Molecular Mechanisms Involved in the Angiotensin-(1-7)/Mas Signaling Pathway in Cardiomyocytes

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Abstract—Recently there has been growing evidence suggesting that beneficial effects of angiotensin-(1-7) [Ang-(1-7)] in the heart are mediated by its receptor Mas. However, the signaling pathways involved in these effects in cardiomyocytes are unknown. Here, we investigated the involvement of the Ang-(1-7)/Mas axis in NO generation and Ca^{2+} handling in adult ventricular myocytes using a combination of molecular biology, intracellular Ca^{2+} imaging, and confocal microscopy. Acute Ang-(1-7) treatment (10 nmol/L) leads to NO production and activates endothelial NO synthase and Akt in cardiomyocytes. Ang-(1-7)–dependent NO raise was abolished by pretreatment with A-779 (1 μmol/L). To confirm that Ang-(1-7) action is mediated by Mas, we used cardiomyocytes isolated from Mas-deficient mice. In Mas-deficient cardiomyocytes, Ang-(1-7) failed to increase NO levels. Moreover, *Mas*-ablation was accompanied by significant alterations in the proteins involved in the regulation of endothelial NO synthase activity, indicating that endothelial NO synthase and its binding partners are important effectors of the Mas-mediated pathway in cardiomyocytes. We then investigated the role of the Ang-(1-7)/Mas axis on Ca^{2+} signaling. Cardiomyocytes treated with 10 nmol/L of Ang-(1-7) did not show changes in Ca^{2+}-transient parameters such as peak Ca^{2+} transients and kinetics of decay. Nevertheless, cardiomyocytes from Mas-deficient mice presented reduced peak and slower $[Ca^{2+}]_i$ transients when compared with wild-type cardiomyocytes. Lower Ca^{2+} ATPase of the sarcoplasmic reticulum expression levels accompanied the reduced Ca^{2+} transient in Mas-deficient cardiomyocytes. Therefore, chronic *Mas*-deficiency leads to impaired Ca^{2+} handling in cardiomyocytes. Collectively, these observations reveal a key role for the Ang-(1-7)/Mas axis as a modulator of cardiomyocyte function. **(*Hypertension*. 2008;52:542-548.)**

Key Words: ventricular cardiomyocytes ■ NO ■ Ca^{2+} transient ■ Ang-(1-7), Mas

The renin-angiotensin system plays a major role in the pathogenesis of cardiovascular diseases.[1] Although angiotensin (Ang) II is the major effector of this system, several other peptides are now recognized as being biologically important. Of particular importance is the heptapeptide Ang-(1-7). Accordingly, Ang-(1-7) has been reported to have a pivotal role in the regulation of the cardiovascular system.[2,3] Briefly, this peptide decreased the incidence and duration of ischemia-reperfusion arrhythmias,[4] improved the postischemic contractile function in isolated perfused rat hearts, and prevented Ang II–induced cardiac remodeling.[5] Previously, we have reported that Ang-(1-7) is an endogenous ligand for the G protein–coupled receptor Mas.[6] In keeping with this finding, many of the cardiovascular effects of Ang-(1-7) can be completely blocked by the selective Mas antagonist A-779,[7] suggesting an important role for the Ang-(1-7)/Mas axis in the cardiovascular system. Supporting these findings, *Mas*-deficient ($Mas^{-/-}$) mice present reduced cardiac function characterized by decreased fractional shortening.[8] Overall, these studies indicate that interactions between Ang-(1-7) and Mas play an important role in cardiac function, and genetic deletion of *Mas* leads to cardiac impairment and endothelial dysfunction.[9] However, the cellular mechanism and signaling pathways involved in Ang-(1-7)/Mas actions in the cardiac cell are not defined. Recently, Sampaio et al[10] have demonstrated that Ang-(1-7) actions in endothelial cells cause endothelial NO synthase (eNOS) phosphorylation and result in NO release through the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt)–dependent pathway. Ang-(1-7) via Mas also reduce the overall growth of cardiomyocytes.[11] In this study, we investigated the involvement of the Ang-(1-7)/Mas axis in NO generation and Ca^{2+} handling in adult ventricular myocytes.

Methods

For expanded Methods please see the data supplement available online at http://hyper.ahajournals.org.

Received April 2, 2008; first decision April 21, 2008; revision accepted July 1, 2008.

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Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.108.114280**

