Microalbuminuria *versus* Brain Natriuretic Peptide in Cardiac Hypertrophy of Hypertensive Rats

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Abstract

The objective of this study is to assess a possible link between microalbuminuria (MA), a major risk factor of the cardiorenal syndrome (CRS) and the brain natriuretic peptide (BNP), a marker of cardiac hypertrophy. Two kidney-one clip (2K-1C) renovascular hypertension was induced in 24 male Wistar rats weighing (220-250g). Rats were then randomized into 4 groups for 8 weeks: Sham, not treated; Bos, treated with bosentan; Cap, treated with captopril; Bos/Cap, treated with both drugs. Blood pressure, plasma BNP and transforming growth factor β1 (TGF-β1) concentrations, MA and creatininaemia were ascertained, as well as cardiac mass, BNP, α- and β- myosin heavy chain (MHC) gene expression and kidney histology. Following stenosis, Sham rats developed hypertension ($p<0.001$), an increase in BNP ($p<0.05$) and TGF-β1 ($p<0.005$) concentrations, creatininaemia ($p<0.001$), and urinary albumin ($p<0.001$). Decreases under treatment, in blood pressure ($p<0.001$), creatininaemia ($p<0.05$), plasma TGF-β1 ($p<0.005$) and BNP ($p<0.05$) concentrations, were concomitant with the absence of MA which was significantly correlated with reductions in cardiac mass ($p<0.05$) and hypertrophy markers (BNP and β-MHC gene expression) ($p<0.005$) as well as in renal fibrosis. These findings, suggest a potential link between MA evolution and BNP, as well as a possible effect of MA-lowering therapy on halting the progression, or even inducing the regression of cardiac hypertrophy.

Keywords: MA; BNP; Hypertension; Cardiac hypertrophy.

Introduction

Cardiovascular diseases (CVD) related factors are responsible for the death of more than 50% of patients with chronic kidney disease (CKD) (USRDS 2008) or end stage renal disease (ESRD) (Keith *et al.* 2004); therefore, it is not surprising that 30 to 50% of patients with congestive heart failure (CHF) exhibit a disruption of the glomerular filtration (McAlister *et al.* 2004). The term cardiorenal syndrome (CRS) has been increasingly used without a consistent or a well-accepted definition; it is manifested by renal failure, microalbuminuria (MA), resistance to diuretics, anemia, and tendency towards hyperkalemia and low systolic blood pressure (SBP). Renal failure leads to a diverse pathophysiologic array including: changes in the process of coagulation and fibrinolysis, endothelial dysfunction, anemia, disorders of the phospho-calcium balance, dysfunction of the renin-angiotensin-aldosterone system (RAAS), abnormal lipid metabolism, left ventricular hypertrophy (LVH) and arrhythmias (McCullough 2002).

The CRS can be classified into five subtypes including the vast array of interrelated derangements and reflecting the bidirectional nature of heart-kidney interaction (Ronco *et al.* 2008). In fact, this interaction is a two way process: renal dysfunction causes heart problems, and *vice versa*. Researchers examined the effects of myocardial infarction on the loss of renal function in unilaterally nephrectomised rats (Van Dokkum *et al.* 2004). They suggested that heart failure (HF) worsens renal dysfunction, possibly via neurohumoral signals. This mutual interaction justifies the concept of the CRS (Schrier 2006).

MA appears to be a major risk factor of this syndrome. In fact, it is currently considered as a marker of endothelial dysfunction and vascular permeability (McCullough 2007). It is associated with LVH and hypertension; subjects with MA are highly vulnerable to CVD with a high mortality rate (Wachtell *et al.* 2002).

Although a recent study found a correlation between blood pressure control, MA and BNP (UNO *et al.* 2008), cardiac hypertrophy was not evaluated, and thus the relationship between MA and cardio pathogenesis, including cardiac hypertrophy remains not very clear. There are

no current studies at the cellular or molecular level which prove the existence of a clear and direct relationship between these two entities. It is well established that treatment with angiotensin converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARBs) in CKD, improves renal function and decreases hospitalization risk for a CHF (Brenner *et al.* 2001). In addition, *in vitro* studies in rats have shown that treatment with endothelin receptor blockers (ERBs) more specifically endothelin type A (ET-A) receptor blockade modulates the natriuretic peptides gene expression in renovascular hypertension (Bianciotti and De Bold 2001) and prevents left ventricular hypertrophy and the re-expression of the β-MHC and atrial natriuretic peptide genes on day 2 in the same 2K-1C model, independently of blood pressure effects (Ehmke *et al.* 1999).

Based on these facts, we used a non-selective ERB and an ACEi as pharmacological tools to treat 2K-1C adult hypertensive rats, in order to assess a possible link between microalbuminuria (MA) evolution, a major risk factor of the CRS, and BNP, a marker of cardiac hypertrophy.

Materials and Methods

The protocols in the present study were designed according to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society and were in adherence to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Animals. Thirty male Wistar rats weighing (220-250g) were obtained from the "Centre d'Elevage R. Janvier" (Le Genest-Saint Isle, France). The animals were housed in individual metal wire metabolic cages with constant temperature (25°C). A specific air ventilation system assured an efficient flow within the room to keep the level of humidity within the animals' immediate environment at an acceptable level; thus, the relative humidity was within the range of 50 ± 5%. The rats were exposed to a 12: 12-h light-dark cycle, were fed ordinary rat chow,

had free access to tap water and were acclimatized for at least one week under these conditions before the start of the study.

Induction of 2K-1C hypertension. During ketamine (Interchemie, Holland) and xylazine (Rotex Medica, Germany) anesthesia (75 and 10mg/kg respectively), the right kidney was exposed through a flank incision. The right renal artery was clipped by placing of a rigid U-shaped silver clip (SLS-clips, Vitalitec, France) with an internal opening of 0.25 mm. The left kidney was left intact.

