (Jiang et al., 2003; Chen et al., 2007), which could significantly increase the intracellular ADMA levels compared with control (Jiang et al., 2006).

In conclusion, the results of the present study furnished, to our knowledge, the first evidence that ADMA can induce inflammatory responses in monocyte. Moreover, reinside C could attenuate the increase of TNF-α induced by exogenous ADMA through inhibition of ROS/NF-κB signaling pathway in THP-1. Our data also indicate that reinside C may be a potential antiatherosclerotic compound, and the further study is needed to define its effect in vivo using hypercholesterolemic and atherosclerosis animal models.

References

Wang, Y.N., Che, S.M., Ma, A.Q., 2004. Clinical significance of serum cytokines IL-1beta, sIL-2R, IL-6, TNF-alpha, and IFN-g in acute coronary syndromes. Chin. Exp. Med. 6, 38–44.


