Short-Term Treatment with an Angiotensin II Receptor Blocker Prevents Necrotic Core Formation by Inhibiting Oxidative Stress-Mediated Apoptosis in Macrophages


Abstract

Objective: To study the role of angiotensin II (Ang II) type 1 receptor (AT1R) on necrotic core formation associated with endoplasmic reticulum (ER) stress-induced macrophage apoptosis.

Methods and Results: Eight-week-old ApoE-deficient mice were fed a high-cholesterol diet and administered AT1R blocker (ARB), hydralazine, or vehicle from 15 weeks of age. Histochemical analysis of atherosclerotic lesion at 19 weeks of age showed a marked reduction of necrotic core area in ARB-treated mice. At 17 weeks of age, mRNA expression levels of C/EBP Homologous Protein (CHOP) and the percentage of TUNEL-positive cells were also decreased in ARB-treated mice, concomitant with the significantly lower expressions of NOX2 and NOX4 genes. Free cholesterol (FC)-loaded thioglycollate-induced peritoneal macrophages (TGPM) showed a significant increase in CHOP mRNA expression and the number of annexin V- or propidium iodide (PI)-positive cells; however, these were not affected by cotreatment with Ang II or Ang II plus ARB. In contrast, TGPM isolated from Ang II-treated mice, exhibiting higher expression levels of NOX2 and p22phox genes, showed a more robust increase in CHOP mRNA expression after FC loading.

Conclusions: Our findings demonstrate that ARB treatment inhibits necrotic core formation, in part by alleviating oxidative stress-mediated amplification of ER stress-induced macrophage apoptosis.

Keywords

Atherosclerosis; Renin angiotensin system; Endoplasmic reticulum stress; Apoptosis; Macrophage; Oxidative stress; CHOP; Necrotic core

Introduction

The renin-angiotensin system (RAS) is profoundly involved both in atherosclerotic lesion initiation as well as advanced plaque progression in both animal experiments and human studies of atherosclerosis [1-4]. Treatment with angiotensin II type 1 receptor (AT1R) blocker (ARB) has been reported to inhibit the progression of vulnerable plaque [5-9]; however, the mechanisms underlying this beneficial effect of ARB in reducing the vulnerability of advanced atherosclerotic plaque, especially in terms of necrotic core formation, have not been fully investigated.

Macrophage apoptosis substantially contributes to necrotic core expansion in advanced plaques [10-14], which is accompanied by augmented accumulation of macrophages, thinned fibrous cap, and enhanced proinflammatory response, resulting in plaque disruption and ultimately in acute atherothrombotic vascular disease [15-17]. Endoplasmic reticulum (ER) stress has been profoundly implicated in macrophage apoptosis [18-21]. When ER stress is abnormally prolonged, C/EBP homologous protein (CHOP) is upregulated in plaque lesions, triggering plaque necrosis [22-25]. CHOP-deficient atherosclerosis-prone mice showed a significant reduction in apoptosis and subsequent plaque necrosis [23]. Furthermore, Miyoshi et al. reported a striking relationship between advanced lesion stage, CHOP expression, and apoptosis in human coronary artery lesions [22].

Recent studies have demonstrated that RAS plays a crucial role in the pathogenesis of ER stress-related cardiovascular diseases, such as congestive heart failure, cardiac remodeling after myocardial infarction, and renal disease [26-28]. Although RAS is largely involved in the pathogenesis of atherosclerosis, its effect on ER stress in the context of the development of vulnerable plaque has not been fully elucidated. In this study, we examined the role of RAS in ER stress-related atherosclerotic lesion development using in vivo plaque rupture model. In additional in vitro experiments, we show the possibility that while ATIR is not autonomously involved in ER stress-induced macrophage apoptosis; it amplifies ER stress-induced macrophage apoptosis by enhancing oxidative stress. Our findings suggest that oxidative stress is a potential therapeutic target to reduce ER stress-induced macrophage apoptosis and stabilize advanced atherosclerotic plaques.

