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Abstract

Little is known about the vascular function and expression of endothelial and neuronal nitric oxide synthases (eNOS and nNOS) in Duchenne muscular dystrophy (DMD). Bradykinin is involved in the regulation of eNOS expression induced by angiotensin-converting enzyme inhibitors. We characterized the vascular function and eNOS and nNOS expression in a canine model of DMD and evaluated the effects of chronic bradykinin treatment. Vascular function was examined in conscious golden retriever muscular dystrophy (GRMD) dogs with left ventricular dysfunction (measured by echocardiography) and in isolated coronary arteries. eNOS and nNOS proteins in carotid arteries were measured by western blot and cyclic guanosine monophosphate (cGMP) content was analyzed by radioimmunoassay. Compared with controls, GRMD dogs had an impaired vasodilator response to acetylcholine. In isolated coronary artery, acetylcholine-elicited relaxation was nearly absent in placebo-treated GRMD dogs. This was explained by reduced nNOS and eNOS proteins and cGMP content in arterial tissues. Chronic bradykinin infusion (1 μg/min, 4 weeks) restored in vivo and in vitro vascular response to acetylcholine to the level of control dogs. This effect was NO-mediated through upregulation of eNOS and nNOS expression. In conclusion, this study is the first to demonstrate that DMD is associated with NO-mediated vascular endothelial dysfunction linked to an altered expression of eNOS and nNOS, which can be overcome by bradykinin.

Author Keywords Duchenne muscular dystrophic cardiomyopathy ; endothelial dysfunction ; endothelial nitric oxide synthase ; neuronal nitric oxide synthase ; bradykinin

Introduction

Duchenne muscular dystrophy (DMD) affects one in 3500 male births, making it the most prevalent of muscular dystrophies. Mutations in the dystrophin gene located on the X chromosome (Xp21) lead to loss of sarcolemmal dystrophin (5 , 20 , 24)], disruption of dystrophin-glycoprotein complex and muscle damage. A selective loss of neuronal nitric oxide synthase (nNOS) in skeletal muscles contributes to muscle lesions in DMD (6 , 8 , 32). In golden retriever muscular dystrophy (GRMD) dogs, a mutation in intron 6 of the dystrophin gene causes loss of dystrophin in striated muscles (13 , 37)], resulting in severe muscle degeneration and ambulation disabilities (15 , 31 , 43)]. GRMD dogs develop full spectrum of DMD pathology and clinical symptoms and are thought to be the most relevant DMD model for preclinical investigations (15 , 31 , 43)], including gene transfers (3 , 7 , 21), cell therapy (15 , 31) or pharmacologic therapy (42)]. Similar to DMD patients, GRMD dogs also develop a dilated cardiomyopathy, a lethal cause for DMD patients (10 , 42). Although vascular endothelial dysfunction occurs in many pathological circumstances such as myocardial infarction-induced heart failure (14 , 33), little is known about vascular function in DMD patients as well as in GRMD dogs. A blister-like swelling of vascular endothelial cells was found in the biopsied muscle specimens from DMD patients at the preclinical stage (25). This suggested that vascular endothelial dysfunction may occur in DMD patients, especially at a stage where cardiac function is impaired. In addition, in our preliminary experiments before infusion of placebo or bradykinin, acetylcholine induces smaller pressure-lowering effect in GRMD dogs than in control normal dogs, suggesting vascular endothelial dysfunction in the formers. Therefore, our primary goal was to assess vascular function with a special focus on coronary endothelial function in GRMD dogs.

Bradykinin is an endogenous nonapeptide that exerts multiple functions in cardiovascular system. Physiologically, bradykinin exerts its actions through activation of B2 receptors leading to NO release (26 , 39). Bradykinin regulates human coronary vascular tone (18) and contributes to the improvement of systemic and coronary endothelial dysfunction during angiotensin-converting enzyme (ACE) inhibition (2 , 30 , 38) and to the cardioprotection of myocardial ischemia by preconditioning (12 , 35)] and by ACE inhibitors and angiotensin AT1 receptor blockers (17 , 23 , 44). In dogs with tachycardia-induced dilated cardiomyopathy, endogenous bradykinin exerts a protective effect on cardiac function (9) while a bradykinin analogue improves left ventricular (LV) endocardial flow reserve (28). In addition, chronic bradykinin infusion prevents the progression of heart failure by preserving LV diastolic and systolic functions and vascular endothelial function, which is probably related to a preserved endothelial NOS (eNOS) expression (41). Moreover, bradykinin

pathway seems to be involved in the upregulation of eNOS expression during ACE inhibition (1). Thus, another goal of this research was to examine the effects of chronic bradykinin treatment on the vascular function and on the expression of eNOS and nNOS.

For these purposes, bradykinin was infused into the left atrium for 4 weeks at a dose without significant effect on arterial blood pressure (30). To evaluate the vascular endothelial function, pressure-lowering effect of acetylcholine was examined in vivo before and after 4 weeks of treatment in conscious dogs and concentration-relaxation curves to acetylcholine were established in vitro on isolated circumflex coronary arteries. Since bradykinin stimulates the release of endothelium-derived vasodilator substances such as NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) (26), acetylcholine-induced relaxation was examined in the absence or presence of N ω-nitro-L-arginine (LNA, a NOS inhibitor), indomethacin (a COX inhibitor), or LNA plus indomethacin combination. Because bradykinin exerts its actions essentially through NO (30), the abundance of the two constitutively expressed NOS isoforms, eNOS and nNOS in the carotid artery was determined by western blot. Finally, because cGMP is the second messenger of NO (34), the cGMP content was measured in the carotid arteries of placebo- or bradykinin-treated control and GRMD dogs.

