Effects of nicotine administration in a mouse model of familial hypertrophic cardiomyopathy, α-tropomyosin D175N

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Gaffin RD, Chowdhury SA, Alves MS, Dias FA, Ribeiro CT, Fogaca RT, Wieczorek DF, Wolska BM. Effects of nicotine administration in a mouse model of familial hypertrophic cardiomyopathy, α-tropomyosin D175N. Am J Physiol Heart Circ Physiol 301: H1646–H1655, 2011. First published July 8, 2011; doi:10.1152/ajpheart.00277.2010.—The effects of nicotine (NIC) on normal hearts are fairly well established, yet its effects on hearts displaying familial hypertrophic cardiomyopathy have not been tested. We studied both the acute and chronic effects of NIC on a transgenic (TG) mouse model of FHC caused by a mutation in α-tropomyosin (Tm; i.e., α-Tm D175N TG, or Tm175). For acute effects, intravenously injected NIC increased heart rate, left ventricular (LV) pressure, and the maximal rate of LV pressure increase (+dP/dt) in non-TG (NTG) and Tm175 mice; however, Tm175 showed a significantly smaller increase in the maximal rate of LV pressure decrease (−dP/dt) compared with NTGs. Western blots revealed phosphorylation of phospholamban Ser16 and Thr17 residue increased in NTG mice following NIC injection but not in Tm175 mice. In contrast, phosphorylation of troponin I at serine residues 23 and 24 increased equally in both NTG and Tm175. Thus the attenuated increase in relaxation in Tm175 mice following acute NIC appears to result primarily from attenuated phospholamban phosphorylation. Chronic NIC administration (equivalent to smoking 2 packs of cigarettes/day for 4 mo) also increased +dP/dt in NTG and Tm175 mice compared with chronic saline. However, chronic NIC had little effect on heart rate, LV pressure, −dP/dt, LV wall and chamber dimensions, or collagen content for either group of mice.

Numerous studies have tested the adverse effects of smoking on cardiovascular health in both healthy and diseased hearts (reviewed in Ref. 5). However, many of these adverse effects may be attributable to the “tar” and/or carbon monoxide in cigarette smoke. Nicotine (NIC) is widely heralded as the addictive component of cigarettes, cigars, and pipe tobacco (6, 7), and its impact alone on cardiovascular disease is gaining more recognition due to the presence of a myriad of smokeless tobacco products (reviewed in Ref. 46). Yet, to the best of our knowledge, its impact on familial hypertrophic cardiomyopathies (FHCs) has not been studied.

FHCs are defined as autosomal dominant disorders that result in numerous cardiac maladies including hypertrophy, diastolic dysfunction, fibrosis, myocyte disarray, and arrhythmias (9, 34). They are considered to be a leading cause of sudden cardiac death in young athletes (10, 32, 35) and have a prevalence of 1:500 when familial and nonfamilial forms are considered together (16). FHCs have been described as a “disease of the sarcomere” since the majority of cases have been linked to mutations in sarcomeric proteins such as myosin heavy chain, troponin T, myosin-binding protein C, and α-tropomyosin (Tm; Refs. 9, 21, 54, 64). We have chosen to use α-TmD175N, originally described in the Japanese population (43), as our model of FHC in this study. In this FHC-causing mutation, residue 175 of Tm has been mutated from Asp to Asn, and the resulting phenotype produces diastolic dysfunction in mice with little to no cardiac hypertrophy or fibrosis (42). The purpose was to compare the effects of both acute and chronic NIC administration on Tm175 and nontransgenic (NTG) hearts.

Most acute NIC studies on cardiac function have focused on its chronotropic, inotropic, and vasopressor effects but not itslusotropic effects. Tm175 is ideal for testing the latter for it affords a comparison between normal diastolic function (i.e., NTG mice) and diastolic dysfunction (i.e., Tm175 mice) without the confounding effects of either maladaptive hypertrophy or fibrosis. We also sought to address the disparity in results found in many acute NIC studies that appears to stem from the method of administration (bolus vs. sustained infusion) and the concentration of NIC used (2, 4, 37, 38, 47). Unfortunately, most of these reports use abnormally high NIC concentrations that are not normally found in humans, and they administer NIC in a bolus injection. To accurately reproduce the most common use of NIC-containing products (i.e., smoking), one must administer NIC slowly over several minutes to achieve a concentration similar to that seen in the plasma of smokers (29). Our study achieves this by acutely administering NIC in a concentration (29, 52) and time frame commonly seen in cigarette smokers. We also examined the effects of chronic, continuous NIC use (i.e., 4 mo) on hemodynamics and cardiac remodeling in NTG and Tm175 mice.

