Asymmetric dimethylarginine triggers macrophage apoptosis via the endoplasmic reticulum stress pathway

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Abstract Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is emerging as a key contributing factor in atherogenesis, a process in turn known to involve macrophage apoptosis. The aim of this study was to determine the effect of ADMA on macrophage apoptosis, with specific reference to the endoplasmic reticulum (ER) stress pathway. Macrophage apoptosis was evaluated by Annexin V-Propidium iodide (PI) and Hoechst 33258 staining assays. Levels of the ER stress marker glucose regulated protein 78 (GRP78) were characterized by western blot. Levels of the proapoptotic C/EBP-homologous protein (CHOP) were evaluated by western blot and reverse transcription polymerase chain reaction (RT-PCR), and caspase-4 activity was measured using a colorimetric protease assay kit. We observed ADMA dose- and time-dependent increases in macrophage levels of GRP78. Similar ADMA dose- and time-dependent increases were detected in intracellular caspase-4 activity and macrophage apoptosis, all of which were sensitive to treatment with siRNAs for protein kinase RNA-like ER kinase and inositol-requiring protein-1 (IRE1), the ADMA antagonist L-arginine, as well as inhibitors of eukaryotic translation initiation factor-2 (salsalubrinal), IRE1 (irestatin 9389), and c-Jun N-terminal kinase (SP600125). Our results indicate that ADMA triggers macrophage apoptosis via the ER stress pathway.

Keywords Asymmetric dimethylarginine · Endoplasmic reticulum stress · Apoptosis · Macrophages

Introduction

Macrophages play key role in all stages of atherosclerosis [1]. In advanced atheromata, for example, macrophage apoptosis resulting from defective phagocytic clearance leads to plaque necrosis [2], which promotes lesion instability and leads to myocardial infarction, stroke, and peripheral vascular disease [3–7]. Collectively these data indicate that macrophage apoptosis may be a critical step in benign-to-vulnerable plaque transformation. Endoplasmic reticulum (ER) stress has been implicated as an adaptive survival system in macrophages [8] and is thought to play a role in the erosion or rupture of atherosclerotic plaques. In this context, data from human and mouse models of atherosclerotic lesions indicate that advanced “vulnerable” plaques are characterized by increased levels of the ER stress marker (glucose regulated protein 78 (GRP78) and the
proapoptotic C/EBP-homologous protein (CHOP)), as well as by increased macrophage apoptosis [9–11]. Moreover, ER stress has been shown to accompany macrophages apoptosis induced by acetylated low-density lipoprotein or free cholesterol [12, 13]. An understanding of the stimuli that intersect with ER stress to cause macrophage apoptosis and plaque necrosis, and the mechanism by which these effects are exerted are therefore key goals in deciphering the cellular biology of vulnerable plaque formation.

Recent research has identified the l-arginine analog asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), as a key contributing factor in atherogenesis and vulnerable plaque formation. ADMA levels have been shown to have prognostic value for mortality after acute myocardial infarction [14] and to predict all-cause and cardiovascular mortality in stable and acute coronary artery disease (CAD) [15]. Moreover, ADMA has been shown to induce apoptosis in endothelial cells and smooth muscle cells, increase oxidized low-density lipoprotein (ox-LDL) uptake during foam cell formation [16], and enhance plasma triglyceride level and lesion areas in apolipoprotein E (ApoE) -/- mice [17]. These studies along with reports of ADMA triggering of lipolysis and ER stress-related inflammatory response in cultured adipocytes [18] collectively suggest a strong relationship between ADMA and the development of atherosclerosis. Here, in an effort to establish a mechanistic basis for this relationship, we studied the effect of ADMA on macrophage apoptosis, with a specific emphasis on characterizing its role in the induction of ER stress.