Methods

Animals

The experiments were performed on dogs supplied by CEDS (Mézilles, France). The maintenance of animals, including postoperative care, analgesia and anti-infectious prophylaxis, was kept in accordance with the Guide for the care and Use of Laboratory animals (NIH publication No. 86-23, revised 1985). The animals used in the present study were in common with a general study attempting to investigate cardiomyopathy features of GRMD dogs. Based on preliminary echocardiographic data obtained in three control dogs and eight GRMD dogs, we established criteria for the enrollment of GRMD dogs in the study: age ≥ 9 months and LV fractional shortening ≥25% and ≤35%. Accordingly, 14 GRMD dogs were included in the study and were instrumented with a catheter in the left atrium and another in the thoracic descending aorta during a left thoracotomy under general anesthesia induced with propofol (6.5 mg/kg, i.v.) and maintained with isoflurane (1–3% vol in 100% O_2). Preemptive and postoperative analgesia was ensured with morphine (0.1 mg/kg, i.v.) and a fentanyl patch (50 μg/h), respectively. Among them, three died of acute respiratory failure following food inhalation (n=1), bronchopneumonia (n=1) or gastric ulcers (n=1) during postoperative recovery period, and 1 died of pulmonary hemorrhage associated with bronchopneumonia during the second week of infusion with 0.9% saline. Finally, we have obtained the results in ten GRMD dogs and eight age-matched normal dogs. After 2–3 weeks of postoperative recuperation, dogs were randomized into four groups: control-placebo (n =4), control-bradykinin (n=4), GRMD-placebo (n=5) or GRMD-bradykinin (n=5). After echocardiographic and hemodynamic studies at baseline, using a portable pump (Microject, Sorenson Medical) placed in a jacket attached on the back of the animal, a continuous infusion of 0.9% saline (166 μl/min) or bradykinin (1 μg/min, 166 μl/min corresponding to 46 ng/kg/min) was performed through the left atrial catheter for 4 weeks. This dose of bradykinin was shown to affect minimally arterial pressure by our previous study (41).

At the time of killing, the coronary arteries were collected in ice-cold physiological saline solution for isolated vessel studies and the carotid arteries were collected and frozen with liquid nitrogen and stored at −80°C for biochemical study.

Echocardiography and in vivo vasodilator response to acetylcholine

At baseline, conventional echocardiography was performed in unsedated conscious dogs in standing position, using continuous ECG monitoring with a Vivid 7 ultrasound unit (General Electric Medical System, Waukesha, WI). LV diameter, LV free wall and interventricular septal wall thicknesses were measured at end-diastolic and end-systolic points. LV fractional shortening (%) was calculated as (LV end-diastolic diameter − LV end-systolic diameter)/LV end-diastolic diameter × 100%.

To evaluate in vivo vasodilator response, the effects of acetylcholine (0.3–3 μg/kg, iv) on arterial blood pressure measured by aortic catheter were recorded at baseline and after 4 weeks of placebo or bradykinin infusion in conscious control and GRMD dogs.

Study in isolated coronary arteries

To examine vascular function, the circumflex coronary artery freshly dissected from the heart was cleaned of adjacent connective tissue and cut into rings (≈3 mm in length). All rings were suspended in isolated organ chambers for isometric tension recording. The organ chambers were filled with 20 ml of physiological saline solution (pH 7.4, at 37.4°C) containing (mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 , 25 $NaHCO_3$, 0.016 EDTA and 11.1 glucose that was continuously gassed with 95% O_2 /5%CO_2 . The rings were equilibrated for 120 min under a resting tension of 2 g. During equilibration period, the rings were washed every 30 min. Then, a first relaxation to acetylcholine (Ach, 10^{-4} M) was implemented to check the integrity of the endothelium in rings precontracted with the thromboxane A2 analogue, U46619 (10^{-8} M). After rinsing with physiological saline solution to baseline tension, rings were equilibrated for 90 min. Thereafter, coronary artery rings were contracted with U46619 (10^{-6} M). Each ring was randomly assigned to acetylcholine alone, acetylcholine + indomethacin (3×10^{-5} M), acetylcholine + LNA (3×10^{-5} M), or acetylcholine + indomethacin (3×10^{-5} M) + LNA (3×10^{-5} M). Responses to cumulative concentrations of acetylcholine (10^{-10} –10^{-4} M) were then obtained. Relaxations were measured at

the peak decrease in tension for each concentration of acetylcholine using IOX and Datanalyst softwares (EMKA Technologies, Paris, France). Results were expressed as percentage of the maximal tension induced by U46619.

Western blot

Frozen carotid arteries were grinded and then homogenized in buffer containing: 10mM Tris-HCl pH 7.6, 250 mM sucrose inhibitors, 2mM DTT, 20 mg/mL protease and 0.5 % Igepal CA-630. The crude cell lysate was centrifuged for 15 min at 14,000 rpm. 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 and transferred to PVDF membranes using standard technique. Membranes were blocked with 6% milk in Tris Buffered Saline with Tween-20 for 1h at room temperature. Then, membranes were incubated with rabbit polyclonal antibodies anti-NOS1 and anti-NOS3 (sc-8309 and sc-654, respectively, Santa-Cruz Biotechnology, USA) at a dilution of 1:200 and 1:400, respectively, for 1 h. After washing, membranes were incubated with HRP-Rabbit secondary antibody (1:5,000; Dako, France) for 30 min. Antibody-protein complexes were detected using an enhanced EZ-ECL chemiluminescent reagent (Biological Industries, Israel). Rabbit Anti-calnexin monoclonal antibody (1:5,000; Sigma, France) was used as a loading control. Densitometric analysis was performed using ImageJ software (NIH, USA).

Measurement of cyclic guanosine monophosphate

Because the coronary artery was used for the isolated vessel study, the measurement of cyclic guanosine monophosphate (cGMP) was performed in the carotid artery taken from the same dogs as the coronary artery. At the time of assay, frozen carotid segment (stored at −80 °C) was homogenized and sonicated in medium containing acetate buffer (0.05 M, pH=5.8) and a phosphodiesterase inhibitor (3-isobutyl-1-1-methylxanthine, 0.1 mM). The homogenate was centrifuged (2,000 g for 20 min at 4°C) and the supernatant used to determine the levels of cGMP by radioimmunoassay using the Amerlex method (Amersham, RPA525). The results are expressed in pmol per mg of total protein content.

