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Plasma concentrations of matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-1 and osteopontin reflect severity of heart failure in DOCA-salt hypertensive rat

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Abstract

The matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) play a key role in extracellular matrix maintenance and are altered in the failing heart, both in experimental models and in chronic end-stage heart failure in humans. As the common diagnostic markers of heart failure, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) primarily reflect increased pressure loading, determination of soluble, heart-derived MMPs and TIMPs in plasma, as well as the determination of the emerging fibrosis marker osteopontin (OPN) might be valuable tools for detecting heart fibrosis. In this study the effect of spironolactone treatment on plasma MMP-2, TIMP-1 and OPN levels was assessed in a heart failure animal model. Unilaterally nephrectomized Sprague Dawley rats received subcutaneous injection of 100 mg deoxycorticosterone acetate (DOCA) once a week and 1% (w/v) NaCl in drinking water. Blood pressure was monitored weekly and blood samples were collected after 1, 2 and 4 weeks. After 6 weeks, left ventricular contractility (LVC) and heart weight-to-body weight ratio (HW/BW) were assessed. DOCA treatment increased plasma MMP-2, TIMP-1 and OPN concentrations. Alterations of plasma marker levels were correlated with changes of HW/BW and paralleled impaired LVC. Furthermore, beneficial effects of spironolactone treatment were observed. In DOCA-salt hypertensive rats, plasma concentrations of MMP-2, TIMP-1 and OPN reflected heart failure associated with haemodynamic, functional and morphological changes. Based on these findings, it appears reasonable to use plasma markers of fibrosis to monitor the development of heart failure.

Keywords: *Cardiac remodelling, MMP-2, TIMP-1, OPN, DOCA-salt hypertensive rat, spironolactone*

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Introduction

Congestive heart failure (CHF) is a common severe disease which affects about 1% of the population after the age of 65 years in the USA (Rosamond et al. 2007). The prognosis for patients diagnosed with CHF is poor, with an annual mortality of about

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20%. This group of heart failure patients display a six- to ninefold higher risk of dying from sudden cardiac death. Moreover, heart failure has a pronounced socioeconomic impact – the estimated direct and indirect cost in the USA for 2007 was $33.2 billion.

The development and progression of heart failure can be caused by a series of factors. Sudden events such as myocardial infarction or persisting hypertension induce, among other effects, neurohormonal disorders (Bristow 1984), local and systemic inflammation (Dibbs et al. 1999), apoptosis of cardiomyocytes (Olivetti et al. 1997, Guerra et al. 1999) and alteration of the renal water and sodium excretion (Schrier 1990). As a consequence of enduring the aforementioned deleterious effects extracellular matrix turnover proceeds and leads to heart hypertrophy and cardiac remodelling (Cohn 1995). Structural reshape of cardiac tissue is mainly controlled by the activity of matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Failing hearts display an increased expression of the Zn^{2+}-dependent endopeptidase MMP-2 and its endogenous inhibitor TIMP-1 on mRNA and protein level (Spinale 2002, Polyakova et al. 2004). In addition, a correlation between the severity of heart failure, as defined by NYHA class, and plasma levels of MMP-2 and TIMP-1 has been shown in human patients (Noji et al. 2004, Yamazaki et al. 2004, George et al. 2005, Ahmed et al. 2006).

Besides these proteases and protease inhibitors, all proteins which are expressed and secreted by cardiac fibroblasts under conditions of heart failure might be potential biomarkers for monitoring cardiac fibrosis. In particular, osteopontin (OPN), a secreted multifunctional phosphoprotein, has been suggested in several recent publications to play an important role in the pathogenesis of CHF (Collins et al. 2004, Okamoto 2007). First, OPN mRNA and protein are hardly detectable in healthy myocardium, but expression of the OPN gene is induced upon cardiomyocyte injury and pressure load in cardiac myocytes and interstitial fibroblasts (Ashizawa et al. 1996, Graf et al. 1997). Second, in humans diagnosed with CHF and in animal models of heart failure cardiac tissue fibrosis is associated with locally increased OPN expression (Singh et al. 1999). Third, OPN interferes with the processes of extracellular matrix degeneration by inhibition of cytokine-induced expression and activation of MMP-2 (Xie et al. 2003, 2004). However, in murine mammary cancer cells, OPN mediates metastatic behaviour, in part, through upregulation of MMP-2 (Mi et al. 2006). Finally, OPN knockout mice are less susceptible to angiotensin II-induced cardiac fibrosis (Matsui et al. 2004).