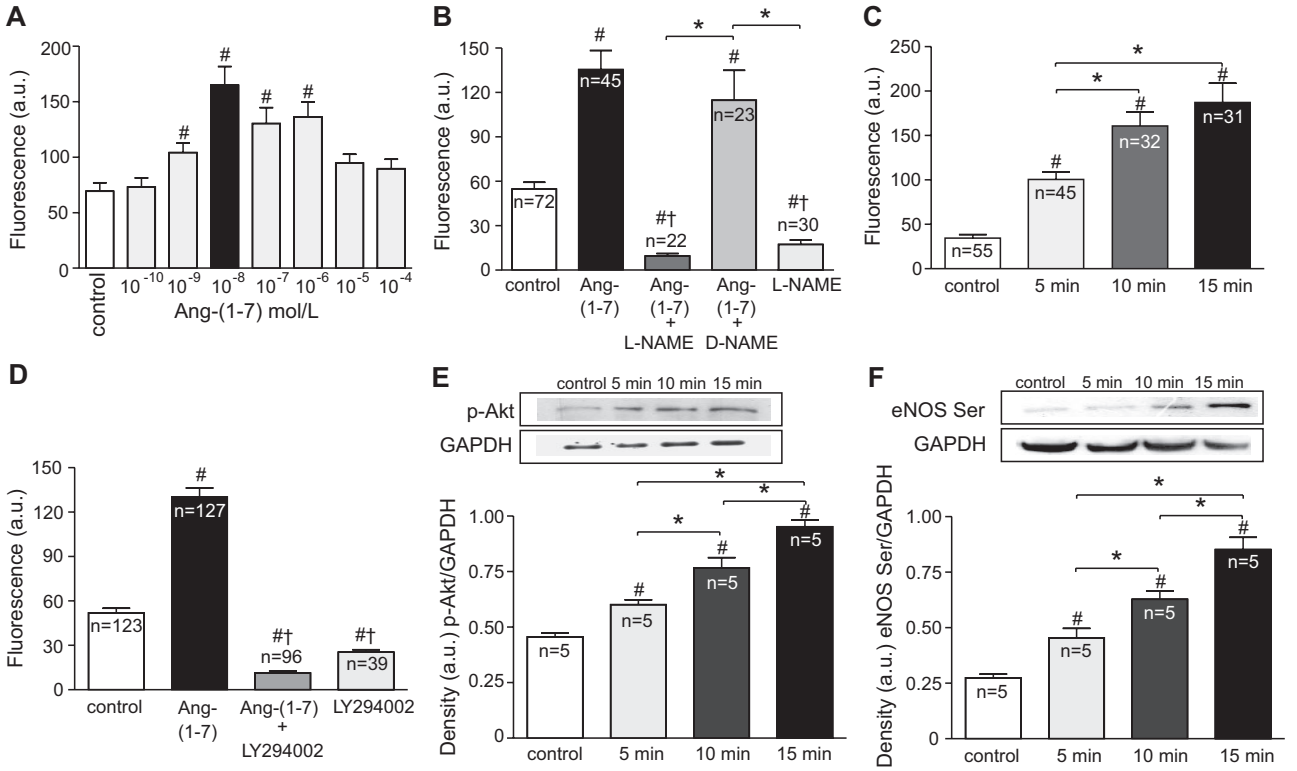

Figure 1. Ang-(1-7) signaling pathway involved in NO generation in cardiomyocytes. A, Concentration-dependent effect of Ang-(1-7) on NO generation in ventricular cardiomyocytes. Each bar represents data from ≥35 cells. B, Averaged-bar graph represents NO generation in adult ventricular myocytes after acute Ang-(1-7) treatment (10 nmol/L; 15 minutes). In N^G-nitro-L-arginine methyl ester–pretreated cells, Ang-(1-7)–dependent NO raise was abolished. C, Time course of NO generation after acute Ang-(1-7) treatment. D, Pretreatment of cardiomyocytes with PI3K inhibitor (LY294002) prevented Ang-(1-7)–stimulated NO raise. In A through D, n=number of cardiomyocytes. E and F, Representative Western blot (top) and bar graph show the time-dependent increase in Akt and eNOS phosphorylation levels after Ang-(1-7) stimulation of cardiomyocytes (bottom). n indicates the number of cardiomyocyte homogenates. GAPDH expression levels were used as a loading control. #$P<0.05$ vs control; *$P<0.05$. In B and D, †$P<0.05$ vs Ang-(1-7)–treated cells.

Animals

Twenty-five wild-type (WT) and 22 *Mas* knockout ($Mas^{-/-}$) on FVB/N genetic background male mice (3 to 6 months old) were obtained from the transgenic animal facilities of the Federal University of Minas Gerais Laboratory of Hypertension. All of the experimental protocols conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by our institution.

Cardiomyocyte Isolation and Confocal Imaging

Freshly isolated ventricular myocytes were stored in DMEM (Sigma Aldrich) until they were used[12] (within 4 hours). Confocal images were obtained using the ZEISS Meta confocal microscope (Zeiss Germany) from CEMEL (Biological Sciences Institute, Federal University of Minas Gerais).

Results

Ang-(1-7) Increases NO Production in Ventricular Cardiomyocytes

To investigate whether Ang-(1-7) is able to generate NO in adult ventricular myocytes, we used the fluorescent indicator 4-amino-5methylamino-2′,7′-difluorofluorescein (DAF-FM) to monitor cellular NO generation. Cells were exposed to Ang-(1-7) for 15 minutes, and maximal DAF fluorescence was observed at 10 nmol/L (Figure 1A), showing a concentration-dependent increase on NO generation by Ang-(1-7) in cardiomyocytes. Figure 1B shows that Ang-(1-7)–dependent (10 nmol/L; 15 minutes) NO raise is abolished by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (10 μmol/L; 15 minutes). On the other hand, the inactive isomer N^G-nitro-D-arginine methyl ester had no effect on Ang-(1-7)–induced NO raise, indicating that Ang-(1-7) stimulates NO generation in cardiomyocytes. To further confirm the specificity of the DAF probe, we incubated cardiomyocytes with the NO donor sodium nitroprusside (Figure S1). Ang-(1-7)–induced NO generation presented a time dependence, as shown in Figure 1C, and significant NO generation occurred 5 minutes after Ang-(1-7) stimulation. LY294002, a PI3K inhibitor (10 μmol/L), blocked the Ang-(1-7)–induced increase in NO fluorescence in cardiomyocytes, suggesting the involvement of the PI3K/Akt pathway in Ang-(1-7) effects in cardiomyocytes (Figure 1D). To confirm whether Ang-(1-7) stimulation in cardiomyocytes leads to Akt activation, we examined the time course of Akt phosphorylation in Ang-(1-7)–treated cells. As shown in Figure 1E, Akt phosphorylation induced by Ang-(1-7) increased significantly in a time-dependent manner. Both endothelial NO synthase (eNOS) and neuronal NO synthase participate in NO production in the heart. The fact that Ang-(1-7) via Akt mediates eNOS activation in endothelial cells prompted us to investigate whether Ang-(1-7) stimulation also results in eNOS activation in cardiomyocytes. eNOS activity is regulated through coordinated phosphorylation of specific sites. Phosphorylation of eNOS at Ser 1177 (stimulatory site) results in

