The role of AGEs-RAGE in the regulation of VSMC apoptosis in arterial dissection

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Abstract: After being combined with a receptor for advanced glycation end products (RAGE), advanced glycation end products (AGEs) activate a series of signaling pathways to produce large amounts of reactive oxygen species (ROS) and cause vascular lesions. Vascular smooth muscle cell (VSMC) apoptosis is related to the pathogenesis of aortic dissection (AD). This study investigated the relationship of AGEs-RAGE with VSMC apoptosis and the pathogenesis of AD. An AD rat model was established to detect Caspase-3 activity. The ROS levels were measured by flow cytometry. The Malondialdehyde (MDA) levels were measured with a kit. The tissue AGEs content was detected by ELISA. RAGE and NOX1 expressions were tested by qRT-PCR and Western blot. VSMCs were treated with different concentrations of AGEs and divided into 3 groups: the control group, the AGEs (80 mg/mL) group, and the AGEs (80 mg/mL) + RAGE antibody blocking agent (8 μg/mL) group. Compared with the control group, Caspase-3 activity, ROS, MDA, AGEs, and RAGE and NOX1 expressions were significantly increased in the vascular media tissue of AD rats. Compared with the control group, AGEs treatment obviously induced apoptosis and ROS production, elevated RAGE and NOX1 expressions in a dose-dependent manner. After being blocked by the RAGE antibodies, ROS production, NOX1 expression, and apoptosis were markedly reduced in VSMCs. AGEs upregulation activates NOX1 expression, induces apoptosis and ROS production in VSMCs, and plays a role in the pathogenesis of AD. Blocking RAGE can antagonize the activation of NOX1 by AGEs and reduce VSMC apoptosis and ROS production, which may play a protective role in AD.

Keywords: AD, apoptosis, ROS, AGEs-RAGE, VSMC

Introduction

Aortic dissection (AD) is caused by aortic medial atrophy and endovascular membrane rupture. Blood flows enter the middle layer of the aorta through the endometrium. It tears the middle layer of the aorta, forming a hematoma or false lumen, eventually leading to stratification of the blood vessel wall to form a "double-cavity aorta" and even aortic rupture [1-3]. Several studies showed that increased apoptosis of the aortic medial VSMC plays a crucial role in the development of AD [4, 5].

AGEs are the relatively stable glycosylation products formed by Amadori reaction rearrangement of Schiff bases, which are produced by the non-catalytic enzyme reactions of the amino moieties of proteins, lipids, and nucleic acids with the carbonyl moieties of reducing sugars, such as glucose, fructose, and pentose [6, 7]. AGEs can exert biological effects by modifying proteins directly to change the structure and function of proteins, and interacting with specific receptors on the cell surface to affect oxidative stress, inflammatory response, cell proliferation, and apoptosis. The receptor-mediated signal transduction is the main pathway for AGEs to exert their effects. The body contains many types of AGEs receptors, including type I, type II scavenger receptors, AGE-R1, AGE-R2, AGE-R3, and RAGE, etc. [8-10]. RAGE, one of the members of the immunoglobulin superfamily on the cell membrane surface, is an AGEs-specific receptor and widely expressed in the heart, lungs, skeletal muscle, brain, vascular endothelial cells, monocytes, smooth muscle cells, and nerve cells [11, 12]. It is currently believed that RAGE is the major transduction mechanism that mediates the biological effects of AGEs [13-15]. At present, there is no study on the relationship between AGEs-RAGE...
and AD. This study aimed to investigate the relationship of AGEs-RAGE with VSMC apoptosis and the pathogenesis of AD.

Materials and methods

Main reagents and materials

SD rats (3 weeks old, weighing 55-60 g) were purchased from Shanghai Slack Experimental Animals Co., Ltd.; Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, Type II collagenase, and trypsin were purchased from Gibco (USA). A QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Germany). β-amino propionitrile (BAPN) was purchased from Sigma (USA). An Annexin V/PI Apoptosis detection kit, a Caspase-3 activity kit, and HRP-labeled secondary antibody were purchased from Beyotime Biotechnology (Nantong, China). MDA and CAT detection kits were purchased from Nanjing Jiancheng Bioengineering Research Institute; AGEs-BSA was purchased from Biovision (USA). Goat anti-Rat RAGE IgG polyclonal antibody and β-actin antibody were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-Rat NOX1 IgG polyclonal antibody was purchased from Abcam (Cambridge, MA, USA). An AGEs ELISA Kit was purchased from Wuhan Huamei Biological Engineering Co., Ltd.