MATERIALS AND METHODS

In Situ Measurements for Acute and Chronic NIC Studies

We used TG mice (FVB/N strain) expressing the D175N mutation in α-TM and NTG littermates for both acute and chronic NIC studies. All experiments were conducted and the care of animals provided in compliance with animal care policies according to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985) and approved by the Institutional Animal Review Board of the University of Illinois at Chicago. TG mice were generated as originally described (42). In situ experiments were performed using a modified method as recently described (17, 45).
In acute experiments, mice (males = 5, females = 3 for both NTG and Tm175) were anesthetized with an initial intraperitoneal dose of etomidate (10 mg/kg), morphine (1 mg/kg), and urethane (750–1,000 mg/kg). Plane of anesthesia was monitored by toe pinch, and subsequent injections (one-fifth of initial dose) were administered until a reflex withdrawal of the toe was no longer observed. Mice were placed on a thermally controlled table and maintained at 37°C via rectal monitoring of body temperature.

A tracheotomy was performed using a solid steel intubation cannula (1.2-mm diameter; Hugo Sachs Electronic-Harvard, Germany) inserted into the trachea and secured with suture. The right common carotid artery was then isolated, the distal end tied off with 6–0 suture, and the artery cannulated with a 1.4 F MIKRO-TIP pressure transducer (model SPR-671; Millar, Houston, TX). The transducer was advanced retrogradely down the right carotid artery, into the aorta, through the aortic valve into the left ventricle (LV). Heart rate (HR), LV pressure, the maximum rate of pressure development (+dP/dt) and −dP/dt were continuously monitored and digitally recorded on Chart software (v. 5.5; AD Instruments).

To gain venous access for intravenous infusion of NIC, the right femoral vein was isolated, the distal end tied off, and the proximal end catheterized with stretched PE-10 tubing. This tubing was connected to a 250-μl glass syringe mounted on a model 355 microinfusion pump (Sage Instruments, Cambridge, MA), which allowed for precise delivery of NIC hydrogen tartrate salt in various doses: 2.5, 5, and 10 ng·g⁻¹·min⁻¹ delivered over 8 min at 1 μL/min. The NIC concentrations used (20, 40, and 80 ng/g) correlate to the amount of NIC absorbed by a human after smoking 0.25, 0.5, or 1 cigarette (20, 29, 47, 52, 55). In control experiments, we utilized saline for femoral injections. At the end of all experiments, the pressure catheter was removed from the LV, and the heart was excised, quickly weighed, and flash frozen in liquid nitrogen for molecular studies.

In chronic experiments, similar procedures were used except isoproterenol (ISO; 0.16 ng·g⁻¹·min⁻¹) was injected into the femoral vein instead of NIC. In addition, NTG and Tm175 animals were both chronically exposed to NIC for 4 mo before in situ measurements were taken. Chronic NIC exposure was accomplished via Azlet osmotic pumps (model 2004) placed subdermally along the back in 2-mo-old male mice and replaced approximately every 30 days for 4 mo. The concentration of NIC used in the pumps was 6 mg NIC tartrate salt·kg⁻¹·day⁻¹, a dose that produces [nicotine]plasma of 30–40 ng/ml (18, 19). This concentration corresponds to the [nicotine]plasma found in heavy smokers (8).

**Transtracheal Echocardiography**

Mice used in chronic NIC studies were also subjected to echocardiography 1 day before osmotic pump placement (2 mo of age) and then again after 4 mo of chronic NIC treatment (6 mo of age). Mice were anesthetized with isoflurane (0.5–1.0%) in 100% oxygen using a face mask. Animals were maintained in the supine position, and body temperature was monitored rectally and maintained at 37°C using a heating pad. HR was also monitored continuously. Transtracheal echocardiographic recordings were then obtained using a 30-MHz high resolution transducer and an integrated rail system (Vevo 770 High-Resolution Imaging System; Visualsonics, Toronto, ON, Canada) as previously described (48).

