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Endothelial nitric oxide synthase decreases β-adrenergic responsiveness via inhibition of the L-type Ca^{2+} current

Honglan Wang, **Mark J. Kohr**, **Debra G. Wheeler**, and **Mark T. Ziolo**
Department of Physiology and Cell Biology, The Ohio State University, Columbus, Ohio

Abstract

Signaling via endothelial nitric oxide synthase (NOS3) limits the heart's response to β-adrenergic (β-AR) stimulation, which may be protective against arrhythmias. However, mechanistic data are limited. Therefore, we performed simultaneous measurements of action potential (AP, using patch clamp), Ca^{2+} transients (fluo 4), and myocyte shortening (edge detection). L-type Ca^{2+} current (I_{Ca}) was directly measured by the whole cell ruptured patchclamp technique. Myocytes were isolated from wild-type (WT) and NOS3 knockout ($NOS3^{-/-}$) mice. $NOS3^{-/-}$ myocytes exhibited a larger incidence of β-AR (isoproterenol, 1 μM)-induced early afterdepolarizations (EADs) and spontaneous activity (defined as aftercontractions). We also examined I_{Ca}, a major trigger for EADs. $NOS3^{-/-}$ myocytes had a significantly larger β-AR- stimulated increase in I_{Ca} compared with WT myocytes. In addition, $NOS3^{-/-}$ myocytes had a larger response to β-AR stimulation compared with WT myocytes in Ca^{2+} transient amplitude, shortening amplitude, and AP duration (APD). We observed similar effects with specific NOS3 inhibition [L-N^5-(1-iminoethyl)-ornithine (L-NIO), 10 μM] in WT myocytes as with NOS3 knockout. Specifically, L-NIO further increased isoprot-erenol-stimulated EADs and aftercontractions. L-NIO also further increased the isoproterenol-stimulated I_{Ca}, Ca^{2+} transient amplitude, shortening amplitude, and APD (all $P < 0.05$ vs isoproterenol alone). L-NIO had no effect in $NOS3^{-/-}$ myocytes. These results indicate that NOS3 signaling inhibits the β-AR response by reducing I_{Ca} and protects against arrhythmias. This mechanism may play an important role in heart failure, where arrhythmias are increased and NOS3 expression is decreased.

Keywords

endothelial nitric oxide synthase; cardiac myocytes; early afterdepo-larizations; action potential

Stimulation of the β-adrenergic (β-AR) pathway is an important regulator of cardiac contractility, leading to positive inotropic and lusitropic effects (7). Activation of the cAMP-dependent protein kinase (PKA) leads to phosphorylation of several myocyte proteins, including the L-type Ca^{2+} channel, phospholamban, the ryanodine receptor, myosin-binding protein C, and troponin I. PKA-dependent phosphorylation of the L-type Ca^{2+} channel causes an increase in Ca^{2+} influx, which contributes to the enhanced Ca^{2+} cycling and the positive inotropic effect.

Nitric oxide (NO), produced via NO synthase (NOS), is also an important regulator of cardiac contractility (54). Cardiac myocytes constitutively express neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3). Recent studies have shown that NOS1 and NOS3 differentially regulate the response to β-AR stimulation (4). NOS1 signaling has been found

Address for reprint requests and other correspondence: M. T. Ziolo, Dept. of Physiology & Cell Biology, The Ohio State Univ., 304 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210 (e-mail: ziolo.1@osu.edu).

to potentiate the response to β-AR stimulation (4). The observed effects of NOS3 signaling on the β-AR response are the opposite of NOS1 (30), that is, studies have shown that NOS3 signaling depresses the functional response to β-AR stimulation. For example, mice with specific knockout of NOS3 ($NOS3^{-/-}$) have an increased response to β-AR stimulation (4, 11,19,20,47). Similarly, transgenic mice with cardiac myocyte-specific NOS3 overexpression have a decreased response to β-AR stimulation (9,23). Although the vast majority of studies found the above effects, it should be noted that a few studies observed dissimilar results (29, 46). In addition, there are limited studies investigating the mechanism of the NOS3-induced reduction of the β-AR response.

The regulation of β-AR stimulation by NOS3 may be via modulation of the L-type Ca^{2+} channel, since one study has shown that nonspecific NOS inhibition can further increase the cAMP-stimulated L-type Ca^{2+} current (I_{Ca}) (33). However, specific NOS isoforms were not examined in this study. Exogenous NO (i.e., NO donors) has also been found to decrease β-AR-stimulated I_{Ca} (48). NOS3 is localized to the caveolae, along with the L-type Ca^{2+} channel and the β_2-AR receptor (3,17). In addition, a study showed that myocytes from female mouse hearts have smaller β-AR -induced I_{Ca} and a higher association between NOS3 and caveolin-3 compared with myocytes from male mouse hearts (43). Indirect evidence also suggests that NOS3 can regulate I_{Ca} via activation of the β_3-AR receptor (4,47,53). These studies have led groups to hypothesize that NOS3 regulates I_{Ca} (4,28,43). However, this has yet to be observed in $NOS3^{-/-}$ myocytes (21,46).

High sympathetic activation and specifically increased I_{Ca} can be detrimental to cardiac myocytes, leading to arrhythmias (24). Interestingly, transgenic mice overexpressing NOS3 demonstrated a lower incidence of spontaneous arrhythmic contractions in cultured neonatal myocytes (32). In addition, $NOS3^{-/-}$ mice had a higher incidence of arrhythmias (28,39). However, the mechanism of the antiarrhythmic effect of NOS3 is unknown. The regulation of I_{Ca} by NOS3 would also affect the action potential (AP) waveform, and AP prolongation may play a role in reentrant arrhythmias (37). There are no studies that we are aware of examining the AP waveform in isolated myocytes from $NOS3^{-/-}$ mice. Therefore, the purpose of this study is to examine the effects of NOS3 knockout or acute inhibition on AP, Ca^{2+} transients, and myocyte shortening. We also directly measured I_{Ca}. We hypothesize that NO produced via NOS3 is protective against arrhythmias (defined as early afterdepolarizations and aftercontractions) by modulation of the β-AR-stimulated I_{ca}. This reduction in I_{Ca} will also result in a decrease in action potential duration (APD) measured as time to 90% repolarization (APD_{90}), Ca^{2+} transients, and myocyte shortening amplitude.