Methods

Reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Hyclone (Thermo Fisher Scientific Inc, UT, USA). ADMA, l-arginine, inositol-requiring protein-1 (IRE1) inhibitor Irestatin 9389, the c-Jun N-terminal kinase (JNK) inhibitor SP600125, and eukaryotic translation initiation factor-2α (eIF2α) inhibitor salubrinal were purchased from Sigma-Aldrich (Sigma-Aldrich Co, St. Louis, MO, USA). Antibodies for GRP78, IRE1, phospho-JNK1 (p-JNK1), JNK1, protein kinase RNA (PKR)-like ER kinase (PERK), eIF2α, phospho-eIF2α (p-eIF2α), and CHOP were purchased from Abcam (Abcam, Cambridge, UK), Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA), and Cell Signaling Technology (Cell Signaling Technology Inc, MA, USA). The revertAidTM first strand cDNA synthesis kit was obtained from Fermentas (Thermo Fisher Scientific Inc, UT, USA). The polymerase chain reaction (PCR) mix was purchased from Takara (TaKaRa Holding Inc, Tokyo, Japan). The caspase-4 activity assay kit was purchased from Biovision (Biovision Inc, Milpitas, CA, USA). Fluoresceinisothiocynate (FITC)-Annexin V and Propidium Iodide (PI) were obtained from Jingmei Biotech (Shenzhen, China). All other biochemicals used in this study were of the highest purity available.

Cell culture

The monocyte cell line THP-1 (ATCC TIB-202) was seeded in RPMI 1640 medium containing 10 % FBS, 50 μg/ml penicillin, and streptomycin. For differentiation of macrophages, THP-1 cells were treated with 100 nm PMA (Sigma) for 48 h. After differentiation, total RNA or cell lysates were prepared for further analysis.

RNA interference of IRE-1 and PERK

Transfection with Lipofectamine RNAiMAX (Invitrogen) was performed according to the manufacturer’s instructions. Briefly, macrophages which had been differentiated for 48 h were transfected with 10 nm Stealth Select RNAi directed against IRE-1 (HSS140846; HSS176615) or PERK (HSS114059; HSS190343) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and incubated for 6 h. Levels of protein targets were evaluated by Western blot analysis.

Apoptosis determination

Apoptosis was determined using an Annexin V-PI double staining assay with flow cytometry and Hoechst 33258 staining assay (see Supplement 2 for details).

Western blotting analysis

Cells were collected and lysed for 30 min at 4 °C in RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate (SDS)] (Beyotime, Jiangsu, China). Total protein (50–100 μg) was loaded and separated by 10 % SDS–polyacrylamide gel electrophoresis for 2 h, prior to transfer to a polyvinylidene fluoride membrane. Primary antibodies for GRP78 (1:1,000), IRE-1 (1:1,000), PERK (1:200), eIF2α (1:1,000), p-eIF2α (1:1,000), JNK (1:1,000), CHOP (1:1,000), or β-actin (1:5,000) were diluted with 5 % bovine serum albumin and incubated with membranes at 4 °C overnight. After washing, horseradish peroxidase-conjugated secondary antibodies (1:10,000) diluted in 5 % non-fat milk were incubated with membranes at room temperature for 1 h. Bands were visualized by adding enhanced chemiluminescence reagent and analyzed with a gel documentation system (Gel Doc1000 and Multi-Analyst version 1.1, Bio-Rad, Hercules, CA, USA).
Fig. 1 ADMA induces apoptosis and triggers ER stress activation in macrophages. Macrophages were exposed to ADMA at various concentrations (0, 1, 3, 10, or 30 µM) for 24 h and the effects on macrophage apoptosis (a,b) caspase 4 activity (c), and GRP78, PERK and IRE1 protein expression (d) were observed. Macrophages were also exposed to 30 µM ADMA for various times (0, 6, 12, or 24 h) and the effect on apoptosis (e,f), caspase 4 activity (g), and expression of GRP78, PERK, and IRE1 (h) were observed. The data were expressed as the mean ± SEM, n = 5. Compared with control, *P < 0.05; **P < 0.01.
Reverse transcription polymerase chain reaction (RT-PCR)

CHOP mRNA levels were analyzed using RT-PCR (see Supplement 3 for details on the procedure). PCR products were separated using 1.5% agarose gel electrophoresis and visualized using nucleic acid dyestuff gelview (BioTeke Corporation, Beijing, China) on a UV transilluminator (Kodak, Rochester, NY, USA). Intensity values were normalized to those of GAPDH reference mRNA.

Measurement of caspase-4 activity

Lysate caspase-4-like protease activity was measured using a colorimetric caspase-4 assay kit according to the manufacturer’s protocol. Briefly, macrophages were collected and lysed with chilled cell lysis buffer on ice for 10 min. The reaction mixture contained 50 µl of cell lysate, 50 µl of reaction buffer, and 5 µl of LEVD-pNA substrate, and the assay was performed in a 96-well plate. The mixture was incubated for 90 min at 37 °C, and the absorbance was read at 405 nm using a Beckman Coulter DXT-880 (Beckman Coulter, Inc, CA, USA). Activity was expressed as the optical density at 405 nm.

