NFATc3-Induced Reductions in Voltage-Gated $K^+$ Currents After Myocardial Infarction

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Abstract—Reductions in voltage-activated $K^+$ (Kv) currents may underlie arrhythmias after myocardial infarction (MI). We investigated the role of β-adrenergic signaling and the calcineurin/NFAT pathway in mediating the reductions in Kv currents observed after MI in mouse ventricular myocytes. Kv currents were produced by the summation of 3 distinct currents: $I_{Na}$, $I_{Klow1}$, and $I_{Klow2}$. At 48 hours after MI, we found a 4-fold increase in NFAT activity, which coincided with a decrease in the amplitudes of $I_{Na}$, $I_{Klow1}$, and $I_{Klow2}$. Consistent with this, mRNA and protein levels of Kv1.5, 2.1, 4.2, and 4.3, which underlie $I_{Klow1}$, $I_{Klow2}$, and $I_{Na}$, were decreased after MI. Administration of the β-blocker metoprolol prevented the activation of NFAT and the reductions in $I_{Na}$, $I_{Klow1}$, and $I_{Klow2}$ after MI. Cyclosporine, an inhibitor of calcineurin, also prevented the reductions in these currents after MI. Importantly, Kv currents did not change after MI in ventricular myocytes from NFATc3 knockout mice. Conversely, chronic β-adrenergic stimulation or expression of an activated NFATc3 decreased Kv currents to a similar extent as MI. Taken together, these data indicate that NFATc3 plays an essential role in the signaling pathway leading to reduced $I_{Na}$, $I_{Klow1}$, and $I_{Klow2}$ after MI. We propose that increased β-adrenergic signaling after MI activates calcineurin and NFATc3, which decreases $I_{Na}$, $I_{Klow1}$, and $I_{Klow2}$ via a reduction in Kv1.5, Kv2.1, Kv4.2, and Kv4.3 expression. (Circ Res. 2004;94:1340-1350.)

Key Words: calcineurin ▪ arrhythmias ▪ heart failure

Arrhythmias are a major cause of death after myocardial infarction (MI). Mounting evidence suggests that modification of the ventricular action potential (AP) underlies the increased probability of developing lethal arrhythmias after MI. Changes in the AP, including reductions in the rate of depolarization, peak depolarization, and an increase in the AP duration have been reported in regions proximal to and removed from the infarct zone. These changes in the AP are produced by reductions in the magnitudes of Na and Kv currents as well as changes in Ca$^{2+}$ signaling proteins of ventricular myocytes. Some of these changes can occur rapidly; decreased Kv currents have been reported 3 days after MI. However, the signaling mechanisms underlying these profound electrical changes in the heart are unclear.

Recent studies have suggested a role for increased β-adrenergic receptor (βAR) signaling in the development of the hypertrophic phenotype post-MI, including changes in Kv currents. Accordingly, catecholamine levels in the heart, as well as protein kinase A (PKA) activity in ventricular myocytes, have been found to increase after MI. Augmentation of PKA activity can elicit profound changes in myocardial Ca$^{2+}$ signaling, including increased Ca$^{2+}$ currents and sarcoplasmic reticulum (SR) Ca$^{2+}$ release. Furthermore, chronic activation of PKA causes cardiac hypertrophy and increases the probability of arrhythmogenesis and sudden death.

The Ca$^{2+}$-activated phosphatase calcineurin has been hypothesized to play a pivotal role in translating altered Ca$^{2+}$ signaling into changes in the transcription of genes linked to the development of hypertrophy and heart failure. On activation, calcineurin dephosphorylates NFAT, allowing translocation into the nucleus, whereupon it modulates the transcription of multiple genes. Expression of an activated form of NFATc4 is sufficient to induce robust hypertrophy in transgenic mice. However, NFATc3-null, but not NFATc4-null, mice exhibit decreased cardiac hypertrophy in response to calcineurin activation. These findings suggest that while NFATc4 can induce hypertrophy, it is NFATc3 that plays an essential role in the pathway leading to cardiac hypertrophy after calcineurin activation. A recent study found that calcineurin and NFATc3 are activated after MI and that inhibition of calcineurin prevented activation of this transcription factor and decreased cardiac hypertrophy post-MI. At present, however, it is unclear if calcineurin/NFATc3 signaling is involved in the electrical remodeling of the heart after MI.

In this study, we examined the role of βAR stimulation, calcineurin, and NFATc3 in mediating reductions in Kv currents after MI. Our data suggest that increased β-adrenergic signaling activates NFAT after MI. We propose that activation of NFATc3 decreases Kv currents via changes...
in gene expression. These findings suggest that βARs, calcineurin, and NFATc3 play a crucial role in the pathway leading to reduced Kv currents post-MI.

Methods

Generation of Myocardial Infarcts
Infarcts were generated as previously described17 in accordance to the guidelines of the Institutional Animal Care and Use Committee. NFATc3 knockout and NFAT reporter mice were kindly provided by Drs Laurie Glimcher (Harvard University, Cambridge, Mass) and Mercedes Rincón, (University of Vermont, Burlington), respectively.

Isolation, Short-Term Culture, and Adenoviral Infection of Adult Mouse Ventricular Myocytes
Ventricular myocytes from adult mice were isolated from regions remote to the MI (ie, right ventricle, noninfarcted section of the left ventricle and septum) 48 hours post-MI. For control experiments, cells were dissociated from regions of the heart similar to those used from infarcted hearts. Adenoviral infection and short-term culture of adult ventricular myocytes were performed as described elsewhere.18

Electrophysiology and Confocal Microscopy
Intracellular Ca²⁺ ([Ca²⁺]i) imaging was performed with a confocal microscope. Currents and membrane potentials were recorded using an Axopatch 200B amplifier.

Administration of CsA, FK506, and Metoprolol
Metoprolol (≈350 mg/kg per day) was administered via drinking water.19 CsA (25 mg/kg per day) and FK506 (3 mg/kg twice per day) were administered intraperitoneally.

Real-Time Reverse-Transcription Polymerase Chain Reaction
Reverse-transcription (RT) was performed using the Superscript First-strand Synthesis system (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with the TaqMan 5′ nuclease assay and TaqMan One-step PCR Mastermix.

