Original Paper

The Effect of Electroacupuncture on Insulin-Like Growth Factor-I and Oxidative Stress in an Animal Model of Renal Failure-Induced Hypertension

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Key Words

Abstract

Background/Aims: The present study was performed to demonstrate the effect of electroacupuncture (EA) and acupuncture on renal failure (RF)-induced hypertension. **Methods:** We stimulated the Zusanli (ST36) and Taixi (KI3) acupoints in a rat model of RF-induced hypertension. **Results:** EA significantly reduced RF-induced hypertension ($p < 0.05$). In a histopathological study, increments in RF-induced glomerulosclerosis and tubulointerstitial fibrosis were attenuated by EA treatment ($p < 0.05$). The increase in albuminuria in the RF group was also reduced by EA treatment ($p < 0.05$). In blood analysis, the increment in RF-induced serum BUN and creatinine concentrations were decreased by EA ($p < 0.05$), and the decreases in RF-induced insulin-like growth factor-I (IGF-I) mRNA and protein levels were increased by EA in both the kidney and the serum ($p < 0.05$). The increases in RF-induced oxidative stress, e.g. inducible nitric oxide synthase, heme oxygenase and thiobarbituric acid-reactive substance expression, were significantly decreased in the RF-EA group ($p < 0.01$). **Conclusion:** These findings suggest that the anti-hypertensive mechanism of EA could be related to the effects of oxidative stress on IGF-I in RF-induced hypertension.

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Introduction

The kidney, which controls fluid metabolism, plays an important role in the body's homeostatic balance, including electrolyte composition, body fluids, blood pressure and acid-base balance. Increased renin-angiotensin system activity is related to hypertension during kidney dysfunction [1–5]. Specifically, renal failure (RF)-induced hypertension is a frequent occurrence and considered to be one of the most important major diseases and disorders [6]. It can be a secondary cause of many diseases, such as stroke, ischemic heart disease and arteriosclerosis for example [7–12]. RF-induced hypertension has been treated medically, by acupuncture and by combined therapy [8, 13, 14]. Although many chemical drugs have been studied and proven to be of therapeutic benefit, evidence of side effects still remains [15].

In the last decade, many publications have addressed the therapeutic effects of acupuncture or electroacupuncture (EA), and its curative effects have been increasingly recognized as an effective approach to treatment in several medical fields [16–18]. Acupuncture has been used in the treatment and prevention of cardiovascular diseases and hypertension [8, 13, 14]. Hypertension is an important part of renal disease and is probably one of the most

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important factors contributing to the progression to chronic kidney disease (CKD) [19].

Recent studies have demonstrated that acupuncture at Zusanl (ST36) induces upregulation of neuronal nitric oxide synthase (NOS) and that this response can control the cardiovascular system. It has also been reported that acupuncture at ST36 reduces blood pressure [20]. Renal blood flow changes have been clearly documented and are related to specific acupuncture points, which can be observed with stimulation at Taixi (KI3) [21].

In RF, insulin-like growth factor-I (IGF-I) increases the glomerular filtration rate by stimulating the kidneys, and the glomerular filtration rate is also directly increased by growth hormone from the pituitary gland [22]. IGF-I has been implicated in the proliferation, differentiation, survival, apoptosis and cellular protective effects that are related to oxidative stress, including reactive oxygen species (ROS), inducible NOS (iNOS), heme oxygenase (HO-1/2) and thiobarbituric acid-reactive substances (TBARS) [23–29]. ROS are balanced by natural antioxidant enzymes. They act as secondary cellular messengers that shift iNOS and HO-1/2 toward IGF-I signaling, thereby activating or inactivating cellular mechanisms [30, 31]. However, the relationship between IGF-I signaling and acupuncture is still unclear. The aim of this study was to investigate the effects of acupuncture and EA at the ST36 and KI3 acupoints, which are related to oxidative stress associated with IGF-I in an experimental model of progressive CKD.

Materials and Methods

Animals
Male Sprague-Dawley rats (Dae Han Biolink, Enumsung, South Korea) were housed in colony cages with free access to food and water, and maintained in a temperature- and light-controlled room (23 ± 2°C, 12/12 h light/dark cycle with lights on at 08:00). All of the methods used in this study were approved by the Institutional Animal Care and Use Committee of Chonbuk National University and conformed to NIH guidelines (NIH publication No. 86-23, which was revised in 1985).

Acupuncture Protocols
All experimental rats were anesthetized with inhalational isoflurane. Ventilation was set to obtain an expired isoflurane concentration of 1.5% in a 3:7 O_2/N_2O mixture using a Tabletop Research Anesthesia with N_2O/O_2 flowmeter failsafe system (V702004, SurgiVet, Waukesha, Wisc., USA). Following isoflurane anesthesia, the rats were treated with acupuncture and EA at the ST36 and KI3 acupoints using a stainless steel needle (diameter, 0.25 mm; length, 30 mm; Dongbang Acupuncture, Seoul, Korea). The needles were inserted into each point (depth, 5 mm for the ST36 point and 2–3 mm for the KI3 point). The electrode needles were also inserted near each point (at the same depth) using a two-way guide tube. After insertion, the acupuncture handle of the verum acupuncture and the reference acupuncture were connected to the crocodile clip of the EA and stimulated (2 Hz, 0.1 mA) for 10 min using a pulse generator (PG-306, Ito, Fukuoka, Japan).