Statistical analysis

Results were expressed as the mean ± standard error of mean. The data were analyzed using Analysis of Variance followed by a Newman-Student-Keuls test for multiple comparisons. The significance level was established at $P < 0.05$.

Results

ADMA induces ER stress and apoptosis in macrophages

Incubation of macrophages with ADMA induced cellular levels of GRP78, PERK and IRE1, as well as caspase-4 activity, and the percentage of apoptotic cells in both dose (0, 1, 3, 10, or 30 µM ADMA for 24 h, Fig. 1a–d) and time (30 µM ADMA for 0, 6, 12, and 24 h, Fig. 1e–h)-dependent manners in THP-1 macrophages.
**Fig. 3** The role of the ER stress sensor IRE1 in ADMA-induced apoptosis in macrophages. Expression of IRE1 was effectively suppressed by IRE1 siRNA (a). IRE1 siRNA markedly suppressed ADMA induction of its downstream effector JNK1 (b) and CHOP (c, d). Consistent with these results, IRE1 siRNA also inhibited ADMA-induced increases in the percentage of apoptotic cells (e) and caspase 4 activity (f). Non-targeting siRNA and IRE1 siRNA alone had no effect. The data were expressed as the mean ± SEM, n = 5. Compared with the control, *P < 0.05; **P < 0.01. Compared with ADMA (30 µM), #P < 0.05; ##P < 0.01.

**Fig. 4** The role of the unfolded protein response in ADMA-induced apoptosis in macrophages. Macrophages were treated with the ADMA inhibitor L-arginine, specific eIF2α inhibitor salubrinal, specific IRE1 inhibitor irestatin 9389, and specific JNK inhibitor SP600125. These inhibitors suppressed ADMA-induced cellular levels of CHOP (a, b), proapoptotic caspase 4 activity (c), and the percentage of apoptotic macrophages (d). The data were expressed as the mean ± SEM, n = 5. Compared with the control, *P < 0.05; **P < 0.01. Compared with ADMA (30 µM), #P < 0.05; ##P < 0.01.
ER stress is involved in ADMA-induced apoptosis in macrophages

ER stress is characterized by the activation of ER stress sensors (PERK and IRE1), resulting in an unfolded protein response (UPR), and subsequent induction of apoptosis [19]. To investigate the role of PERK or IRE1 in mediating ADMA-induced apoptosis, macrophages were transfected with IRE-1 siRNA or PERK siRNA for 6 h, then exposed to ADMA (30 μM). As anticipated, treatment with PERK and IRE1 siRNAs, respectively, reduced PERK (Fig. 2a) and IRE1 (Fig. 3a) protein levels. Moreover, ADMA-induced increases in levels of the proapoptotic protein CHOP, downstream targets phospho-eIF2α (PERK) and phospho-JNK1 (IRE1), as well as intracellular caspase-4 and the percentage of apoptotic cells, were all specifically inhibited by pretreatment with PERK (Fig. 2b–f) and IRE1 (Fig. 3b–f) siRNAs, but not non-targeting siRNA.

To further characterize the role of UPR in ADMA-induced apoptosis in macrophages, cells were pretreated with the ADMA antagonist L-arginine, the eIF2α inhibitor salubrinal, the IRE1 inhibitor irestatin 9389, or the JNK inhibitor SP600125 prior to treatment with ADMA induction of macrophages apoptosis, cellular CHOP levels, and caspase activity was reversed under all these conditions (Fig. 4a–d), confirming the critical role played by ADMA regulation of the ER stress pathway in macrophage apoptosis (Fig. 5).

Discussion

In this study, we have shown that ADMA induces apoptosis in macrophages in a dose- and time-dependent manner, and that this process involves the initiation of ER stress, as evinced by PERK/eIF2α- and IRE1/JNK1-dependent increases in cellular levels of the ER stress marker GRP78 (Fig. 5).

