

## Early Effects in Perivascular Nerves and Arterial Media Following Renal Artery Denervation

Franziska Schlegel\*, Sait Sebastian Daneschnejad\*, Mikhail Mavlikeev, Sara Klein, Marcel Vollroth, Aida Salameh, Bruno Andrea, Friedrich Wilhelm Mohr, Gerd Hindricks, Stefan Dhein

### To the Editor:

In the past years 2 different innovative methods of hypertension treatments were investigated. The first is the promising examination of the electrical activation of the carotid baroreceptors, which is now in the phase III studies,<sup>1,2</sup> and second the usage of selective renal sympathetic denervation (RSD) as an alternative treatment in therapy-resistant hypertension. RSD is thought to be based on alteration of the sympathetic innervation of kidney and secondary effects on the renin-angiotensin-aldosterone system. The renal nerves are located in close vicinity to the renal artery wall. Their activation could be important for the progression of therapy-resistant hypertension. In this context, Krum et al<sup>3</sup> showed the proof of principle of a percutaneous, catheter-based trail to destroy renal sympathetic nerves by the introduction of a RF catheter into the lumen of the main renal artery and its subsequent connection to a radiofrequency generator. Until recently, the renal denervation seemed to be a promising therapy option for antihypertensive treatment. However, the simplicity HTN-3 study as recently announced<sup>4</sup> indicated a lack of effect of RSD. Nevertheless, at present, the underlying molecular mechanisms remain unclear during RSD. RF injury might damage the tissue by thermal coagulation but may also cause apoptosis as observed in colon cancer.<sup>5</sup> Thus, we made up the hypothesis that RSD may induce apoptosis in the perivascular nerves. The initiation of apoptosis is still not entirely understood. One important pathway is the release of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space together with cytochrome *c*.<sup>6</sup> Subsequently, AIF translocates to the nucleus and induces chromatin decondensation and DNA degradation, likely by endonucleases. Typically, when inducing apoptosis, AIF and caspases act together. However, AIF may induce cell death in a caspase-independent manner.<sup>7</sup> The extrinsic pathway of apoptosis leads to an activation of the initiator caspase 8. However, within the intrinsic pathway various types of cell stress, including DNA damage and oxidative stress (and many more), cause mitochondrial release of cytochrome *c* and other factors, resulting in activation of caspase 9. These initiator caspases enable activation of so-called effector caspases, such as caspase 3, subsequently activating the apoptotic program.

Here, we wanted to investigate how acute RSD affects perivascular nerves and renal arteries, and whether apoptosis in nerves and vessel wall might be induced.

### Methods

A detailed outline of the methods is given in the online-only Data Supplement.

All methods were approved by an institutional ethical board and are in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health).

In anaesthetized pigs (n=5; 76.2±2.13 kg; midazolam, thiopental, and fentanyl), radiofrequency ablation (4F electrode-tipped catheter,

Symplcity Catheter System, Medtronic Ardian, Mountain View, CA) was performed. Under angiographic control the catheter was inserted within the main stem of right renal artery (first ablation: near aorta; second: near kidney; Figure S1 in the online-only Data Supplement). Ablations (90 s, 8 W, 60°C) were performed accordingly.<sup>3</sup> Blood clotting was prevented by intravenous heparin. To allow manifestation of early apoptosis after RSD animals were kept for 3 hours under general anesthesia. Finally, renal arteries were excised (right renal artery with RSD; left as control without RSD).

Renal arteries (n=10 ablation points and n=10 pieces of control arteries) and perivascular nerves were fixed with neutrally buffered formalin, embedded in paraffin, cut in 4-µm thick sections, stained with hematoxylin and eosin. To investigate early apoptotic signals in nerves, immunohistochemical AIF staining with anti-AIF rabbit polyclonal (1:100, Santa Cruz) antibody was performed (detection: EnVision FLEX/AEC+Substrate-Chromogen [Dako]) with hematoxylin nuclear counterstaining. Activation of apoptosis was defined as the translocation of AIF into the nuclei. The same procedure was performed with anti-cleaved caspase 3 (Asp 175) rabbit polyclonal (1:250, Cell signaling) antibody to detect caspase-dependent apoptosis. Sections were analyzed by a blinded observer.