Experimental Protocol
The rats used in this study were divided into five groups: the control group; sham group; RF group; RF-A (the acupuncture-treated RF) group, and the RF-EA group. Each group consisted of 10 male rats (160–180 g). In summary, using surgical methods for RF modeling, the right kidney was entirely removed as well as 3/4 of the left kidney [32].

After removing 7/8 of the kidney, blood pressure was monitored for 3 weeks, and then we performed acupuncture or EA for 10 days (daily for 10 min) in the rats with RF-induced hypertension. All groups received isoflurane anesthesia (1.5%) to reduce the stress of EA stimulation during the experiments. Following blood pressure determination, the rats were sacrificed after 3 weeks. In each case, blood, urine and tissue samples were collected.

Blood Analysis, and Histological and Semiquantitative Analysis of the Kidney
To confirm the effects of acupuncture on the RF model, blood and urine analysis (blood pressure and serum chemistry), hematoxylin-eosin (HE) staining and histological analysis were performed. In brief, to confirm RF-induced hypertension, blood pressure (NIBP System; Ad Instruments, Sidney, N.S.W., Australia) was assessed and blood biochemistry parameters were analyzed (VetTest 8008 clinical biochemistry analyzer, Idexx Laboratories, Mass., USA) after 0, 1, 2 and 3 weeks. The rats were placed in individual metabolic cages and 24-hour urine was collected. Albuminuria was measured by ELISA (Bethyl Laboratories, Montgomery, Tex., USA). Then the RF-A and RF-EA groups were treated with acupuncture or EA for 10 days (daily for 10 min), respectively. Systolic (SBP) and diastolic blood pressure (DBP) were monitored in the conscious rats by the tail-cuff method [33] (NIBP System; Ad Instruments) once per week. In the final experiment, each kidney tissue sample was fixed with 10% neutral formalin and stained with HE solution for histopathological examination. All morphological evaluations were performed in a blinded manner by a single observer. Glomerular damage was evaluated on the basis of the percentage of glomeruli that were sclerotic or collapsed. At least 20 glomeruli from each rat were randomly examined. The degree of lesions in each glomerulus was graded on a scale from 0 to 4+ using the following scoring system [34]. Grade 0 represents a normal glomerulus; grade 1, 1–25% loss of capillaries in the tuft (minimal sclerosis); grade 2, 26–50% loss (moderate sclerosis); grade 3, 51–75% loss (moderate-severe sclerosis), and grade 4, 76–100% of the tuft sclerosed (severe sclerosis). For each animal, severity of renal damage was reported as the average score of the glomerulosclerosis index. Tubulointerstitial injury was defined as inflammatory cell infiltration, tubular dilution and/or atrophy, and fibrosis. Injuries were examined in ≥20 areas using the following scoring systems.

TBARS Assay
For ROS measurements, we used TBARS (expressed as nmol malondialdehyde/mg protein) using spectrophotometric detec-
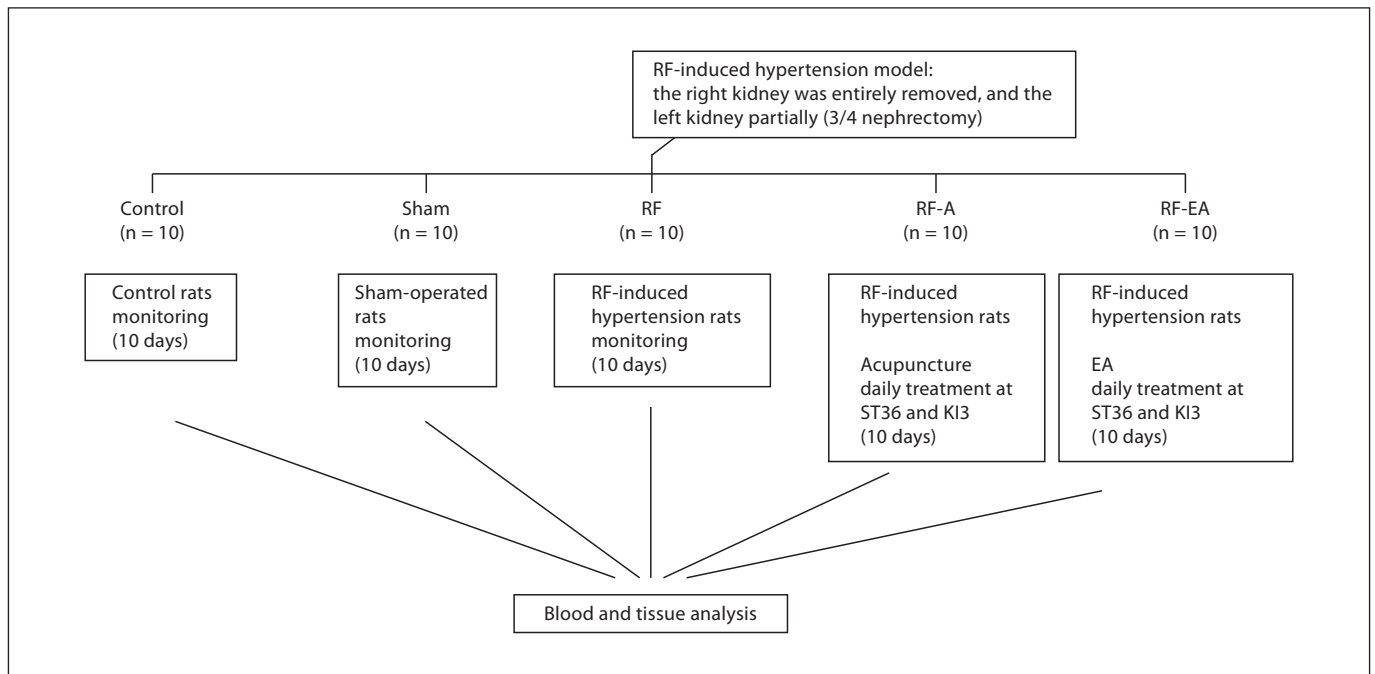
Diagram of the experiment design