**Isolated Mouse Cardiomyocytes**

Cells were isolated and measurements of cell shortening and Ca²⁺ transients were performed as previously described (14, 53, 61, 62).

**Cell isolation.** Hearts from anesthetized (pentobarbital sodium; 50 mg/kg) and heparinized (5,000 U/kg) mice were quickly removed and put into ice cold, nominally Ca²⁺-free perfusion buffer (PB) of the following composition (in mM): 113 NaCl, 4.7 KCl, 0.6 Na₂HPO₄, 0.6 KH₂PO₄, 1.2 MgSO₄, 0.032 phenol red, 12 NaHCO₃, 10 KHC₃O₃, 30 taurine, 10 HEPES, 5.5 glucose, 10 and 2.3-butanediol monoxime (pH 7.4; 37°C). The aorta was cannulated and the heart mounted on a Langendorff perfusion system. Hearts were perfused for 4 min with Ca²⁺-free PB and subsequently for 8–12 min with digestion buffer (DB) containing PB and 12.5 μM Ca²⁺ together with 0.15 mg/ml blendzyme 2 (Roche) and 0.14 mg/ml trypsin (Invitrogen, Carlsbad, CA). Hearts were then removed and transferred to a dish containing PB, and the ventricles were cut into small pieces and gently triturated. At the end of the trituration period, the cell suspension was filtered through a mesh collector and placed into centrifuge tubes, and the digestion process was stopped with an equal volume of PB containing 12.5 μM Ca²⁺ plus 10% bovine calf serum (vol/vol). The cells were then permitted to settle under gravity for 5–7 min. The supernatant fraction was removed, and the cells were resuspended in fresh PB containing 12.5 μM Ca²⁺ and 5% bovine calf serum (vol/vol). Cells were allowed to settle under gravity, the supernatant was removed, and the cells were resuspended in fresh control solution (CS) of the following composition (in mM): 133.5 NaCl, 4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 10 HEPES, and various Ca²⁺ concentrations; first, 200 μM Ca²⁺ followed by 500 μM and then 1 mM Ca²⁺. The cells were stored at room temperature (22–23°C) until used.

**Loading myocytes with fura-2 AM.** After isolation, the cells were placed in a small perfusion chamber mounted on the stage of an inverted microscope. Cells were loaded for 10 min at 30°C in loading solution containing CS and 1 mM Ca²⁺, 1 mg/ml BSA, and 2.5 μM fura-2 AM. After being loaded, extracellular dye was washed out for 5 min by perfusion with fresh CS containing 1.5 mM Ca²⁺. Cells were then stimulated at 0.5 Hz with platinum electrodes placed close to the myocytes.

**Measurement of intracellular Ca²⁺ transients.** Fura-2 AM-loaded myocytes were alternately excited with 340 and 380 nm of light from a monochromator (Photon Technology International). The excitation light was transmitted to the cell under study by a 400 nm dichroic mirror located beneath the microscope nosepiece and a Nikon oil-immersion objective lens. The resulting fluorescence was collected by the objective and transmitted to a multi-image module of the microscope, where it is separated from long wavelength (>600 nm) using a 580-nm dichroic mirror and passed via a 510 ± 25 nm emission filter to a photomultiplier tube. The 340-to-380 ratio (R) was calculated after subtracting the background fluorescence, and the peak amplitude of R and tau for the relaxation phase of the calcium transient were calculated.

**Measurement of cell shortening.** To measure cell shortening, myocytes were illuminated with red light using a special red filter (>600 nm). The cell image was collected using a ×40 Nikon objective and transmitted to the multi-image module, where it was separated from the fluorescence signal by a 580-nm dichroic mirror. Output from the camera was projected onto a TV monitor and a video-edge detector was used to monitor cell length. To characterize contractile parameters from cell length signals, fractional shortening (expressed as %resting length) and tau for the relaxation phase of cell relaxation were calculated.