For statistics ANOVA and Student *t* test with Bonferroni correction were performed on level of significance  $P \leq 0.05$  and data were presented as mean value±SEM.

### Results

In ablated arteries we always found AIF and cleaved caspase 3-positive perivascular nerves. However, not all nerves in a section were positive: after RSD 33 of 64 nerves were positive for AIF. AIF-positive nerves (between 1 and 10 nerves per sample) were located in tunica adventitia (n=31) or in the border zone between tunica adventitia and tunica media (n=2) and occupied an average area of 0.031±0.016 mm<sup>2</sup> with an average diameter of 198.67 µm. Inside the perivascular nerves we found 18.6±1.25% AIF-positive nuclei that indicated early induction of apoptosis. In nonablated control vessels only 1 of 31 AIF-positive nerve was detectable (Figures 1A, 1B, and 2A). Interestingly, the majority (n=20) of these AIF-positive perivascular nerves was >1000 µm away from the endothelium, whereas only 1 was located <500 µm from the endothelium. The remaining perivascular nerves were found 500 to 1000 µm distant to the endothelium (n=12; Figure 2B).

CC3 staining showed similar results (Figure 1C and 1D). Twenty of 55 nerves were positive for CC3 (control: 3 of 24 nerves CC3 positive). One to 5 positive perivascular nerves were detected within each sample, located in the tunica adventitia. The majority of CC3-positive nerves was larger than 5000 µm<sup>2</sup> and >1000 µm far from the endothelium of the respective vessel. The area of the nerves averaged 0.020±0.009 mm<sup>2</sup> with a diameter of 159.73 µm. About 12.38±1.9% of the nuclei within the nerves were CC3 positive (Figure 2).

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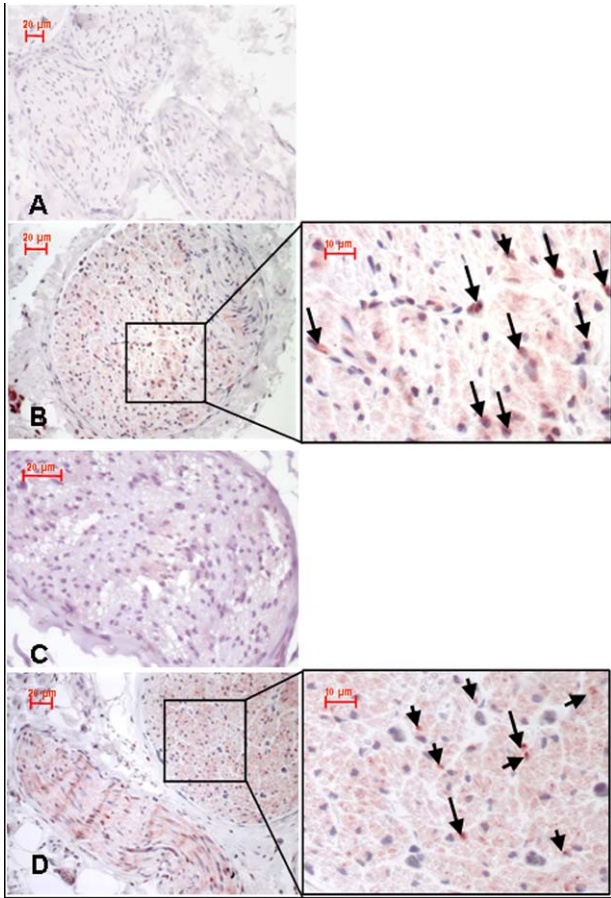
The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.03241/-/DC1>.

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**Figure 1.** Immunohistochemical staining of perivascular nerves. **A**, Apoptosis-inducing factor (AIF) in control: without any positive nuclei. **B**, AIF after renal sympathetic denervation (RSD): with AIF-positive nuclei (black arrows). **C**, CC3 in control: without any positive cells. **D**, AIF after RSD: with CC3-positive cells (black arrows).

In addition, we found that the integrity of the endothelium and the intima was not destroyed or dissociated in all specimens. We also found minor areas of media with hypereosinophilic cells, which may be indicating damage (Figure 3). Further analysis of tunica media indicated AIF and CC3-positive fields in the media (AIF, 54.72±8.7%; CC3, 42.4±10.0%) in which 38.67±5.3% of counted cells were CC3 positive and 28.67±4.02% showed AIF translocation (Figure 4).