tion at 532 nm, as described previously [35]. In brief, each sample was mixed with 20% acetic acid and 0.08% thiobarbituric acid (Sigma, St. Louis, Mo., USA) followed by heating at 105°C for 1 h. After cooling, the mixture was extracted into a butanol-pyridine solvent (Sigma).

IGF-I Radioimmunoassay

The samples were dehydrated after elimination of any IGF binding proteins using a Bio-spin P-10 column [36]. IGF-I analysis was conducted using a radioimmunoassay [37]. Briefly, 50 μl of a rat polyclonal IGF-I antibody (1:1,500; Gro-Pep, Adelaide, S.A., Australia) were added to 100 μl of each sample/standard and then incubated for 1 h at room temperature. Next, [125I]-IGF-I was added to the samples at 20,000 cpm and then incubated for an additional 18 h at 4°C. Next, 50 μl of horse serum (Sigma) and PEG No. 8000 (1 ml) was added to the sample, which was then centrifuged at 3,000 rpm for 30 min at 4°C. After discarding the supernatant, the radioactivity of the precipitates containing bound [125I]-IGF-I was measured with a γ-scintillation counter (Wallac, Turku, Finland). All reagents for routine analysis were obtained from Sigma and Gibco-BRL (Grand Island, N.Y., USA). All assays were performed in triplicate. The intra- and interassay coefficients of variation for IGF were 8 and 10%, respectively.

Reverse Transcriptase-Polymerase Chain Reaction (Quantitative Real-Time PCR)

RNA was extracted from the kidney using the TRIzol reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. In brief, cDNA was synthesized from 3 μg of total RNA by RT-PCR. Second-strand cDNA synthesis was conducted by combining 2 μl of each sample with 10 μl of Hotstart-IT SYBR Green qPCR master mix and 0.1 μM of each primer (IGF-I sense, 5′-CACAGGGTATGGCTC-3′; IGF-I antisense, 5′-CTTCTGG-GTCTTGGG-3′). Next, water was added to achieve a final volume of 20 μl, and PCR was performed. The DNA-binding dye (SYBR Green I) that incorporated into the double-stranded DNA during PCR amplification emits fluorescence whose intensity increases with each cycle number. After 40 cycles of amplification, the products were quantified by a real-time PCR melting curve program (65–95°C, with a heating rate of 0.1°C/s and continuous fluorescence measurements) and cooled down to 15°C. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a housekeeping gene, was used as a control.