As the common markers of heart failure, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) reflect primarily increased pressure loading (Yasue et al. 1994), secreted heart-derived MMP-2, TIMP-1 and OPN might be valuable plasma biomarkers for detecting heart fibrosis in experimental heart failure models and human patients. The purpose of the present study was to assess the appropriateness of MMP-2, TIMP-1 and OPN plasma levels to monitor disease progression in an animal heart failure model. Unilaterally nephrectomized Sprague Dawley rats were administered deoxycorticosterone acetate (DOCA) and sodium chloride, which is known to induce heart hypertrophy and myocardial fibrosis (Brilla et al. 1990, Robert et al. 1995, Young et al. 1995, Sun et al. 1997, Takeda et al. 2000). As several lines of evidence indicate that spironolactone treatment prevents myocardial fibrosis in animals with secondary hyperaldosteronism (Brilla et al. 1993, Nicoletti et al. 1995, Lal et al. 2005) and moreover reduces hospitalization rate and increases survival rate

in CHF patients (Pitt et al. 1999, Weber 1999), we evaluated in the present study, whether a reduction of fibrosis by a mineralocorticoid receptor antagonist is reflected by plasma concentrations of MMP-2, TIMP-1 and OPN.

Materials and methods

Animal experiments

Experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German legislation on animal welfare. Male Sprague Dawley rats (Charles River Laboratories, Sulzfeld, Germany) weighing 200 g were housed in a facility with a constant temperature of 24°C and a 12-h/12-h light/dark cycle. Rats were divided into three groups ($n = 10$ per group). Two groups were unilaterally nephrectomized under isofluran (2.0%) and buprenorphine (0.3 mg kg^{-1}) anaesthesia. DOCA suspension (Sigma-Aldrich, Munich, Germany) was injected subcutaneously once a week (100 mg kg^{-1}), and drinking water was substituted by 1% sodium chloride solution. One DOCA group received spironolactone (50 mg kg^{-1}) (Sigma-Aldrich) subcutaneously once daily, whereas in the placebo group, only the vehicle was administered. Control rats underwent anaesthesia with incision, but without nephrectomy and received tap water. Systolic blood pressure (BP) was measured in unanaesthetized animals weekly by tail-cuff method (Process Control Blood Pressure 209000; TSE Technical & Scientific Equipment, Bad Homburg, Germany). Serial blood samples were collected retro-orbitally after 1, 2 and 4 weeks (approximately 500 μL). At the end of week 6 of treatment, haemodynamic measurements were made and blood was collected by cardiac puncture. After rats were euthanized heart weight was determined.

Determination of plasma biomarker concentration

Blood was collected in heparinized vials. Samples were mixed thoroughly and centrifuged at 1000*g* at a temperature of 4°C for 10 min. Plasma was separated and frozen immediately at −80°C. Plasma concentrations of MMP-2, TIMP-1 and OPN were determined using sandwich enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Wiesbaden Germany). Determination of plasma concentration of ANP was performed using a commercially available enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Inc., Belmont, USA). The TIMP-1 ELISA and the ANP EIA are specific for the respective rat biomarkers. For the detection of OPN a mouse OPN ELISA was applied, which has been validated by the manufacturer for the determination of natural rat OPN in plasma. Concentration of MMP-2 was assessed with a human/mouse specific ELISA. Assays were performed according to the manufacturer's recommendations. The mean minimal detected concentration (MDC) for analytes were 0.16 ng ml^{-1} for MMP-2, 3.5 pg ml^{-1} for TIMP-1, 5.7 pg ml^{-1} for OPN and 5.0 pg ml^{-1} for ANP. The intra-assay precision determined as the coefficient of variation (CV) for the MMP-2 ELISA was 3.4% at 17.4 ng ml^{-1} ($n = 20$), for the TIMP-1 ELISA was 2.4% at 1.3 ng ml^{-1} ($n = 20$), for the OPN ELISA was 4.0% at 1.1 ng ml^{-1} ($n = 20$) and for the ANP EIA was 2.8% at 15.8 ng ml^{-1} ($n = 20$).