**NIC perfusion protocol.** After the cells were loaded with fura-2 AM, steady-state recordings of cell contractions and calcium transients were made. This was followed by a 3-min perfusion with 1.07 μM NIC tartrate (375 nM NIC base), which is enough time to ensure all cells in the perfusion chamber were adequately exposed to NIC to obtain steady-state recordings with NIC. Preliminary measurements were also conducted to ensure there was no rundown of cell shortening over the time period necessary for the protocol.

**Western Blots**

Apexes of the LV from NTG and Tm175 mice were homogenized in RIPA buffer (ThermoScientific) with protease and phosphate inhibitors (Upstate) as previously described (31, 60, 63). Protein concentration was determined using a modified Bradford protein assay.
and blotting protocols were modified from those previously described (14, 30, 56).

For all gels, 15–25 μg of protein were separated on either 10, 12, or 15% SDS/polyacrylamide gels and transferred to either 0.1 or 0.2 μm nitrocellulose using a wet transfer method. Membranes were incubated overnight at 4°C in the appropriate primary antibody [anti-phospho calcium-calmodulin kinase II (CaMKII) Thr286 (1:1,000; Abcam, MA); anti-CaM kinase II (1:500; Cell Signaling); anti-phospho phospholamban (PLB) Thr17 (1:2,500; Badrilla); anti-phospho PLB Ser16 (1:1,000; Upstate); anti-PP2A (1:5,000; Upstate); anti-phospho protein phosphatase (PP)α Thr320 (1:500; Cell Signaling); anti-PP1α (1:1,000; Cell Signaling); anti-phospho PP2A Tyr307 (1:2,000; Epitomics); anti-PP2A (1:1,000; Millipore); anti-phospho troponin I (Tnl) Ser23, 24 (1:500; Cell Signaling); and anti-Tnl (1:5,000; Abcam)]. All primary antibody solutions contained 0.1–1.0% BSA and TBS-T. After washes in TBS-T, secondary antibody (either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase; Promega) was applied to the membranes (1:5,000–1:20,000 as appropriate in 2.5% nonfat milk for 1 h) and detection achieved using chemoluminescence (ECL Plus; Amersham). As necessary, membranes were stripped (Restore Plus; Pierce) at 35°C for 30–40 min and reprobed. For all blots, expression levels were determined via densitometry using Adobe Photoshop, v. 7.0.

Hydroxyproline Assay

Hydroxyline assays were conducted as previously described (44).

Histology

Excised hearts from 1-yr-old mice were cannulated via the aorta, and the coronary were arteries retrogradely perfused with ice-cold saline followed by several milliliters of neutral-buffered formalin (Sigma). The heart was then transversely sliced into four pieces, and each piece was placed into a cassette. Cassettes were kept in formalin (Sigma) for 24 h at room temperature, then placed into graded ethanol substitutions (up to 100%), then Clear Rite (xylene substitute; Thermo Scientific), and finally paraffin at 60°C (with 1 change). Hematoxylin and eosin and picrosirius red staining was conducted on 5-μm sections.

Statistical Analyses

All results are presented as means ± SE. Statistical analyses were conducted using a Student’s unpaired t-test for echocardiography at 2 mo of age, two-way repeated measures ANOVA with Tukey’s post hoc test for acute in vivo studies and isolated cardiomyocytes, and two-way ANOVA with Tukey’s post hoc test for Western blot densitometry, chronic in vivo studies, heart weight/tibia length, heart weight, and the hydroxyproline assay. P ≤ 0.05 was regarded as statistically significant.