MATERIALS AND METHODS

Isolation of ventricular myocytes

Ventricular myocytes were isolated from $NOS3^{-/-}$ mice (42, 52; Jackson Laboratories, Bar Harbor, ME) and corresponding wild-type (WT) mice (C57BL/6J) as previously described (27). Briefly, the heart was mounted on a Langendorff apparatus and perfused with modified MEM (37°C, bubbled with 95% O_2-5% CO2; Sigma, St. Louis, MO). Blendzyme type IV (0.077 mg/ml; Roche Applied Science, Indianapolis, IN) was then added to the perfusate. After 10–15 min, the heart was taken down, the ventricles were minced, and myocytes were dissociated by trituration. Subsequently, the myocytes were filtered, centrifuged, and resuspended in MEM containing 200 μM Ca^{2+}. Myocytes were used within 6 h after isolation. All animal protocols and procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Measurement of I_{Ca}

Whole cell voltage-clamp was used to measure I_{ca} using an Axopatch-200B amplifier and pClamp 8.1 software (Axon Instrument, Foster City, CA), as described previously (56). Electrodes (borosilicate glass tubing), with a resistance of 1.5–3 MΩ, were filled with (in mM): 120 CsCl, 6 $MgCl_2$, 10 EGTA, 10 HEPES, and 2 MgATP, pH 7.2 adjusted with CsOH. The bath solution consisted of (in mM): 120 NaCl, 4 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 5 HEPES, and 1 L-arginine, pH 7.4 adjusted with CsOH or HCl. l_{Ca} was elicited by 200-ms pulses to 0 mV from a holding potential of −80 mV (following a prepulse to −40 mV) at a frequency of 0.2 Hz. This procedure isolates the I_{ca} by inactivation of the Na^+ current with the prepulse, and replacement of K^+ with Cs^+ eliminates the K^+ current. Measurements were performed at room temperature.

Simultaneous measurement of Ca^{2+} transients, cell shortening, and AP

Myocytes were loaded at 22°C with fluo 4-AM (10 μM; Molecular Probes, Eugene, OR) for 30 min and washed out, and then 30 min were allowed for intracellular deesterification. The instrumentation used for cell fluorescence measurement was a Cairn Research Limited (Faversham, UK) epifluorescence system. Intracellular Ca^{2+} concentration was measured by fluo 4 epifluorescence with excitation at 480 ± 20 nm and emission at 535 ± 25 nm. The illumination field was restricted to collect the emission of a single cell. Data were expressed as $\Delta F/F_0$, where F is the fluorescence intensity and F_0 is the intensity at rest. Uneven indicator loading, photobleaching, and motion artifact errors may be introduced by using the singal wavelength Ca^{2+} indicator, fluo 4. However, since each myocyte served as its own control, these errors should be minimized. Simultaneous measurement of shortening was also performed using an edge detection system (Crescent Electronics, Sandy, UT). Data were expressed as cell shortening (μm). AP were recorded with the whole cell current-clamp technique using an Axopatch-200B amplifier and pClamp 8.1 software (Axon Instrument). The pipettes, resistance of 9–11 MΩ, were filled with (in mM): 8 NaCl, 10 KCl, 140 potassium aspartate, 5 HEPES, and 2 MgATP, pH 7.2 adjusted with KOH or HCl. A Grass S48 stimulator gated the amplifier for current injection to activate the AP, triggered by a 1- to 5-ms, 2-nA current injection. Measurements were performed at room temperature.

Solution and drugs

Normal Tyrode solution consisted of (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 5 HEPES, and 1 L-arginine, pH 7.4 adjusted with NaOH or HCl. Isoproterenol (ISO, 1 μM, Sigma), a nonselective β-AR agonist, and L-N^5-(1-iminoethyl)-ornithine (l-NIO, 10 μM, Sigma), a specific NOS3 inhibitor (50) were prepared fresh each experimental day.

Statistics

Myocyte data were averaged per heart and presented as means ± SE. Differences between multiple groups were evaluated for statistical significance using an ANOVA (followed by Neuman-Keuls test) or by paired or unpaired Student's *t*-test for two groups. Statistical significance was accepted at the level of $P < 0.05$.

RESULTS

NOS3 and arrhythmogenesis

Previous studies have shown that NOS3 knockout leads to increased arrhythmias in ouabaintreated myocytes or with digoxin in vivo (28,39). Thus we investigated if NOS3 knockout or inhibition increases the β-AR-stimulated arrhythmogenesis at the level of the myocyte. Figure 1 shows representative examples, in the presence of β-AR stimulation (ISO, 1 μM), of early afterdepolarizations (EADs) in $NOS3^{-/-}$ and WT myocytes with NOS3

inhibition (L-NIO, 10 μM) (Fig. 1*A*). Summary data are shown in Fig. 1*B*. No EADs were observed in WT or $NOS3^{-/-}$ myocytes during control (basal) stimulation. In the presence of ISO (1 μM), 57 ± 12% of $NOS3^{-/-}$ myocytes per heart vs only 8 ± 5% ($P < 0.05$ vs $NOS3^{-/-}$) of WT myocytes per heart displayed EADs. Acute inhibition of NOS3, in the presence of ISO, increased the incidence of EADs in WT myocytes per heart (37 ± 11%, $P < 0.05$ vs. ISO alone). Spontaneous activity (defined as aftercontractions, ACs) was also observed in $NOS3^{-/-}$ myocytes during β-AR stimulation (Fig. 1*C*). Summary data are shown in Fig. 1*D*. No ACs were observed in WT or $NOS3^{-/-}$ myocytes during basal stimulation. However, during β-AR stimulation, 69 ± 10% of $NOS3^{-/-}$ myocytes per heart vs. only 6 ± 6% ($P < 0.05$ vs $NOS3^{-/-}$) of WT myocytes per heart had ACs. Acute inhibition of NOS3, in the presence of ISO, increased the incidence of ACs in WT myocytes per heart (35 ± 9%, $P < 0.05$ vs. ISO alone). During β-AR stimulation, we observed EADs and ACs in myocytes isolated from all $NOS3^{-/-}$ hearts (100%), whereas myocytes from only 25% of WT hearts exhibited spontaneous activity. These data suggest that NOS3 signaling can protect cardiac myocytes from β-AR-stimulated arrhythmias.