In hematoxylin and eosin and AIF and CC3 staining of control group, we did not find any destruction of media and induction of AIF translocation into nuclei in medial fibroblasts. In ≈12% of fields of vision, we found 15.18±3.76% CC3-positive medial fibroblasts.

As expected, blood pressure and heart rate remained unaltered after RSD (within 3 hours; Table S1 in the online-only Data Supplement).

### Discussion

To reduce renal sympathetic afferent and efferent nerves in patients with resistant hypertension, catheter-based radiofrequency ablation of renal sympathetic nerves was positively investigated.<sup>3,8,9</sup> In contrast, recent statements on simplicity HTN-3 study indicated no significant difference in blood pressure between sham-treated and RSD-treated patients after 6 months.<sup>4</sup> However, as long as the underlying mechanisms are mostly unclear, these data are difficult to interpret.

To the best of our knowledge, our data show for the first time that RSD initiates apoptosis in perivascular nerves, as indicated by AIF translocation and cleavage of caspase 3. According to the current understanding, after caspase 3 activation (cleavage) the cell is doomed to die within the next hours to days. Interestingly, in those clinical RSD studies which showed a positive effect, the blood pressure lowering was also not seen immediately after RSD but several days later.<sup>3</sup> Another interesting next aspect is that mainly nerves in >1000 µm distance from the endothelium were affected. This may be because of local factors affecting the RF energy: at the catheter tip the blood flow absorbs parts of the RF energy thereby cooling intima and inner parts of the vessel wall. In outer parts (although energy decays with  $\frac{1}{r^2}$ ) RF energy seems to be high enough to induce damage (Figure 5).

However, we found nuclear AIF translocation and CC3, early markers for initiated apoptosis in only ≈50% of the perivascular nerves 3 hours after ablation. Thus, there is no complete denervation (at least with the energy as used here, which is identical with the study by Krum et al<sup>3</sup>). Although it is tempting to speculate that such an incomplete denervation may be compensated by surviving nerves, a conclusion on this point requires a chronic detailed study including 6-month follow-up blood pressure measurements.

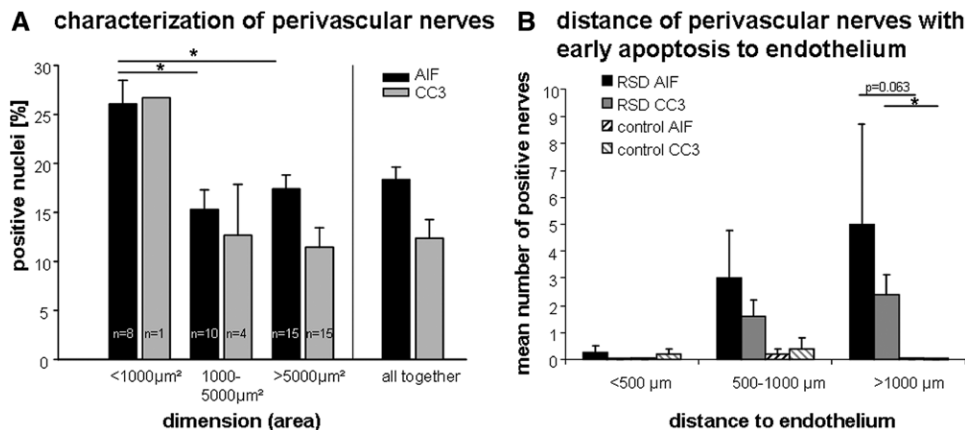
In consequence, one could consider the use of higher energy to optimize the RSD effect. In this case it is important that we also found AIF translocation into nuclei and CC3-positive cells in the media of renal arteries indicating beginning media apoptosis. Thus, further increase in RF energy probably will also cause more media damage.

