Effects of angiotensin (1-7) on the expression of tissue factor induced by angiotensin II and its mechanism in vascular endothelial cells

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Received February 5, 2007

Abstract

Objective. To evaluate the effects of angiotensin II on the expression of tissue factor (TF) in the cell line ECV304 and the effects of angiotensin (1-7) on induced TF angiotensin II expression. Methods. ECV304 cells were cultured in RPMI-1640 medium. The procoagulant activity (PCA) was determined with one-stage clotting assay and TF mRNA examined by semi-quantitative RT-PCR. Immunohistochemical analysis was performed to evaluate the activation of NF-κB. Results. Compared with the control, angiotensin II (10⁻¹⁰ – 10⁻⁶ mol/L) significantly increased PCA of ECV304 cells and TF mRNA expression in a dose-dependent manner (P < 0.01, r = 0.9631) and the peak concentration was 10⁻⁶ mol/L. Angiotensin (1-7) (10⁻¹⁰ – 10⁻⁷ mol/L) alone could not change the PCA, but pretreated the ECV304 cells with angiotensin (1-7), which remarkably inhibited the inductive effects of angiotensin II on TF activity in a dose-dependent pattern (P < 0.05), and the peak concentration was 10⁻⁷ mol/L. Angiotensin (1-7) markedly decreased the amount of TF mRNA induced by angiotensin II (P < 0.05). Immunohistochemical analysis demonstrated that NF-κB immigration from cytoplasm to nucleus was found after treatment of ECV304 cells with angiotensin II. Angiotensin (1-7) significantly inhibited angiotensin II-induced NF-κB immigration. Discussion. Angiotensin II can increase the expression of TF in ECV304 cells, which can be inhibited by angiotensin (1-7); angiotensin (1-7) and angiotensin II may affect the expression of TF through activating of NF-κB. [Life Science Journal. 2007; 4(2): 25 – 27] (ISSN: 1097 – 8135).

Keywords: angiotensin (1-7); tissue factor; vascular endothelial cell; angiotensin II; cell signal transduction

1 Introduction

Many studies showed that the coagulation process may play an important role in the development of some diseases. Tissue factor (TF) is the trigger in initiation of the blood coagulation process[1]. Normally, almost no TF is expressed in human umbilical vein endothelium cell (HUVEC). Stimulated by pathological factor, TF is expressed largely and leads to thrombosis. Renin-angiotensin system (RAS) is linked with many important functions of the system. Recently a new and important member of RAS is found – angiotensin (1-7) [Ang (1-7)]. It is cut from angiotensin I [Ang I] or angiotensin II [AngII], depending on different enzymes[2]. Ang (1-7) is counteracted to AngII in many ways, such as relaxing blood vessel, reducing blood pressure and inhibiting cell proliferation[3], and so on. It has been reported that AngII can affect and accelerate the blood coagulation. However, up to now, it is unclear that whether Ang (1-7) has the reverse function with AngII on blood coagulation, especially on the expression of TF. This study specifically investigated the effect of Ang (1-7) on the expression of TF induced by AngII in human umbilical vein endothelium derived cell line ECV304 and observed the role of NF-κB in the regulation of Ang (1-7) on TF expression induced by AngII in ECV304.

2 Materials and Methods

2.1 Materials

RPMI 1640 cell culture medium and fetal bovine serum were from Gibco (Grand Island, NY, USA). Ang II and
Ang (1-7) was from Sigma (St Louis, MO, USA). β-actin was purchased from Sangon (Shanghai, China). Classic total RNA minipreps kit and DEPC were purchased from Hyclone-Pierce (USA). NF-κB antibody was purchased from Santa Cruz (USA).

2.2 Cell culture

ECV304 from China Center for Type Culture Collection (CCTCC, Wuhan, China) was used in this study. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 50 µg/ml gentamycin under an atmosphere with 5% CO₂. For experiments, cells were washed twice with RPMI and viability checked by trypan blue exclusion (> 95% viable before each experiment).

2.3 Determination of TF activity

After incubation of the cells (24-well plates, 1.5×10⁵ cells/well) with Ang II in the presence or absence of Ang (1-7), the cells were washed 3 times with D-Hank’s and scrape-harvested into 300 µl of D-Hank’s. The cells were frozen in –80ºC, then thawed in 37ºC, repeated for 3 times. The procoagulant activity (PCA), which reflects TF activity, was determined with the one-stage clotting assay measuring total cellular procoagulant activity[4].

2.4 Analysis of TF mRNA

Total RNA of HUVECs was prepared by classic total RNA minipreps kit. The ECV304 were treated with Ang II in the presence or absence of Ang (1-7). TF mRNA was assessed by RT-PCR. The sequences of the primers used for TF cDNA amplification were referenced from the reports[5], as follows: reverse primer: 5’-CACTGCAATATAGCATATTGCAGTAGC-3’, and forward primer: 5’-CTACTGTTTCAGTGTTCAAGCAGTGA-3’. RT-PCR also was performed with control primers, β-actin mRNA. The gel image was captured and densitometrically quantified by HEMA Bio-Image analyzer (Hema Inc, China). The amount of TF mRNA was determined by densitometric analysis.

2.5 Immunocytochemistry analysis

To observe the activation of NF-κB, immunohistochemical analysis was performed. Cells were grown on coverslip glass for 24 hours as above and then washed in PBS. Chilled H₂O₂ was applied to samples and blocked with 10% goat serum. Incubation at room temperature followed with anti-human NF-κB as primary monoclonal antibody. A strepavidin-biotin complex system with diaminobenzidine as chromogen was used for colour development. Sections were finally counterstained with haematoxylin and examined under a light microscope.