Western Blotting

The samples were analyzed using Western blotting, according to the method of Hossenlopp et al. [37]. Briefly, kidney tissue was collected and sonicated for 5 min in lysis buffer. Each sample was centrifuged at 12,000 rpm for 10 min at 4°C. Next, the supernatant was collected, and the protein concentrations were estimated using a BCA (bicinchoninic acid) protein assay kit (Pierce, Bonn, Germany). Approximately 20–40 μg of protein of each sample were subjected to 10% SDS-PAGE and then transferred from the gel onto a PVDF membrane (Bio-Rad, Hercules, Calif., USA). The blots were incubated with specific antibodies (1:750–1:1,000) against IGF-I, iNOS, HO-1/2 and β-actin overnight at 4°C. The immunoblots were then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:3,000). The specific protein bands were visualized using an enhanced chemiluminescence kit (Cell Signaling, Beverly, Mass., USA). The band intensities were quantified by densitometry using AlphaImager software (Alpha Innotech, San Leandro, Calif., USA).
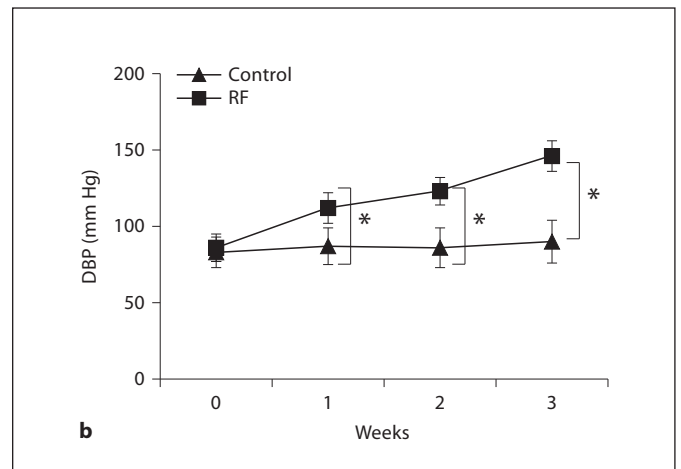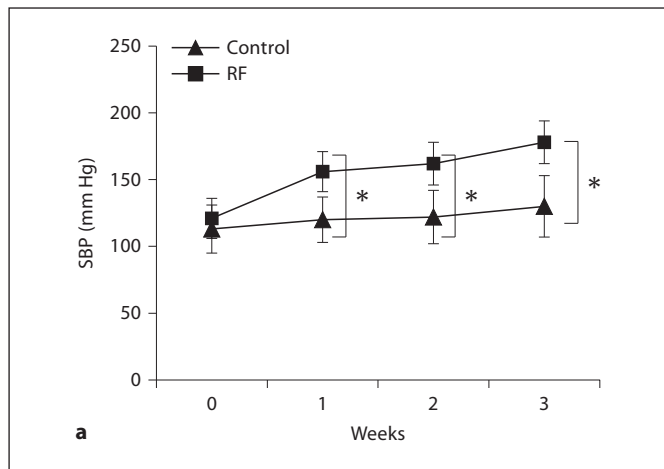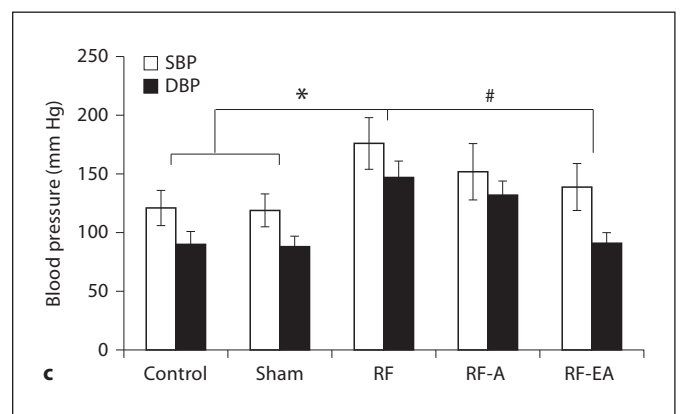
Fig. 1. Effects of EA on SBP and DBP in rats with RF-induced hypertension. After removing 7/8 of the kidney, the changes in SBP (**a**) and DBP (**b**) were monitored for 1–3 weeks. **c** After established RF-induced hypertension rats, the changes in SBP and DBP following acupuncture or EA for 10 days (daily for 10 min) were determined. * $p < 0.05$, vs. controls. # $p < 0.05$, vs. RF.

Statistical Analysis

All data were expressed as the mean ± SD based on triplicate determinations. Data were analyzed for statistical significance using one-way ANOVA, followed by Tukey's test as a post hoc test with SPSS software (SPSS for Windows, version 10). A value of $p < 0.05$ was defined as statistically significant.

Results

The Effects of Acupuncture and EA on Blood Analysis and Pressure

Figure 1 depicts the increases in SBP and DBP in the RF group 1, 2 and 3 weeks after RF modeling compared to the control group (fig. 1a, b; $p < 0.05$). At 3 weeks, the RF group showed maximal increases in SBP and DBP (fig. 1a, b, $p < 0.05$; SBP of 178.9 ± 19.04 mm Hg and DBP of 146.65 ± 9.24 mm Hg vs. SBP of 130.1 ± 25.27 mm Hg and DBP of 90.98 ± 23.01 mm Hg, respectively).