Effects of NOS3 on I_{Ca}

Abnormal L-type Ca^{2+} channel activity can cause EADs (24). Thus we examined the effects of NOS3 signaling on the I_{Ca} in myocytes from WT and $NOS3^{-/-}$ mice. Our data showed that there was no statistical difference in basal I_{Ca} between the two groups (WT: 3.5 ± 0.2 pA/pF, $NOS3^{-/-}$: 3.8 ± 0.4 pA/pF). Figure 2, *A* and *B*, shows representative current traces and time plots of the effects of β-AR stimulation (ISO, 1 μM) and NOS3 inhibition (L-NIO) in a WT myocyte and a $NOS3^{-/-}$ myocyte. Figure 2*C* shows $NOS3^{-/-}$ myocytes had a significantly larger response to β-AR stimulation compared with WT (increased 88 ± 10% of control in $NOS3^{-/-}$ vs. 58 ± 7% of control in WT, $P < 0.05$). In addition, specific NOS3 inhibition with l-NIO, in the presence of ISO, further increased I_{Ca} in WT myocytes (26 ± 12% from ISO alone) but had no effect in $NOS3^{-/-}$ myocytes (decreased 7 ± 3% from ISO alone, $P < 0.05$ vs. WT; Fig. 2*D*). This small decrease was most likely due to the rundown of Ca^{2+} current. These data indicate that the L-type Ca^{2+} channel is an important end target of NOS3 and are consistent with a NOS3-mediated reduction in I_{Ca} being protective against arrhythmias.

Effects of NOS3-derived NO on myocyte function

We also tested the effects of NOS3 on myocyte function. Functional experiments were performed on isolated myocytes from $NOS3^{-/-}$ and WT mice in which AP, Ca^{2+} transients, and shortening were simultaneously measured at a stimulation frequency of 1 Hz. Representative examples of AP (*top*), myocyte shortening (*middle*), and Ca^{2+} transient (*bottom*) traces from a WT and $NOS3^{-/-}$ myocyte are shown in Fig. 3 and summarized in Fig. 4 and Fig. 5 .

After reaching steady state, the myocytes were perfused with ISO (1 μM). In WT myocytes, β-AR stimulation increased APD, measured as time to 90% repolarization (APD_{90}) (49 ± 9 vs. 72 ± 10 ms), shortening amplitude (1.2 ± 0.1 vs. 7 ± 2 μm), and Ca^{2+} transient amplitude (0.34 ± 0.04 vs. 1.3 ± 0.1 $\Delta F/F_0$) and hastened the rate of Ca^{2+} decline, measured as time to 50% relaxation (RT_{50}) (267 ± 20 vs. 147 ± 8 ms) (all $P < 0.05$ vs. control). In $NOS3^{-/-}$ myocytes, ISO also increased APD_{90} (49 ± 12 vs. 119 ± 12 ms), shortening amplitude (2.6 ± 0.6 vs. 14 ± 2 μm), and Ca^{2+} transient amplitude (0.5 ± 0.1 vs. 1.9 ± 0.3 $\Delta F/F_0$) and hastened the rate of Ca^{2+} decline (Ca^{2+} transient RT50: 276 ± 39 vs. 124 ± 7 ms; all $P < 0.05$ vs. control). However, there was a larger response to β-AR stimulation in myocytes from $NOS3^{-/-}$ compared with WT mice in APD_{90} ($P < 0.05$), shortening amplitude ($P < 0.05$), and a trend in Ca^{2+} transient amplitude ($P = 0.06$). These data suggest that myocytes from $NOS3^{-/-}$ mice have a larger response to β-AR stimulation compared with WT myocytes.

After steady state, the bath solution was switched to ISO (1 μM) plus L-NIO (10 μM). In $NOS3^{-/-}$ myocytes, there was no effect of L-NIO on APD_{90} (124 ± 14 ms), Ca^{2+} transient amplitude (1.9 ± 0.3 $\Delta F/F_0$), or shortening amplitude (15 ± 1 μm) compared with ISO alone. Thus L-NIO had no effect on these aspects of $NOS3^{-/-}$ myocyte function. However, L-NIO further increased β-AR-stimulated APD_{90} (99 ± 15 ms), Ca^{2+} transient amplitude (1.5 ± 0.1 $\Delta F/F_0$), and shortening amplitude (9 ± 1 μm) in WT myocytes (all $P < 0.05$ vs. ISO alone). These data demonstrate that the increased response to β-AR stimulation in myocytes from $NOS3^{-/-}$ mice is due to the deletion of NOS3 and not to phenotypic adaptation.

We also observed no change in the response to β-AR stimulation in the Ca^{2+} transient RT50 between $NOS3^{-/-}$ and WT myocytes (WT: decreased 56 ± 4% from control with ISO; $NOS3^{-/-}$: decreased 52 ± 10% from control with ISO). In addition, in β-AR-stimulated WT myocytes, L-NIO had no effect on Ca^{2+} transient RT_{50} (decreased 5 ± 5% from ISO alone). Thus these data suggest that NOS3 signaling does not modulate sarcoplasmic reticulum (SR) Ca^{2+} uptake. Overall, our myocyte functional data suggest that NOS3 deletion or inhibition leads to an increased response to β-AR stimulation in terms of APD_{90}, Ca^{2+} transient amplitude, and shortening amplitude.

