Muscarinic and β-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase

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Nitric oxide (NO) is an ubiquitous signaling molecule produced from L-arginine by NO synthase (NOS). In the vasculature, NO mediates parasympathetic endothelium-dependent vasodilation. NO may also mediate the parasympathetic control of myocardial function[1]. This is supported by the observations that NOS3, the endothelial constitutive NOS, is expressed in normal cardiac myocytes from rodents[2] and human[3], and NOS and/or guanylyl cyclase inhibitors antagonize the effect of muscarinic agonists on heart rate[4,5], atrio–ventricular conduction[6], contractility[2,4,7] and L-type calcium current[1,2,5,6]. Here we examine the autonomic regulation of the heart in genetically engineered mice deficient in NOS3 (NOS3-KO)(ref. 8). We show that the chronotropic and inotropic responses to both β-adrenergic and muscarinic agonists were unaltered in isolated cardiac tissue preparations from NOS3-KO mice, although these mice have a defective parasympathetic regulation of vascular tone[8,9]. Similarly, β-adrenergic stimulation and muscarinic inhibition of the calcium current did not differ in cardiac myocytes from NOS3-KO mice and those from wild-type mice. RT–PCR did not demonstrate upregulation of other NOS isoforms. Similarly, G_i/G_o proteins and muscarinic receptor density were unaltered. These data refute the idea that NOS3 is obligatory for the normal autonomic control of cardiac muscle function[10].

We studied wild-type (WT; $n = 20$) and NOS3-KO mice ($n = 24$) matched for age and body weight. There was no significant difference in the absolute 'wet weight' of the ventricles in these groups, but NOS3-KO mice had an almost 10% increase in the ratio of ventricular weight to body weight (4.7 ± 0.08 mg/g in NOS3-KO mice compared with 4.3 ± 0.08 mg/g in WT mice; $P = 0.02$) and an almost 25% increase in cell membrane capacitance (205 ± 16 pF in NOS3-KO mice ($n = 27$) compared with 167 ± 13 pF in WT mice ($n = 35$); $P = 0.06$), indicating a small amount of ventricular hypertrophy. We assessed the chronotropic effects of isoprenaline (ISO), a β-adrenergic agonist, and carbachol (CCh), a muscarinic agonist, in spontaneously beating isolated right atria. In both groups of mice, application of ISO at concentrations greater than 1 nM increased the beating frequency in a concentration-dependent manner, with a maximal stimulation of about 70% obtained at 100 nM (Fig. 1*a*). Half-maximal effects (EC_{50}) were reached at 7.7 ± 3.9 nM ISO in WT mice and at 3.2 ± 0.31 nM ISO in NOS3-KO mice; the difference was not statistically significant ($P = 0.24$). CCh started to decrease beating frequency at a concentration of 0.03 μM, and the effect was maximal at 10 μM, at which concentration most muscles stopped beating (Fig. 1*b*). The effect of CCh on beating frequency was identical in NOS3-KO and WT mice for both maximal effect and potency (half-maximal inhibition at 0.40 ± 0.08 μM in WT mice and 0.43 ± 0.07 μM in NOS3-KO mice).