AD modeling

After one week of adaptive feeding, SD rats were divided into two groups: the control group was given normal feed for 8 weeks, and the AD model group was fed with the same feed containing 0.25% BAPN for 8 weeks. After 8 weeks, the rate of AD formation in the two groups of rats was observed, and the diameter of AD was measured and recorded.

The rats were anesthetized by an intraperitoneal injection of chloral hydrate. After the aortic dissection was completed, the adventitia and vascular intima were removed. The media were preserved and digested with type II collagenase and 0.05% trypsin. The cells were cultured in a DMEM medium containing 20% FBS, 1% penicillin, and placed in a 37°C and 5% CO2 cell incubator (371 gas sheath type, Thermo, USA). The cells were passaged at 1:4 ratio and used on the 5-6th generation.

VSMC grouping and treatment

The 5th generation of rat aortic VSMC cells was used for the experiments. The cells were divided into 4 groups: the control group, the AGEs-BSA 20 mg/mL group, the AGEs-BSA 40 mg/mL group, and the AGEs-BSA 80 mg/mL group. After 48 hours, the cells were harvested and tested for ROS, apoptosis, and gene and protein expressions.

The VSMCs were divided into 3 groups: the control group, the AGEs (80 mg/mL) group, and the AGEs (80 mg/mL) + RAGE antibody blocking.
agent (8 μg/mL) group. After 48 hours, the cells were harvested and tested for ROS, apoptosis, and gene and protein expressions.

**qRT-PCR**

A QuantiTect SYBR Green RT-PCR Kit was used for the q-PCR reaction. The qPCR reaction system contained 10.0 μL 2 × QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL Forward Primer (0.5 μM), 1.0 μL Reverse Primer (0.5 μM), 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH2O. The reverse transcription conditions were 50°C for 30 minutes. The qPCR reaction conditions were predenatured at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s on the Bio-Rad CFX96/CFX connect Real-Time PCR Detection System.

**Western blot**

The cells were lysed by RIPA and quantified using the BCA method. 50 μg proteins were separated by 10% SDS-PAGE gel and 4% concentrated gel. Then the protein was transferred to PVDF membrane at 300 mA for 100 min. Next, the membrane was blocked with 5% skim milk at room temperature for 1 h and incubated in primary antibodies (RAGE (R&D Systems), NOX1 (Abcam), and β-actin (R&D Systems) at 1:1000, 1:800, and 1:8000, respectively) at 4°C overnight. After that, the membrane was incubated with an HRP-conjugated Goat anti-Rabbit IgG (H+L) secondary antibody (1:10000) at room temperature for 60 min and finally detected by ECL chemiluminescence.

**Cell apoptosis detection**

The cells were digested and resuspended in a 500 μL 1 × binding buffer. Then we added 5 μL of Annexin V-FITC and 5 μL of PI Solution in sequence to the cells, and the solution was incubated in the dark for 15 minutes. After supplementation with 400 μL binding solution, the cells were tested on a Beckmann Gallios flow cytometer.

**Statistical analysis**

All data analyses were performed on SPSS 18.0 software. The measurement data were expressed as the mean ± standard deviation and compared by a t-test or a one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Significant AD formation, obvious oxidative stress, and increased caspase-3 activity in model rats**

The rats in the control group were generally in good condition and no AD formation was found during the experiment. Seven rats in the model group were found to have developed AD (7/10, AD formation rate 70.0%). The AD diameter was 4.13 ± 0.77 mm, and the length was 7.36 ± e1.29 mm. Ultraviolet spectrophotometry showed that the MDA content in the media tissue of the AD model group was significantly higher than the MDA content in the control group (Figure 1A). Compared with the control group, caspase-3 activity was obviously increased in the middle vascular tissue of the AD model group (Figure 1B). Flow cytometry revealed that the ROS content in the middle vascular tissue of the AD rats was markedly higher than it was in the control group (Figure 1C).

**AGEs and RAGE expressions were elevated in the middle vascular tissue of the AD model rats**

An ELISA assay showed that, compared with the control group, the AGEs content in the middle vascular tissue of the AD model group was significantly increased (Figure 2A). qRT-PCR demonstrated that the expressions of RAGE and NOX1 mRNA in the media of the tunica media of the AD model group was obviously higher than that of the control group (Figure 2B, 2C). Western blot exhibited that, compared with the control group (0.38 ± 0.02 for RAGE and 0.41 ± 0.03 for NOX1), the expressions of RAGE (0.83 ± 0.05) and NOX1 (0.81 ± 0.03) protein in the middle blood vessels of the AD model group was apparently upregulated (P < 0.05) (Figure 2D).

