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Bradykinin restores left ventricular function, sarcomeric protein phosphorylation, and e/nNOS levels in dogs with Duchenne muscular dystrophy cardiomyopathy.

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Methods

Echocardiography examinations

Conventional echocardiography and 2D color tissue Doppler imaging (TDI) were performed by the examiners blinded to the animals' study group as previously described.¹ Before thoracotomy, 2-3 weeks after surgical intervention as well as after 2 and 4 weeks of infusion, conscious dogs gently restrained in standing position and monitored with a continuous EKG were examined with a Vivid 7 ultrasound unit equipped with 5-7.5 and 2-5MHz phased-array transducers (GE, Waukesha, WI). Left ventricular (LV) diameter, LV free wall and interventricular septal wall thicknesses were measured at end-diastolic and end-systolic points in accordance with the recommendations of the American Society of Echocardiography.² LV fractional shortening (%) was calculated as $(LV \text{ end-diastolic diameter} - LV \text{ end-systolic diameter}) / LV \text{ end-diastolic diameter} \times 100\%$. Pulsed-wave Doppler variables including early (E) and late (A) diastolic mitral inflow velocities were assessed from the left parasternal 4-chamber view and the mitral E/A ratio was calculated.

For 2D color TDI examinations, real-time color Doppler was superimposed on the gray scale with a high frame rate (> 150 frames/sec). Doppler receive gain was adjusted to maintain optimal coloring of the myocardium, and Doppler velocity range was set as low as possible to avoid aliasing. All digital images were analyzed using specific software (Echo Pac 5.4, GE, Waukesha, WI). A 2 x 2 mm sample was used and a tissue velocity profile displayed in each sample location. Myocardial velocities resulting from the radial LV free wall motion were measured using the right parasternal ventricular short-axis view and measurements were made between the 2 papillary muscles in subendocardial and subepicardial segments of the LV free wall. For each myocardial velocity, the mean value was calculated based on the measurements of 3 consecutive cardiac cycles on the same frame. Radial endo-epicardial systolic myocardial velocity gradients (defined as the difference between systolic

subendocardial and subepicardial velocities) were then calculated. Heart rate was also calculated by EKG monitoring during each radial TDI examination by averaging the same 3 cardiac cycles used for the velocity measurements.

Myocyte preparation and single cell experiments

Immediately after euthanasia, LV myocardial samples were placed in an ice-cold modified-Tyrode's solution containing (in mM): NaCl 140, KCl 4.8, NaHCO₃ 2.5, NaH₂PO₄ 1.6, MgSO₄ 5.0, HEPES 12, glucose 12.5, pH 7.4, adjusted with NaOH. Several subendocardial and subepicardial strips (8 mm long, 2 mm wide, and 1 mm thick) were dissected from the same heart. Muscle strips were either frozen in liquid nitrogen for biochemistry or prepared for mechanical experiments as previously described.³ For micromechanical experiments, muscle strips were permeabilized 10 min at 4°C in relaxing solution containing 1% Triton X-100 and protease inhibitors (0.5 mM PMSF, 0.04 mM leupeptin, and 0.01 mM E64). Strips were then placed in fresh ice-cold relaxing solution and mechanically disrupted at 11,000 rpm for 2-3 s (Polytron PT45-80 with a PTA 10TS shaft, KINEMATICA AG, Switzerland), resulting in a suspension of small clumps of myocyte-sized preparations and cell fragments. The homogenized tissue was centrifuged (100 g, 1 min at 4°C). The pellet containing the myocytes was permeabilized in 0.3% Triton X-100 solution for 6 min to remove remaining sarcolemma membranes and then was extensively washed in relaxing solution. Cells were kept on ice and used within the day. Relaxing and activating buffers contained (in mM): phosphocreatine 12, imidazole 30, free Mg²⁺ 1, EGTA 10, Na₂ATP 3.3, and dithiothreitol 0.3 with pCa 9.0 (relaxing solution) and pCa 4.5 (maximal activating solution), pH 7.1 adjusted with acetic acid (osmolarity 280 mOsm). Potassium acetate was added to adjust ionic strength to 180 mM.

The skinned myocyte was attached at either end to a piezoresistive strain gauge (model AE 801, SensoNor a.s., Horten, Norway; 500 Hz unloaded resonant frequency) and a stepper motor driven micromanipulator (MP-285, Sutter Instrument Company, Novato, CA) with thin needles and optical glue (NOA 63, Norland Products, North Brunswick, NJ) that was polymerized by UV illumination for 2 min. The sarcomere length was determined online throughout the experiment (Ionoptix system). Force was normalized by the cross-sectional area measured from the imaged cross-section as described elsewhere.⁴ Force-pCa relationships were established at two sarcomere lengths of 1.9 and 2.3 μm . Active tensions at submaximal activations were normalized to maximal isometric tension (obtained at pCa 5) at the same sarcomere length. The relation between force and pCa was fitted to the following equation: $force = [Ca^{2+}]^{nH} / (K + [Ca^{2+}]^{nH})$, where nH is the Hill coefficient and pCa₅₀, pCa for half-maximal activation equals $-(\log K)/nH$.