Western Blots
Protein (50 μg) was loaded on a 4% to 15% Tris-HCl polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blots were incubated with primary antibodies specific to Kv1.5, 2.1, 4.2, or 4.3, and then with horseradish peroxidase-conjugated secondary antibodies. Kv channel protein was quantified by densitometry.

Luciferase Assay
Luciferase activity was quantified using a commercially available kit (Promega).

Statistics
Data are presented as mean±SEM. Group comparisons were made using Student t test or ANOVA, which, if necessary, were followed-up by a Tukey test.

An expanded Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Results
Changes in AP Duration and Whole-Cell [Ca²⁺]i Transients 48 Hours After MI
We examined [Ca²⁺]i and the AP of ventricular myocytes from control and infarcted hearts. For these experiments, ventricular myocytes from regions remote to the MI were isolated 48 hours after permanent ligation of the left anterior descending coronary artery (typically midway from the apex to base). Although we could not measure the infarct size in the same hearts from which myocytes were isolated, parallel experiments yielded infarcts that reproducibly averaged 38%±5% of the left ventricle.20 Figure 1A shows representative APs and [Ca²⁺]i transients obtained from control and MI myocytes. The peak and maximal rate of depolarization of the AP were similar in MI (peak=46.1±2.7 mV, dV/dt max=253.8±25.1 V/s, n=16) and control cells (peak=48.6±1.6 mV, dV/dt max=246.3±25.1 V/s, n=54; P>0.05). However, AP duration at 90% repolarization (APD90) was nearly 80% longer in MI (198.4±19.8 ms) than in control myocytes (109.5±5.6 ms, P<0.05; Figure 1B). We also found that the amplitude of the AP-evoked whole-cell [Ca²⁺]i transient was ~1.7-fold higher in MI (935.1±55.7 nmol/L, n=18) than in control (548.3±45.4 nmol/L, n=25, P<0.05) myocytes.

Reductions in Ito, I,Kslow1, and I,Kslow2 After MI
One mechanism by which APD could increase after MI is by a decrease in the Kv currents responsible for the repolarization of ventricular cells. Thus, we examined Kv currents in MI (n=26) and control (n=28) ventricular myocytes (Figure 2). Kv currents were evoked by 1.2-second depolarizing pulses from −90 mV to potentials ranging from −60 to +40 mV. The Kv currents evoked by this protocol activated rapidly and then decayed with time (Figure 2A). The decay phase of the Kv currents recorded during depolarization to +40 mV could be fit with the sum of 2 exponential functions.21 At +40 mV, Kv currents from control cells had a fast (τfast) and slow (τslow) time constant of inactivation of 57.2±9.8 ms and 531.9±33.8 ms, respectively. MI cells depolarized to the same potential produced Kv currents with

Figure 1. APs and [Ca²⁺]i 48 hours post-MI. A, Representative AP traces (top), line-scan images (middle), and spatially averaged time course of [Ca²⁺]i (bottom) from control and MI cells. B, Bar plot of the mean±SEM of the APD90 and the amplitudes of [Ca²⁺]i transients in control and MI cells. *P<0.05 vs control.
Figure 2. Decreased $I_{\text{h}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ caused by reduced expression of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 protein 48 hours post-MI. A, Representative Kv currents from a control and MI cell. B to F, Current–voltage relationships of $I_{\text{h}}$, $I_{\text{fast}}$, $I_{\text{Kslow1}}$, $I_{\text{Kslow2}}$, and $I_{\text{h}}$ in control and MI cells. G, Real-time RT-PCR analysis of Kv1.5, 2.1, 4.2, and 4.3 mRNA in control and MI cells. H, Western blots of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 protein in control and MI hearts. The bar plot shows the percent change in Kv protein after MI. *$P<0.05$ vs control.
similar $\tau_{\text{fast}}$ (46.2±3.4 ms) and $\tau_{\text{slow}}$ (616.6±58.3 ms) ($P>0.05$). However, we found that the amplitudes of the transient ($I_{\text{trans}}$; defined as the difference between the peak and sustained current measured at the end of the pulse) and sustained ($I_{\text{sust}}$; defined as the sustained current measured at the end of the pulse) components of $K_v$ ($I_K$) currents were smaller in MI than in control cells at most potentials examined (Figure 2A through 2C). At $+40$ mV, $I_{\text{trans}}$ was $\approx 70\%$ smaller in MI ($13.76 \pm 2.17$ pA/pF) than in control ($47.80 \pm 2.30$ pA/pF) cells ($P<0.05$). At the same potential, $I_{\text{sust}}$ decreased by $\approx 33\%$ after MI (control $=16.88 \pm 1.11$ versus MI $=11.34 \pm 0.92$ pA/pF, $P<0.05$). Cell capacitance, an indicator of cell surface area, increased by $\approx 20\%$ 48 hours after MI (control $=108.2 \pm 4.8$ pF versus MI $=130.2 \pm 8.7$ pF, $P<0.05$).

It is important to note that we found no significant differences in the voltage dependencies of conductance and steady-state inactivation of $I_{\text{fast}}$ and $I_{\text{sust}}$ in control and MI cells ($P>0.05$; data not shown). The voltage at which 50% of $I_{\text{fast}}$ and $I_{\text{sust}}$ were inactivated was similar in MI ($I_{\text{fast}} = -36.7 \pm 1.4$ mV; $I_{\text{sust}} = -52.5 \pm 2.5$ mV, $n = 12$) and control cells ($I_{\text{fast}} = -38.2 \pm 2.4$ mV; $I_{\text{sust}} = -49.6 \pm 3.5$ mV, $n = 16$) ($P>0.05$). Furthermore, $I_{\text{fast}}$ and $I_{\text{sust}}$ reached 50% of their maximal conductance at a similar voltage in MI ($I_{\text{fast}} = 9.7 \pm 4.2$ mV; $I_{\text{sust}} = -34.0 \pm 3.6$ mV) and control ($I_{\text{fast}} = 45.1 \pm 1.8$ mV; $I_{\text{sust}} = -34.9 \pm 3.7$ mV) cells ($P>0.05$). Taken together, these data suggest that a reduction in the amplitudes of $I_{\text{trans}}$ and $I_{\text{sust}}$ contribute to AP prolongation in mouse ventricular myocytes post-MI. The reductions in $I_{\text{fast}}$ and $I_{\text{sust}}$ were not results of changes in the voltage-dependencies of activation or inactivation of these currents post-MI.

