Basic Science

Protective Effect of Tongxinluo on Oxidative Injury Induced by Angiotensin II in Rats

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Purpose: To investigate the effect of Tongxinluo (TXL), a traditional Chinese herbal medicine, on aortic biological variation after angiotensin II (Ang II)-induced vascular oxidative injury.

Methods: Randomly, 50 Sprague-Dawley (SD) rats were divided into three groups: sham, Ang II, and Ang II+TXL. A pump with Ang II was embedded in the rat backs in the Ang II and Ang II+TXL groups, whereas a pump containing physiological saline was emplaced in the sham group. TXL was delivered through a gastric tube in the Ang II+TXL group. Endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α) and nitric oxide (NO) in the plasma were examined 14 days later; the aortic endothelial cells were observed under a scanning electron microscope (SEM); the expressions of endothelial nitric oxide synthase (eNOS), nuclear factor-κB (NF-κB), and vascular cell adhesion molecule-1. (VCAM-1) were measured via immunohistochemistry assays; apoptotic cells were investigated with DeadEnd™ colorimetric TUNEL System; and the NAD(P)H oxidase subunit P22phox mRNA was examined through reverse transcription polymerase chain reaction.

Results: The plasma concentrations of ET-1 and TNF-α increased significantly in the Ang II group in comparison with the sham one, but decreased in the Ang II+TXL group. The same observations occurred regarding NF-κB, VCAM-1, apoptosis and P22phox mRNA expressions in the aortic tissues. However, eNOS expression in the aorta produced a reverse response, which was ameliorated significantly by TXL.

Conclusion: The findings suggested that TXL could play a potential role in inhibiting the oxidative injury induced by Ang II by reducing the inflammation and the apoptotic process via the P22phox pathway.

Key Words: Angiotensin II • Anti-oxidative • Aorta • Tongxinluo

INTRODUCTION

The vascular wall is an active, pliable and integrated organ made up of cellular (endothelial cells, vascular smooth muscle cells and fibroblasts) and noncellular (extracellular matrix) components, which can dynamically change its shape, and respond actively to physiological and pathological stimuli.1

It has been reported that angiotensin II (Ang II) plays a fundamental role in controlling the functional and structural integrity of the arterial wall and physiologically regulating blood pressure. Ang II treatment has been found to cause the upregulation of O2− formation and oxidative stress as implicated in some steps leading to the development of vascular disease. Ang II has been considered one of the proinflammatory cytokines which can stimulate production of vasoconstrictors (e.g., ET-1) and other growth factors for regulating cell growth and apoptosis.2

Tongxinluo (TXL) is a mixture of traditional Chinese
herbal medicines. It is extracted, concentrated, and freeze-dried from a group of herbal medicines, such as ginseng, radix paeoniae rubra, borneol, and spiny jujuba seed, which contain multiple active components that may be responsible for its multiple vasoprotective effects. Based on its multiple ingredients, TXL has been found to have correspondingly multiple actions, such as improving endothelial function, lowering lipid levels, antioxidation, antithrombosis, antiinflammation, anti-apoptosis and enhancing angiogenesis.\(^3\)\(^6\) Recently, it has been suggested that TXL could improve the microcirculation in the brain, protect against ischemic reperfusion injury, and possess neuroprotective properties.\(^7\)\(^8\) However, the exact activation of TXL on the aorta, especially on the thoracic aorta, has not been defined. In the present study, we detected some parameters related to oxidative stress and apoptosis induced by Ang II to analyze the vasoprotective effect and mechanism of TXL and then provide evidence for its clinical efficacy.

**MATERIALS AND METHODS**

**Reagents**

All procedures were performed in conformance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Tongxinluo (TXL) capsule was provided by the integration of traditional and Western Medical Research Academy of Hebei Province (authorization #: Z19980015; catalogue #: 20090213).

Obtained, respectively, were Ang II (PD-123319, Sigma, USA), anti-rat endothelial nitric oxide synthase (eNOS), nuclear factor-κB (NF-κB), and vascular cell adhesion molecule-1 (VCAM-1) antibodies (Santa Cruz, USA); TUNEL kit (KGA-703, Promega, USA); Trizol and RT-PCR kit (Invitrogen Life Technologies, USA); and Osmotic Pump (Model 2 ml, Alzet, Japan); Iodine \(^{[125]}\) Endothelin Radioimmunoassay Kit and Iodine \(^{[125]}\) Tumor Necrosis Factor Radioimmunoassay Kit (Beijing Puerweiy Biotechnology Co., Ltd); enzymatic colorimetric NO assay kit (Nanjing Jiancheng Bioengineering Institute, China).