Drugs used

Acetylcholine (Sigma), dissolved in H_2O; bradykinin (NeoMPS, Strasbourg, France), dissolved in saline solution; LNA, Nω -Nitro-L-arginine (Sigma), dissolved in H_2O; indomethacin (Sigma), dissolved in DMSO; U46619, 9,11-dideoxy-11α,9α -epoxymethanoprostaglandin $F_{2\alpha}$ (Sigma), dissolved in methyl acetate.

Statistical analysis

Results are expressed as mean±SEM. Statistical analysis was performed with StatView software (v5, Abacus Concepts Inc). Nonparametric Kruskal-Wallis test was used to analyze the overall difference between the 4 groups. If there was a significant difference, the Mann-Whitney test was performed to identify differences between two means. When only two means were compared, the Mann-Whitney test was used. A $p < 0.05$ was considered significant.

Results

Baseline characteristics of the control and GRMD dogs

GRMD dogs had a smaller body weight (BW) than age-matched control dogs (Table 1), indicating an abnormal development of GRMD dogs. At baseline, GRMD dogs had significantly higher heart rate, lower systolic and mean arterial blood pressures than the control dogs and they presented an impaired LV contractile function indicated by a significantly reduced fractional shortening as compared with control dogs (Table 1). LV end-diastolic diameter was slightly but not significantly smaller in GRMD dogs than control dogs. There was no difference for each parameter between the dogs randomized into placebo or bradykinin group for control dogs and for GRMD dogs.

Pressure-lowering effect of acetylcholine in GRMD dogs

In conscious control dogs, acetylcholine induced a dose-dependent decrease in arterial blood pressure. This effect was significantly smaller in GRMD dogs than in control dogs (Figure 1a). After 4 weeks of infusion, the pressure-lowering effects of acetylcholine in placebo-treated GRMD dogs remained smaller than in placebo- or bradykinin-treated control dogs, whereas these effects in bradykinin-treated GRMD dogs were significantly improved and were not statistically different from those in placebo- or bradykinin-treated control dogs (Figure 1b).

Impaired arterial endothelial function in GRMD dogs and its normalization by bradykinin treatment

One of the most important information issued from the isolated-vessel study is that acetylcholine did not produce significant relaxation of the coronary arteries in placebo-treated GRMD dogs (Figure 2), indicating an impaired vascular function in these animals. In contrast, acetylcholine relaxed precontracted coronary arteries in bradykinin-treated GRMD dogs to the same extent as in placebo- or bradykinin-treated control dogs (Figure 2), indicating a normalized vascular endothelial reactivity in bradykinin-treated GRMD dogs.

Nitric oxide was responsible for the normalization of endothelial function induced by bradykinin treatment

To determine the role of NO, vasodilator prostaglandins and EDHF in the effects of bradykinin, we examined the effect of acetylcholine in the presence of indomethacin, LNA or indomethacin plus LNA. In the coronary arteries of control dogs, acetylcholine-evoked relaxation was slightly decreased in the presence of indomethacin, suggesting a role of vasodilator prostaglandins. In contrast, the reduction was more pronounced in the presence of LNA, indicating a key role of NO in this setting (Figure 3a). There was a further inhibition in the presence of LNA plus indomethacin, suggesting also the role of vasodilator prostaglandins (Figure 3a). In coronary arteries of bradykinin-treated control dogs, relaxation response to acetylcholine was slightly reduced by indomethacin, but totally blunted by LNA or LNA plus indomethacin, indicating a more pronounced role of NO than vasodilator prostaglandins in this setting (Figure 3b). In coronary arteries of placebo-treated GRMD dogs, acetylcholine did not produce significant relaxation in the presence or absence of indomethacin, LNA or LNA plus indomethacin (Figure 3c). Differently, in the coronaries of bradykinin-treated GRMD dogs, acetylcholine-evoked relaxation was not modified by indomethacin, but was completely blocked by LNA or LNA plus indomethacin, indicating a crucial role of NO in this setting (Figure 3d). Thus, GRMD dogs had an impaired coronary endothelial function that was normalized by bradykinin through a NO-dependent mechanism.

Expression of eNOS and nNOS proteins in the carotid artery

Compared to control dogs, eNOS protein levels were decreased in the carotid artery of placebo-treated GRMD dogs. In bradykinin-treated GRMD dogs, eNOS protein levels were significantly higher than in placebo-treated GRMD dogs and were similar to bradykinin-treated control dogs (Figure 4a, b), indicating an eNOS upregulation during bradykinin treatment.

No significant difference for nNOS protein levels in the carotid artery was observed between placebo- or bradykinin-treated control dogs (Figure 4a, c). In contrast, nNOS levels in the carotid artery were significantly reduced in placebo-treated GRMD dogs (Figure 4a, c). nNOS levels in bradykinin-treated GRMD dogs were not statistically different from those in bradykinin-treated control dogs but were higher than in placebo-treated GRMD dogs (Figure 4a, c).

Changes in cGMP content in the carotid artery in placebo- or bradykinin-treated GRMD dogs

Compared with control dogs, the cGMP content in the carotid artery was significantly decreased in placebo-treated GRMD dogs (Figure 5). In contrast, the cGMP level was similar between the bradykinin-treated GRMD dogs and control dogs treated with placebo or bradykinin, indicating a restoration of cGMP level in bradykinin-treated GRMD dogs.

