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# Mechanism of vasoactive peptide intermedin in vascular collagen remodeling during angiotensin II-induced hypertention

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**Abstract. – OBJECTIVE: Renin-angiotensin axis plays a pivotal role in the cardiovascular system, and Angiotensin II (Ang II) is of great importance in the progression of hypertension. Vasoactive peptide intermedin (IMD) belongs to calcitonin gene-related peptide (CGRP) family, which is involved in the regulation of the cardiovascular function. This study aims to determine the effect of vasoactive peptide intermedin on vascular collagen remodeling caused by angiotensin II-induced hypertension.**

**MATERIALS AND METHODS: 12-week old rats were randomly assigned into three groups, and each group consisted of 12 rats. Rats were administered with Ang II or Ang II+IMD, respectively. Control group received saline administration. Blood pressure of caudal artery was examined two weeks after administration. Serum procollagen I and III were detected by enzyme-linked immunosorbent assay (ELISA). The vascular microstructure was examined via hematoxylin-eosin (HE) staining to evaluate vascular collagen remodeling. Expressions of protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) were tested by using Western-blot and RT-PCR.**

**RESULTS: Compared with the control group (92.2±9.1 mmHg), blood pressure of group Ang II was increased by 88% (173.1±11.2 mmHg) ($p<0.01$). Moreover, blood pressure level in group Ang II+IMD (131.0±10.9 mmHg) was reduced compared to that in group Ang II ($p<0.05$). Compared with that in control group, higher level of serum procollagen, with significantly increasing vascular W/C ratio and collagen area percentage, was found in group Ang II, while all testing indexes above in group Ang II+IMD were lower than that in group Ang II. No differences were detected in the levels of Akt and MAPK mRNA among all three groups. However, highest expressions of phosphorylation Akt and MAPK protein were shown in group Ang II, and the levels were gradually lower in groups of Ang II+IMD and control.**

**CONCLUSIONS: IMD could attenuate the vascular collagen remodeling caused by angiotensin II-induced hypertension via inhibiting phosphorylation of Akt and MAPK.**

Key Words
Angiotensin II, Hypertension, Collagen remodeling, Akt, MAPK.

## Introduction

Hypertension represents the most common cardiovascular disease in clinical practice, resulting in the increasing risk of stroke and other cardiovascular disease[1]. Angiotensin II (Ang II) is a kind of physiologically active substance in the cardiovascular system. It has been demonstrated that Ang II enhanced vasoconstriction via acting on vascular smooth muscle, which finally elevated blood pressure[2]. The previous finding[3] showed that Ang II played pivotal roles in the development of multiple cardiovascular diseases via regulating collagen synthesis in myocardial cells and vascular smooth muscle cells. Moreover, collagen synthesis as a crucial pathophysiologic process significantly participated in various cardiovascular diseases[4].

Vasoactive peptide intermedin (IMD) belongs to calcitonin gene-related peptide (CGRP) family, and functions via interaction with calcitonin receptor-like receptors[5]. IMD also represents an internal environment vascular regulator and an endogenous cardio-renal protection factor, which maintains multiple biological functions, including lowering blood pressure, dilating coronary



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artery, regulating water and electrolytes balance, and mediating pituitary hormone secretion[6-8]. In this study, we aim to investigate the effect of IMD on vascular collagen remodeling and reveal its potential mechanism during angiotensin II-induced hypertension.

## Materials and Methods

### Experimental Animals

Sprague Dawley (SD) rats (laboratory animal center from Second Military Medical University) of 12 weeks with an average weight of (403.4±5.3) were divided into three groups, including Ang II group (n=12), Ang II+IMD group (n=12), control group (n=12). Rats were treated with normal diet and drinking, with 60%±10% humidity. Ang II $10^{-7}$ mol/l (Sigma-Aldrich, St. Louis, MO, USA) were injected into the lower limbs distal sartorius muscle of rats in Ang II group. Ang II $10^{-7}$ mol/l and IMD 150 μg/kg were injected into rats from Ang II+IMD group, and the control rats were injected only with normal saline of the same volume[7,9]. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Chinese PLA General Hospital.

### Blood Pressure Measurement of Tail Arterial

The tail artery of three groups of rats was detected after different treatments for 2 weeks. The tail artery systolic pressure was measured with the method of tail arterial volume. The rats were set in the incubator (37°C) of the blood pressure heart rate detector. Rat-tail was embroiled into gasbag, and then the systolic pressure was repeatedly detected 3 times.