**Animal model and subgroups**

Fifty Sprague-Dawley (SD) rats (aged 8 weeks and weighted 180–200 g) purchased from the Experimental Animal Center of the Chinese Academy of Sciences, stayed in an environment with relative humidity 45%–55%, illuminated 12 h. They were randomly divided into three groups: the sham composed of 10 (the sham), the angiotensin-II-treated group of 20 (Ang II group), and TXL plus Ang II-treated group of 20 (TXL+Ang II group). Ang II osmotic pumps with 200 ng/min/kg were embedded in the rat backs in Ang II and TXL+Ang II groups, whereas physiological saline osmotic pumps were similarly applied to the sham group. TXL at 0.6 g/kg/d was delivered through a gastric tube three days before the Ang II pump was employed in the TXL+Ang II group. In the sham group, the rats were used with the same volume of physiological saline via gastric tube. All the rats were sacrificed following 14-day treatment with Ang II. All animal care and experimental protocols complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001), and the study was approved by the Animal Care Committee of Fudan University. Before being sacrificed, the rats were recorded the carotid arterial blood pressure with a PowerLab 4/25 AD instrument.

**Plasma biochemical indicator assay**

The thoracic cavities of the rats, following anesthesia with ketamine, were opened to collect blood samples from the right ventricle, which were transferred to a chilled tube containing heparin before being centrifuged at 3,000 rpm for 15 min at 4 °C. Endothelin-1 (ET-1), and tumor necrosis factor-α (TNF-α) were examined via radioimmunoassay methods according to the manufacturer’s protocol.

Nitrite production was measured as an assay of NO release. Cell-free supernatant was recovered after incubation, and NO production was analyzed as NO\(_3^-\) and NO\(_2^-\) concentrations using an enzymatic colorimetric NO assay kit according to the manufacturer’s protocol.

**Scanning electron microscope (SEM) observation of thoracic aorta**

The anesthetized animals with ketamine were fixed on the operating table in a supine position. The thoracic cavities of the rats were opened to expose the beating heart. Then a needle was inserted into the left ventricle, through which 150 ml physiological saline and 150 ml
2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer were respectively perfused into the left ventricle. Meanwhile, a small incision in the right atrium was opened in order to let the perfused liquid flow out. Doing so, the aim was to wash away blood cells on the endothelium surface and fix the thoracic aorta tissue. After that, the thoracic aorta was removed out and the aorta was fixed in 2.5% glutaraldehyde at 4°C for 4 hours. Post fixation, the aorta was placed with 1% osmium tetroxide for 1 h, and then dehydrated through an ascending series of ethanol (concentration from 75% to 100%). Afterwards, it was embedded in epoxy resin overnight, and polymerized at 60°C for 48 hours, followed by trimming, mounting, and coating with gold-platinum. The morphology of the endothelial cells in the thoracic aorta was observed under a SEM (Philips XL30E).

**Apoptotic cell in situ test by TUNEL kit and flow cytometry**

The slides from the aortic samples imbedded in paraffin were deparaffinized, rehydrated and antigen-retrieved before being incubated in a moist chamber with the TUNEL mixture (5 µl of TdT+45 µl) for 1 hour at 37°C. Labeled DNA was visualized with 3,3′-diaminobenzidine (DAB) to be observed under a Leica microscope. For negative control, TdT was omitted from the reaction mixture; three slides were measured and in every slide, five fields were randomly observed under microscope. TUNEL-positive spots showed brown, indicating cellular DNA breaks.

The annexin V assay was performed. Simply, aortic tissue was homogenated and filtered. The cells were washed and incubated in 100 µl of binding buffer containing 5 µl of annexin V-FITC (PharMingen). The nuclei were counterstained with propidium iodide (PI), and the fraction of apoptotic cells was determined by flow cytometry.

**Immunohistochemistry**

The expressions of eNOS, VCAM-1 and NF-κB in the aortic tissues were determined by immunocytochemistry. The paraffin-embedded slides were deparaffinized and rehydrated before incubation with rabbit anti-rat eNOS (1:100), VCAM-1 (1:200) and NF-κB (1:200) primary antibody for 2 h at 37°C, respectively, and with the secondary antibody (1:200) for 1.5 hours at room temperature. For negative controls, the primary antibody was replaced with PBS in the process.