Discussion

Vascular endothelial dysfunction is associated with hypertension, diabetes, atherosclerosis, and heart failure. In DMD, pathological characteristics of skeletal muscles have been well recognized. In contrast, little information is available about vascular function in this disease. This study demonstrates that GRMD dogs with LV dysfunction had an impaired vascular response to acetylcholine both in terms of arterial blood pressure reduction in conscious state and relaxant response in isolated coronary arteries. It is known that endothelium-derived NO relaxes vascular smooth muscle cells and reduces vascular tone (27 , 29 , 34)]. An impaired endothelium-derived NO production related to reduced eNOS expression plays an important role in vascular dysfunction occurring in many cardiovascular disorders (16 , 19 , 29 , 30 , 34). However, it remains unknown whether eNOS expression is changed in DMD patients. The second new finding of the present study is a reduced eNOS expression level in the carotid artery of GRMD dogs compared with control dogs, which should be at least partly responsible for impaired vascular endothelial function in these animals. On the other hand, previous studies have shown that nNOS is involved in the local regulation of blood flow in animals and in humans (4 , 11 , 22 , 32 , 36 , 40). In skeletal muscles of DMD patients, a selective loss of nNOS is associated with a reduced blood flow (32). It was supposed that skeletal nNOS-derived NO opposes to α -adrenergic-mediated vasoconstriction to maintain blood flow in the exercising skeletal muscle. Its deficiency in skeletal muscles was thought to be related to abnormal vasoconstrictor responses to vasoactive agents in mdx or nNOS knockout mice (40)]. Another new finding of the current study is that nNOS protein levels are also reduced in arterial vessels in dystrophin-deficiency myopathy, which might, along with reduced eNOS expression, contribute to altered endothelial function in GRMD dogs. The reduced cGMP content in the carotid artery further supports this hypothesis because cGMP is the common second messenger for both nNOS and eNOS.

Remarkably, contrary to placebo-treated GRMD dogs, bradykinin-treated GRMD dogs exhibited a pressure-lowering response to acetylcholine similar to that in placebo- or bradykinin-treated control dogs. The normalized relaxation response to acetylcholine was confirmed in isolated coronary arteries of bradykinin-treated GRMD dogs. This means that bradykinin treatment improved both coronary and systemic vascular function in GRMD dogs. Physiologically, bradykinin exerts its action through activation of G protein-coupled B2 receptors that increase the intracellular Ca^{2+} concentration by mobilizing Ca^{2+} from both intracellular and extracellular pools. The increase in cytosolic Ca^{2+} stimulates NOS to release NO and also contributes to the production of arachidonic acid. The latter can be further metabolized into prostacyclin by COX or into EDHF by cytochrome P450-dependent epoxygenase or monoxygenase (26). Using LNA and indomethacin in isolated coronary artery rings, we found that acetylcholine-evoked relaxation in bradykinin-treated GRMD dogs was essentially NO-mediated with a little role for prostacyclin or EDHF. This implies that coronary NO production was restored by bradykinin-treatment. Although we did not measure e/nNOS protein levels in coronary tissues, this was supported by an upregulation of e/nNOS proteins and normalization of cGMP content in the carotid arteries of bradykinin-treated GRMD dogs. This study is the first one to demonstrate a significantly altered systemic and coronary vascular function in this model of DMD and to show a restoration of vascular endothelial reactivity by chronic bradykinin treatment through increasing eNOS and nNOS expression. This is of particular importance because of the fundamental role of NO in maintaining organ blood flow. An increased eNOS and nNOS expression renders vascular endothelial cells capable to increase NO production to induce vascular smooth muscle relaxation in response to various stimuli. Accordingly, we found similar cGMP levels in the carotid arteries of bradykinin-treated GRMD dogs and in bradykinin-treated control dogs. Although prostacyclin and EDHF may also participate in the bradykinin effects, the fact that indomethacin did not significantly affect the relaxation response to acetylcholine and that the combination of LNA and indomethacin completely blunted the relaxation response to acetylcholine in bradykinin-treated GRMD dogs suggests that the prostacyclin and EDHF pathways are not the major mechanisms involved in the beneficial effect of bradykinin on the vascular endothelial function in GRMD dogs.

In conclusion, our study is the first one to demonstrate that GRMD dogs with LV dysfunction presented a severe vascular dysfunction associated with a marked reduction in eNOS and nNOS expression and a reduced capacity to generate cGMP. Chronic bradykinin infusion restored vascular endothelial function through the NO-mediated mechanism. This was ensured by upregulation of eNOS and nNOS expression and by restoration of cGMP-generating capacity in the arterial vessels. Since the similarity in pathological changes and in origin of disease between GRMD and DMD, the results obtained in this canine model would have clinical relevance for the treatment of DMD.

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Footnotes:

Conflict of interest The authors declare that they have no conflict of interest.

References:

- 1 . Bachetti T , Comini L , Pasini E , Cargnoni A , Curello S , Ferrari R . Ace-inhibition with quinapril modulates the nitric oxide pathway in normotensive rats . J Mol Cell Cardiol . 33 : 395 - 403 2001 ; 10.1006/jmcc.2000.1311
- 2 . Barbe F , Su JB , Guyene TT , Crozatier B , Ménard J , Hittinger L . Bradykinin pathway is involved in acute hemodynamic effects of enalaprilat in dogs with heart failure . Am J Physiol . 270 : H1985 - 1992 1996 ;
- 3 . Bartlett RJ , Stockinger S , Denis MM , Bartlett WT , Inverardi L , Le TT , thi Man N , Morris GE , Bogan DJ , Metcalf-Bogan J , Kornegay JN . In vivo targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide . Nat Biotechnol . 18 : 615 - 622 2000 ; 10.1038/76448
- 4 . Bauser-Heaton HD , Bohlen HG . Cerebral microvascular dilation during hypotension and decreased oxygen tension: a role for nNOS . Am J Physiol Heart Circ Physiol . 293 : H2193 - 2201 2007 ; 10.1152/ajpheart.00190.2007
- 5 . Bonilla E , Samitt CE , Miranda AF , Hays AP , Salviati G , DiMauro S , Kunkel LM , Hoffman EP , Rowland LP . Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface . Cell . 54 : 447 - 452 1988 ; 10.1016/0092-8674(88)90065-7
- 6 . Brenman JE , Chao DS , Xia H , Aldape K , Bredt DS . Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy . Cell . 82 : 743 - 752 1995 ; 10.1016/0092-8674(95)90471-9
- 7 . Cerletti M , Negri T , Cozzi F , Colpo R , Andreetta F , Croci D , Davies KE , Cornelio F , Pozza O , Karpati G , Gilbert R , Mora M . Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer . Gene Ther . 10 : 750 - 757 2003 ; 10.1038/sj.gt.3301941
- 8 . Chang WJ , Iannaccone ST , Lau KS , Masters BS , McCabe TJ , McMillan K , Padre RC , Spencer MJ , Tidball JG , Stull JT . Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy . Proc Natl Acad Sci USA . 93 : 9142 - 9147 1996 ;
- 9 . Cheng CP , Onishi K , Ohte N , Suzuki M , Little WC . Functional effects of endogenous bradykinin in congestive heart failure . J Am Coll Cardiol . 31 : 1679 - 1686 1998 ; 10.1016/S0735-1097(98)00159-4
- 10 . Chetboul V , Escriou C , Tessier D , Richard V , Pouchelon J-L , Thibault H , Lallemand F , Thuillez C , Blot S , Derumeaux G . Tissue Doppler imaging detects early asymptomatic myocardial abnormalities in a dog model of Duchenne's cardiomyopathy . Eur Heart J . 25 : 1934 - 1939 2004 ; 10.1016/j.ehj.2004.09.007
- 11 . Chi OZ , Liu X , Weiss HR . Effects of inhibition of neuronal nitric oxide synthase on NMDA-induced changes in cerebral blood flow and oxygen consumption . Exp Brain Res . 148 : 256 - 260 2003 ; 10.1007/s00221-002-1310-7
- 12 . Cohen MV , Yang XM , Liu GS , Heusch G , Downey JM . Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels . Circ Res . 89 : 273 - 278 2001 ; 10.1161/hh1501.094266