We assessed the inotropic effects of ISO and CCh in electrically driven mouse papillary muscles. As in most other mammalian species, muscarinic receptor activation had no direct negative inotropic effect in mouse ventricle, as CCh at 1 μM had no effect on basal force of contraction in mouse papillary muscle (100 ± 8% of basal; $n = 6$). However, as in other species, CCh produced a substantial indirect negative inotropic effect best demonstrated after β-adrenergic receptor activation with ISO. Cumulative addition of ISO increased contractile force to a maximum of 340% of baseline in papillary muscle from both WT and NOS3-KO mice (Fig. 1*c*). The effect of ISO started at 0.01 μM, was maximal at 0.3 μM and had a similar EC_{50} value in WT and NOS3-KO mice (39 ± 12 ($n = 12$) and 28 ± 4 nM ($n = 16$), respectively; $P = 0.38$). In papillary muscles pre-stimulated with 0.3 μM ISO, the negative inotropic effect of CCh started at 0.01 μM in both groups and was maximal at 10 μM (Fig. 1*d*). At this concentration, the positive inotropic effect of ISO was decreased by 76 ± 5% ($n = 13$) in WT mice and 86 ± 9% ($n = 11$) in NOS3-KO mice. ANOVA analysis of the concentration–response curves indicated a statistically significant difference between NOS3-KO and WT mice, with a higher half-maximal inhibition value in WT mice (1.1 ± 0.3 in WT mice compared with 0.4 ± 0.08 μM in NOS3-KO mice ($n = 11$ each); $P < 0.05$). However, as desensitization of the ISO response over time might interfere with the response to CCh, we did additional 'double bolus' experiments in which only two concentrations (3 and 10 μM) of CCh were tested on a single concentration (0.3 μM) of ISO. These experiments demonstrated no difference between NOS3-KO and WT mice ($n = 12$ for each; not shown). Thus, the indirect negative inotropic effect of CCh was not affected by NOS3 gene disruption. Similarly, there was no difference between WT and NOS3-KO mice in the positive inotropic effect of isoprenaline or the indirect negative inotropic effect of carbachol in the presence of 0.3 μM isoprenaline in electrically driven left atria (six mice and four NOS3-KO mice; not shown).

Because of the determinant role of the L-type calcium current (I_{Ca}) in the initiation and development of cardiac contraction

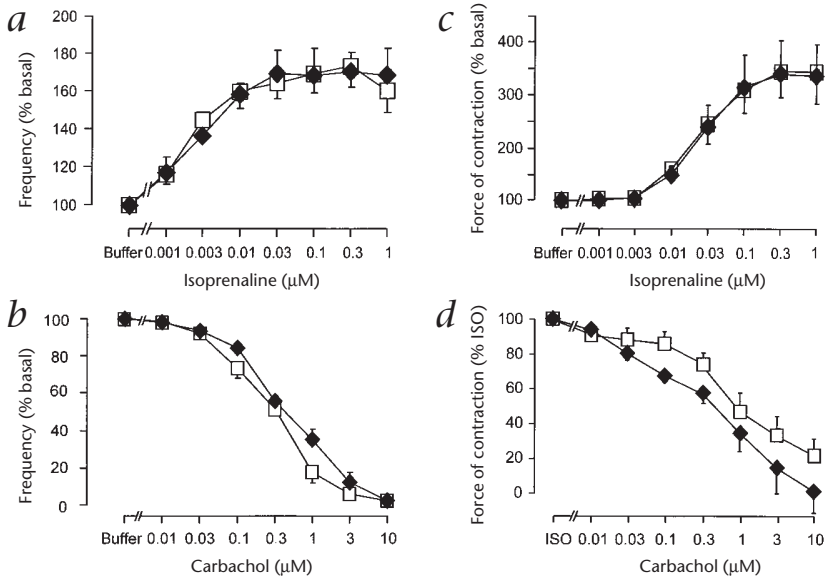

Fig. 1 β-adrenergic and muscarinic chronotropic and inotropic effects. □, WT; ◆, NOS3-KO. Points indicate the mean; vertical lines, the s.e.m. ***a*** and ***b***, Mouse right atrium beating frequency is expressed in % of basal values before addition of the first concentration of ISO (*a*) or CCh (*b*). Basal values were 300 ± 27 bpm, WT (n = 10) and 316 ± 17 bpm, NOS3-KO (n = 11)(*a*), and 352 ± 22 bpm, WT (n = 11) and 341 ± 6 bpm, NOS3-KO (n = 12)(*b*). ***c*** and ***d***, Electrically paced (240/min) papillary muscles were exposed to increasing concentrations of ISO (*c*) or CCh in the presence of 0.3 μM ISO (*d*). *c*, Vertical axis, force of contraction in % of the basal value. Basal values were 7.8 ± 1.6 mg, WT (n = 12) and 9.1 ± 2.1 mg, NOS3-KO (n = 16). *d*, The ISO-mediated increase in contractile force was set to 100%, and the vertical axis indicates force of contraction in % of the ISO-stimulated value. Basal values were 10.5 ± 2.7 mg, WT (n = 13) and 15.4 ± 4.4 mg, NOS3-KO (n = 11). The ISO-mediated increase was 15.6 ± 3.2 mg for WT and 17.6 ± 4.0 mg for NOS3-KO.