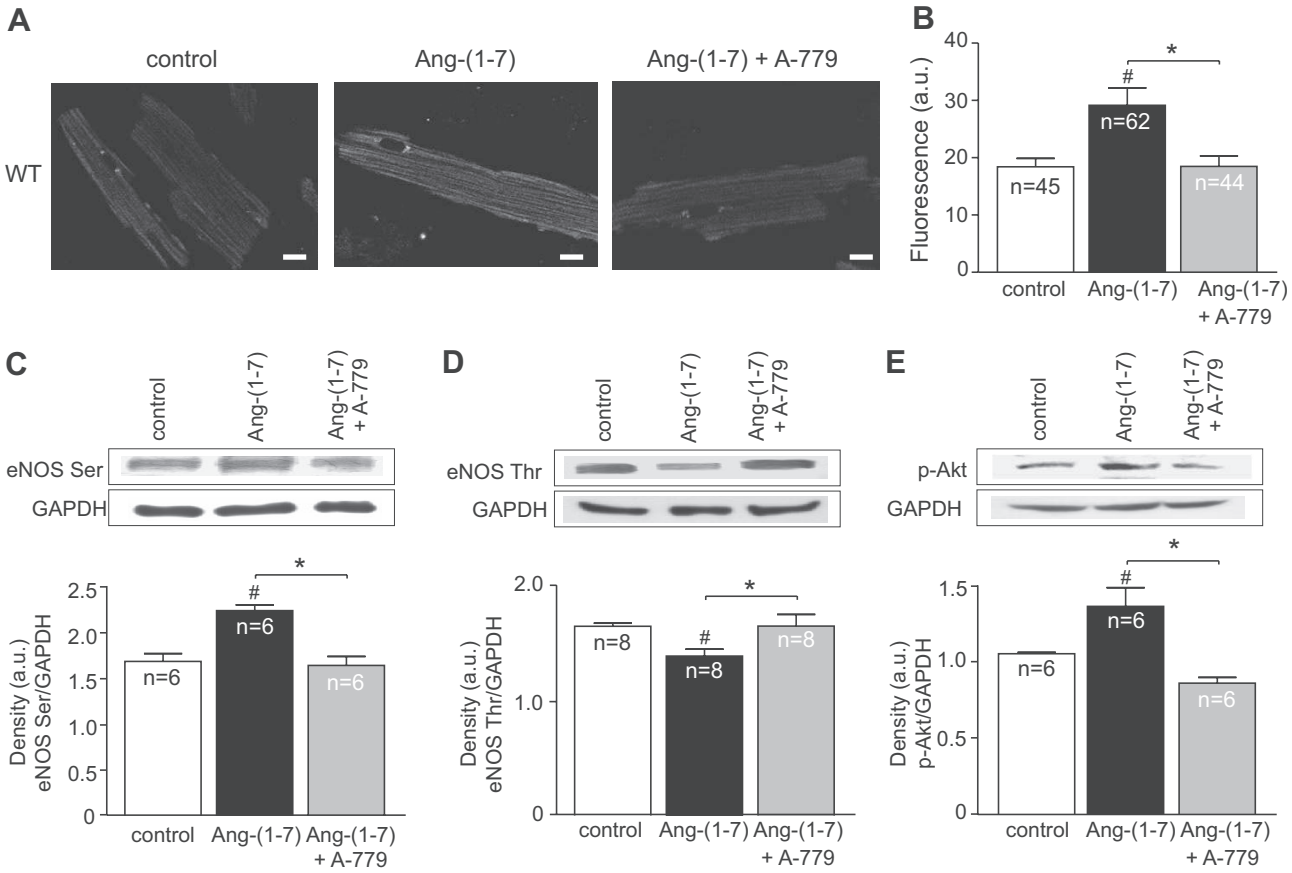

Figure 2. Ang-(1-7) stimulates NO release in cardiomyocytes through Mas. A, Representative confocal images showing DAF-loaded cardiomyocytes from WT mice untreated (control, left), mice treated with Ang-(1-7) (middle), or mice treated with with Ang-(1-7) and the Mas antagonist A-779 (right). Bar=10 μm. B, Averaged DAF fluorescence increase in adult ventricular myocytes after acute Ang-(1-7) treatment (10 nmol/L) for 15 minutes. Application of Mas antagonist A-779 (1 μmol/L) inhibited the increase in DAF fluorescence in Ang-(1-7)–treated cardiomyocytes. n indicates the number of cells. C and D, Top, Representative blots showing Ang-(1-7)–dependent increase in eNOS Ser1177 and concomitant decrease in eNOS Thr 495 phosphorylation levels in WT cardiomyocytes. Preincubation with A-779 prevented Ang-(1-7)–dependent effects in eNOS phosphorylation. Bottom, Averaged bar graph shows the significant increase (C) or decrease (D) in eNOS phosphorylation site levels in Ang-(1-7)—treated cardiomyocytes compared with control and A-779–treated cells. E, Top, Representative blots. Bottom, Averaged bar graph shows the relative increase in Akt phosphorylation by Ang-(1-7). A significant increase in Akt phosphorylation level was observed after Ang-(1-7) treatment. C through E, GAPDH expression levels were used as a loading control. n=number of cardiomyocyte homogenates. #$P<0.05$ vs control; *$P<0.05$.

an increase in enzyme activity and NO production, whereas eNOS phosphorylation at Thr 495 (inhibitory site) reduces enzyme activity and NO production. As shown in Figure 1F, Ang-(1-7) induced eNOS Ser1177 phosphorylation in a time-dependent manner similar to the effects of Ang-(1-7) on Akt activation and NO generation. To verify whether Ang-(1-7) actions on ventricular myocytes occurred via Mas, cells were preincubated with the Mas antagonist A-779 (1 μmol/L; 15 minutes) and then stimulated with Ang-(1-7) (10 nmol/L; 15 minutes). Figure 2A and 2B show that A-779 prevented the Ang-(1-7)–dependent increase in NO. Moreover, the changes in eNOS and Akt phosphorylation levels by Ang-(1-7) were also blocked by A-779 treatment (Figure 2C through 2E).

Ang-(1-7)–Induced NO Generation Is Abolished in *Mas*$^{-/-}$ Cardiomyocytes

To further investigate the role of Mas in Ang-(1-7)–dependent NO generation in cardiac cells, we used cardiomyocytes isolated from *Mas*$^{-/-}$ mice. Figure 3 shows that NO fluorescence failed to augment in *Mas*$^{-/-}$ cardiomyocytes treated with Ang-(1-7) (10 nmol/L; 15 minutes), supporting our findings presented in Figure 2. As expected, the Mas antagonist A-779 had no effect on DAF fluorescence in Ang-(1-7)–treated *Mas*$^{-/-}$ cardiomyocytes. Taken together, Figures 1 to 3 show strong evidence for a role of the Ang-(1-7)/Mas axis in the NO generation of adult ventricular myocytes and reveal a previously unknown signaling pathway in cardiac cells.