Cardiac contractility

Left ventricular contractility (LVC) was assessed after 6 weeks in anaesthetized rats (1.8% isoflurane) using Millar Tip catheter (Millar Instruments Inc, Houston, USA) which was introduced from the right arteria carotis and advanced into the left ventricle. Data were acquired using the Powerlab System and processed with Chart5 Software (ADInstruments Pty. Ltd. Castle Hill, Australia).

Statistics

Data are displayed as mean $\pm$SEM. Statistical significance was estimated by one-way ANOVA (Newman–Keuls post hoc test). A value of $p<0.05$ was considered as significant and $p<0.001$ was considered as highly significant. Correlations between plasma biomarker concentrations and functional or morphological variables were evaluated by linear regression analysis. GraphPad Prism software, version 4.02 (GraphPad Software, San Diego, CA, USA) was applied for all calculations.

Results

Blood pressure

After 6 weeks, blood pressure of placebo-treated DOCA-salt animals increased to 263 ± 2.7 mmHg compared with control animals (160 ± 3.9 mmHg, $p<0.001$) (Figure 1A). At all time points DOCA-salt rats that received spironolactone displayed a significantly lower blood pressure than the ones in the placebo group ($p<0.001$).

Plasma ANP concentration

Two weeks after nephrectomy placebo- and spironolactone-treated animals were characterized by moderately increased plasma ANP concentrations (Figure 1B). ANP levels in the placebo group differed significantly from the control group after 6 weeks (vs $p<0.05$). Furthermore, the elevations of plasma ANP concentrations in DOCA-salt animals were attenuated by spironolactone treatment (control: 16.8 ± 2.5 ng ml^{-1}, placebo: 31.7 ± 2.9 ng ml^{-1}, spironolactone: 21.7 ± 2.4 ng ml^{-1}, all $p<0.05$).

Left ventricular contractility (dp/dt_{max})

As displayed in Table I, the LVC (dp/dt_{max}) of the placebo-treated DOCA-salt animals was diminished significantly after 6 weeks compared with healthy controls ($p<0.05$), whereas spironolactone treatment improved contractility ($p<0.05$).

Heart weight-to-body weight ratio

As detailed in Table I, in DOCA-salt rats the heart weight (HW) and the heart weight-to-body weight ratio (HW/BW) was significantly increased compared with control animals ($p<0.05$ and $p<0.001$, respectively). Although the effect of spironolactone treatment on HW and HW/BW was not significant, a tendency toward reduced HW and HW/BW was observed compared with the placebo group. A significant decline in body weight was observed in the placebo group (Figure 1C).