The next series of experiments investigated the effects of MI on the currents that underlie $I_{\text{fast}}$ and $I_{\text{sust}}$ in mouse ventricular myocytes (Figure 2A). Three kinetically and pharmacologically distinct currents are thought to contribute to $I_{\text{fast}}$ and $I_{\text{sust}}$. $I_{\text{fast}}$ is produced by the rapidly inactivating transient outward current ($I_{\text{o}}$), whereas a noninactivating current ($I_{\text{Kslow}}$) contributes to $I_{\text{sust}}$. A slowly inactivating current ($I_{\text{Kslow2}}$) contributes to $I_{\text{fast}}$ and $I_{\text{sust}}$. Pharmacological tools were used to separate these currents. To isolate $I_{\text{Kslow1}}$, Kv currents were measured before and after the application of 50 μmol/L 4-aminopyridine (4-AP), which blocks this current, but has no effect on $I_{\text{o}}$ or $I_{\text{Kslow2}}$. Subtraction of the currents recorded in the presence of 50 μmol/L 4-AP from the predrug traces results in the 4-AP–sensitive current or $I_{\text{Kslow1}}$. To obtain $I_{\text{Kslow2}}$, currents recorded during superfusion of a solution containing 50 μmol/L 4-AP from the predrug traces results in the 4-AP–sensitive current or $I_{\text{Kslow2}}$. To obtain $I_{\text{Kslow2}}$, currents recorded during superfusion of a solution containing 50 μmol/L 4-AP from the predrug traces results in the 4-AP–sensitive current or $I_{\text{Kslow2}}$. The TEA-sensitive current represents $I_{\text{Kslow1}}$. The remaining current recorded in the presence of 4-AP and TEA corresponds to $I_{\text{o}}$ (defined here as the difference between the peak and sustained current measured at the end of the 1.2-second pulse). $I_{\text{fast}}$, $I_{\text{sust}}$, $I_{\text{o}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ were recorded from the same cells.

In agreement with the data shown in Figure 2B and 2C, $I_{\text{o}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ were significantly smaller after MI than in control cells (Figure 2D and 2F). At $+40$ mV, the amplitudes of $I_{\text{o}}$ and $I_{\text{Kslow1}}$ were nearly 69% and 65% smaller after MI than in control cells, respectively. $I_{\text{Kslow2}}$ decreased by $\approx 30\%$ 48 hours post-MI. These data suggest that the decrease in $I_{\text{trans}}$ observed in MI resulted from equivalent reductions in the magnitude of $I_{\text{fast}}$ and $I_{\text{Kslow1}}$. The decrease in $I_{\text{sust}}$ was produced by reductions in $I_{\text{Kslow1}}$ and $I_{\text{Kslow2}}$.

We examined the molecular mechanisms underlying the reductions in $I_{\text{fast}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ observed post-MI. In the mouse ventricle, $I_{\text{o}}$ is produced by a heterotetramer formed by Kv4.2 and Kv4.3 subunits. Kv1.5 and Kv2.1, in turn, underlie $I_{\text{Kslow1}}$ and $I_{\text{Kslow2}}$. Thus, we used real-time PCR to examine the mRNA levels of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 in control and MI hearts (Figure 2G). Our data indicate that mRNA levels for Kv1.5, Kv2.1, Kv4.2, and Kv4.3 were $\approx 47\%$, 30%, 62%, and 41% lower ($P<0.05$) in MI (n=9 hearts) than in control (n=9 hearts) ventricles, respectively. Additional experiments using actinomycin D (see online data supplement) demonstrated that this decrease in mRNA did not result from an increase in the degradation rate of Kv1.5, Kv2.1, Kv4.2, or Kv4.3 transcripts in ventricular myocytes after MI. Consistent with our electrophysiological and mRNA data, Western blot analysis of fractionated ventricular membrane proteins (50 μg total protein) revealed that Kv1.5, Kv2.1, Kv4.2, and Kv4.3 protein decreased by 45%±10%, 48%±10%, 61%±13%, and 55%±5%, respectively, after MI (n=9 hearts, $P<0.05$) (Figure 2H). These data suggest that the decreases in $I_{\text{fast}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ observed post-MI result from decreases in the expression of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 mRNA and protein.

**Administration of the β-Blocker Metoprolol Prevents Reductions in $I_{\text{fast}}$ and $I_{\text{sust}}$ After MI**

Administration of β-blockers reduces the probability of lethal arrhythmias after MI. Because reductions in Kv currents have been linked to arrhythmogenesis, we investigated whether an increase in βAR stimulation leads to the reduction in $I_{\text{fast}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ observed post-MI. A testable prediction of this hypothesis is that βAR blockers should minimize, or even prevent, the changes in $I_{\text{fast}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ observed post-MI. Thus, the effects of the βAR blocker, metoprolol, on Kv currents after MI were investigated (Figure 3).

For these experiments, mice were given metoprolol beginning 24 hours before infarction (BB-MI). Control (noninfarced; BB) animals received metoprolol for the same period of time. Metoprolol treatment prevented the decrease in $I_{\text{fast}}$ and $I_{\text{sust}}$ amplitudes ($P>0.05$) observed post-MI (Figure 3A and 3B). At $+40$ mV, the amplitudes of $I_{\text{fast}}$ in BB and BB-MI cells were 49.37±3.34 pA/pF (n=23) and 47.78±2.3 pA/pF (n=25), respectively. Accordingly, $I_{\text{fast}}$, $I_{\text{Kslow1}}$, and $I_{\text{Kslow2}}$ were similar in BB-MI and control cells (Figure 3C through 3E). Consistent with this, we found, using real-time PCR and Western blot analysis, that metoprolol prevented the reductions in Kv1.5, Kv2.1, Kv4.2, and Kv4.3 mRNA (n=21 hearts, $P>0.05$) and protein (n=6 hearts, $P>0.05$) observed in the ventricles post-MI (Figure 3F and 3G).