Experimental groups. The operated rats were randomly assigned into four groups of 6: the Bos group treated with the non-selective ERB, bosentan (Actelion, Allschwil, Switzerland); the Cap group treated with the ACEi captopril (Novartis, Switzerland); the Bos/Cap group treated with both drugs and the Sham group which wasn't subject to any pharmacological treatment. Treatment began after the establishment of hypertension two weeks after the stenosis. Every morning drugs were given with a small amount of the drinking water (5 ml) to achieve a final consumption of $10mg.kg^{-1}.day^{-1}$; then each rat was switched back to the tap water as soon as the five milliliters were consumed. The rats were all housed under the same conditions, and since their weights were comparable throughout the study, the necessary time for the 5 ml consumption was approximately the same for all the rats within the same group. The animals were sacrificed after 8 weeks following the stenosis. The development of cardiac hypertrophy in the Sham group was evaluated by comparing the animals' hearts weights to the ones of a fifth group containing six un-operated untreated control rats; these control animals were also kept in metabolic cages under the same conditions throughout the study, and were eventually sacrificed to the purpose of *only* weighing their hearts for the normal baseline values, and therefore assessing the development of cardiac hypertrophy in the other groups.

Measurements of systolic blood pressure SBP. SBP was measured, in all the 24 conscious resting rats before clipping, twice daily for one week for the normal baseline values, and once daily during the development of high SBP, using the tail-cuff method (Swislocki *et al.* 1999;

Kubota *et al.* 2006) with an electrosphygmograph (Model 29 amplifier and sensors, IITC, Woodland Hills, CA). Our measurement system was calibrated weekly using an aneroid sphygmomanometer. Just before blood pressure measurements, rats were placed in acrylic holders (Model 82, IITC). At each time point, two SBP measurements were taken on each animal and averaged. To avoid variations in SBP due to day cycle, all measurements were carried out between 10 and 12 a.m. Preliminary training sessions were performed during one week before starting the experiment.

Measurements of Plasma BNP, TGF-β1 and creatinine. During the week before clipping, three blood samples were taken from each of the 24 rats, one each two days, for normal baseline values; then, after the stenosis, one blood sample was weekly taken from the jugular vein of each rat, till the sacrifice. All blood samples were directly centrifuged at 6000 r.p.m and plasma was kept at -80 °C for later measurements. *ELISA* technique was used for BNP and TGF-β1 measurements: BNP-32 (Rat) kit (Peninsula Laboratories, Bachem Group, USA) was used to measure plasma BNP, and Quantikine Mouse/Rat/Porcine/Canine TGF-β1 kit (R&D Systems, USA) for TGF-β1 measurement. Moreover, plasma creatinine measurement was based on the reaction of creatinine with alkaline picrate as described by Jaffé (Kit: Biolabo, Maizy, France).

Measurement of urinary albumin. During the week preceding the clipping, three 24-hour urine samples were collected once each two days from each of the 24 rats, for normal baseline values. Then after the stenosis, one weekly sample was collected till the sacrifice on the 8th week. All urine samples were conserved at -80 °C for later measurements. The NycoCard U-ALBUMIN kit (Axis-Shield, Norway) was used to evaluate MA.

Cell dissociation for RNA isolation. Following the rats sacrifice, hearts were cut off and weighed. Ventricular myocytes were dissociated from the isolated hearts of sham and treated rats by enzymatic digestion as previously described (Farès *et al.* 1996). Briefly, the rats were injected with 1000 i.u. heparin I.P. (Choay; Sanofi, Gentilly, France) and anaesthetized with the Ketamine/Xylazine mixture; hearts were quickly removed via thoracotomy and transferred to an

ice-cold Tyrode solution. The aorta was cannulated and the heart mounted on a Langendorff apparatus and successively perfused (at 37°C) with the following oxygenated solutions: 5 min with Tyrode solution; 4 min with a nominally Ca^{2+}-free Tyrode solution, and about 20 min with the same solution supplemented with 0.05 % collagenase (type II, Worthington), 0.06 mM $CaCl_2$ and 0.1 % bovine serum albumin (BSA). When the heart was flaccid, it was rinsed with Kraft-Brühe (KB) medium (Isenberg and Klöckner 1982) for 2 min. The ventricles were cut off, chopped into small pieces and gently stirred in KB medium. The isolated cells were filtered on a 200 μm filter, and then instantly homogenized in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) to prevent loss of cells and RNA degradation, and finally kept at - 80°C for later remaining steps of RNA isolation.

Real-time quantitative RT-PCR. Total RNA was extracted from the previously isolated ventricular myocytes by the use of Trizol, and then purified with ethanol 75% (Sigma Chemical CO, St. Louis, USA). The concentration and purity of RNA was determined by measuring the absorbance at 260 nm with the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc, Wilmington, DE, USA). cDNA was synthesized using random primers (250 ng/μl), dNTP (10 mmol/L) and the SuperScript II Reverse Transcriptase kit (Invitrogen). Real-time PCR was conducted using the 7500 Real Time PCR System and the Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). To confirm the specificity of the amplified products, melting curves were performed at the end of the amplification. The amount of PCR products, calculated in reference to the individual calibration curves, were then normalized to that of either TATA Binding Protein (TBP) or Beta Actin (ACTB), determined in the same mRNA sample. In addition, 'no RT' control reactions were performed omitting the reverse transcriptase to confirm the absence of contaminating genomic DNA. The following primers were used: BNP sense 5'-AAGTCCTAGCCAGTCTCCAGAACA-3' and antisense 5'-AGCTCCAGCAGCTTCTGCAT-3'; α-MHC sense 5'-CTTCTGCTGATACCGGTGACAG-3' and antisense 5'-TGAGCCTTTCTTCTTGCCTCC-3'; β-MHC sense

5'-CCTCGCAATATCAAGGGAAA-3' and antisense 5'-TACAGGTGCATCAGCTCCAG-3'; ACTB sense 5'-CGTGAAAAGATGACCCAGATCA-3' and antisense 5'-TGGATGGCTACGTACATGGC-3'; TBP sense 5'-CCACACCAGCCTCTGAGAGC-3' and antisense 5'-ATACAATATTTTGGAGCTGTGGTACAA-3'.