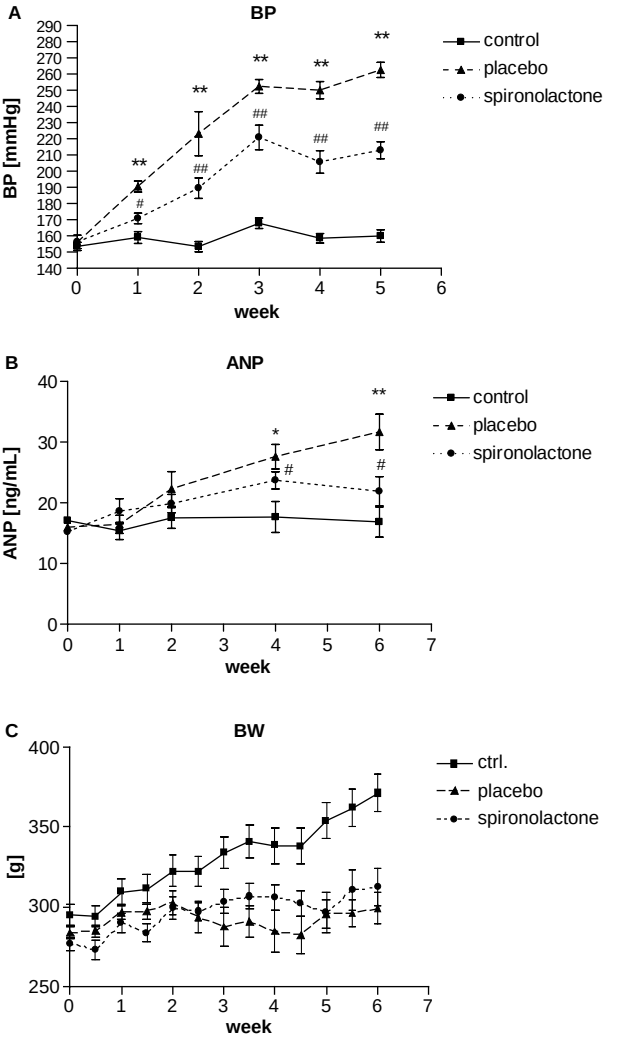

Figure 1. (A) Blood pressure (BP), (B) plasma concentrations of the atrial natriuretic peptide (ANP) and (C) body weight (BW) during the experimental period. Unilaterally nephrectomized rats which received deoxycorticosterone acetate (DOCA; 100 mg kg^{-1}, s.c.) and sodium chloride (1% in drinking water) display increased BP and elevated ANP levels. Spironolactone treatment attenuates rise of BP and ANP concentrations.

*$p<0.05$ DOCA/NaCl vs control; **$p<0.001$ DOCA/NaCl vs control; #$p<0.05$ DOCA/NaCl+spironolactone vs DOCA/NaCl; ##$p<0.001$ DOCA/NaCl+spironolactone vs DOCA/NaCl.

Plasma MMP-2 concentration

At all time points placebo-treated DOCA-salt rats were characterized by a significant increase of plasma MMP-2 concentrations (Figure 2A), whereas spironolactone treatment alleviated the increase of plasma MMP-2 levels (control: 132.5 ± 2.7 ng ml^{-1}; placebo: 188.6 ± 8.3 ng ml^{-1}; spironolactone: 169.4 ± 4.2 ng ml^{-1}; all $p<$ 0.001). However, both, placebo- and spironolactone-treated animals displayed a tendency toward declining MMP-2 levels after six weeks.

Table I. Effect of spironolactone treatment on left ventricular contractility (LVC), heart weight (HW), body weight (BW), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR) of control animals and deoxycorticosterone-acetate (DOCA)-salt hypertensive rat (mean ± SEM).

Group	LVC ($mmHg\ s^{-1}$)	HW/BW ($mg\ g^{-1}$)	HW (mg)	BW (g)	HR ($1\ min^{-1}$)	LVEDP (mmHg)
Control ($n=10$)	8409 ± 431	2.86 ± 0.19	1061 ± 115	365 ± 10	370 ± 9	15 ± 0.8
DOCA ($n=8$)	6296 ± 429*	4.25 + 0.33**	1248 ± 49*	295 ± 8*	292 ± 16**	20 ± 1.6*
DOCA + Spiro ($n=8$)	7687 ± 485#	3.83 + 0.46	1154 ± 29	304 ± 10	325 ± 8	17 ± 1.7

*$p<0.05$ DOCA/NaCl vs control; **$p<0.001$ DOCA/NaCl vs control; #$p<0.05$ DOCA/NaCl + spironolactone vs DOCA/NaCl.