- 13 . Cooper BJ , Winand NJ , Stedman H , Valentine BA , Hoffman EP , Kunkel LM , Scott MO , Fischbeck KH , Kornegay JN , Avery RJ . The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs . Nature . 334 : 154 - 156 1988 ; 10.1038/334154a0
- 14 . Van Craenenbroeck EM , Hoymans VY , Beckers PJ , Possemiers NM , Wuyts K , Paelinck BP , Vrints CJ , Conraads VM . Exercise training improves function of circulating angiogenic cells in patients with chronic heart failure . Basic Res Cardiol . 105 : 665 - 676 2010 ; 10.1007/s00395-010-0105-4
- 15 . Dell'Agnola C , Wang Z , Storb R , Tapscott SJ , Kuhr CS , Hauschka SD , Lee RS , Sale GE , Zellmer E , Gisburne S , Bogan J , Kornegay JN , Cooper BJ , Gooley TA , Little M-T . Hematopoietic stem cell transplantation does not restore dystrophin expression in Duchenne muscular dystrophy dogs . Blood . 104 : 4311 - 4318 2004 ; 10.1182/blood-2004-06-2247
- 16 . Drelicharz L , Kozlovski V , Skorka T , Heinze-Paluchowska S , Jasinski A , Gebska A , Guzik T , Olszanecki R , Wojnar L , Mende U , Csanyi G , Chlopicki S . NO and PGI(2) in coronary endothelial dysfunction in transgenic mice with dilated cardiomyopathy . Basic Res Cardiol . 103 : 417 - 430 2008 ; 10.1007/s00395-008-0723-2
- 17 . Ehring T , Baumgart D , Krajcar M , Hümmelgen M , Kompa S , Heusch G . Attenuation of myocardial stunning by the ACE inhibitor ramiprilat through a signal cascade of bradykinin and prostaglandins but not nitric oxide . Circulation . 90 : 1368 - 1385 1994 ; 10.1161/01.CIR.90.3.1368
- 18 . Groves P , Kurz S , Just H , Drexler H . Role of endogenous bradykinin in human coronary vasomotor control . Circulation . 92 : 3424 - 3430 1995 ; 10.1161/01.CIR.92.12.3424
- 19 . Heusch G , Post H , Michel MC , Kelm M , Schulz R . Endogenous nitric oxide and myocardial adaptation to ischemia . Circ Res . 87 : 146 - 152 2000 ; 10.1161/01.RES.87.2.146
- 20 . Hoffman EP , Brown RH , Kunkel LM . Dystrophin: the protein product of the Duchenne muscular dystrophy locus . Cell . 51 : 919 - 928 1987 ; 10.1016/0092-8674(87)90579-4
- 21 . Howell JM , Lochmüller H , O'Hara A , Fletcher S , Kakulas BA , Massie B , Nalbantoglu J , Karpati G . High-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscle of dystrophic dogs: prolongation of expression with immunosuppression . Hum Gene Ther . 9 : 629 - 634 1998 ; 10.1006/geno.2000.6171
- 22 . Ichihara A , Inscho EW , Imig JD , Navar LG . Neuronal nitric oxide synthase modulates rat renal microvascular function . Am J Physiol . 274 : F516 - 524 1998 ;
- 23 . Jalowy A , Schulz R , Heusch G . AT1 receptor blockade in experimental myocardial ischemia/reperfusion . Basic Res Cardiol . 93 : (Suppl 2) 85 - 91 1998 ;
- 24 . Koenig M , Hoffman EP , Bertelson CJ , Monaco AP , Feener C , Kunkel LM . Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals . Cell . 50 : 509 - 517 1987 ; 10.1016/0092-8674(87)90504-6
- 25 . Miike T , Sugino S , Ohtani Y , Taku K , Yoshioka K . Vascular endothelial cell injury and platelet embolism in Duchenne muscular dystrophy at the preclinical stage . J Neurol Sci . 82 : 67 - 80 1987 ;
- 26 . Mombouli JV , Vanhoutte PM . Kinins and endothelial control of vascular smooth muscle . Annu Rev Pharmacol Toxicol . 35 : 679 - 705 1995 ; 10.1146/annurev.pa.35.040195.003335
- 27 . Moncada S , Higgs A . The L-arginine-nitric oxide pathway . N Engl J Med . 329 : 2002 - 2012 1993 ; 10.1056/NEJM199312303292706
- 28 . Nikolaidis LA , Doverspike A , Huerbin R , Hentosz T , Shannon RP . Angiotensin-converting enzyme inhibitors improve coronary flow reserve in dilated cardiomyopathy by a bradykinin-mediated, nitric oxide-dependent mechanism . Circulation . 105 : 2785 - 2790 2002 ; 10.1161/01.CIR.0000017433.90061.2E
- 29 . Palmer RM , Ashton DS , Moncada S . Vascular endothelial cells synthesize nitric oxide from L-arginine . Nature . 333 : 664 - 666 1988 ; 10.1038/333664a0
- 30 . Prasad A , Husain S , Schenke W , Mincemoyer R , Epstein N , Quyyumi AA . Contribution of bradykinin receptor dysfunction to abnormal coronary vasomotion in humans . J Am Coll Cardiol . 36 : 1467 - 1473 2000 ; 10.1016/S0735-1097(00)00892-5
- 31 . Sampaolesi M , Blot S , D'Antona G , Granger N , Tonlorenzi R , Innocenzi A , Mognol P , Thibaud J-L , Galvez BG , Barthélémy I , Perani L , Mantero S , Guttinger M , Pansarasa O , Rinaldi C , Cusella De Angelis MG , Torrente Y , Bordignon C , Bottinelli R , Cossu G . Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs . Nature . 444 : 574 - 579 2006 ; 10.1038/nature05282
- 32 . Sander M , Chavoshan B , Harris SA , Iannaccone ST , Stull JT , Thomas GD , Victor RG . Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy . Proc Natl Acad Sci USA . 97 : 13818 - 13823 2000 ; 10.1073/pnas.250379497
- 33 . Schäfer A , Fraccarollo D , Pförtsch S , Loch E , Neuser J , Vogt C , Bauersachs J . Clopidogrel improves endothelial function and NO bioavailability by sensitizing adenylyl cyclase in rats with congestive heart failure . Basic Res Cardiol . 106 : 485 - 494 2011 ; 10.1007/s00395-011-0153-4
- 34 . Schulz R , Kelm M , Heusch G . Nitric oxide in myocardial ischemia/reperfusion injury . Cardiovasc Res . 61 : 402 - 413 2004 ; 10.1016/j.cardiores.2003.09.019
- 35 . Schulz R , Post H , Vahlhaus C , Heusch G . Ischemic preconditioning in pigs: a graded phenomenon: its relation to adenosine and bradykinin . Circulation . 98 : 1022 - 1029 1998 ; 10.1161/01.CIR.98.10.1022
- 36 . Seddon M , Melikian N , Dworakowski R , Shabeeh H , Jiang B , Byrne J , Casadei B , Chowienczyk P , Shah AM . Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo . Circulation . 119 : 2656 - 2662 2009 ; 10.1161/CIRCULATIONAHA.108.822205
- 37 . Sharp NJ , Kornegay JN , Van Camp SD , Herbstreith MH , Secore SL , Kettle S , Hung WY , Constantinou CD , Dykstra MJ , Roses AD . An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy . Genomics . 13 : 115 - 121 1992 ;
- 38 . Su JB , Barbe F , Crozatier B , Campbell DJ , Hittinger L . Increased bradykinin levels accompany the hemodynamic response to acute inhibition of angiotensin-converting enzyme in dogs with heart failure . J Cardiovasc Pharmacol . 34 : 700 - 710 1999 ;
- 39 . Su JB . Kinins and cardiovascular diseases . Curr Pharm Des . 12 : 3423 - 3435 2006 ;
- 40 . Thomas GD , Sander M , Lau KS , Huang PL , Stull JT , Victor RG . Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle . Proc Natl Acad Sci USA . 95 : 15090 - 15095 1998 ;
- 41 . Tonduangu D , Hittinger L , Ghaleb B , Le Corvoisier P , Sambin L , Champagne S , Badoual T , Vincent F , Berdeaux A , Crozatier B , Su JB . Chronic infusion of bradykinin delays the progression of heart failure and preserves vascular endothelium-mediated vasodilation in conscious dogs . Circulation . 109 : 114 - 119 2004 ; 10.1161/01.CIR.0000105726.89770.35
- 42 . Townsend D , Turner I , Yasuda S , Martindale J , Davis J , Shillingford M , Kornegay JN , Metzger JM . Chronic administration of membrane sealant prevents severe cardiac injury and ventricular dilatation in dystrophic dogs . J Clin Invest . 120 : 1140 - 1150 2010 ; 10.1172/JCI41329
- 43 . Valentine BA , Cooper BJ , de Lahunta A , O'Quinn R , Blue JT . Canine X-linked muscular dystrophy. An animal model of Duchenne muscular dystrophy: clinical studies . J Neurol Sci . 88 : 69 - 81 1988 ;
- 44 . Weidenbach R , Schulz R , Gres P , Behrends M , Post H , Heusch G . Enhanced reduction of myocardial infarct size by combined ACE inhibition and AT(1)-receptor antagonism . Br J Pharmacol . 131 : 138 - 144 2000 ; 10.1038/sj.bjp.0703544

Figure 1

Changes in mean arterial pressure (MAP) in response to acetylcholine (Ach) at baseline (a) and after 4 weeks (b) of placebo or bradykinin (BK) infusion in conscious control and GRMD dogs. *p<0.05 vs control placebo, †p<0.05 vs control-BK and ‡p<0.05 versus GRMD-placebo.