### Sources of Funding

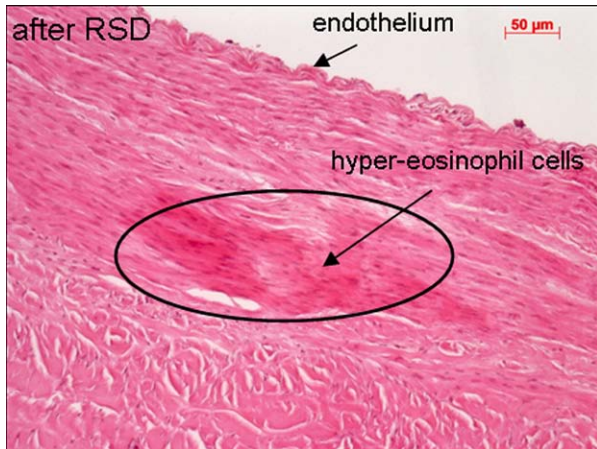
This study was financed by Heart Centre Leipzig.

### Disclosures

None.

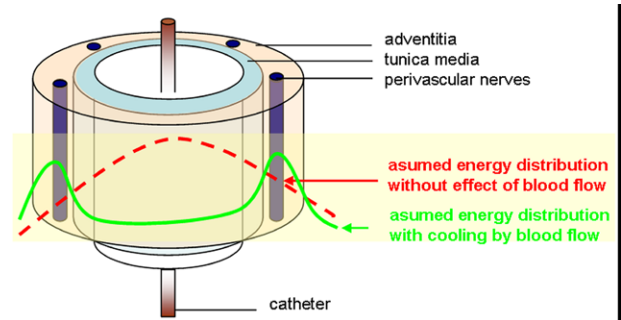


**Figure 2.** **A**, Classification of apoptosis-inducing factor (AIF) and CC3-positive perivascular nerves in area sizes (<1000, 1000–5000, and >5000 µm<sup>2</sup>) and their percentage of positive nuclei. **B**, Mean number of AIF and CC3-positive perivascular nerves in dependence on the distance of the nerve to the endothelium (\*P≤0.05).



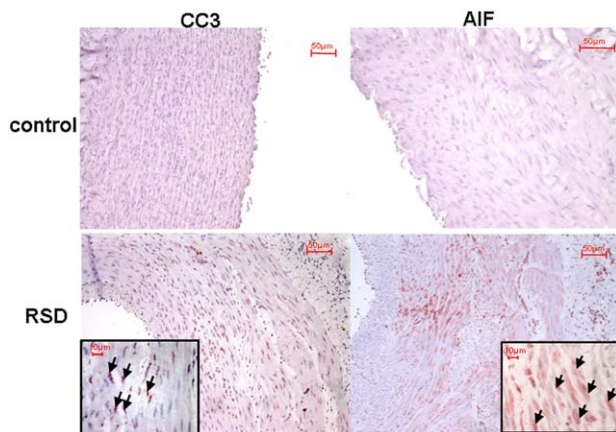
**Figure 3.** Hematoxylin and eosin staining of media after renal sympathetic denervation (RSD) indicated unmodified endothelium and hyper-eosinophilic cells in media.

- Illig K, Levy M, Sanchez L, Trachiotis GD, Shanley C, Irwin E, Pertile T, Kieval R, Cody R. An implantable carotid sinus stimulator for drug-resistant hypertension: surgical technique and short-term outcome from the multicenter phase II Rheos feasibility trial. *J Vasc Surg.* 2006;44:1213–1218.
- Tordoir JHM, Scheffers I, Schmidli J, Savolainen H, Liebeskind U, Hansky B, Herold U, Irwin E, Kroon AA, de Leeuw P, Peters TK, Kieval R, Cody R. An implantable carotid sinus baroreflex activating system: surgical technique and short-term outcome from a multicenter feasibility trial for the treatment of resistant hypertension. *Eur J Endovasc Surg.* 2007;33:414–421.
- Krum H, Schlaich M, Whitbourn R, Sobotka PA, Sadowski J, Bartus K, Kapelak B, Walton A, Sievert H, Thambar S, Abraham WT, Esler M. Catheter-based renal sympathetic denervation for resistant hypertension: a multicentre safety and proof-of-principle cohort study. *Lancet.* 2009;373:1275–1281.
- Dougherty W, Warren J. Medtronic announces U.S. renal denervation pivotal trial fails to meet primary efficacy endpoint while meeting primary safety endpoint. Medtronic Inc web site. 2014. [http://newsroom.medtronic.com/phoenix.zhtml?c=251324&p=irol-newsArticle\\_Print&ID=1889335&highlight=](http://newsroom.medtronic.com/phoenix.zhtml?c=251324&p=irol-newsArticle_Print&ID=1889335&highlight=). Accessed January 9, 2014.