### Enzyme-Linked Immunosorbent Assay (ELISA) Detection

Total of 60 mg/kg water and trichloroaldehyde were intraperitoneally injected for anesthetization. Then rat blood was phlebotomized and centrifuged, and the quantity of I and III procollagen in separated serum was detected by ELISA kit (BioLegend, San Diego, CA, USA), in order to evaluate vascular collagen synthesis and refactoring. 100 μl rat serum above and 100 μl phosphate buffer saline (PBS, pH 7.4) were added into 96 wells ELISA plate for the incubation at 4°C overnight. The plate was rinsed by PBS and blocked by 2% $H_2O_2$-ethanol to inactivate the endogenous peroxidase, then 1% bovine serum albumin (BSA) was added to block the non-specific binding. Being washed by PBS, procollagen I and III specific antibody (mouse) diluted by 1:1000 was added for 2 h at 37°C. Being washed by PBS for 3 times, biotin-labeled goat anti-mouse secondary antibody (1:200) was added and incubated for 1.5 h at 37°C. OPD developing solution was added and treated for 6 min. The reaction was stopped with 0.2 mM $H_2SO_4$, and quantity of I, III procollagen were detected by a microplate reader (Biotek, Winooski, VT, USA).

### Hematoxylin-Eosin (HE) Staining

Total of 60 mg/kg water and trichloroaldehyde were intraperitoneally injected into rats. 0.5 cm blood vessel from mesomere of the thoracic aorta was fixed with 40 g/l paraformaldehyde and embedded with paraffin, and the rest of the blood vessel was cryopreserved with liquid nitrogen. Sections were performed by routine HE staining for firstly baking 30 min at 60°C. Sections were deparaffinized for 15 min in xylene, gradient rinsed with ethanol and rinsed with distilled water twice. After the water was absorbed carefully, the section was stained by hematoxylin for 15 min after drying, rinsed with distilled water twice, and stained by 1% eosin staining solution for 3 min. Automated image analysis system was used to determine thoracic artery wall thickness (W/C) and collagen area percentage[10].

### Western Blot

Frozen tissues were grinded into power in liquid nitrogen, and pre-cooled cell lysis was added into tissues for incubation at 4°C overnight. After 12000-r/min centrifugation for 10 min, the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total protein was extracted and different proteins were separated by SDS-PAGE and transferred into the polyvinylidene difluoride (PVDF) member, then blocked with 5% skim milk powder at 37°C for 1 h. Then, primary antibodies were added (1:1000 p-AKT, 1:1000 p-MAPK specific mouse source antibody) for incubation at 4°C overnight. Extra primary antibodies were rinsed by Tris-Buffered Saline and Tween-20 (TBST), and horseradish peroxidase (HRP) labeled secondary antibody was added for 1 h incubation at room temperature. Diaminobenzidine (DAB) developing solution was added to keep in the dark for 10 min, and then stopped by distilled water. The integral gray values of bands were analyzed by gel document system[11].

**Table I.** RT-PCR primers.

| Name | Sequence | Tm (°C) |
|---|---|---|
| Akt-F | 5'CAGATGGACTCAAGAGGCAGGAAGA 3' | 60.5 |
| Akt-R | 5'GCAGGACACGGTTCTCAGTAAGC 3' | 59.9 |
| MAPK-F | 5'ACCGTACCTCAAGCCTTCCAA3' | 60.1 |
| MAPK-R | 5' CCAGGCCTGTTCAACTTCAATCCT 3' | 59.7 |

### Real-Time PCR

According to sequences of protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) (GeneBank assess number NM_001165894 and X58712 respectively), primers were designed for RT-PCR, shown in Table I. Total RNAs were extracted from vessel tissue by RNAprep pure tissue kit (Qiagen, Venlo, Netherlands) for RT-PCR detection. Reaction condition: 95°C for 3 min, 95°C for 15 s, 60°C for 30 s, 40 cycles. β-actin was regarded as internal reference and analyzed by the Memory Card 9700 PCR system (version 2.02, Shkmsw Bio., Shanghai, China). The results were showed as $2^{-\Delta\Delta Ct}$ [12].

### Statistical Analysis

All test results were summarized and analyzed by SPSS 20.0 software (IBM, Armonk, NY, USA). The results were shown as mean value ± standard deviation. One-way ANOVA was used to compare different groups. $p$-value < 0.05 was considered to be statistically significant.