**Activation of NFAT After MI**

The [Ca$^{2+}$], mRNA, and protein data discussed above raise the intriguing possibility that increased Ca$^{2+}$ signaling could underlie the changes in Kv channel expression observed...
Figure 3. Metoprolol prevents the reductions in $I_{\text{tran}}$, $I_{\text{Kfast1}}$, and $I_{\text{Kfast2}}$ as well as Kv1.5, 2.1, 4.2, and 4.3 mRNA and protein observed post-MI. Current-voltage dependencies of $I_{\text{tran}}$ (A), $I_{\text{Kfast}}$ (B), $I_{\text{Kslow1}}$ (C), $I_{\text{Kslow2}}$ (D), and $I_{\text{i}}$ (E) in control (metoprolol only; BB), MI, and MI-BB cells. The insets in A and C to E show, respectively, representative $I_{\text{Kfast}}, I_{\text{Kslow1}}, I_{\text{Kslow2}},$ and $I_{\text{i}}$ traces recorded during a step depolarization to +40mV. F and G, respectively, show real-time RT-PCR analysis and Western blots of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 in BB, MI, and MI-BB hearts. The bar plot in panel G shows the percent change (relative to BB) in Kv protein after MI in metoprolol-treated hearts. *$P<0.05$ vs MI.
post-MI via the activation of calcineurin/NFAT signaling. Indeed, analysis of the Kv1.5, Kv2.1, Kv4.2, and Kv4.3 genes revealed multiple putative NFAT binding elements (GGAAA) in the 5' untranslated region of these genes (see online data supplement). Thus, we tested the hypothesis that increased AR stimulation activates NFAT post-MI and thereby reduces the expression of Kv1.5, Kv2.1, Kv4.2, and Kv4.3. These experiments involved a transgenic NFAT reporter mouse (NFAT-luc), in which luciferase expression is controlled by multiple NFAT binding sites. We found that 48 hours after MI, luciferase activity in ventricular myocytes from areas remote to the MI was nearly 4-fold higher than in cells from the same region of noninfarcted hearts (NFAT-luc littermates) (control of 2.7 ± 0.2 versus MI of 10.9 ± 2.3 AU; n >100 cells from each of 3 hearts; P<0.05; Figure 4A). Interestingly, this increase in NFAT activity after MI was prevented by metoprolol treatment (BB-MI 2.3 ± 0.3 AU; n >100 cells from each of 3 hearts; P>0.05). Metoprolol did...
not alter NFAT activity in noninfarcted myocytes (BB=2.7±0.2 AU; n>100 cells from 3 hearts; P<0.05). These data suggest that activation of βARs post-MI leads to the activation of NFAT in mouse ventricular myocytes.

**CsA Prevents I_{to} and I_{sust} Reductions After MI**

NFAT is activated by the Ca^{2+}-sensitive phosphatase calcineurin. Thus, we investigated whether activation of calcineurin was necessary to mediate the changes in I_{to}, I_{Kslow1}, and I_{Kslow2} observed post-MI. To do this, Kv currents were recorded from cells dissociated from control (noninfarcted) and infarcted hearts from animals that had been administered a dose of CsA (25 mg/kg per day) shown, in vivo, to inhibit calcineurin activity in the heart. CsA treatment began 48 hours before infarction and continued until hearts were harvested. Noninfarcted mice were treated with a similar regimen. Figure 4B through 4F shows the current–voltage relationships of Kv currents from control (noninfarced, CsA-treated mice; n=21), untreated MI (n=26), and cells dissociated from infarcted hearts treated with CsA (MI–CsA; n=18). CsA prevented the reduction of the amplitudes of I_{to} and I_{sust} (P<0.05; Figure 4B and 4C) after MI. Like metoprolol, CsA also decreased the currents in I_{to}, I_{Kslow1}, and I_{Kslow2} observed post-MI (Figure 4D through 4F). FK506, another calcineurin inhibitor, also prevented the decrease in Kv currents after MI (n=7, P<0.05; data not shown). These data suggest that calcineurin plays a central role in mediating the changes in I_{to}, I_{Kslow1}, and I_{Kslow2} observed post-MI.

**NFATc3 Mediates the Reductions in I_{to}, I_{Kslow1} and I_{Kslow2} Observed After MI**

To further examine the role of NFATc3 in mediating MI-induced reductions in I_{to}, I_{Kslow1}, and I_{Kslow2}, we performed experiments similar to those described above in NFATc3 knockout (NFATc3-KO) mice. Figure 5A shows representative Kv currents from ventricular myocytes dissociated from NFATc3–KO, infarcted NFATc3–KO (NFATc3–KO–MI), and infarcted wild-type (WT–MI) hearts. If activation of NFATc3 is necessary for the reduction in I_{to}, I_{Kslow1}, and I_{Kslow2} observed post-MI, then there should be no reduction in these currents after MI in ventricular myocytes from NFATc3-KO mice. Supporting this hypothesis, Figure 5B through 5F show that although MI decreased I_{to}, I_{sust}, I_{Kslow1}, I_{Kslow2}, and I_{v} in WT cells (WT–MI; n=26), the amplitudes of these currents were similar (P>0.05) in NFATc3–KO (n=26) and NFATc3–KO–MI cells (n=14). Consistent with these data, Kv1.5, Kv2.1, Kv4.2, and Kv4.3 mRNA (n=20 hearts) and protein (n=6 hearts) did not decrease (P>0.05) after MI in NFATc3–KO ventricles (Figure 5G and 5H). These data suggest that NFATc3 plays an obligatory role in the reductions of I_{to}, I_{Kslow1}, and I_{Kslow2} observed 48 hours post-MI. Interestingly, a comparison of Kv currents from WT and NFATc3–KO cells revealed that I_{to} but not I_{Kslow1} or I_{Kslow2} was increased in NFATc3–KO cells, thus suggesting a degree of basal regulation of I_{to} by NFATc3 (see online data supplement).