Western blot analysis

The frozen myocardial tissue was grinded, homogenized and centrifuged (25,000 g, 15 min). The supernatant was used for western blot analysis of nNOS, eNOS, cMyBP-C, phosphorylated cMyBP-C (p-MyBP-C), cardiac TnI, phosphorylated TnI (p-TnI) and two isoforms of VL2 (VLC2, VLC2*) and their phosphorylated forms (p-VLC2, and p-VLC2*). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, France). Equal amounts of extracted proteins were separated on 4-15% SDS-PAGE gels for most proteins studied and transferred to polyvinylidene fluoride membranes using standard technique. After blockade of non-specific epitopes with 5% milk, the blotted membranes were incubated with antibodies against nNOS, eNOS (sc-8309 and sc-654, at a dilution of 1:200 and 1:400, respectively; Santa Cruz Biotechnology, USA), calnexin (rabbit monoclonal antibody, 1:5,000; Sigma-Aldrich, UK), cMyBP-C, phosphor ser²⁸²-MyBP-C (rabbit, 1:1,000; Alexis, France), TnI and phosphor ser^{23/24} TnI (mouse, 1:1,000; Hytest, Finland). Non-

phosphorylated and phosphorylated VLC2 isoforms were separated according to their charge on 10% urea gel and revealed after transfer with VLC2 antibody (rabbit, 1:200; Coger SA, France) and calsequestrin (Rabbit, ABR). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Dako, France). Specific immunoactive proteins were detected by enhanced chemiluminescence (Biological Industries, Isreal or Amersham Pharmacia, UK). Analysis of images was performed using the ImageJ software (NIH, USA). nNOS and eNOS levels were expressed as a ratio to calnexin and the phosphorylation level of cMyBP-C, TnI and VLC2 isoforms was expressed as a ratio of the phosphorylated form/total form.

Results

We have also measured arterial pressure before and after 4 weeks of placebo or bradykinin infusion using aortic catheter. The data showed that before treatment, mean arterial pressure was significantly lower in GRMD dogs (103 ± 3 mmHg, $n=10$) than in control dogs (115 ± 2 mmHg, $n=8$, $p<0.01$). After 4 weeks of placebo or bradykinin infusion, mean arterial pressure was 114 ± 3 , 117 ± 2 , 102 ± 7 and 110 ± 3 mmHg, respectively in placebo-treated control, bradykinin-treated control, placebo-treated GRMD and bradykinin-treated GRMD dogs versus 117 ± 4 , 114 ± 3 , 106 ± 6 , and 101 ± 4 mmHg respectively at baseline, indicating no significant change in mean arterial pressure induced by placebo or bradykinin treatment in control and GRMD dogs.

Table 2. Baseline echocardiographic data obtained 2-3 weeks after surgical intervention in conscious control and GRMD dogs randomized into placebo or bradykinin group

	Control dogs		GRMD dogs	
	Placebo	Bradykinin	Placebo	Bradykinin
N	4	4	5	5
Body weight, kg	26.5±2.6	29.3±3.8	18.8±2.4*†	18.6±2.7*†
LVEDD, mm	46.1±2.6	46.7±3.6	43.1±4.7	43.2±1.4
Fractional shortening, %	34.2±1.5	34.6±2.1	27.3±0.8*†	28.1±1.3*†
LV EDFWTh, mm	9.3±0.2	9.1±0.8	6.9±0.5*†	6.8±0.5*†
LV FWSTh, mm	5.6±0.3	5.4±0.9	2.9±0.8*†	1.9±1.0*†
EDIVSWTh, mm	10.4±0.7	10.5±1.2	7.9±1.0*†	8.1±0.4*†
IVSWSTh, mm	5.0±0.7	5.8±0.6	3.6±1.3*†	3.6±0.9*†
Vendo, cm/s	6.5±0.8	6.9±1.1	5.8±0.5	5.9±0.4
Vepi, cm/s	3.6±0.4	3.9±0.7	4.1±0.6	4.5±0.4
Endo-epi gradient, cm/s	3.0±0.4	3.0±0.3	1.7±0.3*†	1.4±0.1*†
E/A mitral	2.0±0.4	2.0±0.3	1.7±0.1	1.7±0.3

Data are presented as mean±SEM. LV, left ventricular; EDD, end-diastolic diameter;

EDFWTh, end-diastolic free wall thickness; FWTh, free wall systolic thickening;

EDIVSWTh, end-diastolic interventricular septal wall thickness; IVSWSTh, interventricular septal wall systolic thickening; Vendo, radial systolic velocity in the subendocardial region;

Vepi, radial systolic velocity in the subepicardial region; Endo-epi gradient; subendocardial-subepicardial gradient of radial systolic velocity E/A mitral, ratio of early to late diastolic

mitral flow velocity.*p<0.05 vs control placebo group and †p<0.05 vs control bradykinin group.

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