Renal tissue preparation for histopathology. Following the rats sacrifice, the right kidneys were also excised: the renal veins were cut, and kidneys were perfused with ice-cold tyrode solution until all blood was removed, then decapsulated, cut into half through a mid-sagittal plane and fixed with 10% formalin solution. The formalin-fixed tissue was embedded in paraffin, and sections of 4 μm thickness were cut. Paraffin-embedded sections of the kidneys were stained with either hematoxylin and eosin or Sirius red for histopathological evaluation. After staining, the sections were rinsed in distilled water, dehydrated in ethanol/water baths with decreasing water content, and finally rinsed in xylene before being mounted with a permanent mounting medium. Gross examination and histological sections were interpreted by two independent pathologists in a blinded fashion, without knowledge as to how the animals were treated. Eight sections were analyzed for each rat within the four operated groups.

Statistical analysis. Statistical analysis was performed by the use of the one-way ANOVA for repeated measurements. The Mauchly's sphericity test was used to tell if the assumption of sphericity has been violated, then correction was performed by the Greenhouse-Geisser test. To explain the exact difference between group means, the post hoc Bonferroni test was applied.

The relationships among the changes in MA, cardiac mass, plasma BNP and β-MHC and BNP mRNA levels, before and after treatments were assessed by Spearman's correlation coefficient. Results with $p<0.05$ were considered statistically significant. All values are means ± SEM.

Results

SBP, plasma BNP and TGF-β1 variations

Sham group SBP significantly increased as compared to that measured in these same rats before

applying the stenosis (181.8 ± 7.5 *vs* 115.2 ± 2.4 mm Hg, $p<0.001$; Table 1); while those obtained in the Bos (132.1 ± 5.5 mm Hg), Cap (124.1 ± 8.8 mm Hg) and Bos/Cap (116.1 ± 6.7 mm Hg) groups were significantly lower ($p<0.001$) than in the Sham group (Table 1), with no significant differences with the normal values before stenosis.

As shown in Table 1, mean plasma BNP and TGF-β1 levels increased in Sham group as compared to the normal values before stenosis (BNP: 1.96 ± 0.05 *vs* 0.675 ± 0.11 ng/ml, $p<0.05$; TGF-β1: 93.22 ± 10.74 *vs* 22.82 ± 4.26 ng/ml, $p<0.005$). Significant drop offs in BNP were noted under bosentan (1.47 ± 0.02 ng/ml), captopril (1.35 ± 0.03 ng/ml) and bosentan/captoril (1.19 ± 0.04 ng/ml) administrations ($p<0.05$). Furthermore, treatment with bosentan, captopril and their combination significantly decreased plasma TGF-β1 levels (27.74 ± 0.02; 24.27 ± 3.37 and 23.22 ± 3.82 ng/ml respectively, $p<0.005$) and there was no statistically significant differences with the normal baseline before stenosis.

Urinary albumin and plasma creatinine levels

As shown in Figure 1, rats developed MA after banding the right renal artery (17.99 ± 0.2 *vs* 0.36 ± 0.01 mg/L, $p<0.001$), while the low urinary albumin levels observed among groups under treatment with bosentan (< 0.07 ± 0.01 mg/L), captopril (0.67 ± 0.02 mg/L) and the combination of both drugs (< 0.07 ± 0.01 mg/L), indicate the absence of MA.

A concomitant elevation of plasma creatinine was observed after applying the stenosis (0.8 ± 0.11 *vs* 0.49 ± 0.04 mg/dl, $p<0.001$; Figure 1). Creatinine levels obtained under bosentan (0.5 ± 0.03 mg/dl), captopril (0.59 ± 0.003 mg/dl) and bosentan/captopril (0.53 ± 0.009 mg/dl) were significantly lower ($p<0.05$) than in the Sham group (Figure 1). The administration of captopril alone caused a predictable elevation of plasma creatinine compared to the normal value ($p <0.05$).

BNP, α-MHC, ß-MHC gene expression and cardiac mass

As shown in Figure 2, the administration of bosentan, captopril and their combination resulted in significant BNP expression decrease as compared to untreated rats (219 ± 7, 194 ± 17 and

187 ± 14 % respectively *vs* 347 ± 31 %, $p<0.005$). This decrease in BNP gene expression under ERB and/or ACEi treatments was accompanied by an increase in α-MHC gene expression. In fact, α-MHC expression increased under bosentan, captopril and the combination of both (187 ± 6, 201 ± 11 and 219 ± 18 % respectively *vs* 68 ± 3 %, $p<0.005$). On the other hand, a significant decrease in β-MHC expression occurred under treatments (191 ± 16, 180 ± 5 and 141 ± 13 % respectively *vs* 364 ± 8 %, $p<0.005$). A significant increase in cardiac mass was noted in the sham group *versus* control rats (1258.65 ± 103.1 *vs* 860.25 ± 49.12 mg, $p<0.05$; Table 2). Treatment with the non-selective ERB and/or ACEi decreased cardiac mass (1094.03 ± 98.73, 1001.14 ± 90.24 and 986.11 ± 78.46 mg respectively *vs* 1258.65 ± 103.1 mg, $p<0.05$; Table 2).

Correlation of changes in MA with changes in hypertrophy markers

After 8 weeks, as shown in Table 2, there was no significant difference in body weight (g) between the different groups (Control un-operated rats: 234.4 ± 17.2, Sham: 240.2 ± 15.1, bos: 251.5 ± 20.3, cap: 237.8 ± 19.8 and bos/cap: 245.3 ± 16.4); however, the heart weight/body weight ratio (mg/g) was significantly higher in the Sham group as compared to the baseline values of the control un-operated rats (5.24 ± 0.17 *vs* 3.67 ± 0.06, $p<0.005$), suggesting the development of cardiac hypertrophy among banded rats in the sham group. Significant decreases, as compared to Sham rats, in heart weight/body weight ratio (mg/g) were noted under treatments (4.35 ± 0.15, 4.21 ± 0.09 and 4.02 ± 0.04, $p<0.05$).

The mean value of all the MA data, collected throughout the study, of each rat within the same group was reported to the value of the BNP, β-MHC mRNA levels or the heart weight/body weight ratio of this same rat; as shown in Table 3, there was significant positive correlation between changes in MA and changes in cardiac mass, BNP and β-MHC mRNA levels ($r=0.902$, $r=0.87$ and $r=0.93$ respectively, $p<0.05$).