Plasma TIMP-1 concentration

Plasma concentration of TIMP-1 increased progressively in placebo-treated DOCA-salt rats and was highly significantly elevated after 1, 2, 4 and 6 weeks compared with control animals (Figure 2B). In the spironolactone-treated group plasma TIMP-1 levels rose within the first 2 weeks of treatment, but did not further increase until week 6. At the end of the survey plasma TIMP-1 concentrations were significantly lower in the spironolactone-treated animals compared with those receiving placebo (control: 9.4 ± 2.1 ng ml^{-1}; placebo: 28.2 ± 7.2 ng ml^{-1}; spironolactone: 19.2 ± 4.8ng ml^{-1}; all $p<0.001$).

Plasma OPN concentration

In placebo-treated DOCA-salt animals the plasma OPN concentrations rose progressively with time (Figure 2C). The elevation was significant after 1 week ($p<0.05$) and highly significant at 2, 4 and 6 weeks compared with the control group ($p<0.001$).

In the spironolactone-treated group the OPN concentrations were slightly but not significantly decreased after 2 and 4 weeks compared with the placebo-treated group.

At the end of the survey plasma OPN plasma concentrations were significantly lower in the spironolactone-treated animals compared with the placebo group (control: 15.7 ± 0.5 ng ml^{-1}; placebo: 37.4 ± 0.9 ng ml^{-1}; spironolactone: 24.8 ± 3.3 ng ml^{-1}; control vs placebo $p<0.001$; spironolactone vs placebo $p<0.05$).

Correlation of plasma marker concentrations with left ventricular contractility and the heart weight-to-body weight ratio

Linear regression analysis revealed no association between plasma ANP concentrations and the LVC or the HW/BW (Figure 3A,B).

A good correlation between plasma MMP-2 concentration and the HW/BW was found ($r=0.66$, $p<0.001$), whereas the correlation with the LVC was less pronounced and not significant ($r=0.33$, $p=0.12$) (Figure 3C,D).

The plasma TIMP-1 concentration was related to the LVC ($r=0.66$, $p<0.001$) and the HW/BW ($r=0.74$, $p<0.001$) (Figure 3E,F).

Although the plasma OPN concentration was correlated with the HW/BW ($r=0.71$, $p<0.001$), no significant relation was observed with the LVC ($r=0.32$, $p=0.14$) (Figure 3G,H).