RESULTS

In Situ Measurements During Acute NIC Treatment

To evaluate the effects of acutely administered NIC on cardiac function, intravenous injections were given in doses that mimic the effects of smoking 0.25, 0.5, and 1 cigarette (2.5, 5.0, and 10.0 ng·g−1·min−1 NIC tartrate, respectively). Figure 1A shows raw pressure traces obtained from the LV in the absence of NIC, and Fig. 1B shows similar traces in the presence of 10 ng·g−1·min−1 NIC tartrate in a Tm175 mouse. Histograms (Fig. 1, C–F) show that HR (Fig. 1C) and LV pressures (Fig. 1D) increased for both groups of mice upon addition of NIC, but they were not different when comparing NTG to Tm175 mice at any given NIC concentration. +dp/dt (Fig. 1E) also increased for both groups of mice in response to NIC infusion. When comparing NTG to Tm175 mice at each NIC concentration, significant differences were observed at baseline and at 2.5 ng·g−1·min−1 NIC, but their values became similar at the two highest NIC concentrations. Figure 1F shows that –dp/dt was similar for Tm175 and NTG mice at baseline and 2.5 ng·g−1·min−1, yet –dp/dt increased less in Tm175 mice compared with NTGs at the two highest NIC concentrations. Thus NIC exacerbates diastolic function in Tm175 mice compared with NTG since Tm175 –dp/dt values increased less than NTG values following NIC infusion.

Isolated Cardiomyocytes (Acute NIC)

To test whether NIC has direct effects on myocardial cells, calcium transients and cell shortening from isolated cardiomyocytes were measured in the presence and absence of NIC. Figure 2, A and B, shows raw data traces for cell shortening and calcium transients from freshly isolated Tm175 cardiomyocytes in the absence (Fig. 2A) and presence of 375 nM NIC (Fig. 2B). Histograms (Fig. 2, C–F) show that this concentration of 375 nM NIC, a level observed in arterial blood shortly after smoking one cigarette (26), had no effect on myocyte percent shortening (Fig. 2C), tau (i.e., 1/e) of relaxation (Fig. 2D), calcium transient amplitude (Fig. 2E), or tau of calcium transient decay (Fig. 2F).

Western Blots (Acute NIC)

Hearts from acute in situ experiments were flash frozen in liquid nitrogen and then analyzed via Western blots to determine the mechanism for the reduced increase in relaxation in Tm175 hearts compared with NTGs. Four groups were analyzed: 1) NTG injected intravenously with saline (NTG_Saline), 2) Tm175 injected intravenously with saline (Tm175_Saline), 3) NTG injected intravenously with NIC (NTG_NIC), and 4) Tm175 injected intravenously with NIC (Tm175_NIC). We examined the phosphorylation levels of several proteins involved in the heart’s lusitropic response: PLB, TnI, Ca2+/CaMKII and the heart’s most abundant protein phosphatases, PP1A and PP2A (28).

Phosphorylation of PLB was examined at residues Ser16 and Thr17 (Fig. 3, A and B). Both Ser16 and Thr17 phosphorylation levels were increased in NTG_NIC compared with NTG_Saline but not in Tm175_NIC compared with Tm175_Saline. Ca2+/CaMKII is known to phosphorylate PLB at Thr17 (13), and it is made constitutively active when autophosphorylated at Thr286 (36). However, there were no significant differences in its phosphorylation (Fig. 3D) in any of the four groups. In addition, there were no significant differences in the phosphorylation status of PP1A (Fig. 3E), the principal phosphatase of PLB (33).

For Tnl, phosphorylation levels at serines 23 and 24, the sites on Tnl phosphorylated by PKA (31, 66), were increased for Tm175_Saline compared with NTG_Saline (Fig. 3C). NIC increased Ser 23,24 phosphorylation in both NTG_NIC and Tm175_NIC compared with NTG_Saline and Tm175_Saline, respectively. There were no significant differences in the phosphorylation PP2A (Fig. 3F), the principal phosphatase of Tnl (40), among any of the four groups. To summarize, acute NIC increases the phosphorylation levels of PLB at residues Ser16 and Thr17 in NTG mice but not in Tm175.
In Situ Measurements Following Chronic NIC Treatment

