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Effect of Long-term Monotherapy with the Aldosterone Receptor Blocker Eplerenone on Cytoskeletal Proteins and Matrix Metalloproteinases in Dogs with Heart Failure

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Abstract
Purpose Long-term monotherapy with the aldosterone receptor blocker eplerenone in dogs with HF was previously shown to improve LV systolic and diastolic function. This study examined the effects of long-term monotherapy with the aldosterone receptor blocker eplerenone on mRNA and protein expression of the cytoskeletal proteins titin, tubulin, fibronectin and vimentin, the matrix metalloproteinases (MMPs)-1, -2 and -9, and the tissue inhibitors of MMPs (TIMPs)-1 and -2 in left ventricular (LV) myocardium of dogs with heart failure (HF).
Methods HF was produced in 12 dogs by intracoronary microembolizations. Dogs were randomized to 3 months oral therapy with eplerenone (10 mg/kg twice daily, $n=6$) or to no therapy at all (HF-control, $n=6$). LV tissue from six normal dogs was used for comparison. mRNA expression was measured using reverse-transcriptase polymerase chain reaction (RT-PCR) and protein expression using Western blots.
Results Compared to NL dogs, control dogs showed upregulation of mRNA and protein expression for tubulin, fibronectin, MMP-1, -2 and -9, and down-regulation of mRNA and protein expression for total titin. Normalization of mRNA and protein expression for all these genes was seen after treatment with eplerenone. N2BA/N2B-titin mRNA expression ratio increased significantly in dogs with HF treated with eplerenone. No differences in expression for vimentin, TIMP-1 and -2 were observed among groups.
Conclusions In dogs with HF, long-term eplerenone therapy normalizes mRNA and protein expression of key cytoskeletal proteins and MMPs. Reversal of these molecular maladaptations may partly explain the improvement in LV diastolic function seen after long-term therapy with eplerenone.

Key words titin · heart failure · cytoskeletal proteins · reverse remodeling

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Introduction

Aldosterone plays a key role in the pathophysiology of heart failure (HF). It promotes sodium retention and loss of potassium and is implicated in the development of myocardial interstitial fibrosis. Structural remodeling of the interstitial collagen matrix is regulated in part by angiotensin II and aldosterone [1, 2]. It has been documented that aldosterone causes myocardial fibrosis and plays a major role in the development of HF and is therefore, a key target for therapeutic interventions [3]. Thus inhibition of angiotensin II and/or blockade of aldosterone in the setting of HF may attenuate progressive interstitial fibrosis and, in doing so, reverse ventricular remodeling and improve left ventricular (LV) diastolic function and ultimately systolic function.

The cytoskeleton maintains the cell's structural integrity with the help of key cytoskeletal proteins such as titin, tubulin and fibronectin that also modulate myocardial stiffness. The myocardium co-expresses two titin isoforms with distinct stiffness characteristics, the stiff N2B isoform and more compliant N2BA isoform [4]. Accumulation of collagen in the cardiac interstitium or "reactive interstitial fibrosis" (RIF) occurs in HF and contributes to LV dysfunction and

remodeling [5, 6]. The matrix metalloproteinases MMP-1, -2 and -9 are upregulated in HF and contribute to RIF [7, 8]. The cytoskeletal proteins, and collagen network are important determinants of LV compliance properties and alterations in their expression can lead to increased LV chamber stiffness and ultimately to LV diastolic dysfunction. Human studies have confirmed the anti-hypertensive effect and benefits of aldosterone receptor antagonists in congestive heart failure [9]. The "Eplerenone Post-acute Myocardial Infarction Heart Failure Efficacy and Survival Study" (EPHESUS) demonstrated that when eplerenone was added to standard medical therapy in patients with LV dysfunction and clinical evidence of heart failure following acute myocardial infarction there was significant improvement in mortality and morbidity [10, 11]. Although diastolic dysfunction has been recognized in a variety of patients with HF, its molecular basis is not fully understood. We previously showed that eplerenone, in addition to improving LV systolic function, also reduced LV filling pressure and end-diastolic wall stress and stiffness, and improved LV relaxation in dogs with chronic HF [5]. The present study examined the effects of chronic therapy with eplerenone on mRNA and protein expression N2BA and N2B-titin isoforms (mRNA only), total-titin, tubulin-α, tubulin-β, fibronectin, vimentin, MMP-1, MMP-2 and MMP-9 and on tissue inhibitors of matrix metalloproteinases TIMP-1 and TIMP-2 in LV myocardium of dogs with intracoronary microembolization-induced HF.