## Results

### Blood Pressure of Caudal Artery

Blood pressure of caudal artery in three groups was examined two weeks after the administration. Analysis result about blood pressure of caudal artery was showed in Figure 1. Compared with the control group, blood pressure in group Ang II was increased by 88%±8.1% ($p$<0.01). This indicated that exogenous angiotensin II injection induced elevation of blood pressure. Hypertension model of the rat was successfully constructed. Compared with that of group Ang II, blood pressure of group Ang II+IMD was decreased by 24.3%±2.6% ($p$<0.05).

### Examination of Vascular Collagen Remodeling

We performed ELISA to examine serum levels of procollagen I and III so that to evaluate vascular collagen remodeling. Vascular tissue paraffin section was also prepared for HE stain (Figure 2). Artery W/C ratio and collagen volume

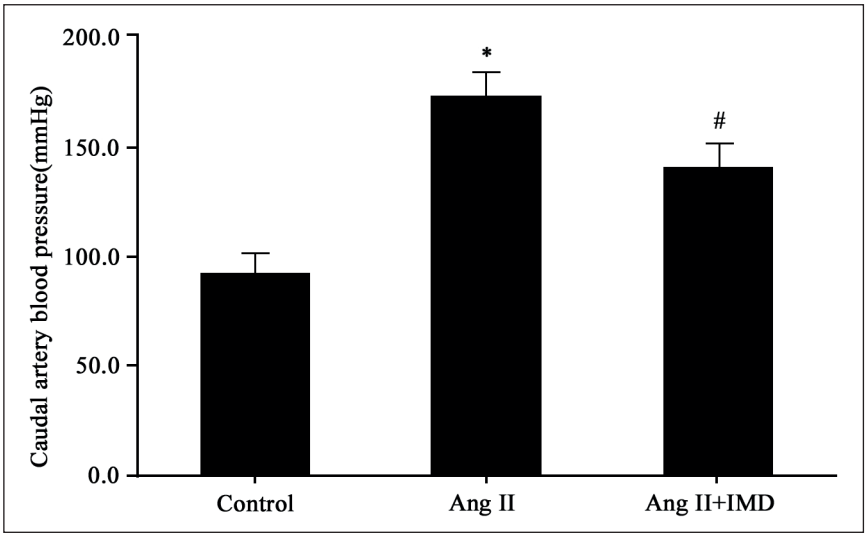


**Figure 1.** Analysis of blood pressure of caudal artery. Asterisk represents statistical difference between Group A and Group C ($p$<0.01), hash symbol represents statistical difference between Group A and Group B ($p$<0.05).

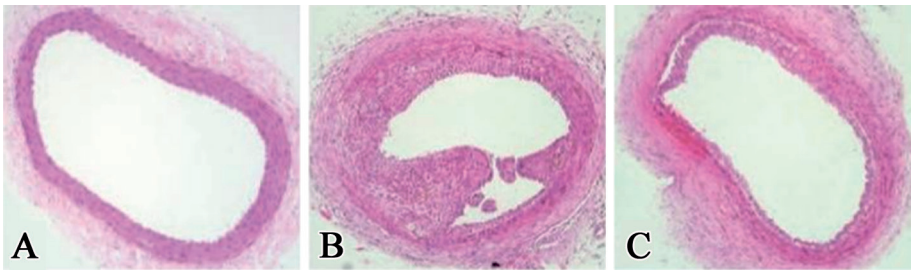


**Figure 2.** HE stain for vascular tissues. Group serial number was marked on left bottom. x40.

fraction (CVF) was showed in Table II. Compared with that of the control group, significantly higher levels of serum procollagen I ($p<0.01$) and serum procollagen III ($p<0.05$) were found in rats of group Ang II. Furthermore, HE stain results showed the diameter of the blood vessel of group Ang II became smaller, suggesting the successful establishment of vascular collagen remodeling (Figure 2B). Compared with Ang II group, result detected from group Ang II+IMD showed that the levels of serum procollagen I ($p<0.05$) and serum procollagen III were significantly reduced ($p<0.05$) (Table II).

### Western Blot

Proteins of vascular tissues were extracted for Western blot analysis, including cell division related protein kinase, Akt, and MAPK. The levels of phosphorylated protein p-Akt and p-MAPK were measured. Relative transcript levels of p-Akt and p-MAPK were examined with β-actin as an internal reference, which were shown in Figure 3. Our data indicated that relative levels of p-Akt and p-MAPK were significantly increased in group Ang II ($p<0.01$), while those in group Ang II+IMD were significantly lower than group Ang II ($p<0.05$).