To evaluate the effects of chronically administered NIC on cardiac function, mice were treated with a high dose of NIC, equivalent to that seen in heavy (2 packs/day) smokers (8), via mini-osmotic pump for 4 mo. Another group of mice received saline via osmotic pumps for the same period of time. Thus there were four groups: 1) NTG_Saline, 2) Tm175_Saline, 3) NTG_NIC, and 4) Tm175_NIC. At the end of the treatment period, the LV was catheterized and hemodynamics were measured before and after ISO. ISO was added to determine if an adrenergic “reserve” existed following a chronic, high dosage of NIC since NIC causes the release of catecholamines from the adrenal medulla (15). Figure 4 shows that HR (Fig. 4A) is not significantly different for any of the four groups at baseline, yet HR increased for each group following ISO. LV pressure (Fig. 4B) was higher in Tm175_NIC at baseline, and only NTG_Saline increased after addition of ISO. +dP/dt (Fig. 4C) increased in both NTG_NIC and Tm175_NIC compared with their saline counterparts, and all four groups increased following ISO addition. −dP/dt (Fig. 4D) exhibited a decrease at baseline for Tm175_Saline compared with NTG_Saline and for Tm175_NIC compared with NTG_NIC.

In addition, all groups increased −dP/dt in response to ISO except NTG_NIC. Thus chronic NIC administration increases +dP/dt but not HR and −dP/dt, yet it does not fully activate the adrenergic system. An adrenergic reserve still exists in the face of prolonged NIC exposure.

Cardiac Remodeling Following Chronic NIC Treatment

Changes in cardiac morphology were assessed via high-resolution echocardiography both before (2 mo of age) and after 4 mo of NIC treatment (6 mo of age; Table 1). At 2 mo of age (before NIC treatment), all morphology parameters were similar between NTG and Tm175 mice except anterior wall thickness during diastole, which was smaller in Tm175 mice compared with NTGs. At 6 mo of age, there were no morphological differences found among any of the groups. Heart weight-to-tibia length ratios were also not significantly different in any of the four treatment groups (Fig. 5A), and body weight was not affected by the prolonged NIC treatment either (Fig. 5B).

Two of three diastolic indexes were also different between Tm175 and NTG (Table 1; isovolumic relaxation time and deceleration time of the E wave were both prolonged) indicat-
Fig. 2. Effects of NIC on cell shortening and calcium transients in freshly dispersed cardiac myocytes. NTG and Tm175 cells were loaded with the calcium-sensitive dye fura-2 and then treated with either saline or 375 nM NIC for 3 min at 30°C with a stimulation rate of 0.5 Hz. A and B: representative traces of cell shortening and calcium transients from isolated Tm175 mouse cardiomyocytes. Histograms (C–F) depict cell shortening (C), tau of cell relaxation (D), peak amplitude of the 340-to-380 ratio (R; E), and tau of calcium transient (F). There were no significant differences for any of the analyzed parameters (n = 10–14 cells from 4–6 animals for each group).

Acute Hemodynamics

NIC’s acute cardiovascular effects include increases in HR, myocardial contractility, and blood pressure, and these are largely due to the release of catecholamines from nicotinic acetylcholine receptors found in peripheral postganglionic sympathetic nerve endings and the adrenal medulla (23). Previous studies have described the effects of acute NIC on hearts with normal function (4) or various other cardiac maladies such as ischemic (47), depressed (37, 38) myocardium, or NIC’s ability to induce arrhythmia (25, 39, 65), but none have reported the effects of NIC in FHC. In addition, few if any studies have detailed the lusitropic effects of acute NIC in either normal or diseased hearts. Most of these published reports indicate that in vivo acute NIC injection increases HR (4, 37, 38) and other cardiac parameters such as LV end diastolic pressure, right atrial pressure, and +dP/dt (37, 38), which is in agreement with our results. However, the concen-
trations of NIC used in these other studies were either sevenfold higher than ours (4) or twice the level and delivered as a bolus (37, 38), which does not replicate the act of smoking as accurately as the current protocol, a steady infusion over 8 min. Here, we utilized NIC concentrations that replicate the effects of smoking 0.25, 0.5, or 1 cigarette (29, 52). Thus it is noteworthy that even one cigarette can produce significant differences in relaxation when comparing normal (i.e., NTG) to FHC hearts. In contrast, Przyklenk (47), using a NIC amount and injection protocol that was nearly identical to ours, showed that NIC had no effect on canine HR. The discrepancy between the latter study and ours may represent species differences and/or the anesthetics used. Unfortunately, the latter study did not detail the effects of NIC on cardiac relaxation.