Mas Ablation Is Accompanied by Changes in Proteins Involved in the Regulation of eNOS Activity

In cardiomyocytes, eNOS is part of a macromolecular complex composed of caveolin-3, heat shock protein 90 (Hsp90), and Akt. Caveolin-3 has been shown to directly interact with eNOS and inhibits its basal activity by preventing calmodulin interaction.[13] Hsp90 acts as a scaffold protein to recruit Akt in the eNOS complex, thereby promoting eNOS phosphorylation–dependent activation.[14] The third component, Akt, phosphorylates eNOS at Ser 1177, rendering the enzyme active.[13] To investigate possible alterations in the proteins involved in

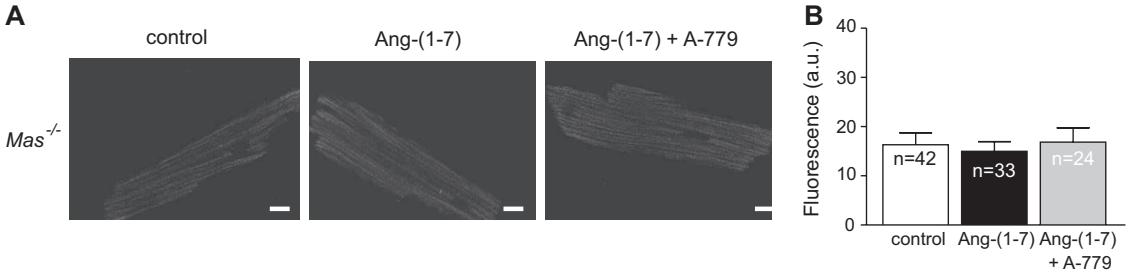

Figure 3. Ang-(1-7) failed to raise NO levels in *Mas*$^{-/-}$ cardiomyocytes. A, Representative confocal images showing DAF-loaded *Mas*$^{-/-}$ cardiomyocytes untreated (control, left), treated with Ang-(1-7) (middle), or treated with Ang-(1-7) and the Mas antagonist A-779 (right). Bar=10 μm. B, Averaged DAF fluorescence in *Mas*$^{-/-}$ cardiomyocytes was unaltered by Ang-(1-7) treatment. n indicates the number of cells.

the regulation of eNOS in *Mas*$^{-/-}$ cardiomyocytes, we performed Western blot analyses in protein homogenates from isolated cardiomyocytes. Immunoblots revealed similar eNOS protein levels in cardiomyocytes from *Mas*$^{-/-}$ mice when compared with control cells (Figure 4A). In contrast, Western blot analysis showed augmented expression of caveolin-3 (by 77%; Figure 4B) in *Mas*$^{-/-}$ cardiomyocytes. We also observed a significant reduction in levels of Hsp90 in *Mas*$^{-/-}$ cardiomyocytes when compared with control cells (Figure 4C). We next evaluated Akt and Akt phosphorylation levels in these cells. Increased Akt levels were observed in *Mas*$^{-/-}$ cardiomyocytes (by ≈56%; Figure 4D). This result was further confirmed by immunofluorescence techniques where cardiomyocytes from *Mas*$^{-/-}$ and WT mice were stained with specific antibodies against Akt. Confocal images in Figure 4E show increased Akt levels in *Mas*$^{-/-}$ cardiomyocytes. To verify Akt activation in *Mas*$^{-/-}$ cardiomyocytes, we assessed phosphorylated Akt levels in these cells. Although Akt expression was higher in *Mas*$^{-/-}$ cells, phosphorylated Akt levels were significantly reduced, suggesting that less active enzyme was found in *Mas*$^{-/-}$ cells when compared with control cardiomyocytes (Figure 4F). These data clearly show changes in expression levels of proteins involving the eNOS complex in *Mas*$^{-/-}$ cardiomyocytes.

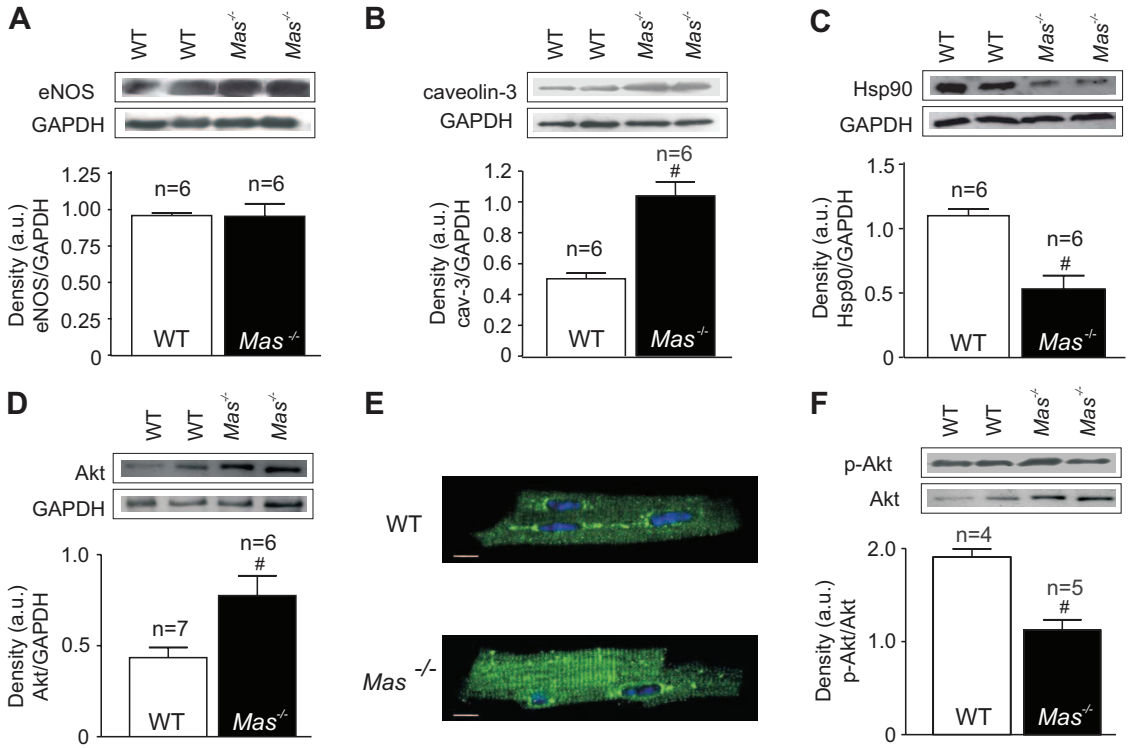

Figure 4. Molecular alterations in proteins involved in eNOS activity regulation in *Mas*$^{-/-}$ cardiomyocytes. Expression of eNOS and its binding partners in adult ventricular myocyte homogenates were assessed by immunoblot techniques with specific antibodies. A, eNOS protein levels were unaltered in cardiomyocytes from *Mas*$^{-/-}$ mice. B, Caveolin-3 expression levels were higher in *Mas*$^{-/-}$ cardiomyocytes when compared with WT cells. In contrast, reduced Hsp90 expression was observed in *Mas*$^{-/-}$ cardiomyocytes (C). D, Akt protein levels were significantly higher in *Mas*$^{-/-}$ cardiomyocytes, whereas phosphorylated Akt levels were lower in these cells (F). E, Immunofluorescence using an anti-Akt–specific antibody showed increased Akt expression (green) in *Mas*$^{-/-}$ cardiomyocytes. The nucleus is stained with 4′,6-diamidino-2-phenylindole (blue). Bar=10 μm. A through F, Top, Representative Western blots; bottom, averaged densitometry. GAPDH expression levels were used as a loading control. #$P<0.05$. n indicates the number of cardiomyocyte homogenates.