DISCUSSION

It is known that NOS3 signaling leads to antiadrenergic effects and is protective against arrhythmias. However, the mechanism(s) of this effect is unknown. Spontaneous activity (EADs and aftercontractions) in our study was observed at the level of $NOS3^{-/-}$ myocytes (NOS3 gene deficiency was confirmed by Western Blot, data not shown) or with acute NOS3 inhibition in WT myocytes. We also investigated I_{Ca} in $NOS3^{-/-}$ myocytes. We are the first to report that $NOS3^{-/-}$ myocytes have an increased I_{Ca} in response to β-AR stimulation. We further demonstrated that acute NOS3 inhibition in WT myocytes also caused a further increase in the β-AR-stimulated I_{Ca}. We simultaneously measured AP, Ca^{2+} transients, and myocyte shortening. We observed that $NOS3^{-/-}$ myocytes had a significantly prolonged APD, increased myocyte shortening, and a trend toward increased Ca^{2+} transient amplitude with β-AR stimulation compared with WT myocytes. These same results were observed with acute NOS3 inhibition in WT myocytes (i.e., significantly prolonged APD, increased Ca^{2+} transient amplitude, and myocyte shortening). Therefore, it is likely that NOS3 signaling modulates I_{Ca} to limit the β-AR response and protect against arrhythmias.

NOS3 and arrhythmogenesis

Previous studies have observed that $NOS3^{-/-}$ mice have an increased incidence of arrhythmias. This was observed as increased ouabain-induced aftercontractions in isolated $NOS3^{-/-}$ myocytes due to an increase in a transient inward current (most likely Na^+/Ca^{2+} exchanger) (28). Similar effects were demonstrated in a study observing electrocardiograms in $NOS3^{-/-}$ and WT mice. Digoxin induced more premature ventricular beats and ventricular tachycardia in the $NOS3^{-/-}$ mice (39). These premature ventricular beats are analogous to the afterdepolarizations observed in their isolated myocyte studies. Our data show that myocytes with NOS3 knockout or acute NOS3 inhibition had an increased incidence of EADs and aftercontractions in response to β-AR stimulation (Fig. 1). Previous work has shown that EADs can result from abnormal I_{Ca} activity (24). In addition to EADs, increased Ca^{2+} influx via I_{Ca} can also lead to aftercontractions. This increased Ca^{2+} influx can lead to SR Ca^{2+} overload and spontaneous release, resulting in aftercontractions (45). Therefore, NOS3 signaling is protective against arrhythmias by inhibiting β-AR stimulated I_{Ca}.

NOS3 and the I_{Ca}

Studies have shown that EADs can be induced using the L-type Ca^{2+} channel agonist BAY K 8644 (15,24). Additionally, studies have shown that L-type Ca^{2+} channel antagonists can significantly decrease the occurrence of EADs (24,44). Exogenous NO (i.e., NO donors) and nonspecific NOS inhibitors have also been shown to regulate β-AR-stimulated I_{Ca} (33,48), and it was hypothesized that this effect was via NOS3. Within cardiac myocytes, NOS3 is localized with the L-type Ca^{2+} channel and the β_2-AR receptor to the caveolae (3,17). However, studies investigating β-AR-stimulated I_{Ca} in $NOS3^{-/-}$ myocytes observed no difference compared with WT myocytes (21,46). We observed an increase in the β-AR-stimulated I_{Ca} from $NOS3^{-/-}$ myocytes compared with WT myocytes (Fig. 2). We also observed that acute NOS3 inhibition in WT myocytes further increased the β-AR-stimulated I_{Ca} (Fig. 2). Thus our data demonstrate that NOS3 is able to modulate the β-AR-stimulated I_{Ca}. We believe that the inability of previous studies to observe a difference in the β-AR-stimulated I_{Ca} in $NOS3^{-/-}$ myocytes was due to the lack of L-arginine (precursor of NO) in the solutions they used. We (55) and others (40) have found for endogenous NO to affect I_{Ca} measured using the ruptured patch-clamp technique, L-arginine must be added to the solution. Additionally, we did not observe a difference in basal I_{Ca} between $NOS3^{-/-}$ and WT myocytes, suggesting that NOS3 specifically modulates the β-AR-stimulated I_{Ca}. Thus the NOS3-mediated reduction in β-AR-stimulated I_{Ca} will reduce the incidence of EADs and aftercontractions and protect the heart from arrhythmias.

In cardiac myocytes, NOS3 is localized in the caveolae with superoxide dismutase (SOD; see Refs. 8 and 17), a superoxide scavenger that which will prevent O_2^- from reacting with NO. Thus NO generated from NOS3 is more likely to activate guanylate cyclase and subsequently form cGMP. The cGMP pathway in cardiac myocytes is primarily via activation of the cGMP-dependent protein kinase (PKG) (49). Previous work has shown that exogenous NO (48), cGMP analogs (26) or exogenous PKG (34) can decrease β-AR-stimulated I_{Ca}. Recent work has shown that PKG can phosphorylate the α_{1C}-subunit of L-type Ca^{2+} channel at position Ser^{533} (25) and that this phosphorylation does occur within cardiac myocytes (51).