**Mechanism**

To elucidate the mechanism for the observed difference in relaxation following acute NIC administration, we tested phosphorylation levels of two myocardial proteins that are critical for cardiac relaxation, PLB and TnI (45, 59). It is well established that NIC infusion elicits the release of catecholamines from the adrenal medulla (15), which then bind to β1-adrenergic receptors, activate PKA, and lead to increased phosphorylation of several intracellular targets including PLB and TnI. Our results showed that infusion of NIC led to increased phosphorylation of PLB Ser16 and Thr17 residues in NTG mice while Tm175 mice showed no significant changes, which at least partially explains the higher relaxation speed (i.e., −dP/dt) in NTG mice vs. Tm175.

Tm175 mice had higher levels of TnI phosphorylation at baseline compared with NTG hearts. We hypothesize that increased TnI phosphorylation in Tm175 TG mice is a compensatory mechanism to ameliorate the observed increased calcium sensitivity (17) caused, presumably, by altered myofilament interactions resulting from the Tm175 mutation. We speculate that if TnI phosphorylation did not increase in Tm175 mice, one might expect an even larger increase in calcium sensitivity than what is actually observed. Nonetheless, both Tm175 and NTG mice displayed significant increases in TnI phosphorylation following acute NIC administration; thus this mechanism does not help explain the difference in relaxation observed between Tm175 and NTG mice following NIC infusion.

However, one additional mechanism that can help explain this difference is the variability in myofilament calcium sensitivity between NTGs and Tm175. In the presence of PKA, which is activated following stimulation of the adrenergic system by NIC infusion (15), Tm175 mice continue to exhibit

![Western blots of signaling proteins affected by acute NIC infusion.](http://ajpheart.physiology.org/)

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increased myofilament calcium sensitivity to calcium compared with NTGs (17). Thus, in the presence of acute NIC, Tm175 mice may effectively bind calcium at lower concentrations than NTG mice thereby prolonging their diastole.

Finally, we found that NIC had no effect on Ca\(^{2+}\) transients or shortening parameters in isolated cardiomyocytes from either NTG or Tm175 mice. These data suggest that NIC does not act directly on the myocytes but must involve the release of other activators, such as catecholamines, from the autonomic nervous system. The dose of NIC we used in isolated cell experiments (1.07 mM NIC tartrate = 375 nM NIC base) is comparable to NIC levels measured in arterial blood 5 min after initiation of smoking a cigarette (i.e., 50–60 ng/ml or 310–370 nM; Ref. 26). Our results are in accordance with previously published work (1) since incubation of isolated rat ventricular myocytes with 100 μM NIC, a nearly 300-fold increase from our dosage, for 4–6 h had no effect on cell shortening or calcium transient amplitude. On the other hand, 270 nM directly blocked transient outward K\(^+\) currents in canine cardiomyocytes (57), which would cause a significant delay in ventricular repolarization. However, we saw no effect of 375 nM NIC on either the time constant (tau) for cell shortening or the tau for calcium transient decay in isolated mouse cardiomyocytes. Other studies (51) have shown that NIC also affects other ionic currents albeit at much higher concentrations. In guinea pig ventricular myocytes, 100 μM NIC blocked both the delayed and inward rectifier K\(^+\) currents but had no effect on I\(_{Ca}\) currents. In summary, the concentration of NIC used in this study on isolated mouse cardiomyocytes had no effect on any of the cell shortening or calcium transient parameters measured.

**Chronic NIC**

We also tested the effects of 4 mo of continuous NIC exposure (via an osmotic pump) in the Tm175 mouse model of...
This length of NIC treatment has been shown to elicit changes in the vascular system of rats (49, 50). Our data showed that the heart’s contractility (i.e., $\frac{dP}{dt}$) increases, while HR, LV pressure, and $\frac{dP}{dt}$ were not affected. However, it should be noted that $\frac{dP}{dt}$ was decreased in Tm175 mice compared with NTGs in both the absence (i.e., Tm175_Saline) and presence of chronic NIC (Tm175_NIC). The difference in these chronic and our acute experiments, where $\frac{dP}{dt}$ was not significantly different between NTG and Tm175 in the absence of NIC, is most likely due to the age of the animals at the time of the recordings (i.e., 4 mo of age in the acute studies vs. 6 mo in the chronic studies). In addition, we have shown that an adrenergic reserve still exists in both NTG and Tm175 mice following long-term NIC exposure since ISO administration caused a significant increase in their HR, contractility, and lusitropy.