**Chronic βAR Stimulation and a Constitutively Active NFATc3 Mimic the Effects of MI on I_{to} and I_{sust}**

To test if activation of NFATc3 was sufficient to account for the changes in I_{to} and I_{sust} observed post-MI, we infected ventricular myocytes from uninjured WT mice with an adenoviral vector expressing a constitutively active EGFP-tagged NFATc3 mutant (ΔNFATc3; Figure 6). Control cells were infected with an adenoviral vector expressing EGFP only. As expected, cells expressing activated NFATc3 showed high levels of fluorescence in their nuclei (Figure 6A). The rate of inactivation of the composite Kv currents at +40 mV was similar in control (τ_{inst}=43.1±8.4 ms, τ_{slow}=623.8±107.9 ms, n=8) and ΔNFATc3 cells (τ_{inst}=44.7±5.8 ms, τ_{slow}=519.5±67.5 ms; n=8, P>0.05). However, expression of ΔNFATc3 reduced (P<0.05) the amplitudes of I_{to} and I_{sust} at most voltages examined. Note that cells cultured for 48 hours in the presence of the β-adrenergic agonist isoproterenol (ISO: 10 nmol/L) had I_{to} and I_{sust} that were smaller than those from cells cultured for the same amount of time, but in the absence of ISO. Interestingly, ISO reduced I_{tran} and I_{sust} to the same extent as ΔNFATc3 (n=6; P>0.05). These findings suggest that activation of NFATc3 and of βARs reduce the amplitude of Kv currents in mouse ventricular myocytes.

**Discussion**

In this study, we provide the first direct demonstration that activation of NFATc3 is essential in the pathway leading to decreased Kv currents post-MI. Our data suggest that increased β-adrenergic signaling plays a pivotal role in the activation of NFATc3 after MI. We propose that activation of NFATc3 after MI downregulates Kv1.5, Kv2.1, Kv4.2, and Kv4.3 gene expression, thereby decreasing the amplitudes of I_{to}, I_{Kslow1}, and I_{Kslow2} in mouse ventricular myocytes. The implications of these findings are discussed below.

**AP and [Ca^{2+}]_{l} After MI**

We found that decreased I_{to}, I_{Kslow1}, and I_{Kslow2} accompanied AP prolongation post-MI. This is consistent with recent studies suggesting that I_{to}, I_{Kslow1}, and I_{Kslow2} are responsible for the repolarization of the mouse ventricle. AP prolongation increases Ca^{2+} influx via t-type Ca^{2+} channels, SR Ca^{2+} load, and SR Ca^{2+} release during excitation–contraction coupling. A similar mechanism may underlie the larger [Ca^{2+}]_{l}, transient observed here 48 hours post-MI. However, it is intriguing to speculate that increased βAR signaling could also contribute to enhanced [Ca^{2+}]_{l} early after MI.

Changes in the maximal rate of depolarization and peak of the AP of control and MI cells were not observed, indicating that the Na^{+} current (I_{Na}) was not changed 48 hours post-MI. In support of this, we found that I_{Na} was similar in control and MI cells (Santana, Rossow, Minami, and Murry, unpublished data). Note, however, that changes in I_{Na} have been observed 5 days post-MI. Thus, decreased Kv currents appear to represent an earlier event in the progression of AP changes post-MI.

**NFATc3 and Kv Expression in Ventricular Myocytes**

The data suggesting that NFATc3 is an essential component of the signaling pathway mediating the changes in I_{to}, I_{Kslow1}, and I_{sust}...
Figure 5. MI does not decrease $I_{to}$, $I_{K_{slow1}}$, and $I_{K_{slow2}}$ in NFATc3-KO ventricular myocytes. A, Representative Kv currents from NFATc3-KO, NFATc3-KO-MI, and WT-MI cells. B to F, Current–voltage relationships of $I_{trans}$, $I_{sust}$, $I_{K_{slow1}}$, $I_{K_{slow2}}$, and $I_{to}$ in NFATc3-KO, NFATc3-KO-MI, and WT-MI cells. G, Real-time RT-PCR analysis of Kv1.5, 2.1, 4.2, and 4.3 mRNA in NFATc3-KO and NFATc3-KO-MI hearts. H, Western blots of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 protein in NFATc3-KO and NFATc3-KO-MI cells. The bar plot shows the percent change (relative to NFATc3-KO) in Kv protein after MI in NFATc3-KO cells. *P<0.05 vs MI.
and I_{Kslow2} after MI are compelling. First, we found that NFAT activity increased nearly 4-fold post-MI. This finding is consistent with a recent study showing NFATc3 hypophosphorylation 4 weeks post-MI.16 Second, prevention of NFATc3 activation by pharmacological (metoprolol and presumably by CsA and FK506) or genetic (NFATc3–KO mice) interventions prevented the decreases in I_{to}, I_{Kslow1}, and I_{Kslow2} observed after MI. Third, expression of an activated form of NFATc3 mimics the actions of MI and chronic βAR stimulation on Kv currents in mouse ventricular myocytes.

Our functional, mRNA and protein data suggest that reduced expression of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 transcripts and proteins is the likely mechanism underlying the decreases in I_{to}, I_{Kslow1}, and I_{Kslow2} post-MI. We found multiple-consensus NFAT-binding elements within the 5' untranslated region of the Kv1.5, Kv2.1, Kv4.2, and Kv4.3 genes. Thus, binding of NFATc3 to these genes could possibly alter their expression. This view is supported by a recent study showing that NFATc3 can downregulate the transcription of CD154, CDK4, IL-4, and IL-5 genes.30 NFATc3 can therefore have a dual role as inducer or repressor of gene transcription.

An intriguing finding in this study is that activation of NFATc3 after MI downregulated I_{to} and I_{Kslow2} to a larger extent than I_{Kslow1}. The molecular mechanisms underlying this difference are unclear, however. We did not detect a significant difference in the relative decrease of Kv4.3, Kv4.2, Kv2.1, and Kv1.5 mRNA and protein post-MI. The difference in the magnitude of the decrease in currents and Kv proteins post-MI could reflect changes in the expression of accessory subunits (eg, KChIPs, KChAPs, Kvβs), trafficking and/or posttranslational processing of these channels. This is important because there are pronounced differences in the amplitude and kinetics of I_{to} within different regions of the mouse heart.23,31 Indeed, the I_{S} reported here have a relatively large amplitude and are fully inactivated by ~200 ms, which suggest that they likely represent the fast I_{to} described in cells from the ventricular epicardium31 and in some cells from the septum.23 I_{Kslow1} is also differentially expressed within the mouse ventricle. Interestingly, Xu et al23 found that I_{Kslow1} was larger in cells with larger fast I_{to}. Future experiments should examine a possible role for NFATc3 in maintaining the regional heterogeneity of I_{to} and I_{Kslow1} in the heart.