NOS3 and myocyte function

I_{Ca}, rigger leading to Ca^{2+} release from the SR, is an important contributor to myocyte contraction (5). For example, increased I_{Ca}, via transgenesis or adenovirally mediated, increases contraction (13,35). Because we observed a difference in β-AR-stimulated I_{Ca}, we also investigated myocyte function by simultaneously measuring AP, Ca^{2+} transients, and myocyte shortening (Fig. 3). We observed that β-AR-stimulated Ca^{2+} transient amplitude (trend) and shortening amplitude (Fig. 5) were significantly greater in $NOS3^{-/-}$ myocytes than WT myocytes. We did not observe any difference in the response to β-AR stimulation in the Ca^{2+} transient RT_{50} between $NOS3^{-/-}$ and WT myocytes. These data suggest that NOS3 does not regulate SR Ca^{2+} uptake. Acute NOS3 inhibition in WT myocytes also led to a further, significant increase in β-AR-stimulated Ca^{2+} transient amplitude and myocyte shortening amplitude. NOS3 inhibition did not change β-AR-stimulated Ca^{2+} transient decline, further supporting the idea that NOS3 does not regulate SR Ca^{2+} uptake. We also observed large increases in APD_{90} with NOS3 knockout or acute inhibition with β-AR stimulation (Fig. 4). This increase can be contributed to increased I_{Ca} leading to increased Na^+/Ca^{2+} exchanger activity. It has also been demonstrated that NOS3 is able to modulate K^+ channels. Specifically, activation of NOS3 leads to an enhancement of slow-delayed rectifier K^+ current and shortening of APD in guinea pig myocytes (2). However, in normal adult mouse ventricular myocytes, the expression of delayed-rectifier K^+ channel is very low (22), and the functional effects of I_{ks} on AP waveform are still undetermined (36). With NOS3 knockout (or inhibition), we could potentially be inhibiting a K^+ current that would result in a prolonged APD. It is known that prolonged APD is a contributing factor to reentrant arrhythmias (38). Thus NOS3

signaling is antiadrenergic in limiting the increase in Ca^{2+} transient and shortening amplitude and also lessens APD. We believe NOS3 signaling may be acting as an endogenous β-blocker (31) protecting against arrhythmias. β-Blockers have been found to reduce sudden cardiac death in heart failure (33a).

L-NIO is a selective NOS3 inhibitor (50) and did not have any effect in $NOS3^{-/-}$ myocytes at 10 μM. Therefore, 10 μM L-NIO did not appear to have any nonspecific (e.g., NOS1 inhibition) effects under our experimental conditions. As discussed, acute NOS3 inhibition with L-NIO in WT myocytes further increased EADs, aftercontractions, I_{Ca}, APD_{90}, Ca^{2+} transient, and shortening amplitude. Because similar results were observed with NOS3 knockout or inhibition, we are confident that NOS3 signaling has antiadrenergic and antiarrhythmic effects.

Although we found significant effects of NOS3 knockout and inhibition on β-AR-stimulated myocyte function, NOS3 signaling may be more important in protecting the heart. A study found that, after chronic pressure overload, $NOS3^{-/-}$ mice had increased hypertrophy, fibrosis, and contractile dysfunction compared with WT mice (41). In addition, mice with cardiac myocyte-specific NOS3 overexpression had limited hypertrophy and contractile dysfunction after pressure overload and myocardial infarction (10,23). Furthermore, it is known that I_{Ca} plays a role in hypertrophy (14). Ca^{2+} influx via I_{Ca} activates calcineurin, leading to nuclear factor of activated T cells dephosphorylation and the activation of hypertrophic signaling. Exogenous NO-mediated inhibition of I_{Ca} can inhibit this calcineurin activation (18). Ca^{2+} influx via I_{Ca} also leads to apoptosis (12) and arrhythmias (1). Thus increased Ca^{2+} influx via β-AR-stimulated I_{Ca} can be detrimental to the myocyte. Interestingly, it has been observed that NOS3 expression is decreased in human heart failure (16). Thus we believe that NOS3 plays an important, protective role against toxic sympathetic activation by reducing the L-type Ca^{2+} current and APD.

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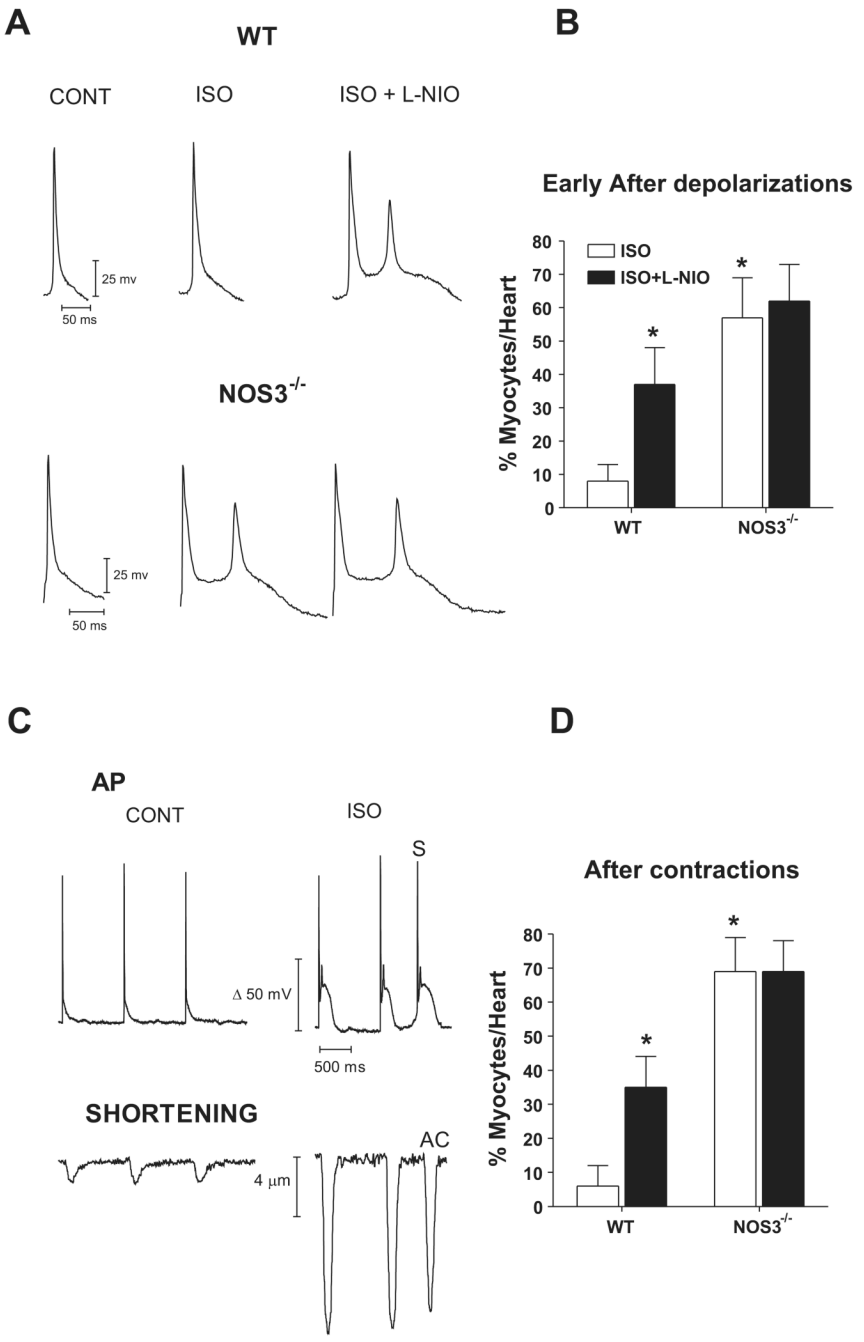