### RT-PCR Results

No significant change of the mRNA levels of Akt and MAPK was found between groups Ang II and control ($p>0.05$), which was the same between groups Ang II and Ang II+IMD ($p>0.05$) (Figure 4). All above-suggested mRNA levels of Akt and MAPK were not affected by the experimental intervention.

## Discussion

To understand the effect of IMD on angiotensin II-induced hypertension and potential mechanism of IMD related to vascular collagen remodeling, we established rat model and found that IMD could effectively antagonize Ang II-induced hypertension and maintain stable blood pressure. Previous studies[13,14] showed that IMD belongs to calcitonin gene-related peptide (CGRP) family, decreased blood pressure via binding RAMPs/CRLR receptor complex and mediating cAMP and other vasoactive factors.

Vascular collagen remodeling represents an important pathophysiologic process in hypertension-induced cardiovascular diseases, including atherosclerosis[15]. To investigate the effect of IMD on vascular collagen remodeling, we performed ELISA to detect changes of vascular microstructure, and analyzed artery W/C ratio and CVF. According to the analysis result, IMD inhibited vascular collagen remodeling in the progression of Ang II-induced hypertension. We further determined Akt and MAPK at protein and mRNA levels, respectively. Consequently, the result unraveled that IMD downregulated the phosphorylation of Akt and MAPK via antagonizing Ang II, and finally inhibited the vascular collagen remodeling.

**Table II.** Data of vascular collagen remodeling.

| Groups | Procollagen I μg/l | Procollagen III μg/l | W/C | VFC |
|---|---|---|---|---|
| Control group | 85.2±9.1 | 46.2±6.1 | 6.1%±0.3% | 4.8%±0.3% |
| Group A (Ang II) | 162.5±11.5* | 63.5±8.3* | 18.7%±0.6%* | 10.3%±0.5%* |
| Group B (Ang II+IMD) | 97.3±7.6#* | 50.9±6.8# | 7.2%±0.5%#* | 6.1%±0.3%#* |

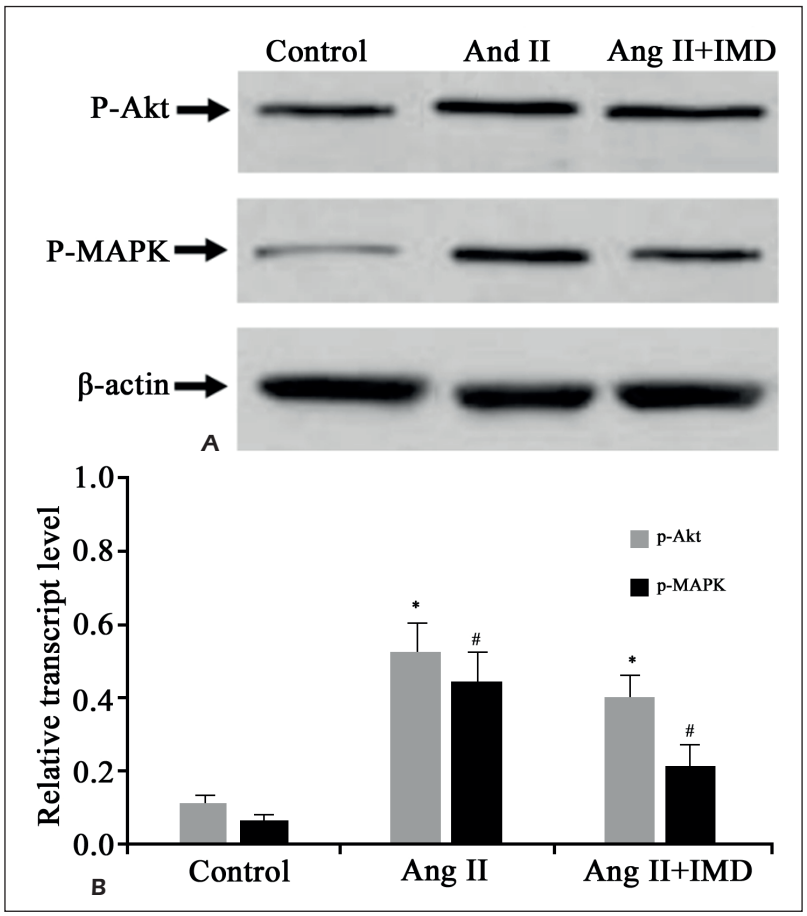


**Figure 3.** Comparison of p-Akt and p-MAPK among three groups. **A**, Western blot protein band. **B**, Relative transcript levels of p-Akt and p-MAPK.