To investigate whether acupuncture or EA affects hypertension, the RF-induced hypertension rats were treated with acupuncture or EA at their ST36 and KI3 acupoints. As shown in figure 1c, acupuncture slightly reduced hypertension in the RF group, especially in the RF-EA group, in which the blood pressure levels decreased to levels of the control and sham groups (SBP of 138.9 ± 26.98 mm Hg and DBP of 91.6 ± 12.78 mm Hg; $p < 0.05$). To confirm the relationship between hypertension and the blood components, we analyzed the serum levels of BUN (blood urea nitrogen) and creatinine. In the RF group, serum BUN and creatinine concentrations were significantly higher compared to the control group (table 1; $p < 0.05$). The RF-A and RF-EA groups had lower concentrations of serum BUN and creatinine compared to the other groups. Specifically, the RF-EA group exhibited reduced concentrations of serum BUN and creatinine, and total cholesterol was also decreased compared to the other groups (table 1; $p < 0.05$). At the end of the experiment, albuminuria was significantly higher in the RF group than in the control and sham groups (table 1; $p < 0.01$), but RF-induced albuminuria decreased following EA treatment (table 1; $p < 0.05$).
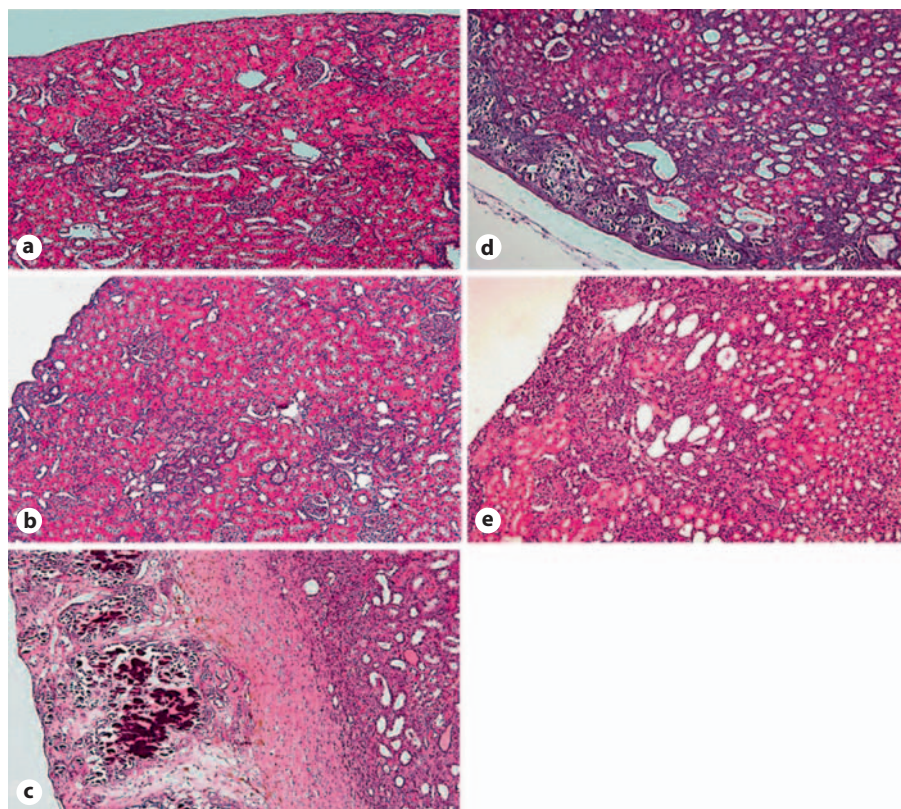
Effect of Electroacupuncture on IGF-I in Hypertension

Fig. 2. Microscopic findings illustrate normal kidneys and kidneys with RF-induced hypertension. HE. ×100. Representative microscopic findings from the normal and sham (**a**, **b**) and RF, RF-A and RF-EA groups (**c**, **d**, **e**, respectively). **c** Severe multifocal tubulointerstitial fibrosis and glomerulosclerosis with cortical calcification in RF-induced hypertension. **d** Moderate-severe tubulointerstitial fibrosis and glomerulosclerosis in the RF-A group. **e** Moderate tubulointerstitial fibrosis and glomerulosclerosis without calcification in the RF-EA group.

Table 1. Blood parameters and albuminuria at the end of the experiment (means ± SD)

Group	Control	Sham	RF	RF-A	RF-EA
Serum levels					
Glucose, mg/dl	201.67 ± 34.2	211.5 ± 21.9	187.7 ± 31.9	180.75 ± 24.0	175.6 ± 30.8
Total cholesterol, mg/dl	119 ± 11.1	120.2 ± 17.5	117.5 ± 14.8	117.5 ± 19.4	79.8 ± 21.7*
BUN, mg/dl	24 ± 9.6	20.89 ± 8.6	68.5 ± 13.2*	44 ± 9.4	30 ± 10.3[a]
Creatinine, mg/dl	0.9 ± 0.23	0.8 ± 0.32	1.175 ± 0.42*	1.05 ± 0.29	0.78 ± 0.32[a]
Total protein, g/dl	4.73 ± 0.5	4.98 ± 0.8	5.625 ± 0.7	5.45 ± 0.8	5.38 ± 0.9
Albumin, g/dl	3.26 ± 0.7	3.3 ± 0.7	3.35 ± 0.54	3.325 ± 0.87	3.52 ± 0.67
Urinary albumin, mg/24 h	15.8 ± 4.34	17.5 ± 5.09	156.9 ± 46.3**	123.9 ± 49.3	84.3 ± 28.6[a]

After confirming RF-induced hypertension, samples were obtained from the RF-A or RF-EA rats for 10 days (daily for 10 min). n = 10 rats/group. * $p < 0.05$, ** $p < 0.01$, vs. control; [a] $p < 0.05$, vs. RF.