Figure 1.

A: top, example of an action potential (AP) that shows early afterdepolarizations (EADs) in the presence of isoproterenol (ISO, 1 μM) and L-N^5-(1-iminoethyl)-ornithine (L-NIO, 10 μM, specific NOS3 inhibitor) in a wild-type (WT) myocyte. *Bottom*, example of an AP that shows EADs in the presence of ISO in a endothelial nitric oxide synthase knockout ($NOS3^{-/-}$) myocyte. *B*: summary data, %myocytes/ heart that showed EADs. *C*: example of a $NOS3^{-/-}$ myocyte that shows spontaneous activity in AP (*top*) and shortening (*bottom*) after ISO stimulation. S, spontaneous AP; AC, aftercontraction. *D*: %myo-cytes/heart that showed aftercontractions. **P* < 0.05 vs. WT ISO; *n* = 8 hearts for WT, *n* = 5 hearts for $NOS3^{-/-}$.

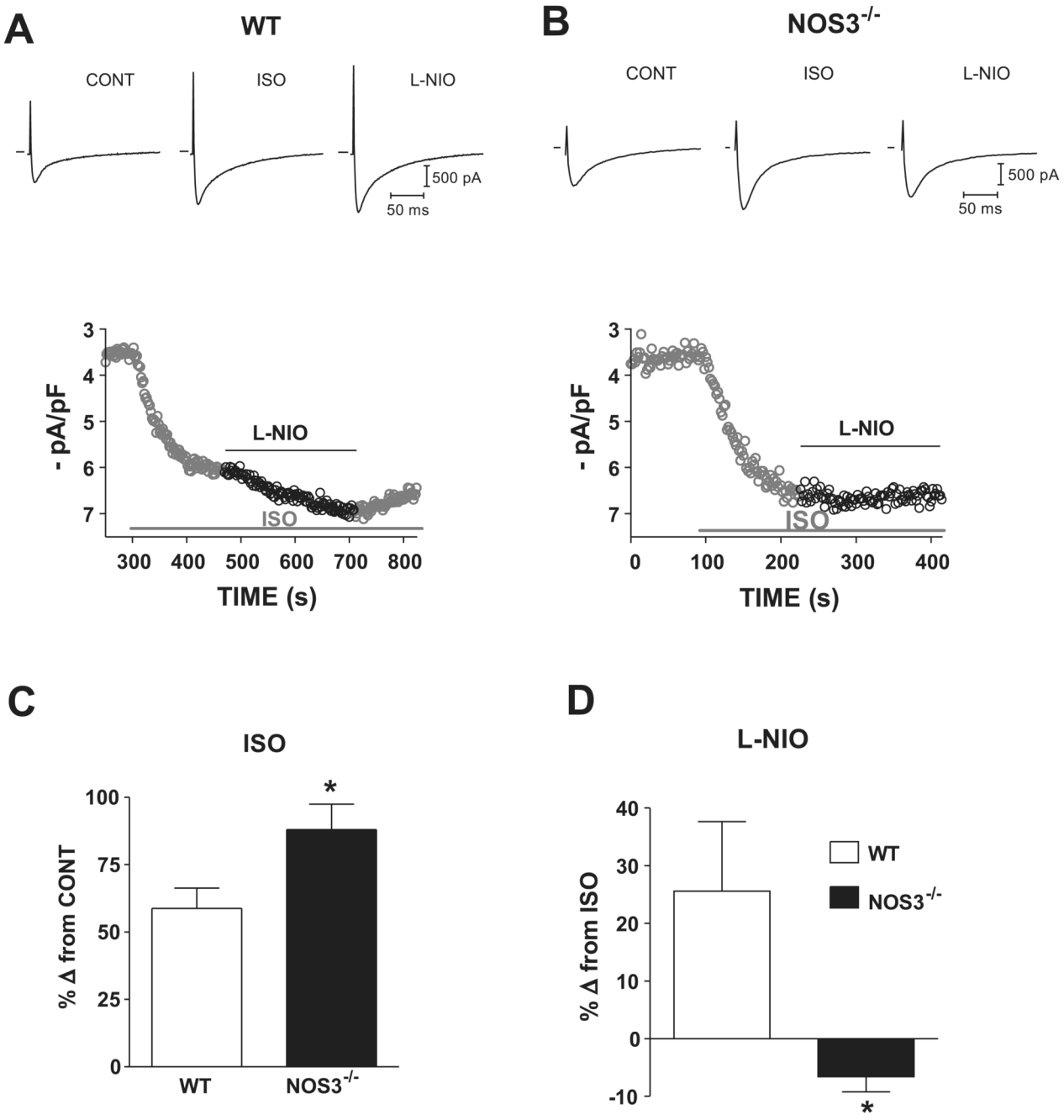

Figure 2.

