Prehypertensive Preconditioning Improves Adult Antihypertensive and Cardioprotective Treatment

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ABSTRACT

Transient prehypertensive treatment (TPT) causes prolonged antihypertensive and cardioprotective effects. Reduced angiotensin II type 1 receptor (AT1R) blockade improves cardiovascular protection of late-onset AT1R blockade in a model of spontaneous hypertensive heart-failure rats (SHHF/Mcc-fa<sup>cr</sup>). TPT (4–8 weeks of age) consisted of AT1R blockade (5 mg/kg candesartan) or vehicle. Candesartan-pretreated SHHF (5 mg/kg/day candesartan; weeks 4–8) received during adulthood (20–28 weeks of age) either candesartan at a dose of 1.5 or 5 mg/kg/day or vehicle. Vehicle-pretreated SHHF received either candesartan (5 mg/kg/day) or vehicle during adulthood. Blood pressure telemetry and longitudinal echocardiography were performed between weeks 20 and 28. Final examination included cardiac and vascular morphometry and measurement of the AT1R signaling and receptor internalization (ATRAP). Combined juvenile and adult AT1R blockade caused lower mean arterial pressure (MAP) than adult AT1R blockade alone (84 ± 5 versus 97 ± 5 mm Hg; P < 0.05). Cardiac and vascular hypertrophy was lower. Juvenile treatments were associated with a reduced cardiovascular AT1R expression and enhanced ATRAP expression. Combined juvenile and reduced adult AT1R blockade resulted in MAP similar to that with adult AT1R blockade alone (92 ± 3 versus 97 ± 5 mm Hg). We conclude that prehypertensive preconditioning improves adult treatment effects in SHHF. Those effects correlate with reduced cardiovascular AT1R expression and enhanced receptor internalization, suggesting reduced angiotensin sensitivity in pretreated SHHF. Moreover, preconditioning allows a reduction of adult AT1R blockade without loss of protection. Therefore, prehypertensive preconditioning may offer a tool to improve treatment efficacy in humans.

The awareness of hypertension is poor and, consequently, is appropriate antihypertensive treatment (Cutler et al., 2008). Therefore, alternative treatment strategies for hypertension and cardiovascular complications are of particular interest. The trial of preventing hypertension (TROPHY) was the first clinical study to demonstrate that a transient prehypertensive treatment (TPT) could successfully delay new onset of hypertension in a cohort of prehypertensive subjects 2 years after withdrawal of an angiotensin II type 1 receptor (AT1R) blocker compared with placebo (Julius et al., 2006). However, TROPHY raised the concern that TPT was a promotion for earlier use of antihypertensive drugs rather than a reliable and efficient addition to antihypertensive treatment (AHT) (Baumann and van den Born, 2006; Grassi, 2006; Meltzer, 2006; Persell and Baker, 2006; Nesbitt, 2007). Indeed, this context has not been investigated in detail. Thus, it remains unclear whether TPT is as effective in blood pressure-lowering and end-organ protection as continuous AHT starting at adulthood. Up to now, the potential preconditioning effects of combined prehypertensive and antihypertensive treatment separated by a period of drug holiday, in particular whether TPT may allow a dose reduction of AHT with consecutive reduction of antihypertensive side effects and costs, have been neglected. Investigating preconditioning in an experimental setting may help us to understand the value of TPT and to estimate the pharmacoeconomic relevance of this treatment strategy (Williams et al., 2008).