**Ca^{2+} Signaling and NFATc3 Activity in the Heart**

In a recent study by Sah et al,32 overexpression of a truncated form of Kv4.2 decreased I_{to} and prolonged the AP of ventricular myocytes of transgenic mice. They found that AP prolongation was associated with increased [Ca^{2+}], and also with activation of calcineurin. These experiments suggested that calcineurin is downstream of changes in Kv currents. However, we overexpressed a constitutively active NFATc3

![Figure 6. Expression of ΔNFATc3 decreases I_{to} and I_{Kslow2}. A, Confocal images showing EGFP and ΔNFATc3 expression in mouse ventricular myocytes. B, Kv currents recorded from EGFP and ΔNFATc3-expressing cells. C, Current-voltage relationships of I_{to} and I_{Kslow2} in control, ISO, and ΔNFATc3 cells. *P<0.05 vs control.](http://circres.ahajournals.org/)

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and found decreased Kv currents, indicating Kv channel transcription is downstream of NFATc3 signaling (Figure 6). Although these 2 studies may initially seem at odds, we suggest a signaling model that is consistent with both observations (Figure 7). We propose that early after MI, βAR-induced changes in [Ca$^{2+}$], result in calcineurin/NFATc3 activation which, in turn, results in decreased Kv channel expression. Reduced Kv channel expression then leads to decreased Kv currents and hence to AP prolongation, which increases [Ca$^{2+}$]. In our model, βAR, calcineurin, and NFATc3 form part of a positive feedback loop that could be activated by increasing [Ca$^{2+}$], directly activating NFATc3, and so on. Initiation of this feedback loop could be prevented by the administration of β-blockers or calcineurin inhibitors.

**Comparison With Other Studies**

Our hypothesis that βARs and NFATc3 play a central role in the downregulation of $I_{\text{Kd1}$, $I_{\text{Kd2}}$, and $I_{\text{Kd3}}$ post-MI is supported by recent work. Zhang et al. suggested that chronic activation of βARs reduce the amplitude of delayed rectifier K$^+$ currents in cultured guinea pig ventricular myocytes. Furthermore, it was reported that mice overexpressing calcineurin show early downregulation of Kv2.1 and Kv1.5 protein levels. Hearts overexpressing calcineurin also had lower Kv4.2 protein expression than controls, but only after the development of heart failure. A recent study by Deng et al. used calcineurin inhibition by CsA as a tool to evaluate the role of this phosphatase in mediating the changes in K$^+$ currents observed in the rat post-MI. Consistent with our results and those of Petrashevskaya et al., they found that CsA administration inhibited calcineurin and prevented the reductions in Kv4.2 and Kv2.1 expression observed post-MI. These findings support the hypothesis that activation of NFATc3 by calcineurin could underlie electrical remodeling of the heart after injury.

In addition to inducing Kv channel downregulation, MI causes hypertrophy. Two recent studies suggested that prevention of calcineurin activation using CsA or by overexpressing the inhibitory calcineurin-interacting protein-1 decreased ventricular hypertrophy by up to 40% after MI. By comparison, our data indicate that preventing NFATc3 activation by pharmacological or genetic means completely prevents Kv current remodeling post-MI. Given that calcineurin/NFAT inhibition only partially blocks hypertrophy, whereas Kv downregulation is completely abrogated, we speculate that there may be less redundancy in the pathways leading to Kv downregulation.

**Summary**

Our results indicate that βARs, calcineurin, and NFATc3 are part of a signaling pathway that reduces Kv currents post-MI. We propose that calcineurin reduces these currents by increasing the expression of Kv1.5, Kv2.1, Kv4.2, and Kv4.3 channels via the activation of NFATc3. These findings provide a mechanistic model for the therapeutic use of β-blockers to reduce arrhythmogenic changes in Kv currents after MI.

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NFATc3-Induced Reductions in Voltage-Gated K⁺ Currents After Myocardial Infarction
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Expanded Methods and Materials

*Generation of myocardial infarcts*

Infarcts were generated as previously described\(^1\). Briefly, adult (6-8 weeks, 25-30 g) male C57BL6/J, NFATc3-KO or NFAT-luc mice were anesthetized and underwent left thoracotomy. The left anterior descending coronary artery was permanently ligated with an 8-0 polypropylene suture (typically midway from the apex to base). Following occlusion and blanching of the myocardium, the chest was closed. Although we could not measure the infarct size in the same hearts from which myocytes were isolated, parallel experiments yielded infarcts that reproducibly averaged 38 ± 5% of the left ventricle\(^2\). Animals were allowed to recover, and infarcted hearts were harvested 48 hours post MI. NFATc3-KO and NFAT-luc mice were provided by Drs. Laurie Glimcher and Mercedes Rincón, respectively. Animals were handled in accordance to the guidelines of the Institutional Animal Care and Use Committee.

*Isolation and adenoviral infection of adult mouse ventricular myocytes*

Ventricular myocytes were isolated from adult mice as previously described\(^3\). Cells were dissociated from regions remote from the MI (i.e. right ventricle, septum, non-infarcted region of the left ventricle). For controls experiments, cells were dissociated from regions of the hearts similar to those used from infarcted hearts. Dissociated cells were maintained at room temperature (25 °C) until used.

The adenoviral construct encoding a constitutively active form of NFAT tagged with an N-terminal EGFP tag (ΔNFAT)\(^4\) was kindly provided by Dr. Martin F. Schneider. Briefly, the
ΔNFAT mutant lacks the first 317 amino acids corresponding to the N-terminal regulatory domain of the protein, but retains the Rel homology and transactivation domains.