and its autonomic regulation[11], we assessed the effects of ISO and CCh on I_{Ca} in single ventricular myocytes isolated from WT and NOS3-KO mice (Fig. 2), both in the absence and presence of N^G-monomethyl-L-arginine (L-NMMA; 1 mM) used to inhibit NOS activity. Basal I_{Ca} density (the amount of calcium current (pA) normalized to cell membrane capacitance (pF); this provides an estimate of the amount of calcium current per surface membrane area) was not different in the two groups of mice (3.9 ± 0.3 pA/pF in WT mice (n = 16) compared with 4.4 ± 0.2 pA/pF in NOS3-KO mice (n = 16); P = 0.19), and was not affected by L-NMMA treatment (not shown). In both groups, exposure of the cells to ISO (30 nM) induced a substantial stimulation of I_{Ca} (Fig. 2*a* and *b*). On average, ISO increased I_{Ca} by 90.7 ± 8.0% in WT mice (n = 14), and the effect was not significantly different in NOS3-KO mice (101.4 ± 7.5% (n = 18); P = 0.34) or after NOS inhibition with L-NMMA (87.7 ± 11.4% (n = 11); P = 0.83). L-NMMA had also no effect on ISO response in NOS3-KO mice (105.6 ± 5.3% (n = 12); P = 0.68). Application of CCh to the myocytes in the continuing presence of ISO produced a profound reduction of the β-adrenergic stimulation of I_{Ca}. In both WT mice (Fig. 2*a*) and NOS3-KO mice (Fig. 2*b*), the effect of CCh was dose-dependent and fully reversible. At all three concentrations tested (10 nM, 100 nM and 1 μM), CCh produced identical effects on I_{Ca} in WT and NOS3-KO mice