Histological analysis

Following stenosis sham rats' kidney sections showed acute tubular necrosis in all cases and tubule cells had detached from basement membrane. Exfoliated tubular cells formed cylinders

within the dilated tubular lumens (Figure 3e); interstitial inflammatory cells (mononuclear cells) also appeared (Figure 3f). Staining with Sirius red also showed elevated levels of interstitial collagen deposition in all of the examined sections (Figure 4e). Rats treated with ACEi, ERB or their combination showed in 75%, 62.5% and 87.5% respectively a normal parenchyma (Figures 3b-d). Glomerulotubular sections were clear with no signs of tubular cell vacuolization or necrosis. The interstitial space was very little visible with no inflammatory infiltration. Marked reductions of interstitial collagen were noticed with Sirius red staining in 75%, 75% and 87.5% of the kidney sections under ACEi, ERB and ACEi/ERB treatment respectively, indicating the regression of fibrosis (Figure 4b-d).

Discussion

In this study, we were able to demonstrate the existence of a possible link between evolution of MA, a major risk factor of the cardiorenal syndrome (CRS), and BNP, a marker of cardiac hypertrophy.

After applying unilateral renal artery stenosis, banded rats under no treatment of any sort developed hypertension (Table 1): in fact, renal artery stenosis causes a glomerular filtration rate (GFR) reduction and thus setting off the RAAS cascade in order to maintain a constant GFR by efferent renal artery constriction. Bosentan significantly decreased SBP which is consistent with previous works (Krum *et al.* 1998); several authors have reported no change in blood pressure using different selective and non-selective ET-1 antagonists in 2K-1C hypertension (Ehmke *et al.* 1999; Hocher *et al.* 1999) but most of these studies were carried out during the early phase in the development of hypertension. Others found attenuation of the rise in blood pressure following treatment with selective ET-A antagonists, even at 2 days after clipping (Schricker K *et al.* 1995). Captopril administration alone was more effective than the bosentan in reducing BP; in fact, ACEi remain the first line of renovascular hypertension treatment (Ruggenenti *et al.* 2006). Additive hemodynamic effects of combined non-selective

ERB and ACEi therapy were noted in the bos/cap group, which is previously reported (Teerlink *et al.* 1994).

BNP is the lead marker of ventricular hypertrophy (Scardovi 2004). MA, a marker of systemic inflammation and endothelial dysfunction (Stehouwer 2004) is associated with a high CVD risk in patients with diabetes (Dries *et al.* 2001), and hypertension (Wachtell *et al.* 2003), as well as in seemingly healthy individuals (Hillege et al. 2002), and the link between MA and hypertension is mediated by inflammation (Kalra *et al.* 2005; Wang *et al.* 2005). Moreover, MA is even a risk factor contributing to the proximal renal tubules inflammation resulting in renal fibrosis (Shankland 2006). MA is considered not only a predictor of CVD, fibrosis and CKD, but also a therapeutic target (Ibsen *et al.* 2005).

The predictive effect of albuminuria in CVD extends its limits to the general population where even low levels of urinary albumin, below the MA threshold, predict the development of a composite of cardiovascular events, including HF, as showed by Arnlov *et al.* in middle-aged nonhypertensive and nondiabetic individuals.

In our study, high BP in banded rats was accompanied by MA development and increased BNP levels (Figure 1, Table 1). Bosentan and/or captopril administration eliminated MA and reduced plasma BNP which was accompanied by the regression of cardiac mass (Table 2) suggesting the regression of cardiac hypertrophy under the non-selective ERB and/or ACEi. This parallel evolution of the two parameters evokes a strong positive correlation (Table 3) between them and suggests a cardioprotective role of MA-lowering. In a recent study, a correlation was found between blood pressure control, MA and BNP (Uno *et al.* 2008), but cardiac hypertrophy was not evaluated, and thus the relationship between MA and cardio pathogenesis, including cardiac hypertrophy remains vague.

The relationship between MA and hypertension leading to cardiac hypertrophy and HF is very complex; prior cross-sectional studies indicate that MA may be a feature of hypertension and a marker of target-organ damage (Cirillo *et al.* 1998), whereas others showed that urinary albumin

excretion predicts blood pressure progression in nondiabetic, nonhypertensive individuals incrementally over established risk factors and at levels well below the conventional threshold for MA (Wang *et al.* 2005). Since the present findings focused on cardiac hypertrophy, in the *first stages* leading to HF, cardioprotective antihypertensive treatments led to a decrease in SBP parallel to the decline in MA; but other large-scale RCTs showed that albuminuria is a risk predictor of *HF* irrespective of antihypertensive treatment (Arnold *et al.* 2003), which leads us again to our conclusion on the cardioprotective role of MA-lowering in the first stages of the disease, with MA being the most important predictor of cardiac hypertrophy and eventually HF.

Angiotensin II (AngII) and Endothelin-1 (ET-1) stimulate TGF-β1 gene expression (Sung *et al.* 1994). AngII can activate collagen I gene in aorta and renal cortex *in vivo* by a mechanism(s) requiring participation and/or cooperation of ET-1 and TGF-β1 (Fakhouri *et al.* 2001). ACE inhibition and endothelin receptors ET-A/B blockade results in cardiac and renal fibrosis decline (Kuwahara *et al.* 1999). Similarly, our study shows first an increase in TGF-β1 levels after clipping the renal artery parallel to the increase of interstitial fibrosis and tubular injury, then a drop-off under ACEi and ET receptor blocker with improvement of renal histology (Table 1, Figures 3 and 4). But we were able to demonstrate a parallel evolution between TGF-β1, BNP, MA and high BP, and that MA-lowering has a reno- and cardioprotective effect manifested by TGF-β1 decrease, and thus a reduced fibrogenesis.

A significant creatinine increase was noted after stenosis, showing a renal function disruption linear to BNP and MA. Creatinine decrease occurred after treatment with bosentan and bosentan/captopril, justifying the renoprotective role of MA-lowering (Figure 1). Creatinine elevation, as compared to normal, under ACEi was already expected and demonstrated by several authors (Textor and Wilcox 2001).