Materials and methods

Animal model

The canine model of chronic HF used in this study has been previously described in detail [12]. In this study, chronic LV dysfunction was produced by multiple sequential intracoronary embolizations with polystyrene latex microspheres (70 to 102 μm in diameter) which results in loss of viable myocardium. The model manifests many of the sequelae of HF seen in humans, including marked depression of LV systolic and diastolic function, reduced cardiac output, and increased LV filling pressures [12]. In the present study, 12 mongrel dogs weighing between 20 and 31 kg underwent serial coronary microembolizations to produce HF. Embolizations were performed 1 to 3 weeks apart and were discontinued when LV ejection fraction (EF) was 30% to 40%. Microembolizations were performed during cardiac catheterization under general anesthesia and sterile conditions. Anesthesia consisted of a combination of intravenous injections of oxymorphone (0.22 mg/kg), diazepam (0.17 mg/kg), and sodium pentobarbital (150 to 250 mg to effect) [13]. The present study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee and complied with the "Position of the American Heart Association on Research Animal Use."

Study protocol

Two weeks after the last microembolization, dogs underwent a pre-randomization left and right heart catheterization. One day later, dogs were randomized to 3 months of oral therapy with eplerenone (10 mg/kg twice daily, n=6) or to no therapy at all (HF-control, n=6). An additional group of six normal (NL) dogs did not undergo any microembolizations and served as NL LV tissue donors. In all HF dogs, final hemodynamic and angiographic measurements were made at the end of 3 months of therapy. While under anesthesia, the dog's chest was opened, the heart was removed, and LV tissue was prepared for biochemical evaluation.

mRNA expression

Total RNA was isolated from frozen LV tissue in RNA Stat-60 using the guanidinium thiocyanate phenol-chloroform method according to the manufacturer's instructions (Tel-Test Inc. Friendswood, TX) and as previously reported [14–16]. Briefly, concentration and quality of the isolated RNA in each sample were determined spectrophotometrically. RNA with an absorbance ratio (260 nm/280 nm) above 1.7 and that exhibited three major bands namely, 28S, 18S, and 5.8S on 2% agarose with 28S being much stronger than 18S, was considered of good quality. Approximately 4 μg RNA was reverse-transcribed into cDNA in an assay volume of 80 μl. The assay contained (final concentration) 3.6 mM of each dNTP (dATP, dTTP, dGTP, and dCTP), 40 U recombinant RNasin (RNase inhibitor, Promega), 6 μM oligo (dT) primer, and 1 U MMLV reverse transcriptase in 10 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM DTT and 3.0 mM $MgCl_2$. Assay tubes were incubated at 42°C for 60 min and then at 96°C for 10 min for denaturation. For each PCR reaction, 2 μl first-strand cDNA was added to 18 μl of a reaction mixture containing 20 pmol of each gene-specific forward and reverse primer, 200 μM of each dNTP, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X100 and 3.0 mM $MgCl_2$. After heating the tube to 95°C for 5 min, 1-U platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) was added and the PCR was allowed to proceed for 20 to 40 cycles. PCR products were analyzed by subjecting 20 μl of each reaction mixture to electrophoresis on 1%–1.5% ethidium-bromide–agarose gels. Band size of the products was compared with standard DNA size markers and confirmed by sequencing. The primers for total titin, N2BA-titin, N2B-titin, tubulin-α, tubulin-β, fibronectin, vimentin, MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and GAPDH, a house keeping gene reportedly unchanged in HF, were based on the gene sequences reported to Gene Bank

(Table 1). Band intensity was quantified in arbitrary densitometric units (du) using a Bio-Rad Gel densitometer.