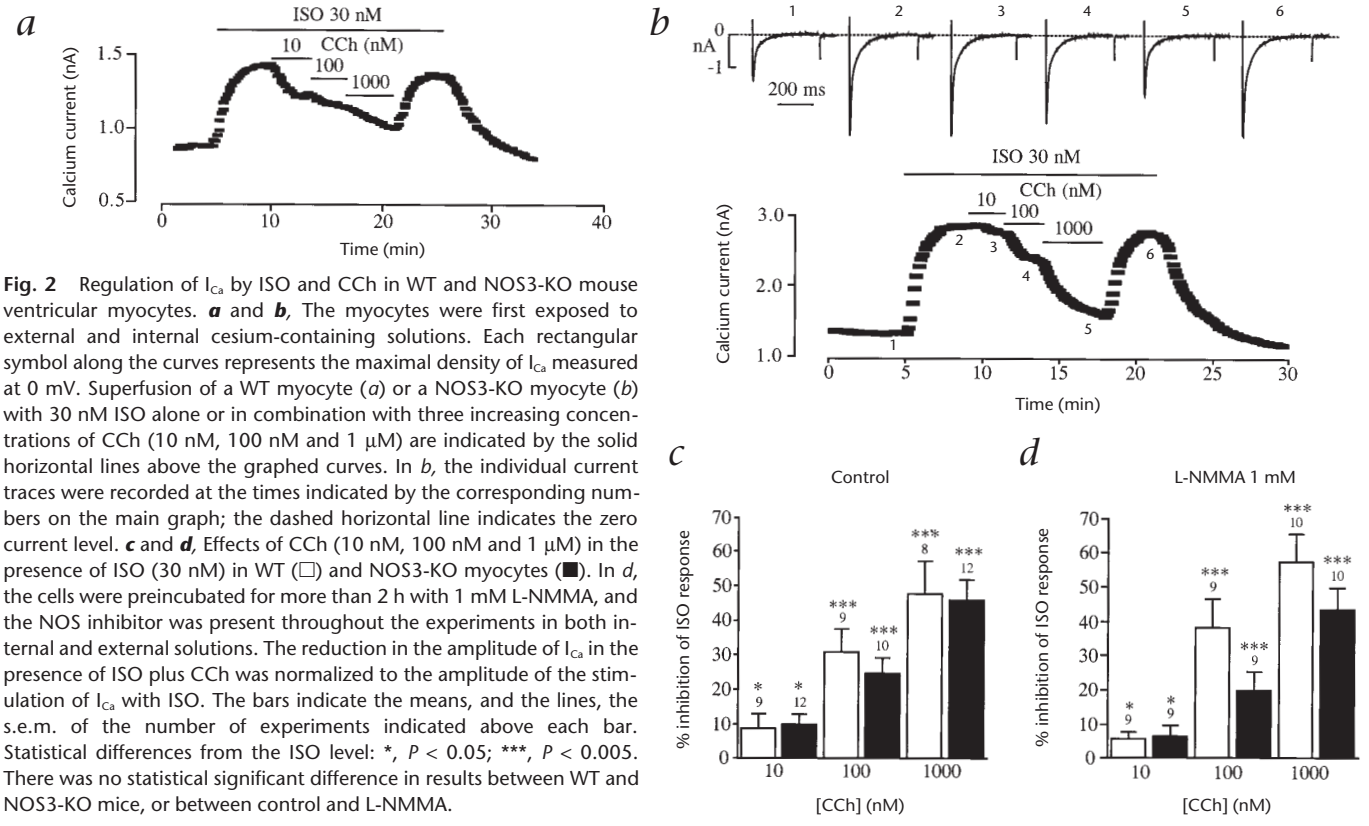

Fig. 2 Regulation of I_{Ca} by ISO and CCh in WT and NOS3-KO mouse ventricular myocytes. ***a*** and ***b***, The myocytes were first exposed to external and internal cesium-containing solutions. Each rectangular symbol along the curves represents the maximal density of I_{Ca} measured at 0 mV. Superfusion of a WT myocyte (*a*) or a NOS3-KO myocyte (*b*) with 30 nM ISO alone or in combination with three increasing concentrations of CCh (10 nM, 100 nM and 1 μM) are indicated by the solid horizontal lines above the graphed curves. In *b*, the individual current traces were recorded at the times indicated by the corresponding numbers on the main graph; the dashed horizontal line indicates the zero current level. ***c*** and ***d***, Effects of CCh (10 nM, 100 nM and 1 μM) in the presence of ISO (30 nM) in WT (□) and NOS3-KO myocytes (■). In *d*, the cells were preincubated for more than 2 h with 1 mM L-NMMA, and the NOS inhibitor was present throughout the experiments in both internal and external solutions. The reduction in the amplitude of I_{Ca} in the presence of ISO plus CCh was normalized to the amplitude of the stimulation of I_{Ca} with ISO. The bars indicate the means, and the lines, the s.e.m. of the number of experiments indicated above each bar. Statistical differences from the ISO level: *, $P < 0.05$; ***, $P < 0.005$. There was no statistical significant difference in results between WT and NOS3-KO mice, or between control and L-NMMA.