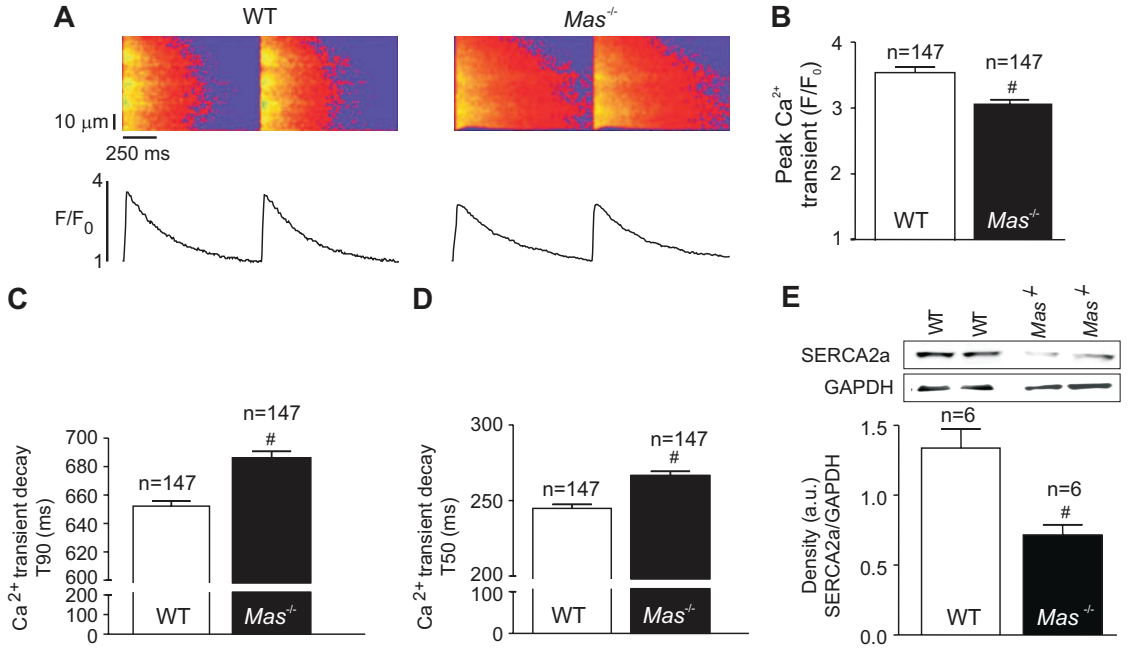

Figure 5. Ca^{2+} signaling defects in *Mas*$^{-/-}$ cardiomyocytes. A, Sample line-scan image (top) and Ca^{2+} transient profile (bottom) from WT and *Mas*$^{-/-}$ cardiomyocytes. B, Bar graph shows averaged-peak Ca^{2+} transient from electrically stimulated cardiomyocytes (1 Hz). *Mas*$^{-/-}$ cardiomyocytes presented significantly reduced Ca^{2+} transients than WT cardiomyocytes. C and D, Averaged Ca^{2+}-transient kinetics of decay. *Mas*$^{-/-}$ cardiomyocytes presented slower Ca^{2+}-transient kinetics of decay than WT cardiomyocytes. T50 and T90 represent the time from peak Ca^{2+} transient to 50% and 90% decay, respectively. n indicates the number of cardiomyocytes. E, Significantly reduced SERCA2a levels were observed in *Mas*$^{-/-}$ cardiomyocytes when compared with WT cells. GAPDH expression was used as a loading control. n indicates the number of cardiomyocyte homogenates. #$P<0.05$.

Increased Apoptosis in the *Mas*$^{-/-}$ Heart

Recently, evidence was presented that NO plays an important role in apoptotic cell death.[15,16] There is evidence suggesting that high concentrations of NO produced from inducible NO synthase induce apoptosis, whereas low concentrations of NO produced from eNOS reduce apoptosis.[15] Given the potential protective effect triggered by eNOS activation in cardiac cells,[14] we hypothesized that *Mas*$^{-/-}$ hearts could exhibit a higher apoptosis rate, because these hearts present significant alterations in proteins involved in eNOS regulation. To investigate this issue, *Mas*$^{-/-}$ hearts were stained using a TUNEL assay, and the total fluorescence was quantified. Figure S2 shows that prominent apoptosis was evident in the left and right ventricles of *Mas*$^{-/-}$ mice when compared with WT hearts. Mean fluorescence levels were significantly higher in the ventricle of *Mas*$^{-/-}$ mice when compared with WT mice, suggesting that differences in apoptotic cell death contribute significantly to *Mas*$^{-/-}$ cardiac defects.