The significant decrease in heart weight/body weight ratio, BNP and β-MHC gene expression under the non-selective ERB, through its ET-A receptor blockade as shown by (Bianciotti and De Bold 2001), and/or ACEi (Figure 2) was correlated with MA absence (r =0.902, r =0.87 and

r=0.93 respectively, p<0.05). These results justify the cardioprotective role of MA-lowering, manifested by the gene expression decline of BNP and β-MHC, markers of cardiac hypertrophy. Simultaneous increase in α-MHC expression supports the last obtained result for the cardioprotective role of MA-lowering; α-MHC being the myosin isoform with the more pronounced ATPase activity (Lowes *et al.* 1997).

In conclusion, we found an existing link between MA evolution and ventricular hypertrophy in the CRS; moreover, MA-lowering therapy has both renoprotective effect as evaluated by microalbuminuria and creatinine levels, and cardioprotective effect as evaluated by cardiac mass, plasma BNP and α-, β-MHC and BNP mRNA levels.

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References

ARNLOV J, EVANS JC, MEIGS JB, WANG TJ, FOX CS, LEVY D, BENJAMIN EJ, D'AGOSTINO RB, VASAN RS: Low-grade albuminuria and incidence of cardiovascular disease events in nonhypertensive and nondiabetic individuals: the Framingham Heart Study. *Circulation* **112**:969-975, 2005.

ARNOLD JM, YUSUF S, YOUNG J, MATHEW J, JOHNSTONE D, AVEZUM A, LONN E, POGUE J, BOSCH J; HOPE Investigators: Prevention of heart failure in patients in the Heart Outcomes Prevention Evaluation (HOPE) Study. *Circulation* **107**: 1284-1290, 2003.

BIANCIOTTI LG, DE BOLD AJ: Modulation of cardiac natriuretic peptide gene expression following endothelin type A receptor blockade in renovascular hypertension. *Cardiovasc Res* **49(4)**: 808-816, 2001.

BRENNER BM, COOPER ME, DE ZEEUW D, KEANE WF, MITCH WE, PARVING HH, REMUZZI G, SNAPINN SM, ZHANG Z, SHAHINFAR S; RENAAL Study Investigators:

Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* **345**: 861-869, 2001.

CIRILLO M, SENIGALLIESI L, LAURENZI M, ALFIERI R, STAMLER J, STAMLER R, PANARELLI W, DE SANTO NG: Microalbuminuria in nondiabetic adults: relation of blood pressure, body mass index, plasma cholesterol levels, and smoking: the Gubbio Population Study. *Arch Intern Med* **158**: 1933-1939, 1998.

DRIES DL, SWEITZER NK, DRAZNER MH, STEVENSON LW, GERSH BJ: Prognostic impact of diabetes mellitus in patients with heart failure according to the etiology of left ventricular systolic dysfunction. *J Am Coll Cardiol* **38**: 421-428, 2001.

EHMKE H, FAULHABER J, MUNTER K, KIRCHENGAST M, WIESNER RJ: Chronic ETA receptor blockade attenuates cardiac hypertrophy independently of blood pressure effects in renovascular hypertensive rats. *Hypertension* **33**: 954-960, 1999.

FAKHOURI F, PLACIER S, ARDAILLOU R, DUSSAULE JC, CHATZIANTONIOU C: Angiotensin II activates collagen type I gene in the renal cortex and aorta of transgenic mice through interaction with endothelin and TGF-β. *J Am Soc Nephrol* **12**: 2701-2710, 2001.

FARES N, GOMEZ JP, POTREAU D: T-type calcium current is expressed in dedifferentiated adult rat ventricular cells in primary culture. *C R Acad Sci III* **319(7)**: 569-576, 1996.

HILLEGE HL, FIDLER V, DIERCKS GF, VAN GILST WH, DE ZEEUW D, VAN VELDHUISEN DJ, GANS RO, JANSSEN WM, GROBBEE DE, DE JONG PE, Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group: Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation* **106**: 1777-1782, 2002.

HOCHER B, GEORGE I, REBSTOCK J, BAUCH A, SCHWARZ A, NEUMAYER HH, BAUER C: Endothelin system-dependent cardiac remodelling in renovascular hypertension. *Hypertension* **33**: 816-822, 1999.

IBSEN H, OLSEN MH, WACHTELL K, BORCH-JOHNSEN K, LINDHOLM LH,

MOGENSEN CE, DAHLOF B, DEVEREUX RB, DE FAIRE U, FYHRQUIST F, JULIUS S, KJELDSEN SE, LEDERBALLE-PEDERSEN O, NIEMINEN MS, OMVIK P, OPARIL S, WAN Y: Reduction in albuminuria translates to reduction in cardiovascular events in hypertensive patients: Losartan Intervention for Endpoint Reduction in Hypertension Study. *Hypertension* **45**: 198-202, 2005.

ISENBERG G, KLOCKNER U: Calcium currents of isolated bovine ventricular myocytes are fast and of large amplitude. *Pflügers Arch* **395(1)**: 30-41, 1982.

KALRA V, MAHAJAN S, AGARWAL SK, TIWARI SC: Cardiorenal disease: A clinical intersection. *International Urology and Nephrology* **37**: 175-184, 2005.

KEITH DS, NICHOLS GA, GULLION CM, BROWN JB, SMITH DH: Longitudinal follow-up and outcomes among a population with chronic kidney in a large managed care organization. *Arch Intern Med* **164**: 659-663, 2004.

KRUM H, VISKOPER RJ, LACOURCIERE Y, BUDDE M, CHARLON V, for The Bosentan Hypertension Investigators: The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. *N Eng J Med* **338:** 784-791, 1998.

KUBOTA Y, UMEGAKI K, KAGOTA S, TANAKA N, NAKAMURA K, KUNITOMO M, SHINOZUKA K: Evaluation of blood pressure measured by tail-cuff methods (without heating) in spontaneously hypertensive rats. *Biol Pharm Bull*. **29(8)**: 1756-1758, 2006.

KUWAHARA F, KAI H, NAGATA T, SHIBATA R, NIIYAMA H, IMAIZUMI T: Inhibition of TGF-.BETA prevents Cardiac Fibrosis and Diastolic Dysfunction in Hypertensive Rats. *Int Soc Heart Res Annu Meet Jpn Sect Program Abstr*. **16th:** 45, 1999.