Protein immunoblot analysis

Protein levels of total titin, tubulin-α, tubulin-β, fibronectin, vimentin, MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured in LV homogenate by Western blot. Briefly, LV homogenate was prepared from approximately 100 mg LV powder as described previously [17, 18] and protein assay was determined by Lowry's method [19]. Approximately 20–100 μg protein of each dog sample was separated on 4–20% SDS–polyacrylamide gel (Bio-Rad) and the separated proteins were electrophoretically transferred to a nitrocellulose membrane. The accuracy of the electrotransfer was confirmed by staining the membrane with 0.1% amido black. Titin was separated on 4% SDS–polyacrylamide gel and similar method was used to identify the protein. For identification of the desired protein, the nitrocellulose blot was incubated with the appropriately diluted primary antibody specific to each protein, based on the supplier's instructions (Table 2). Antibody-binding protein(s) was visualized by autoradiography after treating the blot with horseradish peroxidase-conjugated secondary antibody and ECL color developing reagents according to the supplier (Table 2). Band intensity was quantified using a Bio-Rad GS-670 imaging densitometer and expressed as du. In all circumstances, we made sure the antibody was present in excess over the antigen and the density of each protein band was in the linear scale.

Data analysis

Comparisons among study groups were carried out using one way analysis of variance (ANOVA), with α set at 0.05.

Table 2 Antibody source and identity

Antibody	Source	Identity
Total-titin	Mouse monoclonal	Abcam, Inc. Cambridge, MA (ab7034)
Fibronectin	Mouse monoclonal	Sigma-Aldrich, St.Louis, MO (F0916)
Tubulin-α	Mouse monoclonal	Abcam, Inc. Cambridge, MA (ab7750)
Tubulin-β	Mouse monoclonal	Chemicon, Inc. Temecula, CA (MAB3408)
Vimentin	Mouse monoclonal	Sigma-Aldrich, St.Louis, MO (V6630)
MMP-1	Mouse monoclonal	Chemicon, Inc. Temecula, CA (MAB3307)
MMP-2	Mouse monoclonal	US Biologicals, Swampscott, MA (M2420-50)
MMP-9	Rabbit polyclonal	US Biologicals, Swampscott, MA (M2425-01)
TIMP-1	Mouse monoclonal	Chemicon, Inc. Temecula, CA (MAB3300)
TIMP-2	Rabbit polyclonal	Chemicon, Inc. Temecula, CA (AB19029)

If significance was achieved, pair wise comparisons were performed using the Student–Neuman–Keuls test. For this test, a probability value $p<0.05$ was considered significant. All data are expressed as means±SEM.

Results

mRNA expression of GAPDH was not significantly different in LV myocardium of NL, HF-controls, and eplerenone treated HF dogs.

Table 1 Primer sequences and product sizes

Genes	Sequences	Product size	Accession number
GAPDH	F: 5′–ACCACC ATG GAG AAGGCT GG-3′ R: 5′-CTCAGT GTA GCC CAGGAT GC-3′	528	AB038240
Total-titin	F: 5′-GTA CCA GAG CCA CCC CAA A-3′ R: 5′-TCT CCA CCA CTT CCG GTC T-3′	368	XM_535982
N2BA-Titin	F: 5′-GCT CTC CGT TCT TGA ACC TG-3′ R: 5′-CTG CTG ACC TCG TTT CCT TC-3′	435	AY136513
N2B-titin	F: 5′-GTA CCA GAG CCACCTCCA AA-3′ R: 5′-TCT CCA CCA CTT CCT GGT CT-3′	368	U82221
Tubulin-α	F: 5′-CCA CCA TGC GTG AGG TAT C-3′ R: 5′-TGC ACT TGT CAG CCG TTT C-3′	393	XM_848429
Tubulin-β	F: 5′-GGC CAT CAT GTT CTG GAG T-3′ R: 5′-CTT ACA ACG CCA CCT GTC-3′	359	XM_845405
Fibronectin	F: 5′-ATG CTA CCG AGACCACCA TC-3′ R: 5′-CCT TCC AAT CAG AGGCTC AC-3′	494	U52106
Vimentin	F: 5′-ATG CTT CTC TGG CAC GTC TT-3′ R: 5′-ACG AGC CAT CTC TTC CTT CA-3′	500	XM_844313
MMP-1	F: 5′-GGG CTG TAG GCT TGCAGTAG-3′ R: 5′-CTT GCC TGT CCA GTG TCT CA-3′	442	DQ279458
MMP-2	F: 5′-CTG GCT GTG CAA TACCTGAA-3′ R: 5′-CAG GGT CCA TAG CTCATC GT-3′	501	AF177217
MMP-9	F: 5′-ATC GCG GAG ATCAGGAACTA-3′ R: 5′-GTC CAC CTG GTT CAC CTC AT-3′	507	NM_001003219
TIMP-1	F: 5′-TAC TCC CCC TGC CTA TTC CT-3′ R: 5′-CCA ACC TGG GGAAATACTCA-3′	495	AB016817
TIMP-2	F: 5′-CAA AGG ACCAGACAAGGACA-3′ R: 5′-GAA GGG ATG TCAGAGCTGGA-3′	502	AF112115