Methods

Experimental animals

All experiments were performed in strict adherence to the “Directive 2010/63/EU” of the European Parliament and to the Guidelines for Animal Experiments of the Kyoto Prefectural University of Medicine, following approval by a local university ethics review board.

Apolipoprotein E deficient (ApoE−/−) mice (C57BL/6) were obtained from Taconic Co., Ltd. (Germantown, NY, USA). Eight-week-old male ApoE−/− mice were fed a high-cholesterol diet (13.6% fat, 1.25% cholesterol; Oriental Yeast Co., Tokyo, Japan). From 15 weeks of age, ARB (olmesartan, 3 mg/kg/day), hydralazine (50 mg/kg/day), or saline were administered using an osmotic minipump until 19 weeks of age. For implantation of an osmotic minipump, mice...
were anesthetized using isoflurane (2%, 0.2 mL/min) throughout the surgery. The depth of anesthesia was confirmed by lack of tail pinch response. The animals were housed in a room maintained at 22°C under a 12-hr light/dark cycle and were provided with drinking water ad libitum. At 19 weeks of age, the area of the atherosclerotic lesion in the brachiocephalic artery was evaluated. Before harvesting the tissues, mice were euthanized by trans-cardiac perfusion under anesthesia induced by isoflurane.

**Hemodynamic analysis**

Systolic blood pressure and heart rates were measured under conscious and unrestrained conditions using a programmable sphygmmomanometer (BP-98A; Softron, Tokyo, Japan).

**Serum lipid analysis**

Measurements of total cholesterol, triglyceride, low density lipoprotein cholesterol, and high density lipoprotein cholesterol were outsourced to SRL, Tokyo, Japan.

**Quantitative analysis of atherosclerotic lesions**

Mice were euthanized, and brachiocephalic arteries were embedded in paraffin. Sections were cut every 10 μm along the brachiocephalic artery from the proximal end. Atherosclerotic lesions were examined at 3 different locations. Imaging and analysis of Miller’s elastin/van Gieson-stained artery was accomplished using Image J Software (http:rsbweb.nih.gov/ij/).

**Immunohistochemistry**

Brachiocephalic artery was quickly removed after PBS perfusion, embedded in paraffin, and immunofluorescently labeled. For Mac-2 staining, a combination of anti-mouse Mac2 antibody (CEDARLANE, Burlington, Ontario, Canada) and Alexa Flour 488-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA) was used. Apoptosis in atherosclerotic lesions was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using the tetramethylrhodamine red in situ cell death detection kit (Roche) following the manufacturer’s protocol. Nuclei were labeled using DAPI solution (DOJINDO, Kumamoto, Japan). The TUNEL-stained sections were analyzed using a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany), and quantification was performed using Image J. Five fields of cells for each condition (~1000 cells) were counted. In this protocol, apoptosis is defined as those cells that stain with annexin V or PI, and the data are expressed as the percentage of these cells per total cells counted.

**Statistical analysis**

All data are expressed as mean ± SE. Mean values were compared using ANOVA. If a statistically significant effect was found, Tukey-Kramer test was performed to analyze the differences between the groups. P<0.05 was considered statistically significant.

