Down-Regulation of Calpain 9 is Linked to Hypertensive Heart and Kidney Disease

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Key Words

Calpain 9 • Dahl rats • Experimental hypertension • Gene expression • Hypertrophy, hypertensive target organ damage

differential regulation of calpain 9 may play a role in hypertensive target organ damage.

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Abstract

Calpains are a family of 14 intracellular calcium-dependent proteases, which have been implicated in cardiovascular diseases. We aimed to analyze specifically the expressional regulation of the different calpain isoforms in hypertensive target organ damage. Using real-time PCR, we found calpain 6 and 9 down-regulated by more than 50% and the endogenous calpain inhibitor calpastatin up-regulated by 225%, respectively, in the hearts of Dahl salt-sensitive rats on a high salt (4% NaCl) compared to normal salt diet. On the protein level, calpain 9 but not calpastatin was regulated in the hypertensive target organs heart and kidney. Moreover, the myocardial expression of calpain 9 protein was inversely linked to left ventricular mass ($r = -0.93$, $p < 0.01$), and renal expression of calpain 9 protein correlated inversely with albuminuria ($r = -0.82$, $p < 0.05$). In the aorta, there was no regulation of calpain 9 on the protein level. We conclude that

Introduction

Calpains are a family of intracellular cysteine proteases, which are increasingly recognized to play a major role in cardiovascular disease. Among the hitherto 14 discovered mammalian family members, calpain 1 and 2 are best studied. They have been linked to myocardial infarction [1], ischemia/reperfusion injury [2] and renal cell death [3]. Calpain activation during ischemia/reperfusion is supposed to cause contractile dysfunction by degrading sarcoplasmic reticulum proteins and cardiac troponin I [4, 5], whereas treatment with the calpain inhibitor leupeptin or overexpression of the endogenous inhibitor calpastatin led to cardioprotective effects. In addition, earlier reports have demonstrated altered calpain 1 or 2 activity in different tissues during arterial hypertension [6, 7]. Little information, however, is available regarding the biochemical properties, mechanisms of regulation, and downstream pathways of

nearly all other calpain isoforms. Whereas certain calpains (like calpain 1, 2, and 9) form hetero-dimeric complexes with the regulatory subunit calpain 4, others are functionally active without further subunits. Our limited knowledge of the non-classical calpains demonstrates the current gap between identification of a gene and the full exploration of its tissue expression and protein function. A good example is calpain 14, which has been discovered recently, but seems to be not expressed in up to 76 tissues examined [8].

In the present study we aimed to analyze a possible involvement of calpain isoforms in hypertensive end organ damage. To that end, we investigated the expression of all calpain isoforms and the endogenous inhibitor calpastatin in the myocardium of Dahl salt-sensitive rats on high versus normal salt diets. In a second step, calpain 9 which we found to be differentially expressed in the hypertensive heart and kidney, was further analyzed on the protein level in heart, kidney and aorta in relation to functional parameters of hypertensive target organ damage.

Materials and Methods

Dahl salt-sensitive rats

Animal experiments were carried out in accordance with the German law for protection of animals. Twenty male Dahl salt-sensitive rats aged 6 weeks (Charles River, Sulzfeld, Germany) were housed individually in standard cages and received chow and tap water ad libitum. They were randomly assigned to either low-salt (n=10) or high-salt (n=10) chow (standard diet #1320 with or without additional 4% sodium chloride, Altromin, Lage, Germany) for 17 weeks. One animal in the low-salt group died during the study after a hemorrhagic infection.

In vivo and ex vivo measurements

Systolic blood pressure was measured by the tail cuff method (TSE-Systems, Bad Homburg, Germany). Food and water uptake over 24h as well as urine excretion (albuminuria) were determined, using a fluorescence dye-binding assay (Mikroflural, Progen Biotechnik, Heidelberg, Germany). Metabolic characterization was performed by collecting urine in metabolic cages (#3701D000, Tecniplast Gazzaada S.a r.l., Buguggiate, Italy) over 24 h.