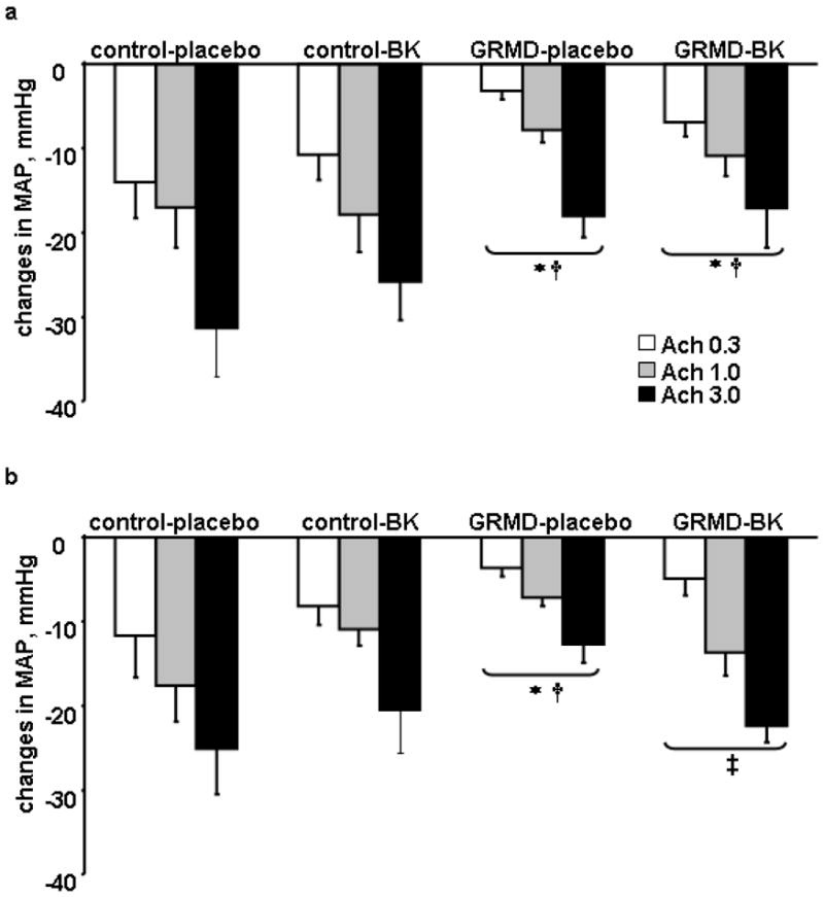

Figure 2

Relaxant effects of acetylcholine (Ach) on coronary arteries in control and GRMD dogs.

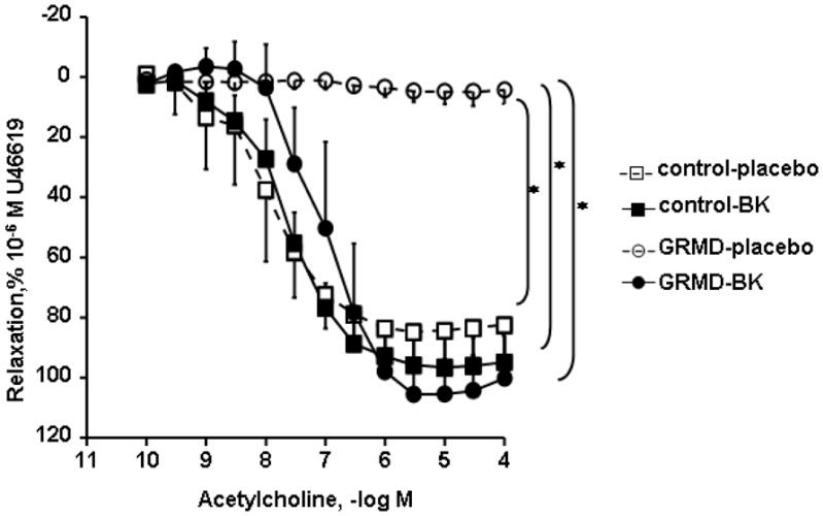

Figure 3

Relaxant effects of acetylcholine (Ach) in the absence and presence of indomethacin (INDO), N ω-nitro-L-arginine (LNA) or LNA plus indomethacin were examined in placebo-treated control dogs (a), in bradykinin (BK)-treated control dogs (b), in placebo-treated GRMD dogs (c) and in bradykinin-treated GRMD dogs (d). *p<0.05.

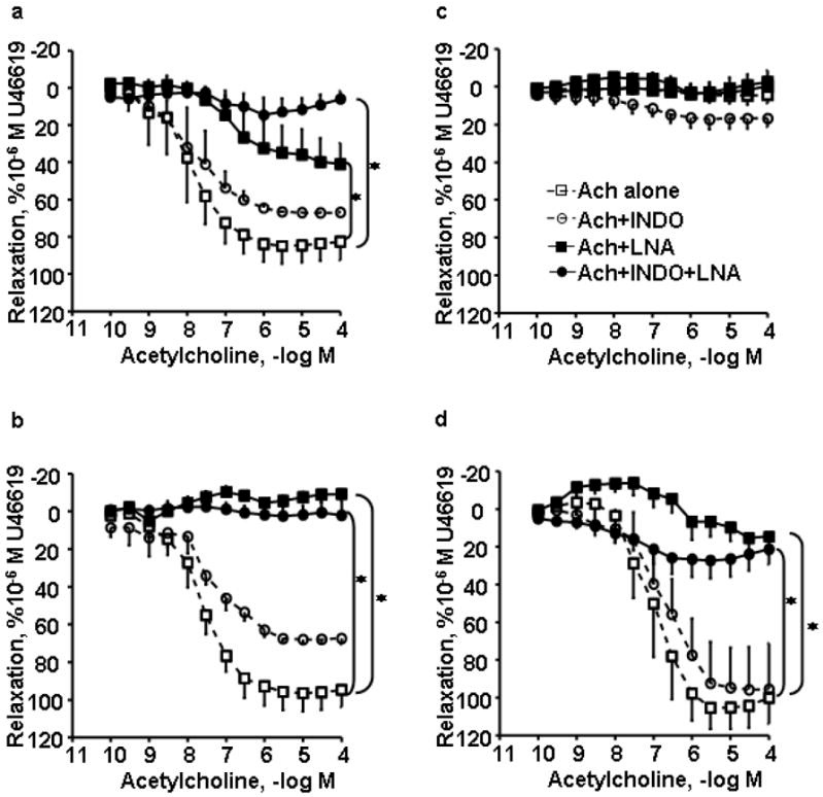

Figure 4

eNOS and nNOS levels in the carotid arteries of control and GRMD dogs (a) A typical example of Western blot of eNOS, nNOS and calnexin protein expression in one dog of each group. (b) Mean values of eNOS in each group. (c) Mean values of nNOS in each group. MW: molecular weight. *p<0.05 versus placebo-treated control dogs, †p<0.05 vs bradykinin (BK)-treated control dogs and ‡p<0.05 vs placebo-treated GRMD.