**Real-time RT-PCR**

Total RNA was extracted from from the aorta using Trizol (Invitrogen; Carlsbad, CA), and cDNA was synthesized using an Invitrogen kit. Polymerase chain reaction (PCR) was performed in triplicate using the SYBR Green PCR Master Mix kit (Applied Biosystems; Foster City, CA); GAPDH was used as an internal control. The amplification program consisted of activation at 95°C for 10 min, followed by 40 amplification cycles, each consisting of 95°C for 15 s, then 60°C for 1 min. The primers and probes were as follows: rat NAD(P)H oxidase subunit P22phox: forward, 5′-CGGGCTTGTCCTCCACTTAC TGc-3; reverse, 5′-TGATGCGCTTCAACCTG C G-3′ (178 bp); P22phoxprobe: FAM-5′-CGACCTCGAGGT AAGGGATCCTG-3′-T A MRA. RatGAPDH: forward, 5′-ACCACAGTCCATGCCATC AC-3′; reverse, 5′-TCC ACCACCTGTTCCTGTGA-3′ (452 bp). GAPDHprobe: FAM-5′-TGAGCTCCACTCG CTCT TCA-3′-TAMRA. Gene expression values were calculated based on the comparative threshold cycle (Ct). A threshold cycle (Ct) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the ΔCt values and expressed as 2−ΔCt. The value of each control sample was set at 1 and used to calculate the fold change of target genes.

**Statistical analysis**

All the determinations were set up in triplicate, the data presented as mean values ± SDs, which were compared using one-way ANOVA as indicated and followed by a post hoc LSD test. Significance was considered if \( p < 0.05 \).

**RESULTS**

**Plasma biochemical indicator**

The levels of ET-1 and TNF-α in the Ang II group increased significantly, and the level of NO decreased significantly compared with that of the sham group, which could be significantly attenuated by TXL (Figure 1).
Among the sham, AngII and Ang II+TXL groups, the carotid arterial blood pressure had significant difference (114.33 ± 22.50, 172.11 ± 19.12, and 129.51 ± 14.75, respectively, p < 0.05).

**Apoptotic measurement**

Strong TUNEL-positive cells were detected in the Ang II group (Figure 2A); apoptotic ones, were observed both in the endothelium and in the smooth muscle layer. It was found that the number of TUNEL-positive cells increased significantly in the Ang II group compared with those in the sham and Ang II+TXL groups (57.5 ± 5.03% vs. 1.75 ± 0.95%, and 20 ± 4.65%, p < 0.05). It was similar to the result of the flow cytometry (Figure 2B).

**SEM observations of thoracic aorta endothelium**

The surface of the thoracic aorta endothelium in the sham group was mostly flat without protrusion, and the endothelial contours appeared to be elongated. In the Ang II group, a large number of endothelial cells were desquamated, exposing the internal elastic membrane. In the Ang II+TXL group, most endothelial cells were repaired, showing a feeble injury (Figure 3).

**Immunohistochemistry**

In the sham group, NF-κB expressions occurred weakly in endochylema and nucleus, and none of VCAM-I in the aorta wall. However, both NF-κB and VCAM-I increased significantly in the Ang II group, and were significantly ameliorated in the Ang II+TXL group.
group. In the sham group, moreover, significant eNOS expression occurred in the endothelial and smooth muscle cells (SMCs), decreased nearly to none in the Ang II group, and showed a little positive enhancement in the Ang II+TXL group (Figure 4).

Reverse transcriptase polymerase chain reaction analysis (RT-PCR) for p22phox mRNA expression

The expression of p22phox in the aortic tissues increased significantly in the Ang II group compared with...
that of the sham group (p < 0.01), showing a significant decrease in the case of TXL (Figure 5).

**DISCUSSION**

In the present study, we observed an inhibitory effect of Tongxinluo, a traditional Chinese herbal medicine, on aortic biological variations when Ang II had induced vascular oxidative injury, finding out that the levels of ET-1, TNF-α, NF-κB and VCAM-1 increased significantly following a 14-day treatment with Ang II, that the severing of endothelium injury of the aorta was significantly raised after Ang II treatment; and that apoptotic cells and NAD(P)H oxidase p22phox, a marker of oxidative injury and apoptosis, increased significantly in the aortic tissues. However, all the conditions mentioned above were improved following TXL treatment, suggesting that TXL possessed a curative effect on the deteriorated activation induced by Ang II in the rats.

Two decades ago, TXL was developed for the treatment of coronary artery disease (CAD). As a compound traditional Chinese medicine, TXL has been successfully used in thousands of patients with chronic CAD to reduce the occurrence of acute coronary events or sudden death.\(^9\) Zhang\(^10\) and Li\(^11\) et al. reported that TXL dose-dependently enhanced the stability of vulnerable plaques and prevented plaques from rupturing in aortic atherosclerosis in a rabbit model by inhibiting the expression of cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMP).

Ang II, an important component of the renin-angiotensin system, has been suggested to be involved in the development and progression of atherosclerosis,\(^12\) and this hypothesis has been supported by recent studies on Ang II receptor type 1 (AT\(_1\)) inhibitors, which have shown an anti-atherosclerotic effect in the experimental studies and a reversal of endothelial dysfunction in patients with CAD.\(^13\) Ang II, as the intracellular signaling pathway molecule, contains the interconnected molecular cascades that transmit information from the cell membrane receptor to the intracellular proteins that regulate such cellular activities such as contraction, cell growth, mitogenesis, apoptosis, differentiation, migration, and other functions.\(^14\) In the present study, the animal model, widely employed in investigations on the endothelial dysfunction of early atherosclerosis nowadays, was established by embedding the osmotic pump with Ang II in the rat backs to imitate a marked injury of the aorta in vivo with Ang II.