The majority of animal models dealing with TPT used spontaneously hypertensive rats (SHR) (Berecek et al., 1984; Wu et al., 1994), which are characterized by late cardiac failure (Slama et al., 2004). The SHHF/Mcc-fa<sup>cr</sup> rat (SHHF) is a model of spontaneous hypertension and congestive heart failure; AHT, antihypertensive treatment; SHR, spontaneously hypertensive rats; SHHF/Mcc-fa<sup>cr</sup>, SHHF, spontaneous hypertensive heart-failure rats; RAS, renin-angiotensin system; ATRAP, AT1R signaling and receptor internalization; W/liter, wall/lumen ratio; TNF-α, tumor necrosis factor-α; MAP, mean arterial pressure; EDV, end-diastolic volume; HW/BW, heart weight/body weight; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
failure, which demonstrates characteristic symptoms as early as 4 to 6 month of age (Bergman et al., 1999; Reffelmann and Kloner, 2003). Similar to that in humans and SHR, development of congestive heart failure in SHHF is associated with activation of the renin-angiotensin system (RAS) (Heyen et al., 2002). Angiotensin II-mediated pathways induce hypertrophy of cardiac myocytes, and treatment of SHHF with an AT1R blocker reduces blood pressure and cardiac mass (Liang et al., 2006). Therefore, we concluded that SHHF is a feasible model for TPT with an AT1R blocker similar to SHR with the advantage that relevant cardiac changes appear earlier in SHHF (Reffelmann and Kloner, 2003; Baumann et al., 2007b).

Furthermore, the mechanistic background of TPT remains uncertain. Several authors suggested that reduced angiotensin sensitivity is involved in the long-lasting cardiovascular protection (Bergström et al., 2002; Baumann et al., 2009). Angiotensin signaling is related to the expression level of the AT1R, but it is also regulated by its internalization which is controlled by the (AT1R)-associated protein (ATRAP). It is known that this internalization ameliorates cardiomyocyte hypertrophy and is regulated by AT1R blockade (Tanaka et al., 2005; Baumann et al., 2007b; Shigenaga et al., 2008).

The primary aim of this study was to investigate the efficiency of TPT alone or as preconditioning treatment for AHT compared against AHT with respect to blood pressure and cardiac effects. Second, in this study we investigated whether the antihypertrophic or anti-inflammatory effects of the AT1R blockade are leading factors involved in the protection by TPT and whether a reduction in the AT1R or its receptor internalization is involved in these effects. Third, the pharmacoeconomic relevance was investigated by adding a group with prehypertensive preconditioning and reduced adult AT1R blockade.

Materials and Methods

Four-week-old male lean SHHF (Charles River Laboratories, Boston, MA) were randomly assigned to five weight-matched groups (n = 9). All animals were housed in a room lit 12 h/day (6:00 AM–6:00 PM) at an ambient temperature of 22 ± 1°C. Animals had access to rodent diet and tap water ad libitum during the experiment. All experiments were approved by the animal ethics committee of the regional government and performed in accordance with institutional guidelines.

Juvenile TPT was defined between 4 and 8 weeks of age during which all 45 SHHF received daily gavage. Twenty-seven seven SHHF received 5 mg/kg/day of candesartan cilexetil dissolved in 100 μl of vehicle (tap water), whereas 18 SHHF received only vehicle. All SHHF underwent a “drug holiday” from the end of juvenile treatment until adulthood. The adult period was defined as 20 to 28 weeks of age. Starting from 20 weeks of age candesartan-pretreated SHHF received either 5 mg/kg/day candesartan (high dose), 1.5 mg/kg/day candesartan (low dose), or vehicle, whereas the other rats received 5 mg/kg/day of candesartan or vehicle (n = 9 in each group).

The Dataquest IV telemetry system (Data Sciences International, St. Paul, MN) was used for measurement of mean arterial pressure and heart rate as described previously. At an age of 11 weeks five rats of each group received transmitter devices (model TAU11PA; Data Sciences International), and measurement started from week 20 as described previously (Baumann et al., 2007a). Transthoracic echocardiography was performed on rats under isoflurane anesthesia at 20, 24, and 28 weeks of age as described previously using a Vivid 5 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) system fitted with a 12-MHz transducer. Hearts and aorta were harvested, and samples were either obtained in formaldehyde for paraffin embedding or stored at −80°C. The paraffin-embedded specimens were cut into 4-μm-thick sections and stained with hematoxylin and eosin. Individual surface areas of at least 20 horizontal cut endocardial cardiomyocytes were calculated, and average values were used for analysis. Capillary staining was performed and quantified as described by Rakusau et al. (1994) including the capillary/ cardiomyocyte ratio (Baumann et al., 2007b). Epicardial arteries were analyzed for lumen diameter, media thickness, and wall/lumen ratio (Winter) (Lutz et al., 2008). Infiltrative perivascular cells were counted and are given in a ratio with the lumen diameter. In addition, cardiac CD68 immunohistochemical analysis was performed to assess the number of interstitial macrophages. All measurements were performed under a light microscope (Olympus BX20; Olympus, Hamburg, Germany), digitalized using a charge-coupled device video camera (Sony, Berlin, Germany), and quantified with Image Pro Plus 5.0.