Effect of eplerenone therapy on cytoskeletal proteins

Results of mRNA expression of cytoskeletal proteins are shown in Table 3 and Fig. 1. mRNA expression of total titin was significantly downregulated where as, mRNA expression of tubulin-α, tubulin-β and fibronectin was significantly upregulated in HF-control dogs compared with NL dogs. Three months of eplerenone therapy restored mRNA expression of total titin, tubulin-α, tubulin-β and fibronectin to near normal levels. Results of protein expression of cytoskeletal proteins are shown in Table 4 and Fig. 2. Protein expression of total titin was significantly downregulated and protein expression of tubulin-α, tubulin-β and fibronectin was significantly increased in HF-control dogs compared with NL dogs. Eplerenone therapy for 3 months increased protein expression of total titin and reduced the expression of tubulin-α, tubulin-β and fibronectin to near normal levels. No significant changes were observed in mRNA and protein expression of vimentin between NL dogs, HF-control dogs and HF dogs treated with eplerenone.

mRNA expression of the N2BA-titin isoform was significantly reduced and that of the N2B-titin isoform was significantly increased in HF-control dogs compared to NL dogs. Long-term therapy with eplerenone significantly increased mRNA expression of N2BA-titin isoform and decreased mRNA expression of N2B-titin isoforms to near NL levels (Figs. 3 and 4). A decrease in N2BA/N2B-titin mRNA expression ratio occurred in HF-control dogs compared to NL dogs and this reduction was reversed after long-term eplerenone monotherapy (Fig. 5).

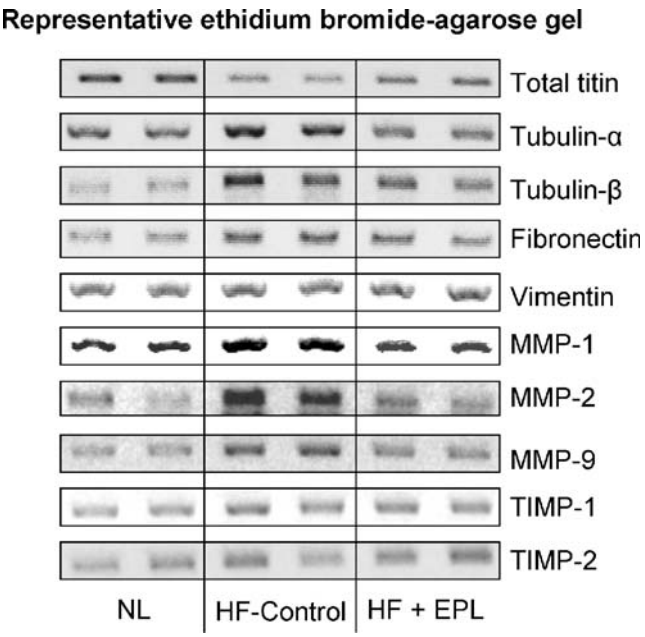

Fig. 1 Representative ethidium bromide–agarose gel showing mRNA encoding total titin, tubulin-α, tubulin-β, fibronectin, vimentin, MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and GAPDH in LV myocardium of two normal dogs (NL), two dogs with HF that are not treated (HF-control), and two dogs with heart failure treated with eplerenone (HF+EPL)

Effect of eplerenone therapy on MMPs and TIMPs

mRNA expression of MMP-1 and the gelatinases MMP-2 and MMP-9 were increased significantly in HF-control dogs compared with NL (Table 3, Fig. 1). Chronic eplerenone therapy restored mRNA expression of MMP-1, MMP-2 and MMP-9 to near normal levels. Similarly, protein expression

Table 3 mRNA expression for cytoskeletal proteins, matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in left ventricular myocardium of normal dogs (NL), dogs with HF that are not treated (HF-control) and dogs with HF treated with eplerenone (HF+EPL)