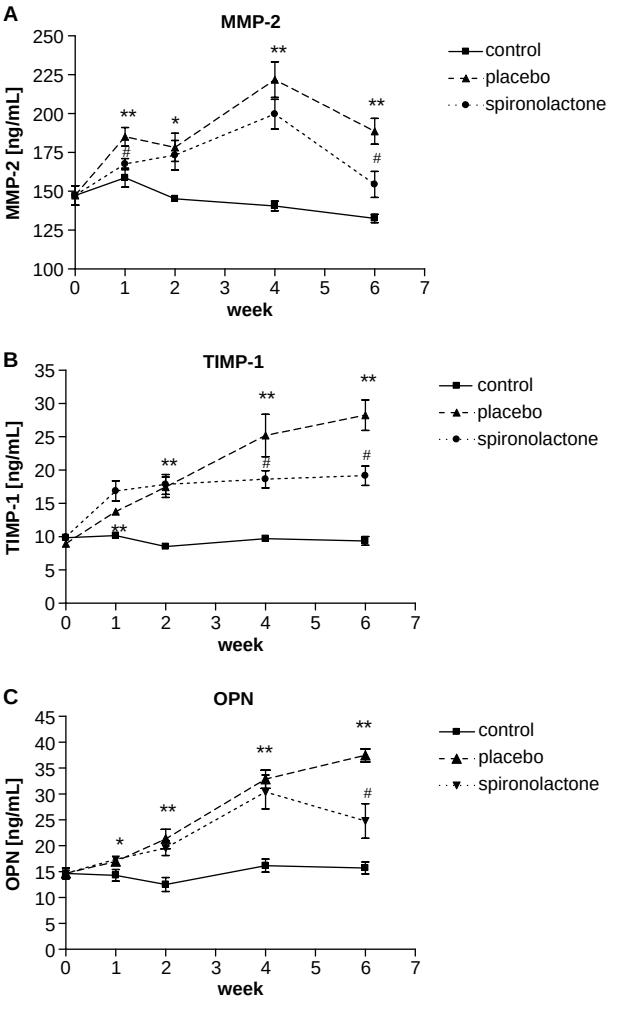

Figure 2. Plasma concentrations of (A) matrix metalloproteinase-2 (MMP-2), (B) tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and (C) osteopontin (OPN) were determined as described in Materials and methods section. In deoxycorticosterone acetate (DOCA)-salt hypertensive rats the plasma concentrations of MMP-2, TIMP-1 and OPN were increased compared with healthy control animals, whereas administration of spironolactone (50 mg kg^{-1} per day, s.c.) attenuated the DOCA/NaCl effects.
#$p<0.05$ DOCA/NaCl vs control; *$p<0.05$ DOCA/NaCl vs control; **$p<0.001$ DOCA/NaCl vs control; #$p<0.001$ DOCA/Nacl+spironolactone vs DOCA/Nacl.

Discussion

Degradation of extracellular matrix and deposition of collagen fibres in cardiac tissue are the major processes that cause impaired cardiac contractility and lead to manifestation of heart failure. These mechanisms are mainly controlled by the expression and activity of MMPs and their endogenous inhibitors. The aim of our study was to investigate whether or not the status of local tissue remodelling in the heart, which leads to impaired left ventricular contractility and increased heart weight, is mirrored by plasma levels of extracellular matrix-processing proteins. As the common circulating markers of heart failure, ANP and BNP primarily reflect