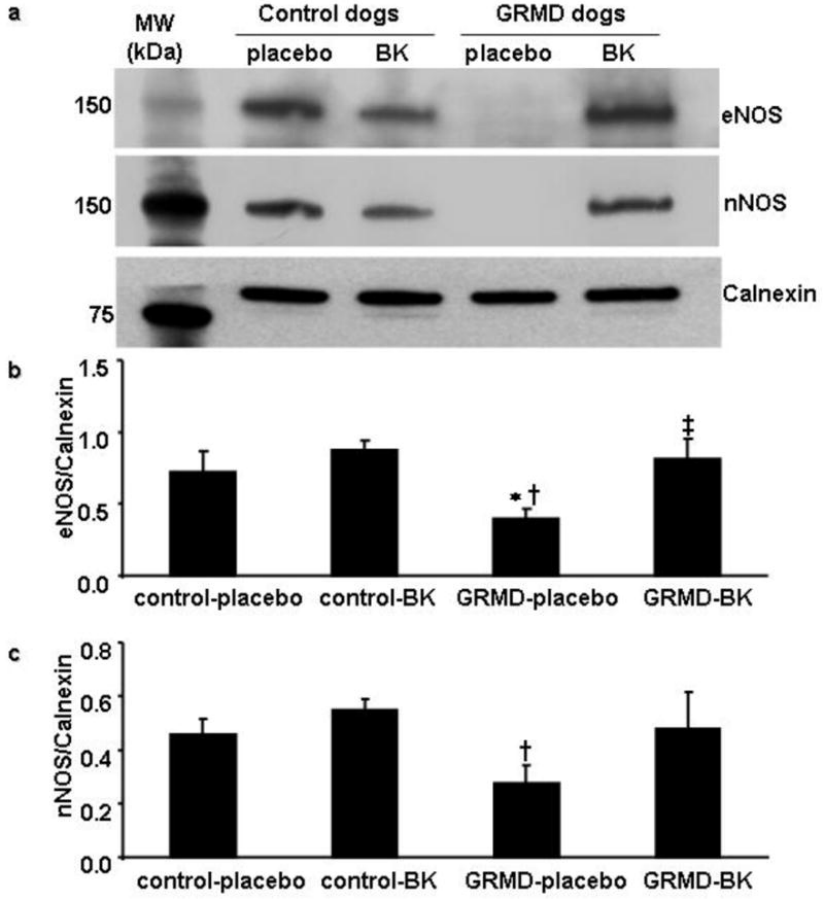

Figure 5

cGMP concentrations in the carotid artery obtained in control and GRMD dogs treated with placebo or bradykinin (BK). *p<0.05 vs placebo-treated control dogs.

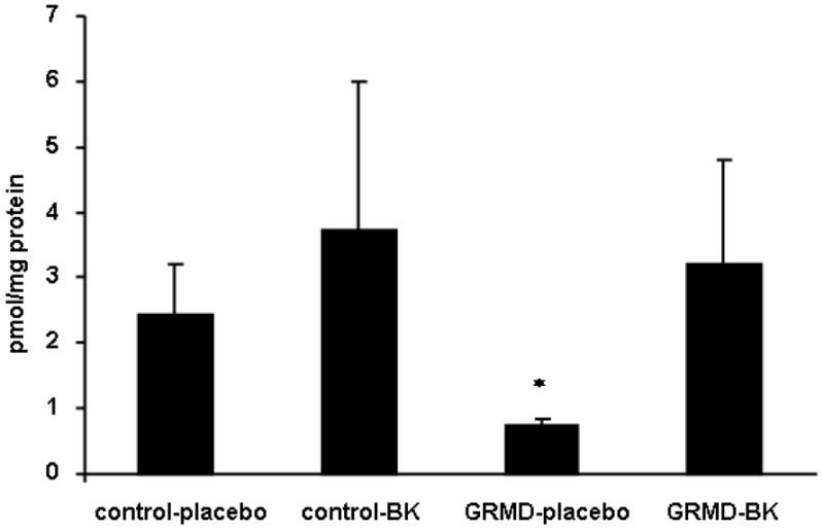

Table 1

Baseline parameters of control and GRMD dogs

	Control dogs		GRMD dogs	
	Placebo	**Bradykinin**	**Placebo**	**Bradykinin**
N	4	4	5	5
Age, month	12.3±0.7	12.9±1.0	12.0±1.4	12.5±0.8
Body weight, kg	26±2	29±3	19±2 * †	19±2 * †
Hear rate, bmp	73±4	80±4	91±3 * †	94±8 *
SAP, mmHg	138±5	138±4	125±7	125±4 * †
MAP, mmHg	117±4	114±3	106±6	101±4 * †
LVEDD, mm	46.1±2.6	46.7±3.6	43.1±4.7	43.2±1.4
Fractional shortening, %	34±2	35±1	27±1 * †	28±1 * †

SAP: systolic arterial pressure; MAP: mean arterial pressure; LVEDD: left ventricular end-diastolic diameter.

* $p<0.05$ vs control placebo group and

† $p<0.05$ vs control bradykinin group.