RNA was isolated from snap-frozen cardiac and aortic material with an RNasy Mini Kit (Qiagen, Hilden, Germany), further purified (Ultraspec-II RNA; Biotech Laboratories, Houston, TX), and transcribed into cDNA with Superscript III reverse transcriptase, using 250 ng of random primers (Invitrogen, Carlsbad, CA). Primers and probes against AT1R, brain natriuretic peptide, and TNF-α were amplified and detected (ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, CA) relative to the expression level of glyceraldehyde-3-phosphate dehydrogenase (Heyen et al., 2002) (Table 1).

Protein expression of the AT1R (Abcam Inc., Cambridge, MA) and ATRAP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in heart and aorta was analyzed by Western blot. Protein extracts were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA) by electroblotting. Immunoreactive bands were visualized through the use of enhanced chemoluminescence (Kacimi and Gerdes, 2003).

All parameters are expressed as means ± S.D. Parameters were compared using an analysis of variance and between-group differences were determined by a post hoc least significance difference test. P < 0.05 was regarded to indicate statistical significance.

Results

Longitudinal Blood Pressure Effects of Transient Prehypertensive and Antihypertensive Treatment. Juvenile AT1R blockade (weeks 4–8) with 5 mg/kg/day candesartan resulted in 20-week-old adult SHHF in a significantly lower MAP (122.5 ± 5.8 mm Hg) than in vehicle-pretreated SHHF (145.4 ± 6.3 mm Hg, P < 0.001). During adulthood (weeks 20–28) MAP of pretreated SHHF remained significantly lower than that of untreated SHHF, whereas AT1R

### Table 1: Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>AT1R</td>
<td>CCTGCTTCCATCCAGAG</td>
</tr>
<tr>
<td>Down</td>
<td>TGGTTTCTCCATCA</td>
</tr>
<tr>
<td>BNP</td>
<td>GCTGTTGGCCGCAGATAGA</td>
</tr>
<tr>
<td>Up</td>
<td>ACCACCTCCGCCCTTCCAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTATGCTCCTCCTACCCA</td>
</tr>
<tr>
<td>Up</td>
<td>AAATGAGATTTCTAGTCTTGC</td>
</tr>
<tr>
<td>Down</td>
<td>ATGCCTGTGATGTTGTGTTAA</td>
</tr>
</tbody>
</table>

BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
blockade (5 mg/kg/day candesartan) in vehicle-pretreated SHHF resulted in significantly lower MAP than that in pre-
treated SHHF without adult AT1R blockade. Combined ju-
venile and adult AT1R blockade at both doses (5 and 1.5
mg/kg/day candesartan; weeks 20–28) showed significantly
lower MAP in SHHF than in rats only receiving adult AT1R
blockade (Fig. 1).

The blood pressure-lowering effect of the treatment strat-
egies was compared at week 28 of age. SHHF receiving AHT
had lower blood pressure (97 ± 6 versus 133 ± 7 mm Hg)
than vehicle-treated SHHF. In parallel, TPT compared with
combined TPT/low-dose AHT (1.5 mg/kg/day candesartan)
resulted in a significantly stronger blood pressure reduc-
tion (84 ± 4 mm Hg; P < 0.05) (Fig. 2).

**Longitudinal Echocardiography during Weeks 20 to
28.** Longitudinal assessment of ventricular mass is summa-
rized in Table 2. At 20 weeks of age ventricular mass was
higher in vehicle-pretreated SHHF (0.90 ± 0.08 cm³) than in
transiently pretreated SHHF (0.81 ± 0.09 cm³; P < 0.001).
Without adult AT1R blockade ventricular mass increased
between weeks 20 and 28 irrespective of pretreatment (week
28, no TPT, 1.00 ± 0.03 cm³ and TPT, 0.87 ± 0.10 cm³; P <
0.01). In contrast, adult AT1R blockade reduced ventricular
mass irrespective of pretreatment (no TPT/AHT, 0.84 ± 0.05
cm³; TPT/AHT low-dose, 0.80 ± 0.09 cm³; and TPT/AHT
high-dose, 0.82 ± 0.07 cm³).