The rats were anesthetized with pentobarbital (50 mg/kg i.p.), blood was taken via a polyethylene catheter from the jugular vein, collected in chilled tubes containing EDTA and immediately centrifuged at 4°C. The plasma was stored at -80°C until it was used for determination of plasma ACE activity. For this purpose, the hydrolysis of the tripeptide N-[3-2-(furyl)acryloyl]-L-phenylalanyl-glycyl-glycin (FAPPG) into

furylacryloyl-phenylalanine and glycine-glycine was measured spectrophotometrically as the decrease in absorption at $\lambda=340$ nm in comparison to known standards. The heart was excised, the left ventricle dissected in cold saline and rapidly weighed. Aorta and kidney were rapidly removed and rinsed in cold saline. All tissues were flash-frozen in liquid nitrogen; in the case of RNA analysis after addition of RNA later (Qiagen, Hilden, Germany). In previous pilot studies we noticed significant regional differences regarding RNA and protein expression. Therefore, to circumvent these heterogeneities, only whole organs were used; n=5 from each group for RNA analysis and n=4 (low salt) or n=5 (high salt), respectively, for protein isolation. In order to assess endothelial function, aortas were removed, quickly transferred to cold (4°C) oxygenated (95% O₂/5% CO₂) physiological salt solution (PSS), and dissected into 5 mm rings as described previously [9], whereby peri-vascular fat and connective tissue were removed. After 1 h equilibration, aortic ring contractile force was measured isometrically using standard bath procedures as described. The composition of PSS (in mM) was 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 1.2 MgSO₄, 11.1 glucose, 1.6 CaCl₂. The volume of the bath solution was 15 mL. Steady-state tension (100%) was obtained with isotonic external 60 mM KCl and relaxation was measured in response acetylcholine (100nM).

RNA isolation, Reverse transcription and Real-time PCR

RNA was isolated from left ventricles, kidney and aorta (RNeasy Mini Kit, Qiagen) and DNase digested (DNA-free, Ambion, Austin, USA) according to the instructions of the manufacturers. Total RNA was reverse transcribed using Omniscript Reverse Transcriptase (Qiagen). Real-time PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) and the i-Cycler (BIO-RAD, München, Germany). Conditions were as follows: 95°C for 15 minutes, followed by 40 cycles each for 30 sec at 94°C, a gene-specific annealing temperature T_m (see Table 1), and 72°C. In the heart, mRNA expression was determined for all calpain isoforms and calpastatin. In kidney and aorta, only calpain 9 expression was measured. Specificity of the PCR was verified by gel electrophoresis, cloning and sequencing of the PCR products, and melting curve analysis. Absence of contaminations was checked by reactions without template or with RNA instead of cDNA.

Western Analysis

Frozen tissues were homogenized in a buffer consisting of 10mM Tris-HCl, 1% sodiumdodecylsulfate (SDS) adjusted to pH 7.6, containing a set of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany), with a Freezer Mill (C3-Analysentechnik, München, Germany). After sonification and ultra-centrifugation at 100,000xg for 30 min, supernatants were mixed with SDS-sample buffer and heated for 20 min at 70°C. Subsequently, proteins were separated on 4-12% BisTris-NuPAGE gels (Invitrogen, Karlsruhe, Germany) with MOPS running buffer. After immunoblotting onto nitrocellulose membranes (PALL, Dreieich, Germany), membranes were blocked with LI-COR blocking buffer (LI-COR Biosciences, Bad Homburg, Germany), diluted 1:2 with PBS.

Table 1. Primer sequences and annealing temperature T_m

Gene	Forward primer	Reverse primer	T_m [°C]
Calpain 1	5'-gaagaggagattgatgacaact-3'	5'-ctgttcaagatggctctgtage-3'	53
Calpain 2	5'-ataccattaaccaaatagcaaat-3'	5'-acgatgtgtagaagtagaaagga-3'	50
Calpain 3	5'-tgccatctattcagctatcat-3'	5'-tttctctctaggcactcttg-3'	50
Calpain 4	5'-aatcagcatatctacagcatga-3'	5'-atctgaaagcacggaaacat-3'	50
Calpain 5	5'-ggatttcacaggtgggtgttct-3'	5'-tcttctcctctcactcttgct-3'	50
Calpain 6	5'-ctgctgctagattacaacaa-3'	5'-gactctcaaggteccatataga-3'	53
Calpain 7	5'-aaccagagtacaatccctgtgg-3'	5'-gtaccttccttccagcgtaa-3'	53
Calpain 8	5'-gctgctccattgatgtctc-3'	5'-acctcttcgactccagtaacag-3'	50
Calpain 9	5'-agctggatttcgatgactac-3'	5'-actcgtttatgttgatggat-3'	57
Calpain 10	5'-agagttctgggtagaggagag-3'	5'-gtatggcacagcactct-3'	57
Calpain 11	5'-aagtctcaagaagtcataacg-3'	5'-atgtccgtgggagagaaat-3'	50
Calpastatin	5'-aactttctgctgctgttct-3'	5'-agttcttctctcctctcag-3'	50
GAPDH	5'-gagtctactggcgtcttcac-3'	5'-ttgaggagattgtcatattct-3'	53