Ca^{2+} Signaling Defects in *Mas*$^{-/-}$ Cardiomyocytes

To investigate the role of the Ang-(1-7)/Mas axis on cardiomyocyte Ca^{2+} dynamics, isolated cardiomyocytes were loaded with the Ca^{2+} sensitive dye Fluo-4/AM, treated with Ang-(1-7) and visualized by confocal microscopy. Top panels of Figure S3A display representative line-scanning images recorded from electrically stimulated (at 1 Hz) cardiomyocytes treated or not with 10 nmol/L of Ang-(1-7) for 15 minutes. Figure S3B shows that acute Ang-(1-7) treatment failed to increase peak Ca^{2+} transient in WT cardiomyocytes. In a similar way, Ang-(1-7) treatment did not alter Ca^{2+} transient kinetics of decay (Figure S3C). Nevertheless, *Mas*$^{-/-}$ cardiomyocytes showed a significantly smaller peak $[Ca^{2+}]_i$ transient ($F/F_0=3.54\pm0.08$, n=147, in WT versus $F/F_0=3.059\pm0.60$, n=147, in *Mas*$^{-/-}$ cardiomyocytes; Figure 5A and 5B), and slower $[Ca^{2+}]_i$ transient kinetics of decay when compared with WT cardiomyocytes (Figure 5C and 5D). These results were consistent with the depression of contractility that has been observed previously in *Mas*$^{-/-}$ hearts.[8] We also treated *Mas*$^{-/-}$ cardiomyocytes with Ang-(1-7). Peak Ca^{2+} transient and kinetics of decay in *Mas*$^{-/-}$ cardiomyocytes were not altered by Ang-(1-7) treatment (data not shown). In cardiomyocytes, Ca^{2+} ATPases found on the sarcoplasmic reticulum (SERCA2a) permit rapid reuptake of the Ca^{2+} released from the sarcoplasmic reticulum. Therefore, we hypothesized that the slow decline of the $[Ca^{2+}]_i$ transient in *Mas*$^{-/-}$ myocytes may be caused by alterations in SERCA2a activity or expression levels. SERCA2a expression levels were then assessed by immunoblot in WT and *Mas*$^{-/-}$ cardiomyocytes. A significant reduction in SERCA2a (by 53%) was found in cardiomyocytes from *Mas*$^{-/-}$ mice relative to WT cardiomyocytes (Figure 5E). Reduced SERCA2a levels in *Mas*$^{-/-}$ cardiomyocytes can explain, at least in part, the changes in Ca^{2+} handling observed in these cells.

Discussion

Recently, multiple lines of evidence have shown that the beneficial effects of Ang-(1-7) in the heart are mediated by Mas. However, the signaling pathways involved in these effects on ventricular myocytes are unknown. In this study, we have made several novel observations. First, we demon-

strated that Ang-(1-7) via Mas increased NO production and provided direct evidence that eNOS and PI3K/Akt are downstream mediators of Ang-(1-7) signaling pathway in cardiomyocytes. We further confirmed these data by showing that *Mas*$^{-/-}$ cardiomyocytes lacked Ang-(1-7)–induced NO production. Second, we have characterized alterations in expression levels of proteins involved in regulation of eNOS activity in *Mas*$^{-/-}$ cardiomyocytes, revealing an important link between Mas and the eNOS macromolecular complex in cardiac cells. Third, we have characterized a Ca^{2+} signaling dysfunction in *Mas*$^{-/-}$ cardiomyocytes, suggesting an important role for Mas in the long-term maintenance of normal Ca^{2+} handling in the cell. We have also shown that acute Ang-(1-7) treatment failed to alter Ca^{2+} handling in ventricular myocytes excluding a direct alteration of Ca^{2+} handling protein function by Ang-(1-7) in our experimental condition. To our knowledge, this work is the first demonstration of the signaling pathways involved in Ang-(1-7)/Mas effects in ventricular myocytes.

Ang-(1-7)/Mas Signaling Pathways in Adult Ventricular Cardiomyocytes

Sampaio et al[10] have demonstrated that Ang-(1-7) actions in endothelial cells cause eNOS phosphorylation, resulting in NO release through the PI3K/Akt-dependent pathway. Here, we have shown direct evidence that eNOS and PI3K/Akt are downstream effectors in the Ang-(1-7) signaling pathway in ventricular myocytes. Multiple lines of investigation suggest that activation of the PI3K/Akt pathway is a critical determinant of cell survival and growth in a variety of signaling pathways.[17] Given the beneficial effects of Ang-(1-7) in the heart, it seems likely that activation of this pathway plays an important role in Ang-(1-7) cardioprotective effects.

Our data are in agreement with a recent observation by Giani et al[18] showing that Ang-(1-7) via Mas stimulates the phosphorylation of Akt in the heart; however, this previous study was performed using whole heart homogenates. By using isolated ventricular myocytes, we specifically addressed the downstream mediators involved in the Ang-(1-7) signaling pathway in the cardiac cell. Nevertheless, a variety of regulators have been identified that influence NO synthesis by regulation of eNOS phosphorylation, including kinases and phosphatases.[14]

The lack of a significant effect of Ang-(1-7) on Ca^{2+} handling in cardiomyocytes does not exclude the possibility that Ang-(1-7) might indirectly affect Ca^{2+} handling protein function. It is well established that constitutive NO production modulates the function of various components of cardiomyocyte excitation-contraction coupling.[19] Martinez-Moreno et al[20] have shown that a fraction of the total eNOS protein directly associates with RyR2. In keeping with this finding, Petroff et al[21] suggested that eNOS-derived NO enhanced RyR2 open probability in cardiomyocytes in response to stretch. Moreover, there has been some evidence in the literature supporting an interplay among eNOS, cGMP-dependent protein kinase, and Ca^{2+} handling in cardiomyocytes.[22] Accordingly, insulin treatment of cardiomyocytes increased phosphorylation and activity of eNOS, leading to augmented phospholamban phosphorylation and consequent SERCA2a activation in reoxygenated cardiomyocytes. In this context, Ang-(1-7)–dependent NO could modulate the activity of multiple Ca^{2+} handling proteins in the cell. On the other hand, it is possible that alterations in the proteins involving the eNOS complex and consequent reduction in eNOS activity in *Mas*$^{-/-}$ cardiomyocytes contribute to the Ca^{2+} handling dysfunction observed in these cells and might be related to SERCA2a changes. This finding is particularly important because it shows for the first time that the Ang-(1-7)/Mas axis is important for long-term maintenance of normal Ca^{2+} handling in the cardiac cell. Nevertheless, we cannot exclude the possibility that a decreased level of SERCA2a is secondary to the cardiac dysfunction observed in *Mas*$^{-/-}$ hearts. Additional experiments are necessary to elucidate the relationship between the Ang-(1-7)–dependent NO signaling pathway and Ca^{2+} handling in cardiomyocytes.

eNOS-Derived NO in Cardiomyocytes

Both eNOS and neuronal NO synthase participate in NO production in the heart. In this work, we showed direct evidence of eNOS as a downstream factor in the Ang-(1-7) signaling pathway in cardiomyocytes. Moreover, we showed that Ang-(1-7) increased NO production in cardiac cells in a time-dependent manner through the PI3K/Akt-dependent pathway. Although we cannot exclude a role of neuronal NO synthase in NO production in adult cardiomyocytes, we believe that, at least in part, we can attribute NO generated by Ang-(1-7) in adult cardiomyocytes to eNOS. Two sets of data support this finding: Ang-(1-7) treatment of cardiomyocytes leads to eNOS activation, and *Mas*-ablation leads to alterations in proteins involved in eNOS regulation in the cell. Although we did not observe changes in eNOS expression levels between *Mas*$^{-/-}$ and WT cardiomyocytes, we believe that eNOS activity in these cells is reduced (see below). Interestingly, Ang II type 2 receptor–deficient mice showed decreased cardiac eNOS expression.[23] The contribution of neuronal NO synthase to NO generation in control and *Mas*$^{-/-}$ cardiomyocytes awaits elucidation.