EA Reduces Glomerulosclerosis, Tubulointerstitial Fibrosis and Kidney Calcification

To demonstrate the effects of EA on renal histopathology in RF, we performed HE staining on paraffin-embedded kidneys from each group. The kidney tissue of the RF group showed severe multifocal tubular necrosis and cortical calcification with renal fibrosis and cystic tubule formation (fig. 2c), and the kidneys of the RF-A group exhibited moderate-severe focal tubular necrosis, calcification and cystic tubule formation (fig. 2d). Moreover, the RF-EA group showed moderate cystic tubule formation and mild interstitial fibrosis without kidney tissue calcification (fig. 2e). The grade of the glomerulosclerosis and tubulointerstitial fibrosis index were statistically higher
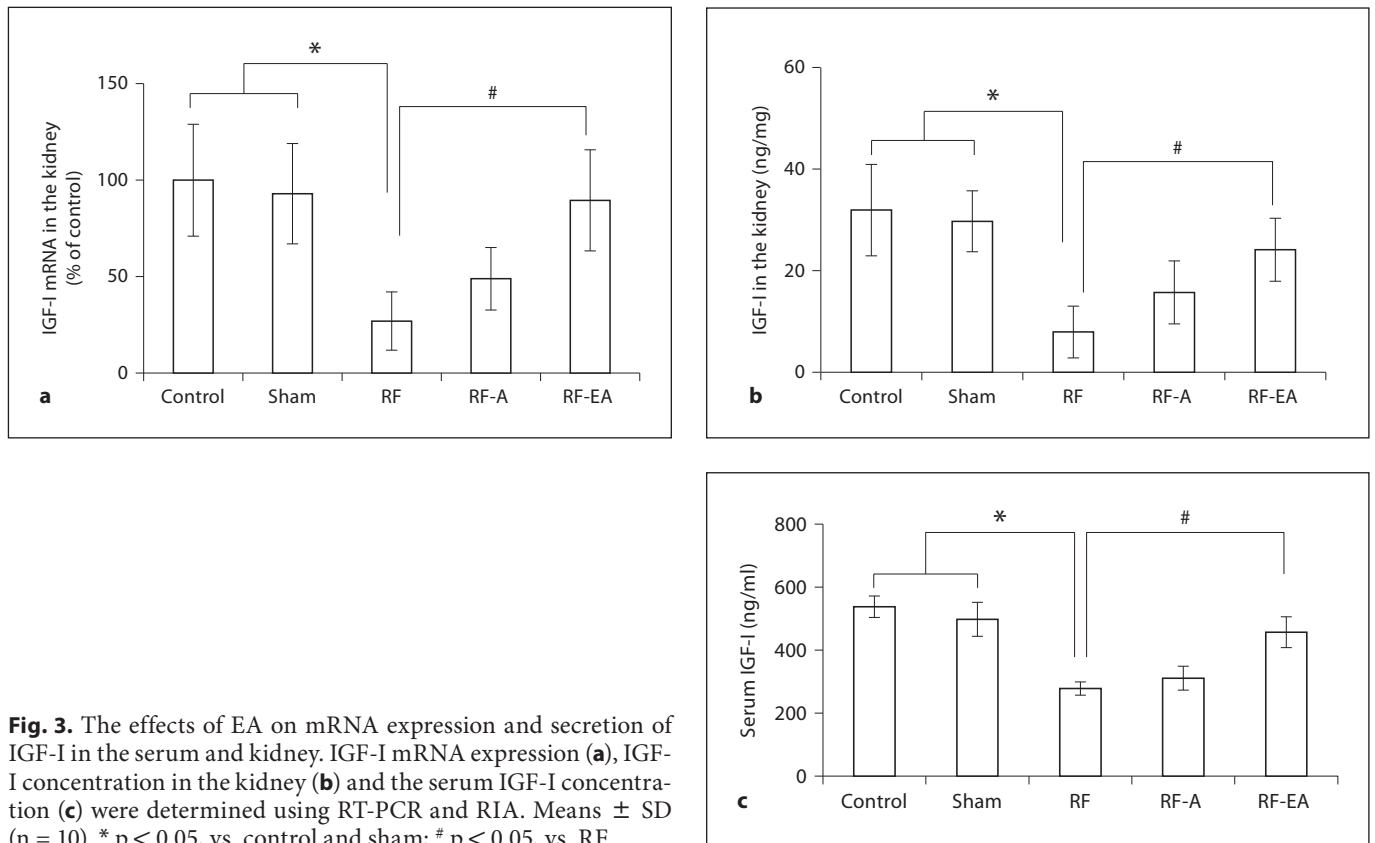
Fig. 3. The effects of EA on mRNA expression and secretion of IGF-I in the serum and kidney. IGF-I mRNA expression (**a**), IGF-I concentration in the kidney (**b**) and the serum IGF-I concentration (**c**) were determined using RT-PCR and RIA. Means ± SD (n = 10). * p < 0.05, vs. control and sham; # p < 0.05, vs. RF.