Antibody incubations were in the same solution including 0.25% Tween-20, with primary antibodies for calpain 9, calpastatin (#RP3Calpain-9, #RP2calpastatin; both TriplePointBiologics, Portland, USA), actin or β -tubulin (#sc-1615(C-11), #sc-9104(H-235); both Santa Cruz Biotechnology, California, USA). Specifically bound primary antibodies were detected with goat-anti-rabbit or donkey-anti-goat secondary antibodies (both labeled with Alexa Fluor 680; MoBiTec, Göttingen, Germany) and the Odyssey Imaging System (LI-COR Biosciences).

Statistical analysis

Results are means $\pm$ SEM. For statistical analysis student's *t* test was used. * denotes $p < 0.05$, ** $p < 0.01$ compared to healthy control animals.

Results

Development of hypertension and subsequent target organ damage in Dahl rats

After four months of feeding with 4% salt in chow, systolic blood pressure raised significantly to 201 ± 5 mm Hg in high-salt Dahl salt-sensitive rats, in comparison to 168 ± 3 mm Hg in low-salt rats ($p < 0.001$). Body weights were comparable; high-salt rats weighed 420.61 ± 8.92 g, low-salt rats 422.54 ± 5.37 g. Weight of left ventricles at the end of the study was substantially increased in high-salt rats, showing occurrence of cardiac hypertrophy (Fig. 1A). Urine albumin secretion was strongly elevated as a clear sign of pathological changes in kidney (Fig. 1B). Since we measured a diminished acetylcholine-induced

relaxation in aortas of high-salt rats (-43.48 ± 3.58 % in high-salt vs. -60.92 ± 6.13 % in low-salt rats, $p < 0.05$), these vessels showed a slight endothelial dysfunction. Plasma ACE activity tended to be lower in high-salt compared to low-salt rats (78.1 ± 7.5 vs. 90.5 ± 10.3 U/L), but the difference was not significant ($p > 0.05$).

Expression of calpain isoforms in Dahl salt-sensitive rat hearts

We analyzed the expression profile of calpain 1 up to calpain 11 in hearts of Dahl salt-sensitive high- and low-salt rats by Real-time PCR. No expression of calpain 8 and 11 could be detected in healthy and diseased hearts. Interestingly, the isoforms 6 and 9 showed a significant down-regulation by more than 50%, whereas calpain 1, 2, 3, 4, 5, 7 and 10 were not regulated (Fig. 2A). The endogenous inhibitor of the calpains, calpastatin, was up-regulated by 225%. The threshold cycle (C_t)-values were higher for calpain 9 ($C_t = 30$ to 32) compared to calpains 1, 2 and 4 ($C_t = 24$ to 26), indicating a less abundant expression. Calpain 9 was significantly down-regulated by more than 50% in the kidney, but not aorta, from high-salt rats (Fig. 2B).

Calpain 9 protein decreases in Dahl salt-sensitive rats

In order to analyze whether not only calpain 9 mRNA but also protein is down-regulated, we performed Western blots with a specific antibody and noticed a statistically significant down-regulation by 50% in the hearts of high-

Fig. 1. Left ventricular weight (A) and urinary albumin excretion (B) in Dahl salt-sensitive rats (low-salt group n=9, high-salt group n=10).

salt rats (Fig. 3A). Among other relevant cardiovascular organs, we observed a significant decrease in calpain 9 protein also in kidney. In lysates taken from aorta, no difference between high- and low-salt rats could be observed. Within the three organs examined, aortas displayed the highest degree of variation of calpain 9 expression even within one group. All blots were probed with actin antibodies and the calpain 9 expression was normalized to actin as housekeeping gene. Signal variations of the actin bands in the heart samples may be due to slight different protein loading. Normalization to α -tubulin expression instead of actin yielded similar results (data not shown).