**AGEs treatment significantly upregulated RAGE expression, induced VSMC cell apoptosis and ROS production**

A qRT-PCR showed that compared with the control group, different concentrations of AGEs significantly upregulated the expressions of RAGE and NOX1 mRNA in VSMCs in a dose-dependent manner (Figure 3A, 3B). Flow cytometry demonstrated that compared with the control group, different concentrations of AGEs markedly elevated the VSMC intracellular ROS content and enhanced apoptosis in a dose-depen-

Figure 1. Obvious oxidative stress and increased caspase-3 activity in AD model rats. A. MDA content detected by the kit; B. Caspase-3 activity detected by spectrophotometry; C. Cell apoptosis detected by flow cytometry. *P < 0.05, compared with control.

Figure 2. AGEs and RAGE expressions elevated in the middle vascular tissue of the AD model rat. A. AGEs content detected by ELISA; B. RAGE mRNA detected by qRT-PCR; C. NOX1 mRNA detected by qRT-PCR; D. Protein expression detected by Western blot. *P < 0.05, compared with control.

(0.35 ± 0.02 for RAGE and 0.39 ± 0.02 for NOX1), different concentrations of AGEs apparently enhanced the VSMC intracellular RAGE (0.43 ± 0.02; 0.63 ± 0.03; 0.81 ± 0.03) and NOX1 (0.45 ± 0.03; 0.58 ± 0.04; 0.86 ± 0.05) protein expressions in a dose-dependent manner (P < 0.05) (Figure 3D).

Blocking RAGE obviously reduced NOX1 expression, attenuated cell apoptosis, and decreased ROS production

A qRT-PCR showed that, compared with the control group, the expressions of RAGE and NOX1 mRNA in the VSMCs of the AGEs-treated group were significantly increased, and the expression of NOX1 mRNA was obviously decreased after the RAGE was blocked by antibodies (Figure 4A). Western blot demonstrated that compared with
the control group (0.47 ± 0.02 for RAGE and 0.51 ± 0.03 for NOX1), the expressions of RAGE and NOX1 protein in the VSMCs of AGEs-treated group were apparently upregulated (0.69 ± 0.03 for RAGE and 0.73 ± 0.03 for NOX1), and the expression of NOX1 protein (0.62 ± 0.02) markedly declined after the RAGE was blocked by antibodies (Figure 4B). Flow cytometry revealed that compared with the control (0.50 ± 0.02 for ROS and 2.25 ± 0.68% for apoptosis), the AGEs treatment significantly induced intracellular ROS production (86.6 ± 6.5) and apoptosis (11.6 ± 1.56%) in the VSMCs. Intracellular ROS production (7.6 ± 1.3) and apoptosis (6.65 ± 1.10%) were obviously reduced in VSMCs after being treated by a RAGE blocking antibody (Figure 4C, 4D).

Discussion

VSMC is the main cellular component in the medial aorta. It plays a role in maintaining vascular wall structure, regulating vasomotor function, and maintaining vascular tone and elasticity. Abnormal changes in the number and function of VSMCs will lead to blood pressure disorders, remodeling of blood vessel wall structure, weakened elasticity, and increased brittleness [4, 16]. A number of studies have shown that increased apoptosis of the aortic medial VSMC plays a crucial role in the development of AD [4, 5].

In general, most types of proteins have short half-lives, which are not sufficient to produce non-enzymatic reactions with carbohydrates to form AGEs. As a result, AGEs in vivo are less accumulated and are at a low level. When the body becomes senescent, the rate of protein clearance by the body slows down, resulting in an increase in the half-life of the protein. Glycosylation occurs in proteins that have not been eliminated, resulting in an increased accumulation of AGEs in the aging body. Therefore, AGEs are products that slowly increase in the body, and the accumulation of AGEs is closely related to the degenerative
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Changes in cells and tissues [17]. For example, an excessive accumulation of AGEs is a disease-causing factor of diabetic vascular disease [18, 19] and cardiovascular diseases [20]. In addition to changing the structure and function of proteins, AGEs can also play a biological function by binding to their specific RAGE receptors, and it is currently thought that the latter is predominant.