**Figure 5.** Hypothetically energy distribution during renal sympathetic denervation (RSD) in renal arteries without/with cooling effect of blood flow: Without cooling effect of blood flow energy seems to effects also tunica media and with a lower influence in the adventitia and perivascular nerves. Cooling by blood flow during RSD could lead to change energy distribution so that the effect on media could be reduced and induced in perivascular nerves.

- Fujisawa S, Romin Y, Barlas A. Evaluation of YO-PRO-1 as an early marker of apoptosis following radiofrequency ablation of colon cancer liver metastases. *Cytotechnology.* 2014;66:259–273.
- Clerk A, Sugden PH. Dying by the way you live: AIF vs. caspases in apoptosis of hypertrophied cardiomyocytes. *Cardiovasc Res.* 2010;85:3–4.
- Joza N, Pospisilik JA, Hangen E, Hanada T, Modjtahedi N, Penninger JM, Kroemer G. AIF: not just an apoptosis-inducing factor. *Ann NY Acad Sci.* 2009;1171:2–11.
- Brandt MC, Mahfoud F, Reda S, Schirmer SH, Erdmann E, Böhm M, Hoppe UC. Renal sympathetic denervation reduces left ventricular hypertrophy and improves cardiac function in patients with resistant hypertension. *J Am Coll Cardiol.* 2012;59:901–909.
- Symplicity HTN-2 Investigators, Esler MD, Krum H, Sobotka PA, Schlaich MP, Schmieder RE, Böhm M. Renal sympathetic denervation in patients with treatment-resistant hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. *Lancet.* 2010;376:1903–1909.



**Figure 4.** Investigation of renal artery media: difference between control vessels (without renal sympathetic denervation [RSD]) and ablated vessels (RSD) on apoptosis-inducing factor (AIF) and CC3 staining. Note the positive nuclei in the media after RSD (black arrows).

# **Early effects in perivascular nerves and arterial media following renal artery denervation**

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## Online Supplement

### **Methods**

#### *Study Design*

All methods were approved by an institutional ethical board and are in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health).

For transport pigs (n=5, 76.2±2.13kg) were pre-medicated with atropine (0.5 mg/kg), midazolam (0.5 mg/kg), ketamin (15 mg/kg). After intubation they were connected to mechanical ventilation (10 ml/kg fresh air, PEEP 0-3 mmHg, O<sub>2</sub> 50-60 Vol-%, AF 15-20/min, PIP 25). Subsequently total intravenous anaesthesia was performed with midazolam (0.2 mg/kg/h), thiopental (20 mg/kg/h), fentanyl (7.5 µg/kg/h). To prevent blood clotting during the procedure, heparin was administered (1000IU/kg BW). Furthermore, the central venous pressure (CVD), arterial blood pressure (see supplementary table S1) and blood gas were controlled over the entire procedure. We used a radiofrequency ablation system (Symplicity® Catheter Sytem™, Medtronic Ardian, Mountain View, CA, USA) consisting of 4 French electrode-tipped catheter (single-use). Under angiographic control the catheter was inserted within the main stem of the right renal artery. The first ablation was near the aorta, the second near the kidney (see supplementary figure S1). Both ablations were performed for 90 sec, 8 Watt (fixed) and 60 °C (fixed) according to Krum et al. 2009 [1]. In order to allow the manifestation of early apoptosis after radiofrequency ablation animals were kept for 3 hours under general anaesthesia. Finally pigs were euthanized (T 61 i.v.) and the renal arteries (right renal artery with RSD; left one as control without RSD served) were excised for pathologic analysis.

#### *Immunohistology and histology*

Renal arteries (n=10 ablation points and n=10 pieces of control-arteries) and perivascular nerves were fixed with neutrally buffered formalin, embedded in paraffin and cut in 4µm thick sections.

In a first approach, slides were stained with haematoxylin & eosin (H&E) using standard protocols to allow detection of hypereosinophilic areas (which can indicate necrosis or thermal damage).