End-diastolic volume (EDV) was significantly larger in ve-
icle-pretreated SHHF compared with pretreated SHHF at
20 weeks of age (no TPT, 0.330 ± 0.028 cm³ versus TPT,
0.282 ± 0.036 cm³; P < 0.01). Adult AT1R blockade reduced
EDV in nonpretreated SHHF significantly (0.370 ± 0.030
versus 0.323 ± 0.047 cm³) but did not reach the level of
pretreated SHHF independent of AHT (0.283 ± 0.035 cm³).
Juvenile-pretreated SHHF had no additional effect on EDV
by adult AT1R blockade throughout the observation period
(Table 3). Fractional shortening and ejection fraction did not
show any significant difference between groups at any time
of observation.

**Cardiac Characteristics in 28-Week-Old SHHF.** The
heart weight/body weight ratio (HW/BW) was highest in
SHHF receiving no treatment (Table 3). All treatment regi-
mens showed significantly smaller HW/BW (P < 0.001).
HW/BW was lowest in SHHF receiving combined juvenile
and adult AT1R blockade (P = 0.019). HW/BW was similar
between SHHF receiving adult AT1R blockade or combined
juvenile and low-dose adult AT1R blockade.

Cardiomyocytes were largest in SHHF receiving no treat-
ment. All treatment regimens showed significantly smaller
cardiomyocytes (P < 0.001). Cardiomyocytes were smallest
in SHHF receiving combined juvenile and adult AT1R blockade.
Capillary density was lowest in SHHF receiving no treat-
ment. All treatment regimens exhibited significantly higher
capillary density (P < 0.001).

Epicardial arteries of SHHF receiving no treatment were
characterized by increased media thickness, higher W/liter,
and more perivascular infiltration (Table 4). Adult AT1R
blockade resulted in significantly thinner media, reduced
W/liter, and less infiltration. Combined juvenile and adult
AT1R blockade showed similar values for media thickness
and W/liter. Moreover, the combined AT1R blockade was
characterized by the larger lumen diameter of the epicardial
arteries.

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**Prehypertensive Preconditioning**

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**TABLE 2**

Longitudinal evaluation of ventricular mass by echocardiography

<table>
<thead>
<tr>
<th>Ventricular mass (cm³)</th>
<th>Vehicle 5 mg/kg/day Candesartan</th>
<th>TPT 1.5 mg/kg/day Candesartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricular mass week 20</td>
<td>0.89 ± 0.1</td>
<td>0.90 ± 0.07</td>
</tr>
<tr>
<td>Ventricular mass week 24</td>
<td>0.94 ± 0.07</td>
<td>0.92 ± 0.11</td>
</tr>
<tr>
<td>Ventricular mass week 28</td>
<td>1.04 ± 0.03</td>
<td>0.84 ± 0.05*</td>
</tr>
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* P < 0.05 vs. placebo/placebo.
Expression of AT1R was quantified in cardiac and aortic tissue of SHHF. Untreated SHHF showed the highest AT1R mRNA expression, whereas combined treatment resulted in the lowest expression pattern. Accordingly, AT1R density was determined by Western blot. Untreated SHHF showed the highest cardiac AT1R density, whereas combined treatment resulted in the lowest density ($P < 0.01$). Aortic tissue showed the same tendency, but results did not reach statistical significance because of the large S.D. In addition, the inhibitory binding molecule of the AT1R (ATRAP) was reduced in prehypertensively treated SHHF ($P < 0.05$) (Fig. 3).

Cardiac interstitial inflammation with respect to CD68-positive cells was not different between the groups. Likewise, serum TNF-α values showed no significant differences. Real-time PCR of cardiac TNF-α expression revealed significantly less TNF-α mRNA in SHHF receiving adult AT1R blockade (Table 3). However, perivascular infiltration of epicardial vessels was significantly reduced in the combined treatment (Table 4 and Fig. 4).