Due to the increased calpastatin mRNA expression in heart of high-salt rats, we investigated additionally the expression of calpastatin protein by Western blotting. No

Fig. 2. Calpain and calpastatin mRNA expression of Dahl salt-sensitive rats (each group n=5). (A) Upper panel: Calpain isoform (C) and calpastatin (CAST) expression in heart of Dahl salt-sensitive rats. mRNA expression in low-salt rats was set to 100%. All values were normalized to GAPDH expression. Calpain 6 and 9 were significantly down-regulated to less than 50%. Lower panel: Calpain 8 and 11 were not detectable in heart whereas positive controls showed a strong signal (as shown for calpain 11 with testis as positive control). The calpain 9 PCR product is shown as representative example of specific PCR conditions for each gene. NTCs (no template controls) and controls with RNA instead of cDNA as template were always negative. (B) Calpain 9 mRNA expression in heart and kidney. Calpain 9 was down-regulated by more than 50% in kidney of high-salt rats whereas the aortic expression remained unchanged.

Fig. 3. (A) Calpain 9 down-regulation in heart and kidney of Dahl salt-sensitive rats. Total protein from heart, kidney and aorta was isolated and used in a Western against a specific calpain 9 antibody. (B) Western analysis of calpastatin (CAST)

protein expression in heart of Dahl salt-sensitive rats. No difference in the expression level between high- and low-salt animals could be detected (low-salt group n=4, high-salt group n=5).

difference in the cardiac calpastatin protein level between high- and low-salt rats could be observed (Fig. 3B). Similarly, kidney and aorta also showed the same calpastatin expression in both groups (data not shown).

To test the correlation between the calpain 9 protein level and the left ventricular weight or albuminuria, respectively, in individual animals, we plotted these values against each other and determined the Pearson's correlation coefficient (Fig. 4). The results show that the calpain 9 protein expression is inversely proportional to the left ventricular weight/100 g body weight with a correlation coefficient of -0.93 ($p < 0.01$), and to the albuminuria with a correlation coefficient of -0.82 ($p < 0.05$).

Discussion

The present study demonstrates that calpain 9 is differentially regulated in the heart and kidney during salt induced arterial hypertension. These data indicate a potential role for this calpain isoform in the development of hypertensive target organ damage.

No information is available so far about the specific regulation of the different calpain isoforms in arterial hypertension. Since expressional changes play often an important role in calpain regulation [10-12], we analyzed the expression profile of all calpain isoforms in Dahl salt-sensitive rats on high and normal salt diets. As expected, the high salt diet led to arterial hypertension, myocardial hypertrophy, proteinuria, and endothelial dysfunction. These pathologies were associated with significant down-regulation of calpain 6 and 9 mRNA in high-salt rats during cardiac hypertrophy.

Fig. 4. (A) Correlation of cardiac calpain 9 expression to left ventricular weight/100 g bodyweight. (B) Correlation of kidney calpain 9 expression to albumin excretion (low-salt group $n=4$, high-salt group $n=5$).

In our further experiments we focused on calpain 9, because calpain 6 lacks critical amino acids in the catalytic triade and therefore is most likely not proteolytically active [13]. Hence, we analyzed specifically the calpain 9 mRNA expression additionally to cardiac tissue in two other cardiovascular target organs, kidney and aorta. We found calpain 9 down-regulated in the kidney, but not aorta. To prove whether the down-regulation of calpain 9 mRNA is mirrored on the protein level, we performed Western Blots with lysates from heart, kidney and aorta. We demonstrated that calpain 9 protein was down-regulated in the hypertensive heart and kidney, whereas aortic expression was unaffected. The striking correlations between calpain 9 expression and myocardial hypertrophy

or proteinuria, respectively, emphasize the potential link between this isoform and hypertensive target organ damage. Although correlations are not sufficient to establish a causal relationship, our findings set the stage for future investigations on the pathophysiological role of calpain 9 in cardiovascular disease.