LOWES BD, MINOBE W, ABRAHAM WT, RIZEQ MN, BOHLMEYER TJ, QUAIFE RA, RODEN RL, DUTCHER DL, ROBERTSON AD, VOELKEL NF, BADESCH DB, GROVES BM, GILBERT EM, BRISTOW MR: Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. *J Clin Investig* **100**: 2315-2324, 1997.

MCALISTER FA, EZEKOWITZ J, TONELLI M, ARMSTRONG PW: Renal insufficiency and heart failure: prognostic and therapeutic implications from a prospective cohort study. *Circulation* **109**: 1004-1009, 2004.

MCCULLOUGH PA: Cardiorenal intersection: Crossroads to the future. *Arq Bras Cardiol* **88(1)**: 100-108, 2007.

MCCULLOUGH PA: Cardiorenal risk: an important clinical intersection. *Rev Cardiovasc Med* **3**: 71-76, 2002.

RONCO C, HOUSE AA, HAAPIO M: Cardiorenal syndrome: refining the definition of a complex symbiosis gone wrong. *Intensive Care Med* **34(5)**: 957-962, 2008.

RUGGENENTI P, PERNA A, GANEVA M, ENE-IORDACHE B, REMUZZI G: BENEDICT STUDY GROUP: Impact of blood pressure control and angiotensin-converting enzyme inhibitor therapy on new on-set microalbuminuria in type 2 diabetes: a post hoc analysis of the BENEDICT trial. *J Am Soc Nephrol* **17(12)**: 3472-3481, 2006.

SCARDOVI AB: Clinical applications of brain natriuretic peptide testing. *Ital Heart J* **5(5 suppl)**: 343-356, 2004.

SCHRICKER K, SCHOLZ H, HAMANN M, CLOZEL M, KRAMER BK, KURTZ A: Role of endogenous endothelins in the rennin system of normal and two-kidney, one clip rats. *Hypertension* **25**: 1025-1029, 1995.

SCHRIER RW: Role of diminished renal function in cardiovascular mortality: Marker or pathogenetic factor? *J Am Coll Cardiol* **47**: 1-8, 2006.

SHANKLAND SJ: The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int* **69**: 2131-2147, 2006.

STEHOUWER CD: Endothelial dysfunction in diabetic nephropathy: State of the art and potential significance for non-diabetic renal disease. *Nephrol Dial Transplant* **19(4)**: 778-781, 2004.

SUNG CP, ARLETH AJ, STORER L, OHLSTEIN EH: Angiotensin type I receptors mediate

smooth muscle proliferation and endothelin biosynthesis in rat vascular smooth muscle. *Pharmacol Exp Ther* **271**: 429-437, 1994.

SWISLOCKI ALM, KINNEY LAPIER TL, KHUU DT, FANN KY, TAIT M, RODNICK KJ: Metabolic, hemodynamic, and cardiac effects of captopril in young, spontaneously hypertensive rats. *Am J Hypertens* **12**: 581-589, 1999.

TEERLINK JR, LOFFLER BM, HESS P, MAIRE JP, CLOZEL M, CLOZEL JP: Role of endothelin in the maintenance of blood pressure in conscious rats with chronic heart failure: acute effects of the endothelin receptor antagonist Ro 47-0203 (bosentan). *Circulation* **90**: 2510-2518, 1994.

TEXTOR SC, WILCOX CS: Renal artery stenosis: a common, treatable cause of renal failure? *Annu Rev Med* **52**: 421-442, 2001.

UNITED STATES RENAL DATA SYSTEM. USRDS 2008, Annual Data Report. Bethesda MD., 2008. National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases.

UNO H, ISHIKAWA J, HOSHIDE S, KABUTOYA T, ISHIKAWA S, SHIMADA K, KARIO K: Effects of strict blood pressure control by a long-acting calcium channel blocker on brain natriuretic peptide and urinary albumin excretion rate in Japanese hypertensive patients. *Hypertens Res* **31:** 887-896, 2008.

VAN DOKKUM RP, EIJKELKAMP WB, KLUPPEL AC, HENNING RH, VAN GOOR H, CITGEZ M, WINDT WA, VAN VELDHUISEN DJ, DE GRAEFF PA, DE ZEEUW D: Myocardial infarction enhances progressive renal damage in an experimental model for cardio-renal interaction. *J Am Soc Nephrol* **15**: 3103-3110, 2004.

WACHTELL K, OLSEN MH, DAHLOF B, DEVEREUX RB, KJELDSEN SE, NIEMINEN MS, OKIN PM, PAPADEMETRIOU V, MOGENSEN CE, BORCH-JOHNSEN K, IBSEN H: Microalbuminuria in hypertensive patients with electrocardiographic left ventricular hypertrophy: the LIFE study. *J Hypertens* **20**: 405-412, 2002.

WACHTELL K, IBSEN H, OLSEN MH, BORCH-JOHNSEN K, LINDHOLM LH, MOGENSEN CE, DAHLOF B, DEVEREUX RB, BEEVERS G, DE FAIRE U, FYHRQUIST F, JULIUS S, KJELDSEN SE, KRISTIANSON K, LEDERBALLE-PEDERSEN O, NIEMINEN MS, OKIN PM, OMVIK P, OPARIL S, WEDEL H, SNAPINN SM, AURUP P: Albuminuria and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: The Life Study. *Ann Intern Med* **139**: 901-906, 2003.

WANG TJ, EVANS JC, MEIGS JB, RIFAI N, FOX CS, D'AGOSTINO RB, LEVY D, VASAN RS: Low-grade albuminuria and risks of hypertension and high blood pressure. *Circulation* **111**: 1370-1376, 2005.

Tables

Table 1: SBP, plasma BNP and TGF-β1 variations in the treated and untreated 2K-1C male adult rats.