Ang II has been reported to stimulate the production of cytokines such as TNF-α and T1 via the Ang II type I receptor, which is present on monocytes, macrophages and vascular smooth muscle cells,\(^15-17\) and can turn on the synthesis of the potent vasoconstrictor peptide ET-1 in different vascular cells, which thus plays an important role in cardiovascular disease and vascular remodeling.\(^18,19\) It was of interest to examine the effects of ET, NO and TNF-α on responses to Ang II; one or more these factors might be involved in inflammatory atherosclerosis and had been implicated in experimental Ang II-induced progression of atherosclerosis.\(^20,21\) It was the first time to explore the relationship between the traditional Chinese medicine TXL and Ang II. It was reported that the mechanism of the upregulation of NF-κB and VCAM-1, after Ang II treatment, was mainly due to the increases of TNF-α and ET-1, which could stimulate the expressions of NF-κB and VCAM-1 in vitro.\(^15,21-23\) In the study, however, TXL could inhibit all the proinflammatory cytokines, suggesting its beneficial effect on early atherosclerosis.

Recent reports have shown that Ang II could assist in producing the membrane-bound nicotinamide adenine dinucleotide, NAD(P)H oxidase, which is capable of generating reactive oxygen species (ROS) in vascular SMCs.\(^24\) Excess NAD(P)H oxidase generation is considered to be a likely initiator of atherosclerotic events, causing the increased synthesis of numerous mitogenic factors that contribute to the hyperproliferation of SMCs and vascular plaque formation.\(^25\) The NAD(P)H oxidase subunit p22-phox, which is linked to the development of atherosclerosis in humans, has been shown to be a critical component in the Ang II-induced hypertrophy in vascular
SMCs. In this study, we semiquantified the formation of NAD(P)H oxidase subunits p22-phox and investigated the p22phox in response to Ang II via RT-PCR, the results showing that TXL could play an anti-oxidative and vasoprotective role by regulating the NAD(P)H oxidase pathway in the aortic tissues. Furthermore, we examined the apoptotic cells in the aortic tissues so that we could verify that TXL could be involved in the signal pathway between the oxidative stress and apoptotic process.

CONCLUSION

The present study provided an insight into the protective effect of TXL on oxidative injury in the blood vessels by proving a decrease in the secretion of inflammatory factors and apoptosis via the p22phox pathway, which could be one of the working mechanisms responsible for a vasoprotective effect.

ACKNOWLEDGEMENT

This research was supported by two National Key Basic Research Development Programs grants, whose numbers 2005CB523302 and 2006CB503803.

DISCLOSURE

There are no financial or other relations that could lead to a conflict of interest.

REFERENCES


SUPPLEMENTAL MATERIAL

1. Name of Traditional Chinese Medicine: Tongxinluo capsule
2. Authorization number: Z19980015; catalogue number: 20090213

**The HPLC and GC fingerprints of TongXinLuo:**

The HPLC and GC fingerprints of TongXinLuo were established to control the quality of the Chinese herbal formula TongXinLuo.

Peak 1: Paeoniflorin
Peak 7: Ginsenoside Rg1
Peak 8: Ginsenoside Rb1
Peak 9: Jujuboside A
Peak 10: Jujuboside B

**The HPLC fingerprint:**

The HPLC system was equipped with an Agilent HP-1100 HPLC instrument. Separation was performed on a Waters symmetry C18 (4.6 × 250 mm, 5 μm) analytical column with mobile phase consisting of acetonitrile and water with gradient elution with the flow rate 1.0 ml/min and the column temperature at 30 °C. The UV wavelength used for detection was set at 203 nm, and the analysis time was 90 min. 13 common peaks on the HPLC fingerprints of TongXinLuo were indicated.

**The GC fingerprint:**

GC analyses were carried out on an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID). Separation was performed on an Agilent DB-624 (30.0 m × 353 μm × 3.0 μm) capillary column with nitrogen as carrier gas; the split ratio was 10:1. The injector and detector temperatures were set at 200 and 250 °C, respectively. The column temperature was programmed isothermally at 50 °C for 2 min, and then increased first at a rate of 10 °C/min to 160 °C and subsequently at a rate of 5 °C/min to 200 °C, where it was held for 5 min of isothermal operation. 8 common peaks on the HPLC fingerprints of TongXinLuo were indicated.