Table 2. Effects of EA on glomerulosclerosis and tubulointerstitial fibrosis in a rat model of RF-induced hypertension

Groups	Control	Sham	RF	RF-A	RF-EA
Glomerulosclerosis score	0.18 ± 0.10	0.20 ± 0.12	3.67 ± 0.62[a]	2.932 ± 0.58	1.89 ± 0.48[b]
Tubulointerstitial injury score	0.16 ± 0.09	0.21 ± 0.15	3.31 ± 0.67[a]	2.92 ± 0.60	1.37 ± 0.43[b]

Means ± SD. [a] p < 0.01, vs. the control group; [b] p < 0.05, vs. the RF group.

in the RF group than in the control and sham groups (table 2; p < 0.01), and this morphological changes also recovered following EA treatment (table 2; p < 0.05).

EA Significantly Increases IGF-I mRNA Levels and IGF-I Secretion in the Kidney

To examine the involvement of IGF-I in RF-induced hypertension, we analyzed IGF-I levels in the serum and kidney. As shown in figure 3, IGF-I mRNA and protein levels in the serum and kidney were significantly decreased in the RF group compared to the control and sham groups (fig. 3; p < 0.05). Acupuncture and EA cor-

rected the decreases in RF-induced IGF-I mRNA and protein levels. Specifically, in the RA-EA group, IGF-I mRNA and protein levels effectively recovered reaching those of the control and sham groups (fig. 3; p < 0.05).

EA Significantly Decreases TBARS, iNOS and HO-1/2 in the Kidney

To confirm the relationship between the effects of acupuncture and oxidative stress, we analyzed TBARS, iNOS and HO-1/2 levels. In the RF group, TBARS production was significantly increased compared to the control and sham groups (fig. 4a; p < 0.01; 8.7 ± 0.6 nmol/mg), but
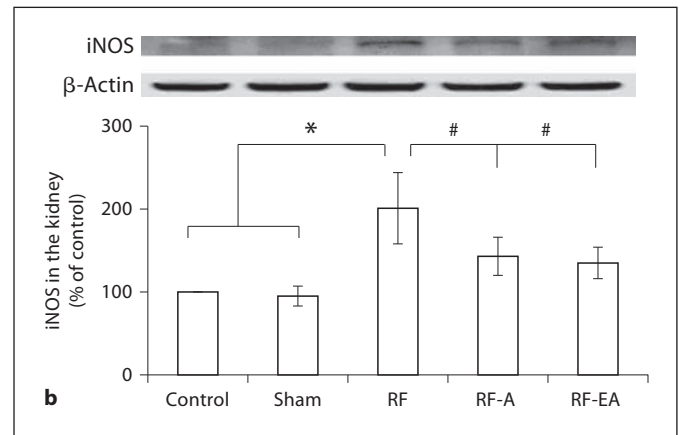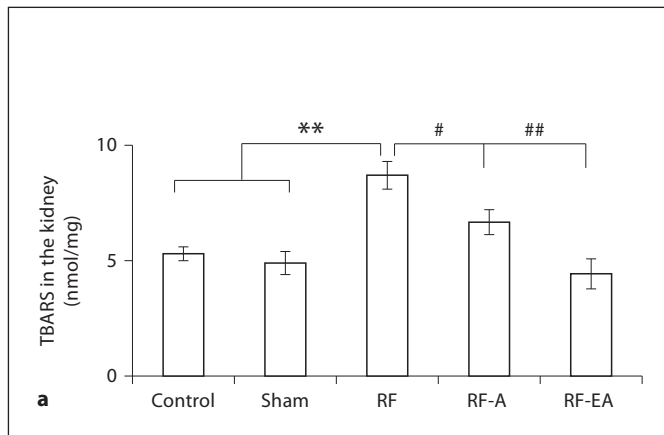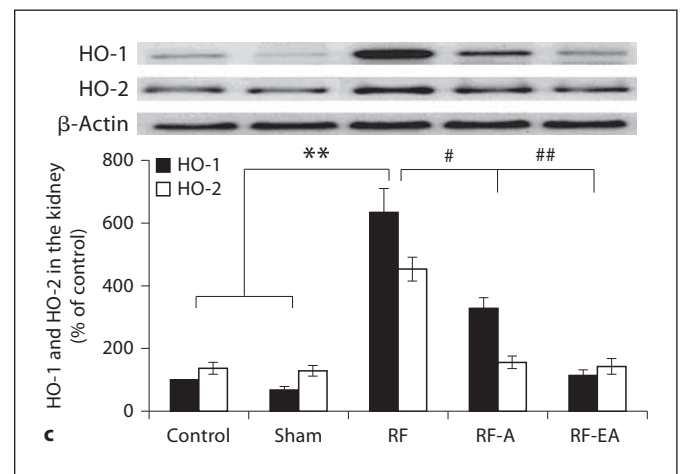
Fig. 4. The effect of EA on TBARS, iNOS and HO-1/2 expression. TBARS (**a**), iNOS (**b**), and HO-1/2 expression (**c**) were determined using TBARS and Western blotting. Mean $\pm$ SD (n = 10). * $p < 0.05$ and ** $p < 0.01$, vs. control and sham; # $p < 0.05$, ## $p < 0.01$, vs. RF group.

acupuncture and EA significantly resulted in a significant attenuation of TBARS levels compared to the RF group. Moreover, TBARS production was significantly decreased in the RF-EA group compared to the RF group (fig. 4a; $p < 0.01$; 4.43 ± 0.65 vs. 8.7 ± 0.6 nmol/mg, respectively). The levels of iNOS and HO-1/2, which are known to be measures of oxidative stress and cell resistance, are also increased in the RF group compared to the control and sham groups (fig. 4b, c; $p < 0.05$, $p < 0.01$, respectively). The increases in RF-induced iNOS and HO-1/2 expression were significantly decreased in RF-A and RF-EA groups (fig. 4b, c; $p < 0.05$; $p < 0.01$, respectively).