	NL (n=6)	HF-control (n=6)	HF+EPL (n=6)
Total titin (du)	16±1	11±1*	15±1**
Fibronectin (du)	10.5±1.5	21.3±1.9*	15.6±0.8*·**
Tubulin-α (du)	29.6±1.6	46.4±1.5*	24.6±3.7**
Tubulin-β (du)	10.9±0.8	41.1±3.3*	27.7±2.9*·**
Vimentin (du)	20.6±1.0	22.6±0.3	22.5±1.5
MMP-1 (du)	39±2	69±1*	43±2**
MMP-2 (du)	37.2±4.6	76.1±13.3*	36.9±4.6**
MMP-9 (du)	13.6±1.5	26.6±2.3*	17.0±1.5**
TIMP-1 (du)	23.4±1.3	24.5±0.7	25.3±2.9
TIMP-2 (du)	25.5±2.4	26.6±3.8	28±2.5
GAPDH (du)	10.7±0.2	10.8±0.1	10.7±0.2

du densitometric units, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of matrix metalloproteinase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase
*p<0.05 vs. NL; **p<0.05 vs. HF

Table 4 Protein expression for cytoskeletal proteins, matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in left ventricular myocardium of normal dogs (NL), dogs with HF that are not treated (HF-control) and dogs with HF treated with eplerenone (HF+EPL)

	NL (n=6)	HF-control (n=6)	HF+EPL (n=6)
Total titin (du)	5,875±211	3,803±59*	4,968±189*·**
Fibronectin (du)	2,980±139	7,149±438*	3,394±304**
Tubulin-α (du)	1,438±293	3,418±446*	1,526±371**
Tubulin-β (du)	2,186±191	3,342±147*	2,157±205**
Vimentin (du)	3,959±119	4,025±155	3,832±222
MMP-1 (du)	1,663±212	3,984±240*	2,373±147*·**
MMP-2 (du)	1,660±216	3,180±350*	1,867±107**
MMP-9 (du)	2,937±304	5,622±323*	3,673±270**
TIMP-1 (du)	5,223±512	5,533±739	4,914±436
TIMP-2 (du)	3,052±144	3,174±188	3,304±128

du densitometric units, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of matrix metalloproteinase. *p<0.05 vs. NL; **p<0.05 vs. HF

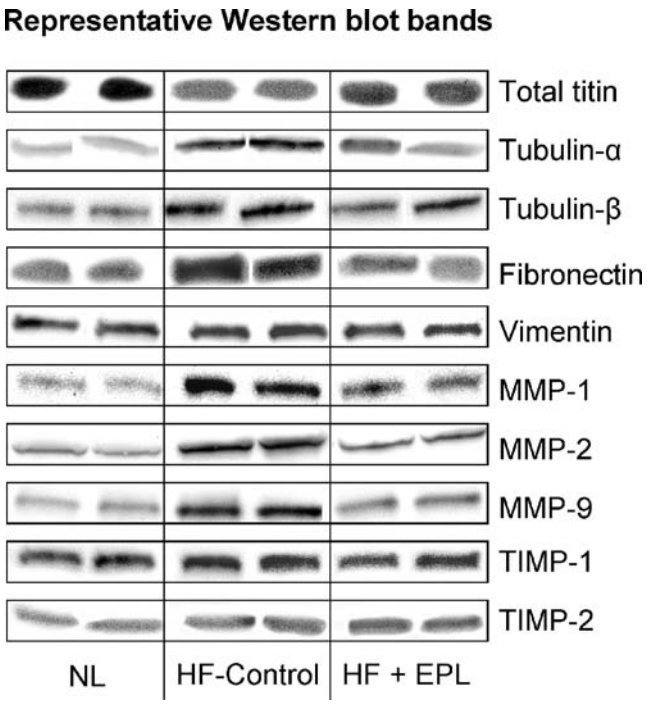

Fig. 2 Representative Western blot bands showing protein expression of total titin, tubulin-α, tubulin-β, fibronectin, vimentin, MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in LV myocardium of two normal dogs (NL), two dogs with HF that are not treated (HF-control), and two dogs with heart failure treated with eplerenone (HF+EPL)