**Discussion**

Prehypertensive preconditioning improves antihypertensive and cardiovascular antihypertrophic effects of a late-onset (adult) AT1R blockade in SHHF. These effects tie in with a reduced cardiovascular AT1R density achieved by juvenile treatment. Furthermore, preconditioning combined with a reduced adult AT1R blockade is as effective as high-dose AT1R blockade alone.

Juvenile RAS blockade during a "critical period" results in prolonged blood pressure-lowering and organ protection that remains for a prolonged time after drug withdrawal (Shigenaga et al., 2008). Reduced angiotensin sensitivity has been attributed as a potential mechanism for this phenomenon. In this study, juvenile AT1R blockade was used in combination with late-onset (adult) AT1R blockade to assess the effects of an adult AT1R blockade after juvenile sensitization of the RAS.

Our data demonstrate that prehypertensive preconditioning combined with adult AT1R blockade is superior to adult AT1R blockade alone because the absolute blood pressure was lower. Moreover, the blood pressure reduction upon adult AT1R blockade was higher after preconditioning. This is suggestive for a sensitization of the RAS in these SHHF. Reduced sensitization of the RAS in SHHF should further affect cardiovascular hypertrophy and remodeling, key characteristics of angiotensin signaling (Julius et al., 2006; Liders et al., 2008). Our data demonstrate a superior antihypertrophic effect of combined juvenile and adult AT1R blockade on cardiac mass, cardiomyocytes, and epicardial arteries. Moreover, these SHHF are characterized by a larger epicardial luminal artery diameter, demonstrating less vascular remodeling with consecutive beneficial hemodynamic effects (Kearney et al., 2005). Taken together, these observations suggest a superior effect on cardiomyocytes and vascular smooth muscle cells after combined prehypertensive preconditioning and adult AT1R blockade in line with the idea of reduced sensitivity of the RAS. In addition, prehypertensive preconditioning and adult AT1R blockade were superior to adult AT1R blockade with respect to perivascular inflammation. This result is suggestive of reduced AT1R-mediated vascular inflammation in SHHF after juvenile treatment based on reduced RAS sensitivity. In contrast, interstitial and systemic inflammation was not primarily involved in the effects.
Because hemodynamic and morphological characteristics are in line with reduced RAS sensitivity after prehypertensive preconditioning and adult AT1R blockade, we aimed to investigate whether the AT1R expression and density pattern were modified. Combined treatment is characterized by reduced AT1R expression in the heart and aorta. This is reproducible with respect to the AT1R protein density. Therefore, our data demonstrate that combined juvenile and adult AT1R blockade reduces the amount of AT1R, which can explain the improved antihypertensive and antihypertrophic effects in SHHF. Moreover, ATRAP was up-regulated in prehypertensively treated SHHF. Because ATRAP has been demonstrated to be an endogenous inhibitor of AT1R signaling in cardiovascular cells (Shigenaga et al., 2008), the up-regulation of ATRAP in cardiovascular tissue may add to the therapeutic benefits of the preconditioning.

Finally, we demonstrated that after preconditioning a reduced dosage of the AT1R blocker during adulthood results in effects comparable to those of adult AT1R blockade alone. This finding is of translational relevance because drug-related side effects of continuous treatment could be reduced, whereas prehypertensive treatment is feasible (Julius et al.,...
In addition, prolonged dose reduction during adulthood may result in fewer side effects and a reduced pharmaeconomic burden (Williams et al., 2008).

In summary, this study revealed that combined juvenile and adult AT1R blockade improves the antihypertensive and antihypertrophic effects of adult AT1R blockade. In context with the reduced cardiovascular AT1R expression and density this finding suggests reduced angiotensin sensitivity in these SHHF. These effects associated with the juvenile AT1R blockade further allow dose-reduction at adulthood without a reduction in treatment efficacy, which may lead to reduced side effects and costs. Future studies are needed to investigate whether combined juvenile/prehypertensive and adult/hypertensive treatment leads to comparable results in humans.

References


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