**Results**

**Necrotic core formation is significantly reduced in ARB-treated ApoE−/− mice**

In preliminary experiments, we examined the atherosclerotic lesions in the brachiocephalic arteries of 16- and 20-week-old ApoE−/− mice fed a high-cholesterol diet from 8 weeks of age. Consistent with the previous findings of Johnson et al. [30], the necrotic core area was found to have extensively expanded from 16 weeks to 20 weeks of age (Supplemental Figure 1). To examine the effect of ARB treatment on the expansion of necrotic core area, olmesartan, hydralazine, or vehicle was administered from 15 weeks of age. At 19 weeks of age, ARB-treated mice showed a marked reduction in the necrotic core area as well as the atherosclerotic plaque area compared with hydralazine- and vehicle-treated mice (Figure 1A and 1B). Lipid profile did not differ between the 3 groups except for high-density lipoprotein cholesterol (Supplemental Figure 2A). Compared to vehicle-treated mice, systolic blood pressure was significantly decreased in ARB- and hydralazine-treated mice; however, there was no significant difference between the 2 groups (Supplemental Figure 2B), suggesting that the inhibitory effect of ARB on necrotic core expansion was independent of its antihypertensive effect. The number of MAC-2-positive cells as well as CD68 mRNA expression levels at 17 weeks of age was significantly reduced in ARB-treated mice accompanied by the decreased mRNA expression of MCP-1 (Figures 1C and 1D), suggesting that ARB treatment attenuates cavities of wild-type mice 4 days after an intra-peritoneal injection of 2.0 mL of 3% sterile thioglycollate medium. After 3 h of incubation in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS, non-adherent cells were removed and fresh medium was added. Flow cytometric analysis showed that MPO of the adherent cells were positive for F4/80.

After 24 hours, cells were incubated with 100 μg/mL acetyl-LDL (Biomedical Technologies Inc., Stoughton, MA) plus the acyl-CoA:cholesterol acyltransferase inhibitor CI976 (Sigma-Aldrich Inc; 10 μmol/L) alone or with Ang II (10−7 M). Olmesartan (10−7 M) or PD123319 (10−5 M) was added 2 h prior to stimulation. When isolating TGPM from Ang II-treated wild-type mice, a peritoneal injection of 1 mL of Ang II (10−6 M) was performed 24, 21, and 18 h prior to cell harvesting, as described previously [29]. After the indicated incubations, 100 μl of 1 x annexin-binding buffer (25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin) containing 5 μl of Alexa-488 annexin V, 1 μl of 100 μg/ml propidium iodide (PI), and Hoechst dye were added to the cells for 15 min at room temperature according to the manufacturer’s instructions (Molecular Probes). Cells were viewed with a 40 x objective using a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany), and quantification was performed using Image J. Five fields of cells for each condition (~1000 cells) were counted. In this protocol, apoptosis is defined as those cells that stain with annexin V or PI, and the data are expressed as the percentage of these cells per total cells counted.
the migration of monocytes/macrophages into the atherosclerotic plaque lesions preceding the necrotic core expansion.

TUNEL-positive cell numbers are markedly reduced in ARB-treated ApoE-/- mice

To examine the effect of ARB treatment on apoptosis in atherosclerotic plaque, TUNEL staining was performed at 17 weeks of age as it would have been difficult to detect apoptotic cells after the formation of the large necrotic core was completed. As expected, ARB-treated mice showed a marked decrease in the percentage of TUNEL-positive cells compared with hydralazine- and vehicle-treated mice (Figures 2A and 2B), suggesting that the inhibitory effect of ARB on plaque apoptosis contributes to the reduced necrotic core expansion in ARB-treated mice.

CHOP mRNA expression is markedly inhibited in ARB-treated ApoE-/- mice

We next examined the mRNA expression levels of CHOP and B-cell lymphoma 2 (Bcl-2), which are involved in apoptosis in atherosclerotic lesions. Although Bcl-2 mRNA expression level was not increased in ARB-treated mice, CHOP mRNA expression level was significantly lower in ARB-treated mice than those in hydralazine- and vehicle-treated mice (Figure 3A), suggesting that ARB treatment inhibits ER stress-induced apoptosis by reducing CHOP mRNA expression. We tried to examine the protein expression levels in the brachiocephalic arteries; however, the amount of protein lysates was too small to perform a conventional Western blotting. Activation of AT1R has been reported to increase oxidative stress [31-33], and CHOP expression is augmented by oxidative stress [34-36]. We therefore examined the mRNA expression levels of Nox2 and Nox4, which encode key NADPH oxidases involved in intracellular reactive oxygen species (ROS) production [32,35]. The mRNA expression levels of Nox2 and Nox4 were markedly reduced by ARB treatment (Figure 3B). These findings suggest that amelioration of oxidative stress by ARB treatment may contribute to the inhibition of ER stress-induced apoptosis in ARB-treated mice.