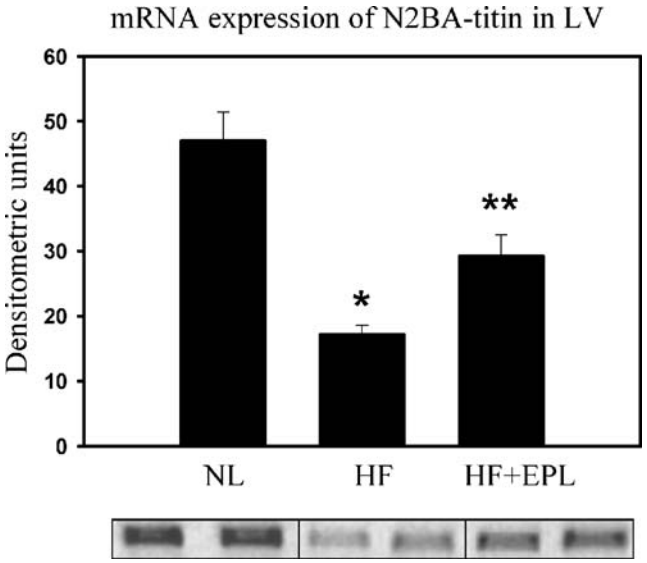

Fig. 3 Bar graph depicting band intensity in densitometric units for mRNA expression of N2BA-titin in LV myocardium of six normal dogs (NL), six dogs with HF that are not treated (HF-control), and six dogs with heart failure treated with eplerenone (HF+EPL). *Bottom*: ethidium bromide-agarose gel showing mRNA encoding N2BA-titin in LV myocardium of two normal dogs, two HF-control dogs, and two dogs with heart failure treated with eplerenone. *p<0.05 vs. NL, **p<0.05 vs. HF-control

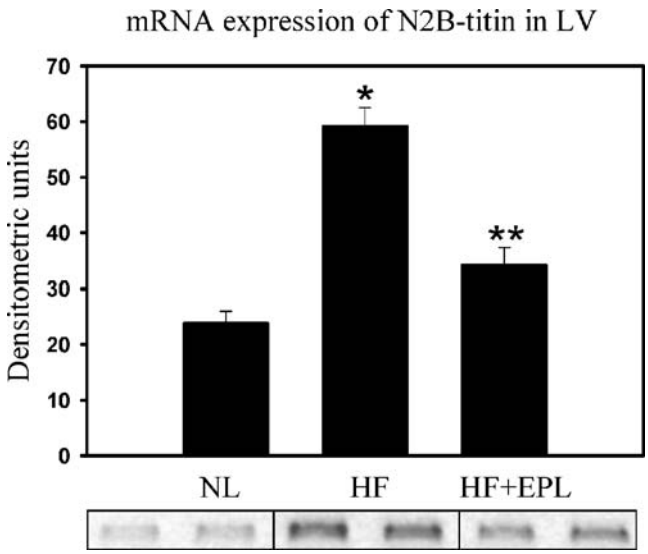

Fig. 4 Bar graph depicting band intensity in densitometric units for mRNA expression of N2B-titin in LV myocardium of six normal dogs (NL), six dogs with HF that are not treated (HF-Sham), and six dogs with heart failure treated with eplerenone (HF+EPL). *Bottom*: ethidium bromide–agarose gel showing mRNA encoding N2B-titin in LV myocardium of two normal dogs, two HF-control dogs, and two dogs with heart failure treated with eplerenone. *p<0.05 vs. NL, **p<0.05 vs. HF-control

of MMP-1, MMP-2 and MMP-9 was significantly upregulated in HF-control dogs compared with NL dogs (Table 4, Fig. 2). A significant downregulation was observed after eplerenone therapy. There were no significant differences among groups with respect to mRNA and protein expression of TIMP-1 and TIMP-2.