Reduced eNOS Activity in *Mas*$^{-/-}$ Cardiomyocytes

Several factors support the finding that eNOS activity is reduced in *Mas*$^{-/-}$ cardiomyocytes, the first being increased caveolin-3 expression and consequent eNOS inhibition. Garcia-Cardena et al[24] have shown that peptides corresponding to the scaffolding domain of caveolin-3 inhibit eNOS in cardiomyocytes. In keeping with this finding NO synthase activity is reduced in caveolin-3–overexpressing hearts.[25] Second, *Mas*$^{-/-}$ cardiomyocytes show reduced Hsp90 levels. Low levels of Hsp90 reduce Akt recruitment to the eNOS complex, thereby preventing eNOS-dependent Akt activation. Third, *Mas*$^{-/-}$ cardiomyocytes present reduced Akt activity because of reduced phospho-Akt levels in these cells. Together, these data reveal an important association between receptor Mas and the eNOS signaling complex in adult cardiomyocytes.

There is evidence in the literature suggesting a cardioprotective role of eNOS-derived NO.[26] Specifically, regulation of NO synthesis in the heart by eNOS represents a critical final common pathway to explain the benefit of several

effective treatments for both acute myocardial ischemia and chronic congestive cardiac failure.[14] Thus, it is possible that the alterations in the proteins involving the eNOS complex and consequent reduction in eNOS activity contribute to the cardiac dysfunction in *Mas*$^{-/-}$ hearts, including the increased apoptosis rate. However, it should be noted that, in addition to the potential cardioprotective effect triggered by eNOS, previous studies also indicated a deleterious effect of eNOS-derived NO during myocardial ischemia/reperfusion.[27]

Perspectives

Ang-(1-7) has been reported to have a pivotal role in the regulation of the cardiovascular system. We now report that Ang-(1-7) via Mas activates the PI3K/Akt pathway and eNOS, leading to NO production in cardiomyocytes. These data suggest that eNOS and Akt are important downstream effectors of the Ang-(1-7)/Mas-mediated pathway in cardiomyocytes and possibly play important roles in Ang-(1-7) beneficial effects in the heart. Our data reveal a previously unknown signaling pathway involved in NO production dependent on the Ang-(1-7)/Mas axis in ventricular myocytes that plays an important role in the regulation of cardiomyocyte function. During the development of pathological conditions, dysregulation of the Ang-(1-7) signaling pathway may contribute to the ventricular dysfunction observed under these conditions.

Sources of Funding

This work was supported by grants from Deutscher Akademischer Austauschdienst (PROBRAL program of DAAD/CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; S.G. and R.A.S.S.), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (S.G.), Instituto Milênio (S.G.), PRPq/UFMG (S.G.), and PRONEX. M.N.M.A. is recipient of CNPq PhD fellowship. M.F.D.-P. and E.R.M.G. are recipients of a CAPES Fellowship. L.G. has a PIBIC/CNPq Fellowship.

Disclosures

None.

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Hypertension
Journal of the American Heart Association

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Hypertension. 2008;52:542-548; originally published online August 11, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.114280
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

MOLECULAR MECHANISMS INVOLVED IN ANGIOTENSIN-(1-7)/Mas SIGNALING PATHWAY IN CARDIOMYOCYTES

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Short title: Ang-(1-7)/Mas signaling pathway in cardiomyocytes

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NO measurement

Measurement of NO production in living cardiomyocytes was done using the membrane permeable fluorescent indicator DAF-FM (Molecular Probes) as previously described[1]. Time-course experiments were performed in Ang-(1-7) 10 nmol/L treated cells for the specified time intervals (5 min, 10 min and 15 min). In some experiments cells were pre-incubated for 15 min with the PI3-K specific inhibitor LY294002 before addition of 10nmol/L Ang-(1-7). 10 μmol/L LY294002 was used to block PI3K pathway according to the literature[2, 3].

Ca^{2+} recording and induction with Ang-(1-7)

Intracellular Ca^{2+} (Ca^{2+}_i) imaging experiments were performed in Fluo-4 AM loaded-cardiomyocytes[1]. Cells were then incubated with Ang-(1-7) (10 nmol/L) for 15 minutes at room temperature, and imaged for $[Ca^{2+}]_i$ as previously described[1, 4, 5].

Western blot

Adult ventricular myocytes were homogenated and protein content was quantified. 40-60 μg of protein were separated by SDS-PAGE, and immunoblotted. Measurement of eNOS and Akt phosphorylation were performed in Ang-(1-7) (10 nmol/L, 15 minutes) treated cardiomyocytes unless otherwise specified. Protein levels were expressed as a ratio of optical densities. GAPDH band was used as a control for any inaccuracies in the protein loading.

Antibodies

Antibodies and their sources are as follows: anti-caveolin 3 (BD transduction), anti-SERCA2a (ABR), anti-Akt, anti-phospho Akt, and anti-eNOS (AbCam), anti-phospho eNOS Thr 495, anti-phospho eNOS Ser 1177, and anti-Hsp90 (Cell Signaling), and anti-GAPDH (Clontech). Immunodetection was carried out using ECL-Plus (Amersham Biosciences).

Immunofluorescence.

Cardiomyocytes were fixed in PFA 4% and permeabilized with saponin 0.5%. Anti-Akt antibody was used at a dilution of 1:100. Anti-rabbit antibody conjugated to Alexa-488 (Molecular Probes) was used at a dilution of 1:1000.

TUNEL Assay

For assessment of apoptosis, specimens were fixed deep frozen in 80% methanol / 20% DMSO solution for six days at -80°C and one day at -20°C. After paraffin embedding 5µm slices were prepared on a Microtome, rehydrated and incubated with TUNEL-Kit (In Situ Cell Death Detection Kit TMR red, Roche), diluted in $CoCl_2$ – Cacodylate Buffer, for 1h[6]. Samples were analyzed with the programs ImageTools and GraphPad Prism[7].