A and *B*: representative traces (*top*) and time plot (*bottom*) of I_{Ca} with ISO (1 μM) and L-NIO (10 μM), selective NOS3 inhibitor, in a WT (*A*) and NOS3$^{-/-}$ (*B*) myocyte. *C*: summary data (means ± SE) of the effects of ISO in WT and NOS3$^{-/-}$ myocytes. *D*: summary data (means ± SE) of the effects of L-NIO in WT and NOS3$^{-/-}$ myocytes. *$P < 0.05$ vs. WT ($n = 5$ hearts for WT; $n = 5$ hearts for NOS3$^{-/-}$).

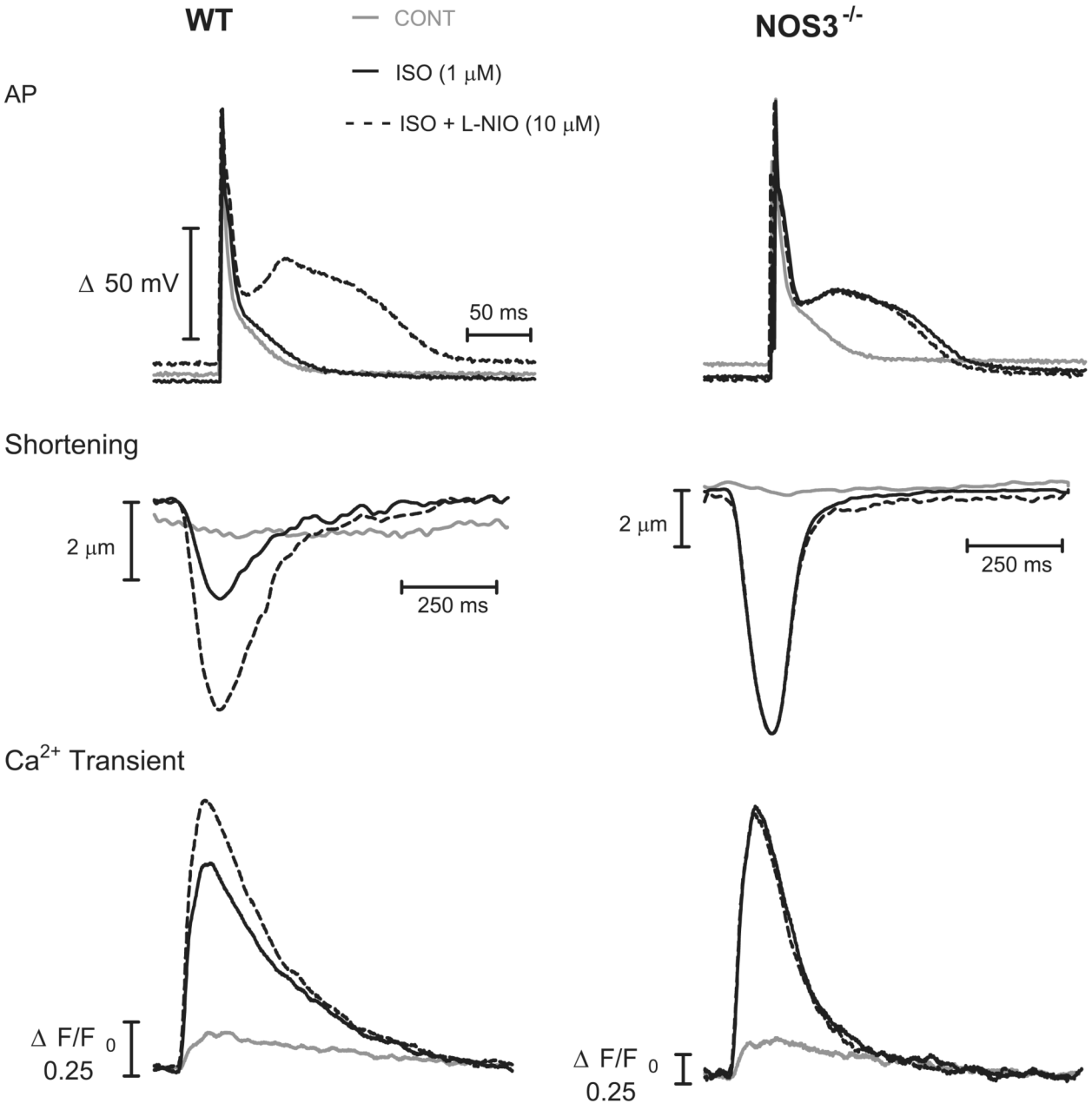

Figure 3.
Representative examples of AP (*top*), shortening (*middle*), and Ca^{2+} transients (*bottom*) from a WT (*left*) and a $NOS3^{-/-}$ (*right*) myocyte.