To investigate early apoptotic signals in nerves immunohistochemical AIF-staining with anti-AIF rabbit polyclonal (1:100, Santa Cruz) antibody was performed. Sections were deparaffinised the sections with Xylol and washed in ascending ethanol series, than sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for blocking endogene peroxidase activity. Thereafter heat-induced antigen retrieval in 0.01M sodium- citrate buffer was performed for 30 min. Tissue sections were blocked for 1h with 2% bovine serum albumin in TBS and incubated with primary antibodies over night. For detection of anti-AIF EnVision™ FLEX Mini Kit (Dako) system was used and the reaction was visualized by AEC+ Substrate-Chromogen (Dako). Haematoxylin was used for nuclear staining. In this case, the activation of apoptosis was defined as the translocation of AIF into the nuclei. The same procedure was performed with anti- cleaved caspase 3 (Asp 175) rabbit polyclonal (1:250, Cell signalling) antibody to detect caspase-dependent apoptosis.

Sections were analyzed by a blinded observer using Axioplan2 Microscope (Zeiss, Jena, Germany) and images digitalized with AxioCam MRC5 (Zeiss, Jena, Germany). AIF and CC3 positive nerves were counted; their location within the artery substructure (tunica adventitia, tunica media or tunica intima) was defined and the cross-sectional area and diameter of the nerves was measured. Furthermore for every AIF and CC3 positive nerves the

distance between the nerve and the arterial endothelium was assessed. To characterize the AIF and CC3 positive nerves more in detail, the number of AIF- positive cell nuclei and CC3- positive cells were detected and related to the whole number of nuclei. For investigation of the tunica media the whole vessel was analysed at 100 x magnification, and AIF or CC3 positive fields of vision were counted and related to the total number of fields. Therefore, we counted the positive cell nuclei in these fields (5 pictures in a magnification 400x, analysed with Image J, 1.47h, National Institutes of Health).

### *Statistics*

Statistical analysis of data was performed using ANOVA and student's t-test with bonferroni correction on level of significance  $p \leq 0.05$  and data were presented as mean value  $\pm$  SEM.

### **Reference**

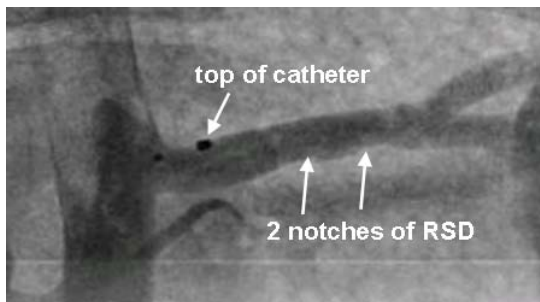
- 1 Krum H, Schlaich M, Whitburn R, et al. Catheter-based renal sympathetic denervation for resistant hypertension: a multicentre safety and proof-of-principle cohort study. *Lancet*. 2009;373:1275–1281.

## Supplementary legend

| pig           | weight [kg]     | gender | CVD    | before RSD     |             |             | 3h after RSD   |             |             |
|---------------|-----------------|--------|--------|----------------|-------------|-------------|----------------|-------------|-------------|
|               |                 |        |        | blood pressure |             | HR          | blood pressure |             | HR          |
|               |                 |        |        | systolic       | diastolic   |             | systolic       | diastolic   |             |
| 1             | 79              | male   | 12     | 110            | 70          | 90          | <b>100</b>     | <b>60</b>   | <b>70</b>   |
| 2             | 82              | male   | 5 to 8 | 160            | 90          | 80          | <b>160</b>     | <b>100</b>  | <b>90</b>   |
| 3             | 73              | female | 7      | 125            | 90          | 90          | <b>110</b>     | <b>80</b>   | <b>80</b>   |
| 4             | 77              | female | 9      | 100            | 60          | 70          | <b>110</b>     | <b>60</b>   | <b>70</b>   |
| 5             | 70              | female | 7      | 105            | 65          | 60          | <b>110</b>     | <b>80</b>   | <b>70</b>   |
| <b>MW±SEM</b> | <b>76.2±2.1</b> |        |        | <b>120±11</b>  | <b>75±6</b> | <b>78±6</b> | <b>118±11</b>  | <b>76±7</b> | <b>76±4</b> |

### Supplementary table S1:

General parameters of the experimental animals before RSD and 3h after RSD: CVD = central venous pressure, HR = Heart rate



### Supplementary figure S1:

Position of the catheter in the renal artery under angiography guidance. In this picture there are also 2 notches after RSD visible (arrows).

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