Ang II could bind the Ang II receptors ($AT_1R$) on the surface of the vascular endothelial cell, and activate the downstream pathways to cause collagenous hyperplasia[16]. Fang et al[17] reported that coupling mode of $AT_1R$ and Gi proteins enhanced hydrolyzation effect of Gi protein on GTP, prompted Gβr dissociation, activated tyrosine kinase to stimulate epider-

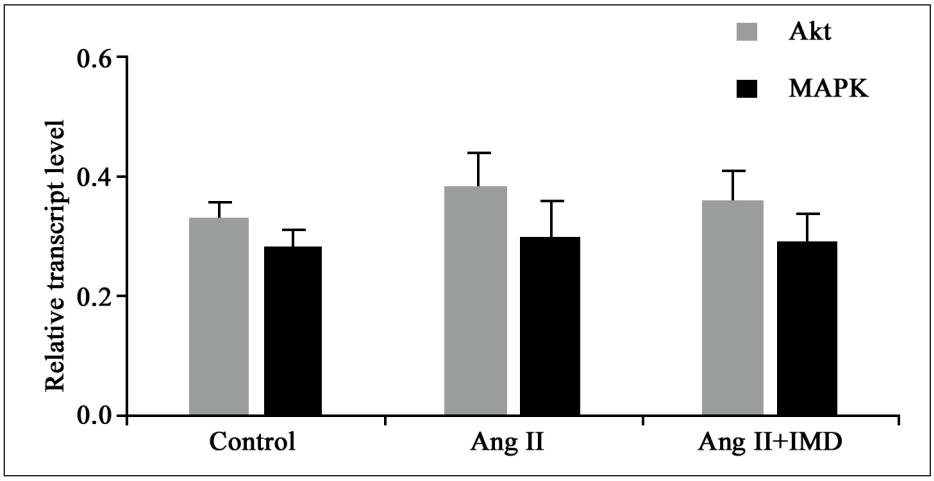


**Figure 4.** Relative mRNA transcript levels of Akt and MAPK.

mal growth factor receptor (EGFR), and finally triggered down-stream Ras/raf/MAPK/ERK pathway and PI3K/Akt pathway. Similarly, the data on protein coupling mode of AT1R and Gi indicated that $Ca^{2+}$/calmodulin was essential for EGFR pathway activated by protein coupling, by which phosphatidylinositol phospholipase C (PIPLC), and latter hydrolyzed membrane-bounded diphosphoinositide (PIP2) was activated to inositol triphosphate (PI3)[18]. As a member of CGRP family, IMD could bind with $Ca^{2+}$/calmodulin to restrict their activity and abrogate activation of EGFR pathway, thus interfering with the activities of down-stream essential proteins, including MAPK and Akt[19]. Ras/raf/MAPK/ERK and PI3K/Akt are two essential cell-signaling pathways stimulated by steroid hormones and growth factors, and they have been implicated in various aspects of cell proliferation and differentiation. Of note, our data indicated that Akt and MAPK were significantly activated by Ang II, which was consistent with previous finding[20,21]. As the treatment of IMD remarkably suppressed levels of phosphorylated protein p-Akt and p-MAPK while no significant changes in the Akt and MAPK mRNAs were present, indicating that IMD attenuated the effect of Ang II via inhibition of Ras/raf/MAPK/ERK and PI3K/Akt signaling pathway.

IMD is an important active peptide of the internal environment. Current researches[22,23] have proved IMD maintained protective effect against oxidative stress injury and it played a vital role in cardiovascular protection. As reactive oxygen species (ROS) production is attenuated by Ang II[24], along with the induction of cell apoptosis[25], the ROS associated enzymatic antioxidant system and the molecules must be mentioned in following studies. In the further study, we would include the evaluation of cell cycle progression, enzymatic antioxidant system, ROS production post the treatment of IMD on vascular collagen remodeling, as well as the clinical application of IMD requires in-depth investigation.

## Conclusions

We showed that IMD served as a potential protective factor against vascular collagen remodeling during angiotensin II-induced hypertension.

### Conflict of Interests:

The Authors declare that they have no conflict of interests.

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