Other studies have also examined the effects of long-term NIC use on the cardiovascular system, but again no lusitropic effects were reported. In fact, most of the reports focus on the effects of NIC in the vasculature (reviewed in Ref. 3). In addition, the cardiovascular effects have been quite disparate, which could be due to the means of NIC administration used, the means of measurement, the vascular bed studied, or the species used. In rats administered 20 µg/ml NIC in their drinking water ad libitum for 4–6 mo, which corresponds to a NIC intake of 2.4 mg·kg$^{-1}$·day$^{-1}$ (the equivalent of smoking 2 packs a day), the iliac resistance actually decreased compared with nontreated animals (50). However, in rats administered 1.2 or 2.3 mg·kg$^{-1}$·day$^{-1}$NIC by the same method, mean systolic pressure (measured by the tail cuff) increased. Surprisingly, 3.4 and 4.6 mg·kg$^{-1}$·day$^{-1}$ decreased mean systolic pressure (58).

Conversely, in rats given 50 mg/l NIC in their drinking water ad libitum, there was no effect on HR or blood pressure measured by an aortic catheter (24). Dogs receiving two intramuscular bolus injections per day (210 µg/kg, equivalent to having smoked $\approx$ 7 cigarettes/day) for 9–22 mo showed decreased $+dP/dt$ along with ejection fraction and contractile velocity while HR and end diastolic pressure did not change (2). NIC administration via mini-osmotic pumps (5.0 mg·kg$^{-1}$·day$^{-1}$) following myocardial
infarction produced atrial flutter in dogs that resembled human atrial flutter (41). Also, in dogs treated with mini-osmotic pumps (1.44 mg·kg\(^{-1}\)·day\(^{-1}\)) for 5–8 wk, there was no change in HR but an increase in mean arterial pressure (27). Thus our data (where NIC tartrate was 6 mg·kg\(^{-1}\)·day\(^{-1}\) = 2.1 mg·kg\(^{-1}\)·day\(^{-1}\) NIC free base) are similar to those who utilized NIC delivery via mini-osmotic pumps in that there was no change in HR. Yet, to the best of our knowledge, no one has studied the ability of the adrenergic system to respond to a challenge (i.e., ISO) in the face of chronic NIC use. We have shown that an adrenergic reserve still exists after chronic NIC exposure, which implies that the adrenergic system is not completely activated.

Lastly, we studied the effects of chronic NIC use on cardiac remodeling. Our data show that prolonged NIC use does not cause adverse cardiac remodeling in terms of septum, ventricular wall, or chamber dimensions. This is in contrast to studies that showed increased LV chamber dimensions in rats following exposure to cigarette smoke for either 4 mo (11, 12) or 5 wk (22). However, these effects may be due to the tar in cigarette smoke as opposed to NIC or due to species differences. In addition, chronic NIC use does not appear to initiate fibrosis in either NTG or Tm175 mice. To the best of our knowledge, we are the first to address cardiac fibrosis in the presence of prolonged exposure to NIC alone.

Conclusion

In conclusion, we have found that acutely delivered NIC increases several heart performance parameters, including relaxation, in both NTG mice and a mouse model of FHC (i.e., Tm175); however, the increase in relaxation rate was attenuated in Tm175 mice compared with NTGs. We propose that the latter observation involves an increase in phosphorylation of PLB Ser 16 and Thr17 residues in NTGs while Tm175 mice harboring mutant tropomyosin linked to hypertrophic cardiomyopathy. NIC delivery via mini-osmotic pumps in that there was no change in HR. Yet, to the best of our knowledge, no one has compared cardiac contractility but has no effect on HR, LV pressure, lusitropy, LV wall and chamber dimensions, or fibrosis in both NTG mice and a mouse model of FHC (i.e., Tm175); however, the increase in relaxation rate was attenuated in Tm175 mice compared with NTGs. We propose that the latter observation involves an increase in phosphorylation of PLB Ser 16 and Thr17 residues in NTGs while Tm175 mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