Cotreatment with Ang II does not increase CHOP mRNA expression and subsequent apoptosis in FC-loaded peritoneal macrophages

We further examined whether AT1 receptor activation and its
inhibition by ARB could modulate CHOP mRNA expression and subsequent apoptosis. First, we isolated thioglycollate-induced peritoneal macrophages (TGPM) from wild-type mice and examined CHOP mRNA expression levels after free cholesterol (FC) loading. As shown in Figure 4A, CHOP mRNA expression showed a time-dependent increase in FC-loaded TGPMs, peaking at 8h. We therefore examined the effect of AT1R on CHOP mRNA expression at 8h after FC loading. While FC loading markedly increased CHOP mRNA expression, cotreatment with Ang II did not affect CHOP mRNA expression (Figure 4B). To exclude the possibility that AT1R counteracts with Ang II type 2 receptor (AT2R), the effects of cotreatment with Ang II plus ARB and PD123319 (a selective AT2R inhibitor) were examined. CHOP mRNA expression levels after FC loading was found to be comparable between the groups (Figure 4B). Similarly, the percentage of annexin V- or PI-positive cells after FC loading was unchanged (Figure 4C), suggesting that neither CHOP mRNA expression nor CHOP activity is autonomously regulated by AT1R or AT2R.

AT1R-mediated oxidative stress amplifies FC-induced CHOP mRNA expression

Next, we examined the possibility that AT1R could amplify FC-
Figure 3B: Quantitative PCR analysis of the mRNA expression levels of Nox2 and Nox4 in the brachiocephalic artery of 17-week-old ApoE<sup>−/−</sup> mice. Values are the mean ± SE relative to those in vehicle-treated mice. Each group consisted of 6 (vehicle), 3 (hydralazine), and 5 (ARB) mice. *P < 0.05 vs. vehicle-treated mice. # P<0.05 vs. hydralazine-treated mice.

Figure 4A: Ang II treatment does not affect the mRNA expression levels of CHOP and apoptosis in peritoneal macrophages.
(A) Quantitative PCR analysis of the time-dependent mRNA expression levels of CHOP in TGPMs from wild-type mice. White bar shows TGPMs without FC loading. Black bar shows FC loaded-TGPMs. Values are the mean ± SE relative to those in TGPMs at 0h. Each group consisted of 4 samples. Each sample was combined from 3 mice. *P<0.05 vs. TGPMs at 0h. TGPM: thioglycollate-induced peritoneal macrophages. CHOP: C/EBP homologous protein. FC: free cholesterol.

Figure 4B: Quantitative PCR analysis of the mRNA expression levels of CHOP in TGPMs at 8h after FC loading. Values are the mean ± SE relative to those in non-FC loaded macrophages at 8h. Each group consisted of 4 samples. Each sample was combined from 3 mice. *P<0.05 vs. TGPMs without FC loading. TGPM: thioglycollate-induced peritoneal macrophages. CHOP: C/EBP homologous protein. FC: free cholesterol. Ang II: angiotensin II. ARB: angiotensin receptor blocker. PD: PD123319, angiotensin II type 2 receptor inhibitor.