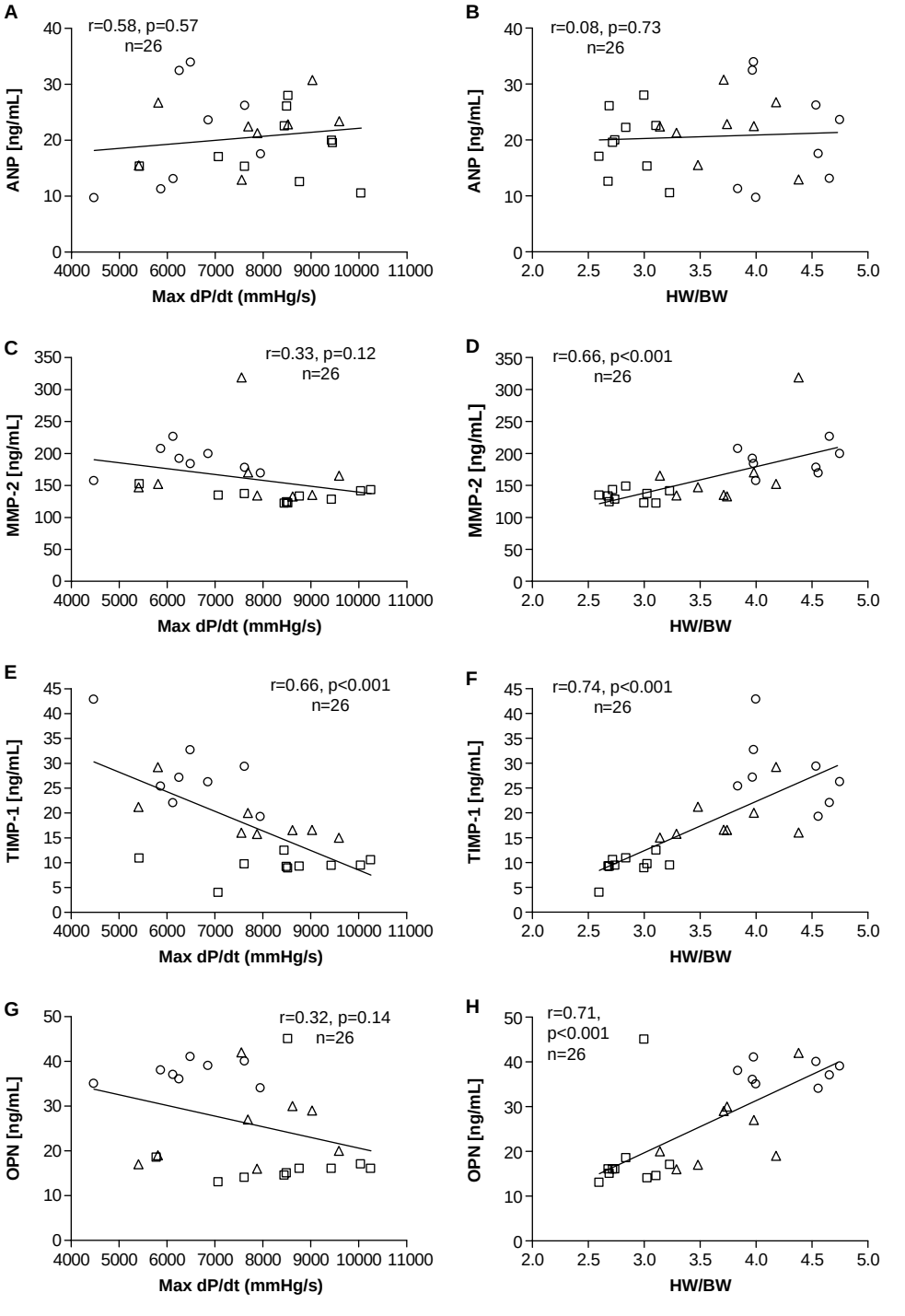

Figure 3 (Continued)

increased pressure loading, we tried to detect heart-derived fibrosis-associated proteins. Therefore experimental heart failure was induced in rats by unilateral nephrectomy, mineralocorticoid treatment (DOCA) and exposure to high concentrations of sodium chloride in drinking water (1%). As the results from different trials gave evidence that treatment with mineralocorticoid receptor antagonists delay the onset of cardiac fibrosis in heart failure (Delyani et al. 2001, De Mello 2006, Mulatero et al. 2006), we investigated the effects of spironolactone in the DOCA-salt hypertensive rat model. We correlated plasma levels of secreted MMP-2, TIMP-1 and OPN with the functional parameter LVC and the HW/BW. In contrast to previously published studies, in which the disease status was investigated only at the endpoint of a trial, in our approach blood was withdrawn at four different time points, which enabled us to determine longitudinal plasma concentrations of potential heart failure markers.

Results of different clinical trials suggest that plasma MMP-2 and TIMP-1 concentrations can be useful to assess disease severity in patients suffering from hypertrophic cardiomyopathy or heart failure (Noji et al. 2004). Furthermore, plasma TIMP-1 was related to indices of LV hypertrophy and systolic dysfunction (Sundström et al. 2004). Prognosis of heart failure patients seems to be correlated with MMP-2 levels (George et al. 2005). It is important to mention that neither plasma MMP-2 nor TIMP-1 levels are influenced by hypertension as long as LVC is normal (Ahmed et al. 2006).

In our study placebo-treated DOCA-salt rats displayed symptoms of severe heart failure: elevated plasma ANP levels, significantly impaired LVC and increased HW/BW. Furthermore, these animals were characterized by a significant increase of plasma MMP-2, TIMP-1 and OPN concentrations at all time points. Prevention of heart failure by spironolactone treatment as determined by less-impaired LVC and an attenuated increase in the HW/BW is associated with lower plasma MMP-2, TIMP-1 and OPN concentrations. As TIMP-1 limits the activity of MMP-2 (Gomez et al. 1997) and OPN regulates expression and activation of pro-MMP-2 (Xie et al. 2003, Mi et al. 2006), it is not surprising that plasma levels of TIMP-1 and OPN are closely associated with the MMP-2 expression.