Discussion

In this study, we investigated the antihypertensive mechanism of EA at the ST36 and KI3 acupoints on RF-induced hypertension to demonstrate the relationship between EA and the regulation of IGF-I and oxidative stress. For this study, we established an RF-induced hypertension model in 3/4 nephrectomized rats, as previous reported [33], and we confirmed systolic and diastolic hypertension after 1–3 weeks [32].

These results demonstrate that EA was more effective than acupuncture in the treatment of RF-induced hypertension. Histopathologically, EA was also more effective than acupuncture in the treatment of RF rats. The rats with RF-induced hypertension showed severe multifocal tubulorinterstitial fibrosis and cortical calcification with renal glomerulosclerosis. In contrast, EA in the rats with RF-induced hypertension resulted in improved moderate tubulointerstitial fibrosis and glomerulosclerosis. Albuminuria was also reduced by EA treatment. Blood analysis showed that EA reduced the increases in RF-induced BUN and creatinine levels in the serum. Moreover, the decrease in the total cholesterol concentration in the EA treatment group (vs. the other groups) was caused by elec-

trical stimulation at the ST36 and KI3 acupoints, which can be explained by an increase in involuntary muscle movements. These results are consistent with those of another study in which EA and moxibustion reduced blood pressure, serum creatinine, diuresis, glomerulosclerosis and tubulointerstitial kidney fibrosis in 5/6 nephrectomized rats [38].

One of the most notable findings of our study was the beneficial effect of EA at the ST36 and KI3 acupoints on 3/4 nephrectomized rats with RF-induced hypertension (vs. traditional acupuncture). Based on these findings, we suggest that EA treatment at ST36 and KI3 acupoints could be a more effective therapy than traditional acupuncture.

IGF-I exerts protective effects on the cell, which are related to cell resistance and oxidative stress, against cytotoxicity and proliferation [23]. Other studies have reported that rats with RF-induced hypertension exhibit apoptosis and oxidative stress in the kidney and that oxidative stress in the kidney and serum decrease IGF-I secretion [39–45]. This work showed an increase in IGF-I levels following EA stimulation at the ST36 and KI3 acupoints, suggesting that EA could activate IGF-I during RF-induced hypertension.

The IGF-I concentration of the kidney induced various cellular responses in 3/4 nephrectomized rats with RF-induced hypertension [46]. Ischemic RF-induced hypertension and cell death are regulated by an oxidative stress mechanism and ROS [47]. The oxidative stress molecules that are involved in apoptosis and survival are iNOS [48], HO-1/2, NF-κB (nuclear factor κB), AP-1 (activator protein 1), Sp1 (specificity protein 1) and Nrf-2 (NF-E2-related factor 2) [49, 50]. NO is an important molecule that plays a role in a variety of physiological functions, including immune modulation and cytotoxicity [51]. There are three isotypes of NO synthase, calcium-dependent endothelial NOS, neuronal NOS and calcium-independent iNOS, which can produce large amounts of NO [48]. It has been reported that therapeutic effects of acupuncture on experimental renovascular hypertension are implicated in decreases in the levels of NO synthases [33]. We previously reported that inducible NOS and HO-1/2 expression is involved in the secretion of IGF-I in MCF-7 cells [52]. Furthermore, expression of the cellular protection and oxidative stress resistance protein HO-1/2 is decreased in kidneys with RF [53] but recovers after EA [54]. However, our results show that rats with RF-induced hypertension respond to oxidative stress, which is reflected by TBARS, iNOS and HO-1/2 levels, and EA treatment inactivates these proteins. This finding suggests that oxidative stress-induced cell protection and resistance are improved by EA in rats with RF-induced hypertension. The antioxidant IGF-I is also related to oxidative stress, implicating that EA treatment in RF-induced hypertension counteracts the oxidative stress induced by changes in IGF-I levels, demonstrating the beneficial effect of EA compared to traditional acupuncture. Therefore, the antihypertensive effect of EA is associated with oxidative stress and IGF-I in an animal model of RF-induced hypertension. Furthermore, further studies on EA-induced changes in the IGF-I- and NO-mediated signaling pathways are needed. However, in the future, studies on NADPH oxidase and superoxide dismutase activity in RF-induced hypertension should be performed to confirm the relationship between decreased ROS and IGF-1- and NO-mediated signaling.

Acknowledgment

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