Adenoviral infection and short-term culture of adult ventricular myocytes was performed as previously described. Briefly, freshly dispersed cardiac myocytes were plated into 35-mm petri dishes one hour prior to exposure to recombinant adenovirus encoding either EGFP (control) or ΔNFAT. The culture medium was aspirated, and 1 mL of serum-free MEM containing adenovirus encoding either EGFP (control) or ΔNFAT was added to the dish. Adenoviral vectors were added at a multiplicity of infection of 10-100. Following 1-2 h of culture, 1 mL of serum-free MEM was added. Positively infected myocytes were identified via fluorescence microscopy 48 hours post infection.

**Electrophysiology and confocal microscopy**

Currents and membrane potentials were recorded using an Axopatch 200B patch-clamp amplifier. K⁺ currents were measured using a pipette solution containing (in mmol/L): 110 K-Aspartate, 30 KCl, 10 HEPES, 5 ATP-Mg and 10 EGTA (pH=7.3). APs were recorded using a similar solution, but with 0.05 fluo-4 and no EGTA. With this solution, the patch electrodes had resistances that ranged from 0.8 to 1.2 MΩ. The external solution used to measure K⁺ currents contained (in mmol/L): 140 N-methyl-D-glucamine, 5 KCl, 10 HEPES, 10 glucose, 0.1 CaCl₂, 2 MgCl₂, 0.01 TTX and 0.01 nifedipine (pH = 7.4). To this solution we added 4-aminopyridine (4-AP; 50 µmol/L) and/or tetraethylammonium chloride (TEA; 25 mmol/L) to pharmacologically isolate the K⁺ current(s) of interest. After TEA and/or 4-AP was added, the pH of the external solution was verified to be 7.4. The series resistance compensation circuitry of the Axopatch amplifier
200B was used in all voltage clamp experiments to compensate for about 60% of the series resistance. All electrophysiological and imaging experiments were performed at 22-25 °C.

Kv currents were analyzed using Clampfit 8 software (Axon Instruments). Normalization of ventricular K⁺ currents was performed by dividing the current amplitudes by the capacitance of the cells from which they were recorded. Analysis was only performed on K⁺ currents that met the following criteria. First, there was no abrupt change in current during the increasing phase of the current-voltage relationship. Second, the voltage drop across the uncompensated series resistance (≈ 0.6 MΩ) was not larger than 3 mV. These criteria are similar to those used by others.6–8

[Ca²⁺]ᵢ imaging was performed with a BioRad Radiance 2100 confocal system. Ca²⁺ sparks were identified using a previously described computer algorithm. Calibration of fluorescence signals was performed using the “pseudo-ratio” method.

Administration of CsA, FK506 and metoprolol

Metoprolol (~350 mg/Kg/day) was administered, via drinking water 24 hours prior to infarct and treatment was continued until the infarcted hearts were harvested. CsA (25 mg/Kg/day) or FK506 (3 mg/Kg twice a day) was administered IP 48 hours prior to infarct and was continued until the infarcted hearts were harvested.

Real Time RT-PCR
RNA was extracted from the right ventricle, septum and non-infarcted region of the left ventricle of MI, MI-BB and NFAT-KO-MI mice. For controls, RNA was extracted from similar regions of the hearts of control, NFAT-KO and BB mice. Real-time, quantitative RT-PCR was performed with the TaqMan 5’ nuclease assay using the ABI 7700 (PE Applied Bio-systems) and TaqMan Onestep PCR Mastermix. Briefly, a standard curve was generated for each pair of primers and probes using log_{10} serial dilutions of total RNA from control hearts. The efficiency of each pair of primers and probes was determined by the slope of the standard curve. The standard curve for each probe and primer set was used to determine the relative quantity of Kv transcript in each tissue relative to the concentration of total RNA, the endogenous β-actin or 18s ribosomal RNA standard in the same sample. Primers and probes for real time RT-PCR were as follows: Kv1.5 (GenBank accession no. NM_145983) sense nt 1673-1698, anti-sense nt 1704-1735, and probe nt 1740-1757; Kv2.1 (GenBank accession no. NM_008420) sense nt 575-597, anti-sense nt 639-660, and probe nt 610-636; Kv4.2 (GenBank accession no. NM_019697) sense nt 1769-1792; anti-sense nt 1823-1840, and probe nt 1793-1822; Kv4.3 (GenBank accession no. NM_019931) sense nt 85-104; anti-sense nt 132-151, and probe nt 106-128; β-actin (GenBank accession no. NM_007393) sense nt 1035-1055; anti-sense nt 1090-1110, and probe nt 1060-1086. 20x pre-developed TaqMan assay reagent for 18s ribosomal RNA primers and probes was obtained from Applied Biosystems. Following reverse transcription at 48°C for 30 min, AmpliTaq polymerase was activated at 95°C for 10 min, samples underwent 40 cycles of amplification in which they were incubated at 95°C for 15 s and 60°C for 1 min. RT(-) and non-template controls were included as a control for non-specific amplification. Samples (n=3 for each tissue) were run in triplicate and mean values generated were compared using Student's t-test.
**RNA time-course experiments**

Freshly isolated cells from control and MI hearts were treated with actinomycin D (10-20 µg/mL) and incubated at room temperature from 0-8 h. This concentration of actinomycin D has been previously used to inhibit transcription in ventricular myocytes. RNA was isolated as described above. In those experiments designed to test the effects of β-adrenergic stimulation on Kv channel transcript degradation, myocytes isolated from control hearts were incubated with isoproterenol (10 nmol/L) and actinomycin for 0-8 hours. Real time quantitative RT-PCR was performed as described above.