In our study after 6 weeks the MMP-2, TIMP-1 and OPN plasma levels were clearly correlated with the HW/BW; thus, concentrations of these markers can be applied to assess severity of heart failure at least in this animal model. In particular, TIMP-1 showed a good correlation with the LVC and therefore might be a useful marker to monitor changes in functional parameters of the myocardium.

Interestingly, MMP-2 protein was less abundant in plasma of DOCA-salt and spironolactone-treated DOCA-salt animals at the end of the trial compared with the

Figure 3. Correlation of plasma marker concentrations with left ventricular contractility (LVC) or the heart weight-to-body weight (HW/BW) ratio. A linear regression analysis was performed to determine the correlation of plasma atrial natriuretic peptide (ANP), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and osteopontin (OPN) concentrations with the LVC or the HW/BW, respectively, of controls, placebo-treated deoxycorticosterone acetate (DOCA)-salt rats and spironolactone-treated DOCA-salt rats. (A,B) No correlation was observed between plasma ANP concentration and the LVC or HW/BW, respectively. (C,D) The plasma MMP-2 levels were associated with the HW/BW, but were not related to the LVC. (E,F) A significant relationship between TIMP-1 and both the LVC and the HW/BW was found. (G,H) The plasma OPN concentration correlated with the HW/BW, but not with the LVC.
□, control, ○, DOCA/NaCl+placebo, Δ, DOCA/NaCl+spironolactone (50 mg kg^{-1} per day).

first weeks. Potentially, induction of MMP-2 gene expression might be an early transient event in the development of heart failure. At later time points in our study or as observed in severe (terminal) heart failure in patients, extracellular matrix degeneration might not play a key role any longer and therefore local MMP-2 synthesis does not show any further increase.

Still it is controversial whether or not plasma ANP reflects changes in morphometric patterns of the heart. Kohno et al. (1992) for example observed a correlation between plasma ANP and the HW/BW in spontaneously hypertensive rat, whereas Cavallero et al. (2007) found no relation between cardiomyocyte diameter and length, and plasma ANP. In our study plasma ANP did not reflect functional or morphological changes of the left ventricle.

As heart failure is characterized by several disorders such as inflammation, pressure overload, left ventricular dilation, cardiac fibrosis, etc., it is reasonable to make use of several plasma markers to assess type, status and severity of this disease in patients and animal models. Furthermore, a combination of several markers potentially can be useful to assess beneficial effects of pharmacotherapy before they become observable by imaging methods or by determining haemodynamic variables. A set of markers that reflect different aspects of the disease can also be useful to decipher off-target or pleiotrophic effects of active substances.

Conclusions

Based on our observations, it seems reasonable to use plasma concentrations of a panel of markers such as MMP-2, TIMP-1 and OPN to monitor the development of heart failure in DOCA-salt hypertensive rat.

It remains to be established whether or not in human patients with heart failure levels of these markers correlate with impairment of cardiac contractility and cardiac fibrosis as determined by imaging methods. In practice, in clinical drug development, determination of MMP-2, TIMP-1 and OPN plasma concentration on the one hand and cardiac imaging on the other hand might complement one another in the assessment of the severity of fibrotic changes in the heart and to reflect potential beneficial effects of pharmacotherapy.

Limitations of our study

Left ventricular function in our study was assessed by invasive catheterization only after 6 weeks. Non-invasive imaging techniques such as small animal MRI or ultrasound offer the possibility for longitudinal correlation of ejection fraction, fractional shortening, mitral valve function and other cardiac variables with plasma biomarker concentration. Future studies should include, for example, echocardiography at different time points during the study protocol.

Furthermore, the histopathological assessment of cardiac tissue at the end of the trial would have provided additional important information.

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