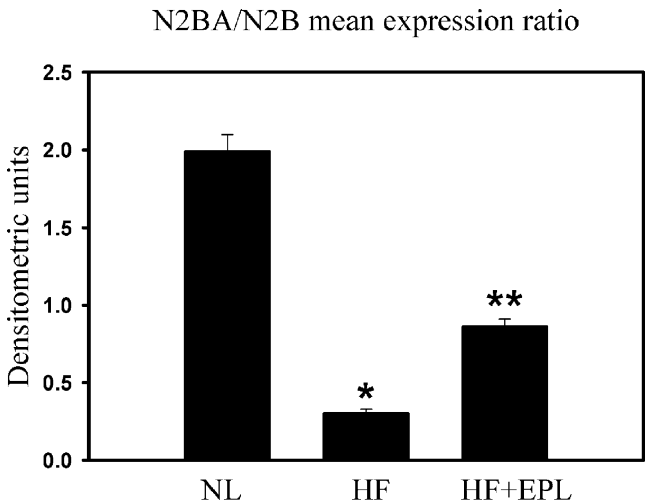

Fig. 5 Bar graph depicting band intensity of N2BA/N2B-titin expression ratio in densitometric units (du) in LV myocardium of six normal dogs (NL), six dogs with HF that are not treated (HF-control), and six dogs with heart failure treated with eplerenone (HF+EPL). Values are mean±SEM. *p<0.05 vs. NL, **p<0.05 vs. HF-control

Discussion

Attenuation of maladaptive ventricular remodeling has become an important goal in the treatment of heart failure. Earlier studies from our laboratory demonstrated that long-term aldosterone receptor blockade with eplerenone in animals with experimentally-induced HF can prevent progressive LV systolic dysfunction and chamber remodeling and improve LV diastolic function [5]. The present study demonstrates that long-term therapy with eplerenone in LV myocardium of dogs with HF normalizes maladaptive mRNA and protein expression of several key cytoskeleton proteins namely, total-titin, N2BA and N2B-titin, tubulin-α and -β, fibronectin, and key matrix metalloproteinases specifically MMP-1, -2 and -9. These genes are involved in the process of LV remodeling as well as in LV diastolic dysfunction that often accompanies the HF state.

The effects of aldosterone on the heart include but are not limited to ventricular hypertrophy, interstitial and perivascular fibrosis, and structural remodeling [20]. Although an inhibition in the production of aldosterone is observed after treatment with ACE inhibitors, the inhibition may be incomplete and escape of aldosterone occurs [21, 22]. Aldosterone receptor antagonism imparts its beneficial effects in patients with heart failure by various mechanisms which include increases in myocardial norepinephrine uptake and vascular compliance [20] and reduction of cardiomyocyte hypertrophy, interstitial and perivascular fibrosis and vascular inflammation [5, 20].

Aldosterone receptor antagonism in patients treated with ACE inhibitor has shown to reduce mortality in patients with congestive heart failure [10]. The utility of spironolactone has been limited due to its anti-androgenic and progestational side effects [9]. Eplerenone is a new selective aldosterone receptor antagonist with decreased side effects compared with spironolactone. Eplerenone when compared to spironolactone, amlodipine, losartan and enalapril effectively reduces blood pressure. Eplerenone increases serum potassium, particularly in patients taking other potassium-sparing drugs such as ACE inhibitors and AT_1 receptor antagonists and in patients with renal insufficiency. Eplerenone also reduces mortality in patients with left ventricular dysfunction [23].

Titin is a 300 kDa cytoskeletal protein that spans in a spring-like fashion from the Z-disc to the M-band and, under normal conditions, ensures elasticity and extensibility of the sarcomere [24, 25]. In the present study, a decrease in the expression of the compliant N2BA-titin isoform and an increase in the expression of the stiff N2B-titin isoform is observed in dogs with HF. Normalization of mRNA expression of N2B-titin and N2BA-titin isoforms in HF dogs treated with eplerenone was observed. We also observed significant increase in the mean N2BA/N2B-titin gene expression ratio after treatment with eplerenone. Differential splicing of titin has been shown to lead to a differential expression of the non-compliant stiff N2B isoform and the compliant N2BA isoform. As alluded to earlier, these titin isoforms have different mechanical properties and play a major role in altering passive stiffness in failing hearts [26–28]. The mechanical properties of titin affect diastolic ventricular filling and systolic ventricular emptying [29, 30]. A decrease in expression of N2BA-titin in response to pressure overload in a spontaneously hypertensive rat model [31], and an increase in titin-N2B expression and decrease in titin-N2BA expression in a canine tachycardia-induced model of dilated cardiomyopathy has been reported [28]. In contrast to results in the present study, an increase in the expression of the compliant N2BA-titin isoform was observed, paralleled by an increase in N2BA/N2B-titin expression ratio in LV myocardium of patients with ischemic cardiomyopathy [27].