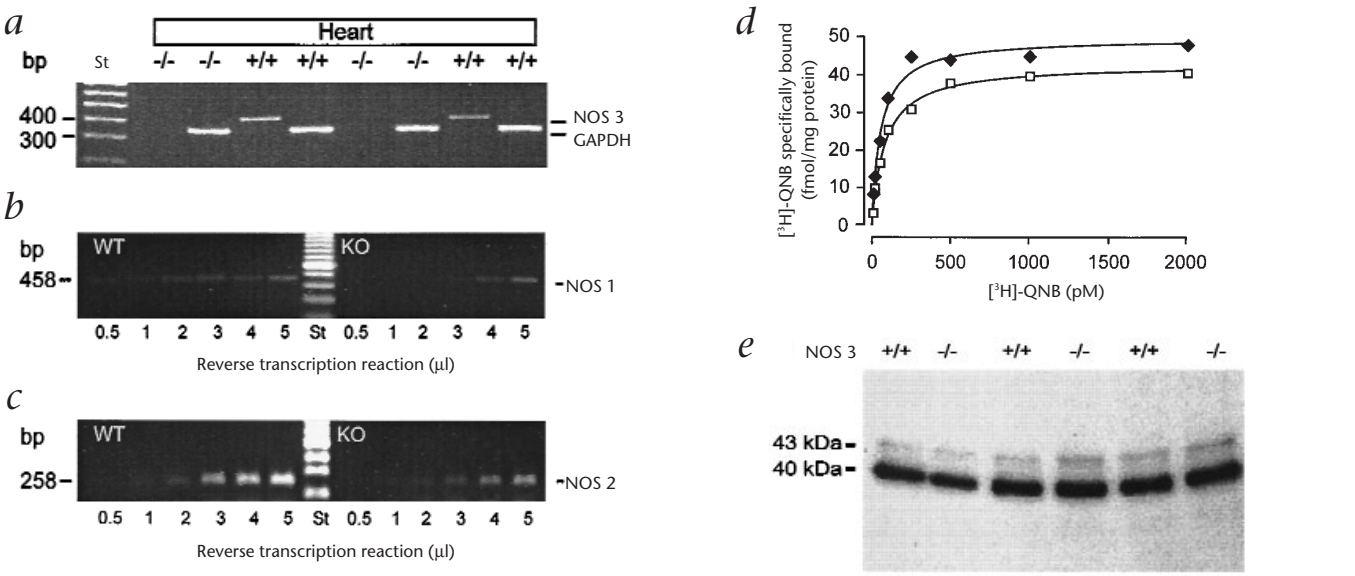

Fig. 3 RT–PCR analysis of NOS isoforms and muscarinic receptor binding and G_i/G_o content in hearts from WT and NOS3-KO mice. ***a*–*c***, Total RNA was prepared from whole heart ventricle homogenates, treated with DNase I and subjected to RT–PCR. Left margins, molecular size; right margins, positions of RT–PCR products. St, standard. *a*, NOS3 and GAPDH (positive control) amplicons in two NOS3-KO (–/–) hearts and two WT (+/+) hearts. *b*, NOS1 amplicons in 0.5–5 µl RT reaction (= 50–500 ng total RNA) of one WT and one KO hearts. *c*, NOS2 amplicons in 0.5 to 5 µl RT reaction (= 50-500 ng total RNA) of one WT heart and one KO heart. *a*–*c* represent two to five experiments with a total of four to ten hearts from each group. ***d***, Representative saturation isotherms of ^{3}H-QNB binding to crude homogenates from WT (□) and NOS3-KO (◆) mouse ventricles. The density (B_{max}) and affinity (K_D) of cardiac muscarinic receptors in WT and NOS3-KO mice were determined in crude ventricular homogenates by radioligand saturation experiments. B_{max} and K_D were 38 ± 4 fmol/mg protein (n = 5) and 70 ± 17 pM (n = 3), respectively, in WT mice, and 37 ± 3 fmol/mg protein (n = 5) and 120 ± 72 pM (n = 3), respectively, in NOS3-KO mice. ***e***, Representative autoradiography of the 40/41-kDa region of SDS–PAGE of crude ventricular homogenates of mice hearts after a pertussis toxin-catalyzed ^{32}P-ADP-ribosylation *in vitro*. Left margin, the apparent sizes of the two PTX-sensitive G proteins $G_{i,\alpha2}$ (40 kDa) and $G_{i,\alpha3}$ (43 kDa). The concentration of G_i/G_o protein α-subunits was determined in crude ventricular homogenates by pertussis toxin-catalyzed ADP-ribosylation in the presence of ^{32}P-NAD as described[18].

(Fig. 2*c*). Moreover, the muscarinic response was similar in the absence (Fig. 2*c*) and presence (Fig. 2*d*) of L-NMMA in both WT and NOS3-KO mice, eliminating the possibility that other NOS isoforms are involved in this response.