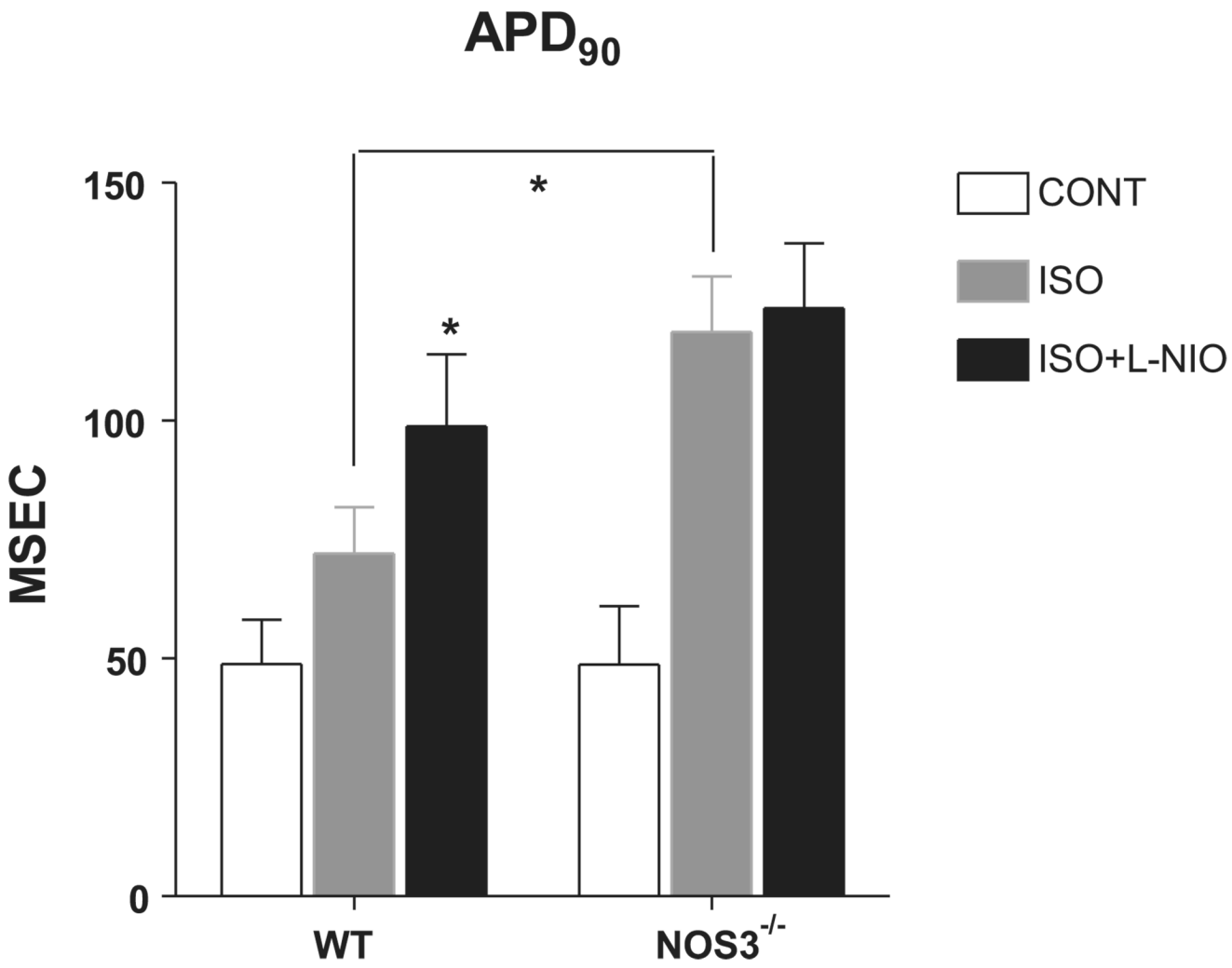

Figure 4.

Summary data (means ± SE) of the effects of ISO (1 μM) and L-NIO (selective NOS3 inhibitor, 10 μM) on action potential duration measured as time to 90% repolarization (APD_{90}). *P < 0.05 vs. WT ISO; n = 8 hearts for WT, n = 5 hearts for $NOS3^{-/-}$.

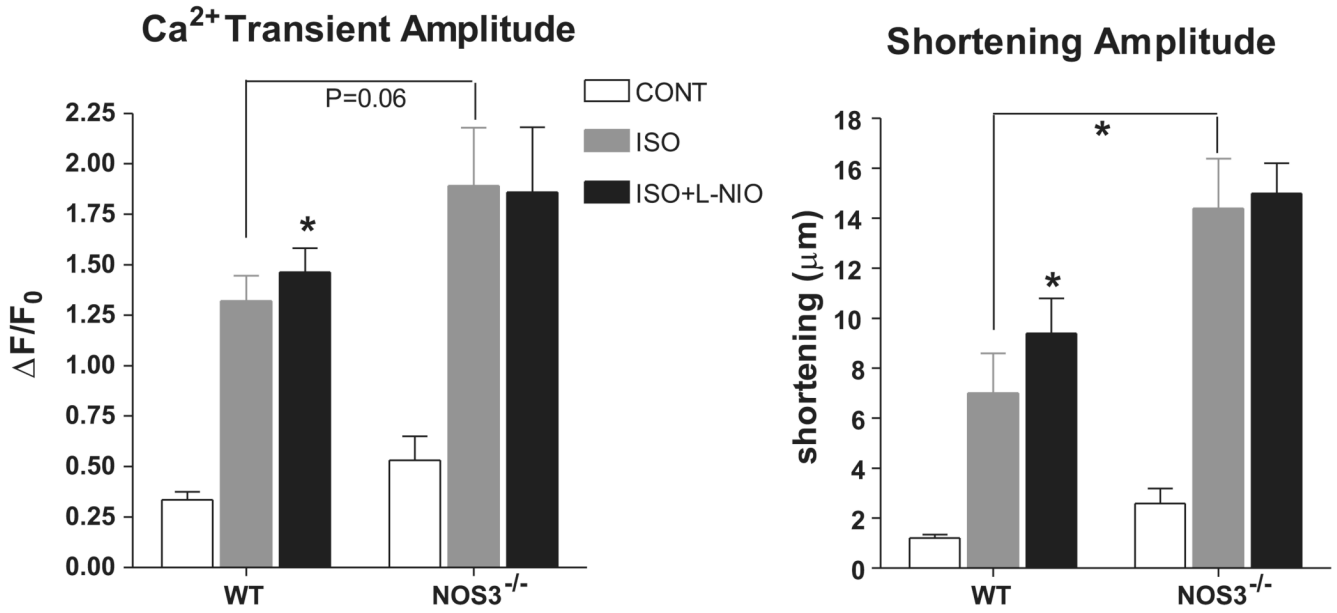

Figure 5.

Summary data (means ± SE) of the effects of ISO (1 μM) and L-NIO (10 μM), a selective NOS3 inhibitor, on Ca^{2+} transients and shortening amplitude. *$P < 0.05$ vs. WT ISO; $n = 8$ hearts for WT, $n = 5$ hearts for $NOS3^{-/-}$.