	Before Stenosis (n=24)	Sham (n=6)	Bos (n=6)	Cap (n=6)	Bos/Cap (n=6)
SBP (mm Hg)	115.2 ± 2.4	181.8 ± 7.5*	132.1 ± 5.5^{+}	124.1 ± 8.8^{+}	116.1 ± 6.7^{+}
Plasma BNP (ng/ml)	0.675 ± 0.11	1.96 ± 0.05**	1.47 ± 0.02^{++}	1.35 ± 0.03^{++}	1.19 ± 0.04^{++}
Plasma TGF-β1 (ng/ml)	22.82 ± 4.26	93.22 ± 10.74 ***	27.74 ± 0.02^{+++}	24.27 ± 3.37 $^{+++}$	23.22 ± 3.82 $^{+++}$

SBP: systolic blood pressure, BNP: brain natriuretic peptide, TGF-β1: transforming growth factor beta 1. Bos: bosentan, Cap: captopril, Bos/Cap: bosentan and captopril. $^{*}p<0.001$, $^{**}p<0.05$ and $^{***}p<0.005$ Sham *vs* Before stenosis; $^{+}p<0.001$, $^{++}p<0.05$ and $^{+++}p<0.005$ Treated *vs* Sham. Data are presented as mean ± SEM. n: number of animals.

Table 2: Cardiac mass variations among the treated and untreated 2K-1C male adult rats.

	Control (n=24)	Sham (n=6)	Bos (n=6)	Cap (n=6)	Bos/Cap (n=6)
Body weight (g)	234.4 ± 17.2	240.2 ± 15.1	251.5 ± 20.3	237.8 ± 19.8	245.3 ± 16.4
Heart weight (mg)	860.25 ± 49.12	1258.65 ± 103.1^{+}	1094.03 ± 98.73*	1001.14 ± 90.24*	986.11 ± 78.46*
Heart weight/ body weight ratio (mg/g)	3.67 ± 0.06	5.24 ± 0.17^{++}	4.35 ± 0.15*	4.21 ± 0.09*	4.02 ± 0.04*

Bos: bosentan, Cap: captopril, Bos/Cap: bosentan and captorpil. $^{*}p<0.05$ Treated *vs* Sham, $^{+}p<0.05$ and $^{++}p<0.005$ Sham *vs* Control. Data are presented as mean ± SEM. n: number of animals.

Table 3: Correlation of changes in MA with changes in cardiac mass, BNP and β-MHC mRNA levels among the 2K-1C rats, before and after treatments.

	MA (mg/dl) (n=24)	
	R	*P* value
Heart weight/body weight ratio (mg/g) (n=24)	0.902	0.05
BNP mRNA (n=24)	0.87	0.05
B-MHC mRNA (n=24)	0.93	0.05

MA: microalbuminuria, BNP: brain natriuretic peptide, β-MHC: beta myosin heavy chain. *p* values were assessed by the Spearman's correlation coefficient. n: number of animals.

Figure legends

Figure 1: Urinary albumin level and creatininaemia variations in the 2K-1C male adult rats, under or without treatment. Bos: bosentan, Cap: captopril, Bos/Cap: bosentan and captopril. **a)** **p*<0.001 *vs* Before stenosis; **b)** ***p*<0.05 *vs* Sham, ^{+}p<0.05 *vs* Before stenosis. Data are presented as mean ± SEM. n: number of animals.

Figure 2: BNP, alpha- and beta-MHC mRNA levels in the ventricular myocytes isolated from the hearts of the 2K-1C male adult rats. Bos: bosentan, Cap: captopril, Bos/Cap: bosentan and captopril, BNP: brain natriuretic peptide, MHC: myosin heavy chain. **p*<0.005 *vs* Sham. Data are presented as mean ± SEM. n: number of animals.

Figure 3: Hematoxylin and eosin staining of the 2K-1C male adult rats kidney sections, under or without treatment. **a)** control, **b)** bosentan, **c)** captopril, **d)** bosentan/captopril, **e)** sham. G: glomerule, T: tubule, L: lymphocytes. Arrows in f) show the interstitial inflammatory outbreaks. Magnification: x200 in (a-e) and x400 in f). Scale bars: 50 µm.

Figure 4: Sirius red collagen staining of the 2K-1C male adult rats kidney sections, under or without treatment. **a)** control, **b)** bosentan, **c)** captopril, **d)** bosentan/captopril, **e)** sham. G: glomerule, T: tubule. Arrows in e) show the interstitial collagen deposite. Magnification: x200. Scale bars: 50 µm.