**Western blot analysis**

The right ventricle, septum and non-infarcted region of the left ventricle were isolated from the hearts of MI, MI-BB and NFAT-KO-MI mice. Similar regions of the hearts of control, NFAT-KO and BB mice were used for these experiments. Tissue was ground under liquid nitrogen and homogenized for 30-60 seconds in PBS with 5mM EDTA, 1% Titon-X 100 and a mammalian protease inhibitor cocktail (Sigma). The homogenate was cleared of cellular debris by centrifugation at 10,000 x g at 4°C, for 10 min. Protein concentration of the supernatant was determined using the bicinchoninic acid method with bovine serum albumin (BSA) as a standard. 50 µg of total protein was loaded on a 4-15% Tris-HCl polyacrylamide gel and run with an appropriate molecular weight standard at 100 mV for 1 hour. Fractionated protein was transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini-trans Blot Cell (Bio-Rad Laboratories) at 100 mV for 1h at 4 °C. The blots were blocked in TBS-Tween (20 mmol/L Tris pH 8.0, 150 mmol/L NaCl, and 0.05% Tween-20) with 5% nonfat milk for 1h at 25 °C. Following blocking, blots were incubated with primary antibodies specific to Kv1.5
Online Data Supplement

Regulation of Kv channels expression after MI

(Upstate), 2.1 (Upstate), 4.2 (Sigma) or 4.3 (Sigma) in TBS-Tween with 1% non-fat milk at 25°C for 1h. Following incubation with the primary antibody, blots were washed 3x with TBS-Tween for 10 minutes and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody diluted in TBS-Tween for 1 hour. This was followed by incubation with Super-signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc). Kv channel protein was quantified by densitometry using an Alphaimager 2200 analysis system and software (Alpha Innotech Corp).

Luciferase assay

For luciferase measurements protein extracts were purified from freshly dispersed ventricular myocytes and quantified using the Bradford Assay \(^3\). NFAT activity in mice genetically engineered to express luciferase following NFAT activation was quantified using a Luciferase Assay Kit (Promega) as per the manufacturers instructions.

Analysis of the 5’ UTR of Kv1.5, 2.1, 4.2 and 4.3

Vector NTI 8.0 software (Informax) was used to identify the presence of multiple NFAT binding elements (GGAAA) within the 5’ UTR of Kv1.5 (NM_145983; 72 – 1243), Kv2.1 (NM_008420; 1040 – 1827), Kv4.2 (NM_019697; 184 – 1960) and Kv4.3 (NM_019931; 136 – 1474).

Statistics

Data are presented as mean ± SEM. Two-sample comparisons were made using Student’s t-test. Multi-group comparisons were performed using an ANOVA, which, if necessary, was followed
by a Tukey test. A $p$ value of less than 0.05 was used as an indicator of significance. The asterisk (*) symbol is used in the figures to illustrate a significant difference between groups. N values represent number of cells. Experimental groups include data from at least 5 hearts.
Additional Figures and Supporting Information

MI or βAR stimulation does not increase the degradation rate of Kv1.5, Kv2.1, Kv4.3 and Kv4.2 mRNA

We investigated whether increased mRNA degradation caused the decrease in Kv transcript observed following MI. For these experiments, cells isolated from control (non-infarcted) and infarcted hearts were exposed to the transcriptional inhibitor actinomycin D. Kv mRNA levels were measured before (time 0) and 8 hours after the application of actinomycin D in control and MI cells using real-time RT-PCR (Fig. 1). We found that eight hours after the inhibition of transcription with actinomycin D, the levels of Kv1.5, Kv2.1 and Kv4.2 mRNA decreased to a similar extent in control and MI cells (n > 100 cells from 3 hearts; p > 0.05). The rate of Kv4.3 degradation was not increased in MI cells. However, compared to controls, Kv4.3 mRNA decreased to a lesser extent in MI cells (n > 100 cells from 3 hearts; p < 0.05). These data indicate that the degradation of Kv1.5, Kv2.1, Kv4.2 and Kv4.3 mRNA is not increased in MI cells.

Larger I_{to} in NFATc3-KO than in WT ventricular myocytes

We examined the possibility that NFATc3 could regulate Kv currents in non-infarcted ventricular myocytes. For these experiments we recorded I_{K}, I_{to}, I_{Kslow1} and I_{Kslow2} from non-infarcted WT and NFATc3-KO ventricular myocytes. Interestingly, we found that NFATc3-KO cells (n = 26) had larger composite Kv currents than WT cells (n = 28) at most voltages examined (p < 0.05; Fig. 2A). Closer analysis of these currents revealed that I_{trans} but not I_{sust}, was increased in NFATc3-KO cells (Fig. 2B-C). Since I_{to} and I_{Kslow1} underlie I_{trans}, we investigated
whether an increase in one, or both, of these currents could account for the larger $I_{\text{tran}}$ in NFATc3-KO cells. Fig. 2C-D shows the current-voltage relationship of $I_{\text{to}}$ and $I_{\text{Kslow1}}$ in NFATc3-KO and WT cells. Note that, at most voltages examined, $I_{\text{to}}$ is larger in NFATc3-KO than in WT cells ($p < 0.05$). Indeed, at $+40$ mV the amplitude of $I_{\text{to}}$ was $41.29 \pm 4.36$ pA/pF in NFATc3-KO cells versus $26.63 \pm 4.07$ pA/pF in WT cells ($p < 0.05$). Although we noticed a tendency for $I_{\text{Kslow1}}$ to be slightly larger in NFATc3 KO than in WT cells, this difference was not statistically significant ($p > 0.05$). Consistent with the observation that $I_{\text{sust}}$ was similar in NFATc3-KO and WT cells, we found no difference in the current-voltage relationship of $I_{\text{Kslow2}}$ in these cells (Fig. 2E). These data suggest that there is greater basal regulation of $I_{\text{to}}$ by NFATc3. Future studies should examine the molecular mechanisms underlying NFATc3 regulation of $I_{\text{to}}$ in the un-infarcted heart.
Fig. 1. MI does not alter the rate of degradation of Kv1.5, Kv2.1, Kv4.3 and Kv4.2 mRNA. Bar plot of the mean SEM Kv mRNA levels in control and MI cells 8 hours after the addition of actinomycin D. Data were normalized to the amount of Kv mRNA before actinomycin treatment.
Fig. 2. $I_{to}$, $I_{Kslow1}$ and $I_{Kslow2}$ in NFATc3-KO and WT ventricular myocytes. (A)

Representative Kv currents from NFATc3-KO and WT cells. (B-F) Current-voltage relationships of $I_{tran}$, $I_{sust}$, $I_{Kslow1}$, $I_{Kslow2}$ and $I_{to}$ in NFATc3-KO and WT cells. *p < 0.05.
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