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme subunit contains a variety of isoenzymes, including NOX1, NOX2, NOX3, NOX4, NOX5, DUO1, and DUOX2. NOX1 is mainly distributed in vascular smooth muscle and the main catalytic enzyme for intravascular ROS production [21]. The combination of AGEs and RAGE can activate the NADPH through a variety of mechanisms, reduce the activity of superoxide dismutase (SOD), and generate large amounts of ROS in cells. ROS can further induce cell proliferation, apoptosis, migration, inflammation infiltration, and the release of inflammatory factors to participate in the development of AD. At present, there is no study that reports on the relationship between AGEs-RAGE and AD. This study aimed to investigate the relationship of AGEs-RAGE with VSMC apoptosis and the pathogenesis of AD.

Figure 4. Blocking RAGE obviously reduced NOX1 expression, attenuated cell apoptosis, and decreased ROS production. A. mRNA expression detected by qRT-PCR; B. Protein expression detected by Western blot; C. ROS content detected by flow cytometry; D. VSMC cell apoptosis detected by flow cytometry. *P < 0.05, compared with control.

After BAPN was given to SD rats for 8 weeks, 70% of rats in the model group exhibited AD in the aorta, indicating high modeling success rate. Compared with the control group, the MDA
content, ROS production, and caspase-3 activity in the middle vascular tissue of the AD model group were significantly increased, revealing that the oxidative stress and apoptosis in the AD tissue were enhanced. Durdu et al. [4] showed that, compared with the control group, the caspase-3 activity, pro-apoptotic protein Bax expression, and apoptosis rate were markedly elevated in VSMCs of AD patients, confirming the apoptosis of VSMCs. An ELISA assay demonstrated that, compared with the control group, the AGEs content in the blood vessels of the AD model group was upregulated significantly. mRNA and protein expression tests exhibited that the expression levels of RAGE and NOX1 in the AD model group were obviously higher than they were in the control group. The results showed that AGEs elevation plays a role in upregulating RAGE expression, activating NOX1 expression, promoting ROS production, accelerating cell apoptosis, and AD pathogenesis. This study further investigated whether AGEs play a role in influencing RAGE, VSMC apoptosis, and oxidative stress in vitro. The results showed that treatment with different concentrations of AGEs significantly induced VSMCs apoptosis and enhanced ROS production in a dose-dependent manner. Moreover, it was found that the administration of RAGE antibody treatment obviously declined the upregulation of NOX1 expression by AGEs, resulting in markedly attenuated apoptosis and ROS production in VSMCs. Gray et al. [21] revealed that high glucose treatment can up-regulate NOX1 expression to induce vascular oxidative stress injury. The downregulation of NOX1 expression can attenuate vascular injury and prevent sugar-induced atherosclerosis. Das et al. [22] reported that the expression of AGES receptor S100A12 was significantly increased in AD vascular smooth muscle cells. The knockout of S100A12 apparently restrained caspase-3 activity and decreased the apoptosis rate of the vascular smooth muscle cells. These studies indicated that activation of NOX1 by AGEs plays a role in mediating apoptotic vascular lesions, which was similar to our study.

In the study of AGEs regulating the biological effects of VSMC, Hegarb et al. [23] showed that AGEs could promote vascular lesions by accelerating VSMC cell proliferation. Li et al. [24] observed that AGEs can upregulate LCN2 expression in human VSMCs and promote cell migration. He et al. [26] exhibited that AGEs bind to RAGE and activate the wnt/β-catenin signaling pathway to promote OPG protein expression and VSMC calcification. At present, there is no published study on the relationship between AGEs-mediated VSMC apoptosis and AD pathogenesis. This study found that the activation of AGEs-RAGE upregulates NOX1 expression and AD pathogenesis. Blocking AGEs-RAGE may play a role in alleviating VSMCs apoptosis, which may have some therapeutic value in the protection of AD. However, whether AGEs-RAGE blocks the effect of reducing AD in vivo still requires further animal studies and a large-scale clinical validation.

Conclusion

AGEs upregulation activates NOX1 expression, induces apoptosis and ROS production in VSMCs, and plays a role in the pathogenesis of AD. Blocking RAGE can antagonize the activation of NOX1 by AGEs and reduce VSMC apoptosis and ROS production, which may play a protective role in AD.

Disclosure of conflict of interest

None.

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