Figure 1

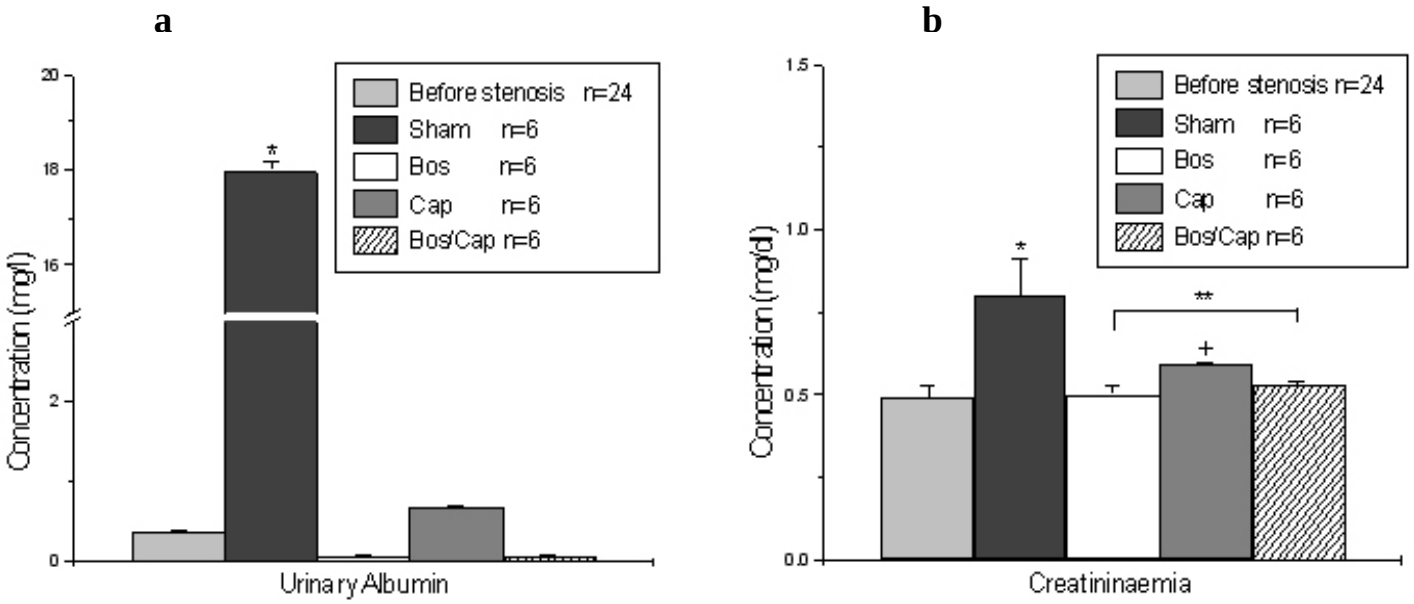

Figure 2

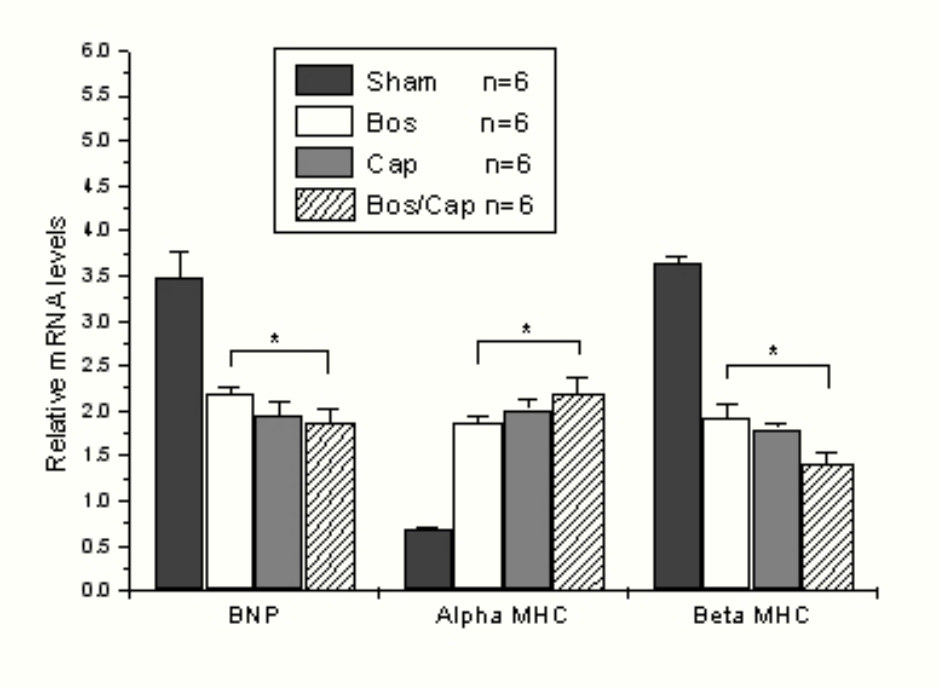

Figure 3

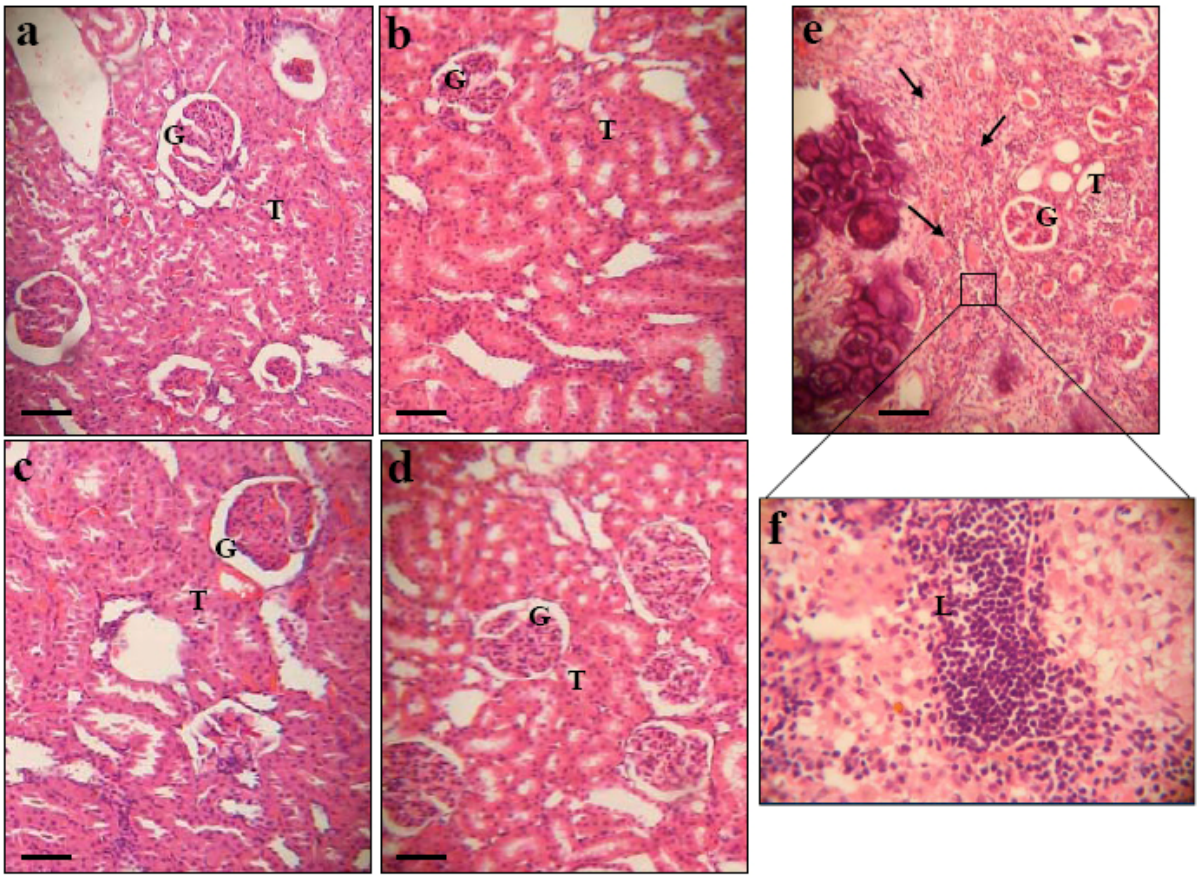

Figure 4