RT–PCR analysis of NOS mRNA proved the lack of NOS3 expression in the hearts of NOS3-KO mice (Fig. 3*a*) and excluded the possibility of upregulation of the other constitutive neuronal NOS1 (Fig. 3*b*) or inducible NOS2 (Fig. 3*c*) genes. Two independent series of experiments on a total of four hearts from each group, done in semiquantitative conditions, demonstrated amplified NOS1 and NOS2 cDNA in all samples, albeit at very low and varying levels (NOS1: 5.37 ± 0.93 arbitrary units [a.u.] in WT compared with 5.0 ± 1.73 a.u. in NOS3-KO, n = 4 each; NOS2: 4.6 ± 1.0 a.u. in WT compared with 3.1 ± 1.2 a.u. in NOS3-KO, n = 4 each). The identity of the amplicons was verified by direct sequencing. Muscarinic receptor densities (Fig. 3*d*) and G_i/G_o protein content (Fig. 3*e*) were also determined to assess whether counter regulatory mechanisms might account for sensitization of the muscarinic receptor pathway in NOS3-KO mice. Muscarinic receptor density and affinity for ^{3}H-QNB (^{3}H-Quinuclidinyl benzilate) were not substantially different in WT and NOS3-KO mice (Fig. 3*d*). Similarly, incorporation of ^{32}P-labeled ADP ribose into the two main G_i/G_o protein α-subunits in the heart ($G_{i\alpha2}$ and $G_{i\alpha3}$) was found to be identical in the two groups of mice (Fig. 3*e*). Thus, neither of these parameters differed substantially for NOS3-KO and WT mice.

Although there is compelling evidence that exogenously applied NO affects cardiac rhythm and contractility[1,11], the role of endogenous NO produced inside cardiac myocytes remains a matter of debate. The muscarinic control of cardiac rate[4,5], contractility[2,4,7] and I_{Ca}[1,2,5,6,11] is attenuated or even abolished when the NO/cGMP pathway is blocked by L-arginine analogues or guanylyl cyclase inhibitors. Similarly, these conditions increase the sensitivity of cardiac preparations towards β-adrenergic stimulation[4,12]. Although these studies support an 'obligatory' role of endogenous NO in the autonomic control of the heart, this is challenged by opposite results[11–17]. To settle the issue, we and others[10] used genetically engineered mice that are deficient in NOS3, the only NOS isoform normally expressed in intact cardiac myocytes[1–3]. However, the results are again contradictory. The muscarinic regulation of I_{Ca} was absent in NOS3-KO mice elsewhere[10], whereas it is absolutely normal here, although the mice used in both studies originated from the same laboratory[8]. Although, we have no obvious explanation for this discrepancy, our further experiments on intact muscle preparations demonstrate that muscarinic and β-adrenergic regulation of beating frequency and force of contraction, two principal physiological indices of heart function, were also similar in WT and NOS3-KO mice. Moreover, basal values of these parameters did not differ in the two groups of mice. Finally, we found no evidence for the involvement of other NOS isoforms or for counter-regulatory mechanisms in the NOS3-KO mice relevant to the autonomic control of the heart. Thus, given this large body of evidence obtained both in vitro and in physiological conditions, we conclude that NOS3 is not obligatory for the postsynaptic autonomic regulation of the heart.

Methods

Isolated muscle preparations and contraction experiments. Adult WT (C57B/6) and NOS3-KO mice 3–6 months of age[8] were anesthetized with CO_2, weighed, and killed by decapitation. The heart, including the great vessels, was removed rapidly into gassed Tyrode's solution (119.8 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 22.6 mM $NaHCO_3$, 0.05 mM Na_2EDTA, 0.28 mM ascorbic acid, 5.0 mM glucose and 30 mM 2,3-butanedione monoxime (BDM), continuously gassed with 95% O_2 and 5% CO_2) as described[18]. Right and left atria and left papillary muscles were dissected from the ventricles, suspended individually in 20-ml organ baths (Tyrode's solution without BDM), and connected to force transducers to record the isometric force of contraction. Data were recorded and evaluated by a PC-based system (BMON; Ingenieurbüro Jäckel, Hanau, Germany). Muscles were equilibrated at a pacing frequency of 30/min (left atria and papillary muscles) for 10–20 min and then carefully stretched to the length of near-maximal force development, which was reached at a baseline tension of (mean) 30 mg for right atria, 24 mg for left atria and 55 mg for papillary muscles. The pacing frequency was then increased to 240/min, which was chosen as the best compromise between the physiological frequency (500–600/min) and experimental stability. The influence of endogenous adenosine was abolished by adenosine deaminase (ADA, 1 μg/ml; Boehringer), neuronal release in paced preparations (left atrium, papillary muscle) blocked by tetrodotoxin (TTX) at 20 ng/ml and ISO-induced automaticity in left atria, papillary muscles blocked by lidocaine (LIDO) at 0.002% (85 μM). After complete equilibration (three changes of Tyrode's solution), the following protocols were used (each concentration step lasted for 5 min or until a steady state condition was reached): To assess the chronotropic effects of CCh and ISO in right atria: ADA, cumulative addition of CCh (0.01–10 μM), three washes, ADA, cumulative addition of ISO (0.001–1 μM. To assess the inotropic effects of CCh and ISO in electrically driven left atria and papillary muscles: ADA + TTX + LIDO, 0.3 μM ISO, cumulative addition of CCh (0.01–10 μM), three washes, ADA + TTX + LIDO, cumulative addition of ISO (0.001–3 μM). To assess the inotropic effect of bolus applications of ISO and CCh in electrically driven papillary muscle: ADA + TTX + LIDO, 0.3 μM ISO, 3 and 10 μM CCh. The experiments were done at 37 °C.