ADMA, the product of an asymmetric methylated protein, is an endogenous inhibitor of NOS. Plasma concentrations of ADMA are maintained at low levels under physiological conditions and are greatly elevated under conditions involving hypertension, hyperlipemia, and hyperhomocysteinemia [20]. An increasing number of studies have shown that ADMA is an atherogenic factor contributing to the initiation and progression of CAD [21, 22]. For example, a recent study showed that plasma levels of ADMA are higher in patients with acute coronary syndrome (ACS) compared to patients with stable CAD [23]. Apoptosis in macrophages plays a critical role in ACS, and we have shown here for the first time that ADMA induces apoptosis in macrophages in a dose- and time-dependent manner, indicating its possible role in the development of ACS.

We also showed that ADMA-induced apoptosis in macrophages involves the initiation of ER stress, a prolonged period of which leads to cell death [24]. Moreover, recent studies have implicated ER stress-associated apoptosis in a variety of human diseases, including cardiovascular disease, neurodegenerative disease, diabetes mellitus, and liver disease [25–27]. GRP78 is a highly abundant ER chaperone which has been associated with the onset of ER stress, and which we found in this study was induced by ADMA in macrophages, further demonstrating the link between ADMA and ER stress.

ER stress involves the activation of the ER transmembrane sensors PERK, IRE1, and activating transcription factor-6 (ATF-6), leading to UPR. IRE1 promotes the phosphorylation of JNK, which promotes the degradation of misfolded proteins [28].

PERK, via phosphorylation of eIF2α, temporally delays protein translation, which allows for correction of any perturbations in the process of protein translation. Moreover, PERK, via p-eIF2α, induces of CHOP, which participates in various corrective functions during transient ER stress [29]. siRNA and inhibitor analysis in the present study implicated PERK and IRE1 in ADMA-induced macrophage apoptosis. In addition, inhibition of the downstream targets of PERK and IRE1, eIF2α, and JNK, using salubrinal and SP600125, respectively, inhibited ADMA-induced apoptosis in macrophages. Collectively, these data demonstrated that ER stress plays a critical role in ADMA-induced apoptosis in macrophages.
Initiation of the ER stress-mediated apoptotic process is promoted by pathways involving caspase-4 and CHOP, a factor whose role in ER-initiated apoptotic signaling is well characterized [24]. CHOP exhibits proapoptotic characteristics by directly regulating the expression of numerous proapoptotic factors, such as members of the caspase family of cysteine-dependent aspartate-specific proteases, which play a major role in the induction of apoptosis. For example, both caspase-12 and caspase-4 have been reported to be localized to the ER and are cleaved in cells treated with ER stress agents [30]. Moreover, cells treated with siRNA against caspase-4 have been reported to be resistant to ER stress agent-induced apoptosis [31]. In the present study, we found that ADMA induction of cellular levels of both CHOP and caspase-4 activity was accompanied by an increase in cell apoptosis. Moreover, treatment of cells with PERK siRNA, IRE siRNA, and the previously discussed inhibitors of cysteine-dependent aspartate-specific proteases, which characterize the ER stress agent-induced apoptosis [31]. In the present study, we found that ADMA induction of cellular levels of both CHOP and caspase-4 activity was accompanied by an increase in cell apoptosis. Moreover, treatment of cells with PERK siRNA, IRE siRNA, and the previously discussed inhibitors of cysteine-dependent aspartate-specific proteases, which characterize the ER stress agent-induced apoptosis [31], further study on the role of NOS in ER stress activation. Collectively, these results indicate that CHOP and caspase-4 play important roles in ADMA-induced apoptosis in macrophages via the ER stress pathway.

The effective concentrations of ADMA used in the present study are similar to that reported in previous studies [32], and are higher than the plasma level ADMA observed in patients with ACS. While it is possible that the intracellular ADMA levels in the endothelium are higher than the reported range for plasma values, these have yet to be determined in ADMA patients. Future studies will investigate the intracellular levels of ADMA and its proapoptotic effects in vivo.

Conclusion

In summary, our study is the first to report that ADMA induces macrophage apoptosis by triggering ER stress via a mechanism involving the PERK/eIF2α and IRE1/JNK1-CHOP/caspase-4-dependent signaling pathways. It should be stressed that the current study has not explored the relationship between NOS signaling and macrophages apoptosis. Although it has been previously reported that expression of NOS is associated with osteocyte apoptosis [33], further study on the role of NOS in ER stress activation and macrophages apoptosis is warranted.

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