Results of the present study also showed a significant increase in the expression of fibronectin, tubulin-α and tubulin-β in LV myocardium of dogs with HF with normalization of these genes following therapy with eplerenone. There is evidence which suggests that derangements of cytoskeletal proteins such as tubulin, vinculin and vimentin contribute to alterations in intracellular signaling, myocytes function and coupling of myocytes to extracellular matrix during cardiac hypertrophy and failure [32]. In HF functional maladaptations may result in structural abnormalities in the heart and may adversely influence global LV contractile performance [33]. Fibronectin is a major cytoskeletal protein and is an important component of the cellular basement membrane [34]. Fibronectin is increased in hearts with dilated cardiomyopathy together with an increase in other cytoskeletal proteins such as tubulin, vinculin and vimentin [35]. An increase in fibronectin was observed in ischemic areas compared to control hearts in a porcine model of HF [36]. Tubulin is a heterodimer 55 kDa molecule consisting of an alpha- and beta-isoforms that form microtubules and transmits mechanical and chemical stimuli within and between cells [37]. Alterations of the cytoskeleton have been also described in hypertrophied and failing animal hearts [38–42]. An increase in stiffness of the cardiomyocytes is a consequence of increased amounts of tubulins. Increases in microtubule content and up-regulation of tubulin mRNA and proteins were observed in cardiac hypertrophy and failure, both in animal models and human biopsies [35, 43]. An increased microtubule density was found by quantitative confocal microscopy in HF [44]. Vimentin is a cytoskeletal protein present in several cell types in the myocardium. The present study showed no changes in the regulation of vimentin in HF. Other studies have shown that vimentin is a more important component of the cardiomyocyte during development rather than in adult life [45].

Accumulation of collagen in the cardiac interstitium or RIF, accompanied by loss of matrix cross-link integrity, occurs in HF and contributes to LV dysfunction. MMPs and TIMPs are present in the myocardium and are involved in ventricular remodeling [46, 47]. MMPs including MMP-2 and MMP-9, are upregulated in the failing heart and are associated with the development of interstitial and perivascular fibrosis [48]. It has been proposed that MMPs degrade normal collagens in the failing heart that are replaced by fibrous deposits that, in turn, weaken and dilate the ventricles [49]. In the present study, treatment with eplerenone decreased MMP-1, -2 and -9 mRNA expression, without affecting TIMP mRNA expression. These findings suggest that an imbalance of MMP activity regulation by TIMP inhibition may lead to increased MMP activity with subsequent development of RIF. MMPs are also involved in angiogenesis, and theoretically, inhibition of MMPs could prevent new vessel formation [50]. However, it was shown that selective inhibition of MMPs attenuates ventricular remodeling without suppression of angiogenesis [51]. Several other studies have also demonstrated that inhibition of MMPs improves function and prevents ventricular remodeling in failing hearts [7, 51].

In conclusion, the results of this study indicate that the cardioprotective actions of eplerenone may be attributable to normalization of mRNA and protein expression of key cytoskeletal genes namely, N2BA-titin, N2B-titin, fibronectin, tubulin-α, and tubulin-β and key matrix metalloproteinases namely, MMP-1, MMP-2 and MMP-9. Findings of this study provide for possible molecular mechanisms by which eplerenone, a novel selective aldosterone receptor blocker, represents a potentially useful therapeutic option to limit LV structural remodeling and potentially improves LV diastolic dysfunction. Multicenter clinical trials are currently underway that evaluate the efficacy of aldosterone receptor blockers in the treatment of patients with HF and preserve LV ejection fraction; the so-called “diastolic HF” patient. There are at least three such trials underway specifically, (1) Trial of Aldosterone Antagonist Therapy in Adults With Preserved Ejection Fraction Congestive Heart Failure (TOPCAT) sponsored by the United States National Heart, Lung, and Blood Institute, (2) Aldosterone Antagonism in Diastolic Heart Failure sponsored by the United States Department of Veterans Affairs, and (3) Eplerenone in Reversing Endothelial and Diastolic Dysfunction and Improving Collagen Turnover in Diastolic Heart Failure (PREDICT) sponsored by the Cleveland Clinic Foundation.

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