Electrophysiology. Isolation of cardiac myocytes from WT (C57B/6 or 129SvEv) and NOS3-KO mice[8] was achieved using to the method described[19]. The whole-cell configuration of the patch-clamp technique was used to record the L-type calcium current (I_{Ca}) in 1.8 mM $CaCl_2$ on calcium-tolerant myocytes with a routine protocol consisting of a depolarizing test pulse to 0 mV (400 ms duration) elicited every 8 s from a holding potential of –50 mV. The control solutions and the protocol used to measure I_{Ca} were as described[14]. The experiments were done at room temperature (19–23 °C).

Detection of NOS mRNA. Total RNA was prepared from frozen ventricular tissue of WT and NOS3-KO mice. Reverse transcription (RT) from 5 μg total RNA was done with random hexamers and the PCR with the specific primers as described[20]. NOS3 was assessed qualitatively (35 cycles) in five WT and five NOS3-KO samples. For quantitative experiments, RNA was treated with DNase I for 15 min at 37 °C and heated to 70 °C for 15 min before RT. NOS1 and NOS2 quantification was done with 0.5, 1, 2, 3, 4 and 5 μl reverse transcriptase, and GAPDH quantification, with 0.01, 0.025, 0.05, 0.1, 0.2 and 0.4 μl of identical reverse transcriptase, to ensure that the reaction was in the linear range. Ethidium bromide signals of PCR products were quantified by densitometry (Molecular Analyst; BioRad, Richmond, California). The GAPDH signal was used for normalization (3,947 ± 719 in WT mice compared with 4,012 ± 932 in NOS3-KO mice, n = 4 for each). The PCR protocol was 2 min at 94 °C; 37 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C; 5 min at 72 °C. Primers (sense and antisense) were: NOS3, 5′–GACATTGAGAGCAAAGGGCTGC–3′ and 5′–CGGCTTGTCACCTCCTGG–3′; NOS1, 5′–GGCAACAGCGGCAATTTG–3′ and 5′–TGGACTCAGATCTAAGGCGGTTG–3′; NOS2, 5′–AATAGAGGAACATCTGGCCAGG–3′ and 5′–ATGGCCGACCTGATGTTGC–3′; GAPDH, 5′–ATGGGAAGCTTGTCATCAACG–3′ and 5′–GGCAGTGATGGCATGGACTG–3′. RT–PCR with RNA but without reverse transcriptase served as negative controls. NOS cDNAs were purified from the gel and directly sequenced to check the identity of each amplicon (Medigene, München, Germany).

Data analysis. The results are expressed as means ± s.e.m. Differences between WT and NOS3-KO mice were analyzed by Student's t-test for unpaired observations. Effects of drugs on the frequency, force of contraction or I_{Ca} were analyzed by Student's t-test for paired observations. Concentration–response curves were analyzed by two-way ANOVA. A P value of less than 0.05 was considered significant. 'Basal' refers to the absence of ISO.

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