2.6 Statistic analysis

Results are expressed as x ± s. Comparison between data from control and experiment groups was performed by t-test. Correlation between data was analyzed with liner regression. Results were considered statistically significant at P < 0.05.

3 Results

3.1 Effects of AngII on TF activity

Treated with AngII for 0, 2, 4, 6, 8, 10, 12 hours, TF activity of ECV304 increased and reached the peak at the 8th hour (P < 0.05) (Figure 1). Compared with the control, AngII (10⁻¹⁰ – 10⁻⁶ mol/L) significantly increased ECV304 cells TF activity in a dose-dependent manner (P < 0.01, r = 0.9631) and the peak concentration was 10⁻⁶ mol/L.

3.2 Effects of Ang (1-7) on TF activity with AngII

Ang (1-7) (10⁻¹⁰ – 10⁻⁷ mol/L) alone could not change the PCA (P > 0.05), but added Ang (1-7) to the ECV304 cells beforehand for 30 minutes earlier, it remarkably inhibited the effects of AngII (10⁻⁷ mol/L) on TF activity in a dose-dependent pattern (P < 0.05, r = 0.9860), and the inhibitory effects reached the peak with 10⁻⁷ mol/L Ang (1-7) (Figure 2).

3.3 Effects of Ang (1-7) on TF mRNA with AngII

As shown in Figure 3, 10⁻⁷ mol/L AngII markedly induced TF mRNA expression after 2 hours of the treatment. Ang (1-7)(10⁻¹⁰ – 10⁻⁷ mol/L) alone could not change the TF mRNA expression by AngII (P > 0.05). When Ang (1-7) and AngII were added, the effects of AngII on TF mRNA expression were dramatically abolished (P < 0.01) (Figure 3).

Figure 1. Effects of Ang(1-7) on TF activity in ECV304 stimulated with AngII at different time. n = 4, x ± s. * vs. control, P < 0.05; ** vs. control, P < 0.01.

Figure 2. Effects of Ang (1-7) on TF activity with AngII. Ang (1-7)(10⁻¹⁰ – 10⁻⁷ mol/L) alone could not change the PCA (P > 0.05), but added Ang (1-7) to the ECV304 cells beforehand for 30 minutes earlier, it remarkably inhibited the effects of AngII (10⁻⁷ mol/L) on TF activity in a dose-dependent pattern (P < 0.05, r = 0.9860), and the inhibitory effects reached the peak with 10⁻⁷ mol/L Ang (1-7) (Figure 2).
Effects of different concentration of Ang(1-7) on TF mRNA in –7

Recently another member of RAS, Ang (1-7), has been found. It is counteracted to AngII in many ways, such as relaxing blood vessel, reducing blood pressure and inhibiting cell proliferation. So in this expriment, AngII was used as stimulator and we observed the relationship between Ang (1-7) and AngII on the expression of TF.

TF initiates the extrinsic pathway of blood coagulation by acting as a cofactor for activating factor VII[6]. Over-expressed TF by endothelial cells and monocytes may be responsible for some thrombus diseases. We observed that Ang (1-7) remarkably inhibited the inductive effects of AngII on TF activity in a dose-dependent manner. In addition, Ang (1-7) also inhibited TF mRNA expression induced by AngII. So our results showed for the first time that Ang (1-7) had the reverse function with AngII on the expression of TF, and Ang (1-7) may reduce the chance of thrombosis, which might be useful for the application in the prevention of thrombosis.

The position of NF-κB in the cells was observed by immunocytochemistry staining. Transcription factor NF-κB plays an important role in signal transduction. The inactivated form of NF-κB is a heterodimer consisted of two subunits, the p65 and p50 proteins, usually binding to IκB which prevents NF-κB from entering the nucleus. The release of IκB from NF-κB results in the immigration of NF-κB from cytoplasm to the nucleus, where it binds to specific DNA sequences in the promoter regions of target genes[7]. We found that, in normal cells, NF-κB was found in the cytoplasm, and AngII could promote the NF-κB immigrating to nucleus; Ang (1-7) counteract the role of AngII on NF-κB immigration. It implied that Ang (1-7) inhibit AngII on the expression of TF by blocking NF-κB activation. NF-κB activation is the last step of signal transduction from cytoplasm to the nucleus. But many passway may be related with the effects of Ang (1-7) on the expression of TF induced by AngII, such as NO and PGI₂.

3.4 Effect of Ang (1-7) on the immigration of NF-κB from cytoplasm to the nucleus

ECV304 without AngII didn’t show colour in nucleus after NF-κB antibody was added. While AngII treated the cells for 45 minutes, the nucleus showed brown after NF-κB antibody was added. Finally pretreated the cells with Ang (1-7) for 15 minutes and AngII was added. 45 minutes later, the brown grain in cells decreased obviously, which showed that Ang (1-7) could inhibit the immigration of NF-κB from cytoplasm to the nucleus stimulated by AngII.

4 Discussion

AngII is the most important and well-known member of RAS, and it can result in contraction of blood vessels. Besides, AngII is also related to thrombosis by activating blood platelet and stimulating the expression of TF. Recently another member of RAS, Ang (1-7), has been found. It is counteracted to AngII in many ways, such as relaxing blood vessel, reducing blood pressure and inhibiting cell proliferation. So in this expriment, AngII was used as stimulator and we observed the relationship be-

References