Calpain 9, originally designated nCL-4 (novel Calpain Large subunit-4), is not ubiquitously expressed in all tissues. Based on the mRNA expression levels (C_t -values), calpain 9 seems to be less abundantly expressed compared to calpains 1, 2 and 4. It must be kept in mind, however, that Real-time PCR does not allow an exact comparison of expression levels between various genes because of potential differences in amplification efficacy. Beside the expression in cardiovascular organs, as demonstrated in this study, calpain 9 is highly expressed in stomach and small intestine [14]. The potential role of calpain 9 in gastroenterology is highlighted by its down-regulation in gastric cancer cells [15]. From a pharmacological point of view, the relatively specific expression of calpain 9 may offer the opportunity for specific intervention to treat cardiovascular disease.

Regarding the functional consequence of the calpain 9 regulation, Liu *et al.* showed that a calpain 9 down-regulation of approximately 50% had a profound regulatory effect because it led to cellular transformation of murine NIH3T3 fibroblasts and tumor formation by injection of these cells into nude mice [16]. Hence, we assume that our finding of calpain 9 down-regulation during hypertension may have a pathological meaning as well. To date, further mechanistic studies on calpain 9 are currently not feasible due to the lack of specific inhibitors. Research on calpain 9 is additionally hampered by the fact that no substrates have been identified up to now. In transfected COS-7 cells a juxtanuclear and/or nuclear staining of calpain 9 has been observed [17]. Therefore it may be speculated that transcription factors could be among the possible targets. E.g., NFAT3, MEF-2 and GATA-4 have been reported to play a role in cardiac hypertrophy [18, 19]. Moreover, fos and jun, known substrates of calpain 1 and 2 [20], can act as causal mediators of hypertrophic growth of cardiomyocytes [21, 22]. The search of substrates for calpain 9, especially in heart and kidney as target organs, may be a key activity in order to understand the mode of action of this calpain isoform in cardiovascular diseases.

The current study demonstrates that we can rule out any involvement of calpain 8 and 11 isoforms, since they were not expressed in hearts and kidneys of healthy

and diseased rats. In contrast, we obtained strong signals with stomach and testis as positive control tissues for calpain 8 and 11, respectively. Furthermore, we could not detect any calpain 8 and 11 mRNA in kidneys and hearts of db/db- and db⁻-mice, a model of diabetic nephropathy (unpublished data). Our finding of calpain 8 absence in the kidney is somehow controversial to results by Braun *et al.* who demonstrated an expression of this isoform in murine kidney by Northern blot [23]. On the other hand, it is supported by Sorimachi *et al.* reporting missing calpain 8 expression in rat kidney [24]. In line with the present calpain 11 data, Dear *et al.* showed missing mRNA expression of calpain 11 in human heart and only minimal traces in kidney by dot blot [25]. Regarding other isoforms, we could clearly demonstrate that calpains 1, 2, 3, 4, 5, 7 and 10 were expressed in cardiac tissue, but their mRNA expression did not change under salt load.

One interesting finding was the up-regulation of calpastatin mRNA by more than 200% in the heart of Dahl salt-sensitive high-salt rats, which does not translate in increased protein amount. Our findings are in good

accordance with results of another rat model of hypertension. Averna *et al.* reported that kidney calpastatin mRNA levels of 30 and 90 days old hypertensive rats from the Milan strain were elevated vs. age-matched normotensive rats [26]. In contrast, hypertensive rats showed the same (30 days) or a smaller (90 days) calpastatin protein amount due to increased calpastatin degradation. These similarities regarding alteration in calpastatin mRNA and protein expression lead us to the conclusion that independent of the origin of hypertension in these different rat models, similar mechanisms in calpain regulation may occur.

Among the different calpain isoforms, we have identified calpain 9 as the most prominent isoform involved in cardiac and renal damage during salt sensitive arterial hypertension. Calpain 9, being a protease, belongs to a target class that is generally considered “druggable” by the pharmaceutical industry. Therefore, pharmacological modulation of calpain 9 may lead to novel treatment options for prevention and treatment of hypertensive target organ damage.

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