Figure 4C: Quantitative analysis of the percentage of annexin V or PI-positive cells in TGPMs at 16h after FC loading. Values are the mean ± SE relative to those in TGPMs without FC loading. Each group consisted of 3 samples. Each sample was combined from 3 mice. *P<0.05 vs. TGPMs without FC loading. TGPM: thioglycollate-induced peritoneal macrophages. CHOP: C/EBP homologous protein. FC: free cholesterol. Ang II: angiotensin II. ARB: angiotensin receptor blocker. PD: PD123319, angiotensin II type 2 receptor inhibitor.
induced CHOP mRNA expression by augmenting oxidative stress. We isolated TGPM from wild-type mice treated with Ang II before harvesting. Nox2 mRNA expression before FC loading was markedly increased compared with that of TGPMs from vehicle-treated mice (Figure 5A). Although Nox4 mRNA expression could not be detected in either TGPM group, mRNA expression levels of p22phox, but not p47phox, were significantly increased in TGPMs from Ang II-treated mice, suggesting that TGPMs from Ang II-treated mice produced more ROS than the control TGPMs. The fold increase in CHOP mRNA expression after FC loading was extensively augmented in TGPMs from Ang II-treated mice compared with that in control TGPMs (4.1-fold vs 2.0-fold, P<0.01) (Figure 5B), suggesting that AT1R amplifies CHOP mRNA expression in FC-loaded macrophages by augmenting oxidative stress.

Discussion

In this study, we demonstrated for the first time that short-term treatment of advanced atherosclerosis lesions with ARB markedly inhibits the large necrotic core formation characteristic of rupture-prone vulnerable plaque [15,16]. We observed a significant reduction in the percentage of TUNEL-positive cells, accompanied by decreased expression of CHOP, which plays a crucial role in ER stress-induced macrophage apoptosis [23-25]. These findings suggest that AT1R profoundly contributes to necrotic core formation by promoting ER stress-induced macrophage apoptosis. Furthermore, results from in vitro experiments using TGPM indicated that cotreatment with Ang II does not affect FC-induced CHOP mRNA expression and subsequent apoptosis; however, the TGPM from Ang II-treated mice, with significantly increased Nox2 mRNA expression, showed exacerbated CHOP expression after FC loading. Our findings demonstrate that short-term treatment with ARB exerts an inhibitory effect on necrotic core formation in advanced plaques, which is likely to be due to the anti-oxidative effects.

Ang II has been reported to promote plaque vulnerability via AT1R activation, which is profoundly inhibited by ARB treatment in animal experiments [6-9]. Aono et al. demonstrated that AT1R deficiency or ARB treatment significantly reduced the incidence of plaque rupture in ApoE−/− mice [9]. They employed the murine plaque deficiency or ARB treatment significantly reduced the incidence of plaque rupture in animal experiments [6-9]. Aono et al. demonstrated that AT1R activation, which is profoundly inhibited by ARB treatment, leads to marked suppression of AT1R amplifies CHOP mRNA expression in FC-loaded macrophages by augmenting oxidative stress.

Figure 5A: Ang II treatment does not affect the mRNA expression levels of CHOP and apoptosis in peritoneal macrophages. (A) Quantitative PCR analysis of the mRNA expression levels of Nox2, p22phox, and p47phox in TGPMs from wild-type mice pretreated with Ang II or vehicle. White bar shows TGPMs from vehicle-treated mice. Black bar shows TGPMs from Ang II-treated mice. Values are the mean ± SE relative to those in TGPMs form vehicle-treated mice. Each group consisted of 3 samples. Each sample was combined from 3 mice. *P<0.05 vs. TGPMs from vehicle-treated mice. TGPM: thiglycolate-induced peritoneal macrophages.

Necrotic core formation is influenced by the balance between apoptosis and effective clearance of apoptotic cells by macrophages, termed efferocytosis [39-42]. Alternatively activated macrophages (M2) are the most superior efferocytic macrophage subtype [43], and the AT1R inhibitor has been reported to modulate macrophage phenotype toward M2 rather than classical activated macrophages (M1) in renal injury animal models [44,45], suggesting that the ratio of M1/M2 macrophage in ARB-treated mice is decreased, thereby contributing to the preservation of effective efferocytosis.
In conclusion, our results showed that short-term treatment with ARB in the advanced stages of atherosclerosis potentially inhibits necrotic core formation due to the anti-oxidative effects of ARB. Our findings suggest that local environmental conditions that modulate ER stress in the vessel wall are critical factors for consideration in the development of new therapeutic targets to regulate plaque vulnerability.

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**References**


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