Reagents

The peptides angiotensin-(1-7) and D-Ala7-angiotensin-(1-7), A-779, were from Bachem. Unless specified, other reagents were obtained from Sigma Chemical Corp.

Statistical analysis

Data are presented as mean ± SEM. Sample comparisons were performed using Student's *t* test or one way ANOVA followed by post-hoc analysis for multiple comparisons. In all statistical tests a $p< 0.05$ was used as a measure of statistical significance.

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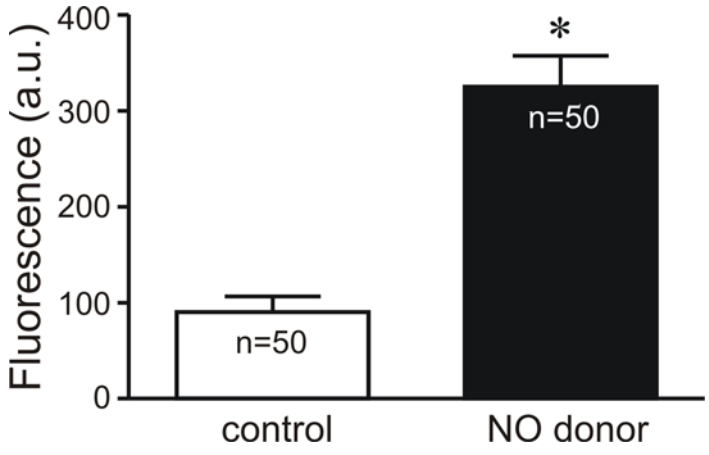

Figure S1: Significant DAF fluorescence increase in cardiomyocytes treated with NO donor sodium nitroprusside (10µmol/L, 15 min). n= number of cardiomyocytes analysed. *$p<0.05$.

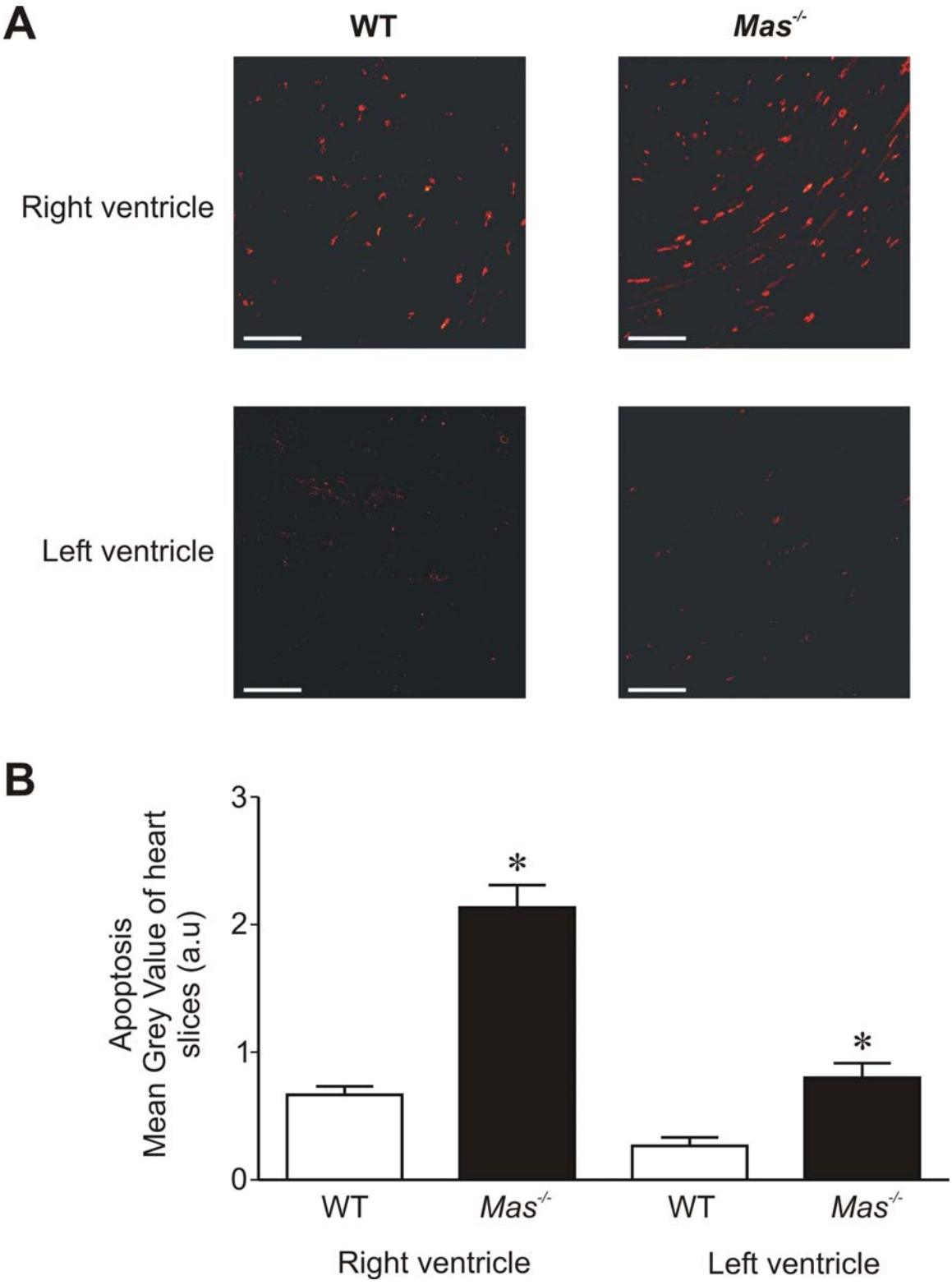
A
WT
Mas-/-
Right ventricle
Left ventricle
B
Apoptosis
Mean Grey Value of heart slices (a.u)
3
2
1
0
*
*
WT
Mas-/-
WT
Mas-/-
Right ventricle
Left ventricle

Figure S2: Cardiac apoptosis is higher in the ventricle of *Mas*$^{-/-}$ mice. **A.** Representative confocal images from right ventricle (top) and left ventricle (bottom) showing TUNEL positive cells. **B.** Quantitative analyses of TUNEL-positive cells in the right and left ventricles of *Mas*$^{-/-}$ mice. Mean fluorescence levels of TUNEL-positive cells was significantly higher in both ventricles of transgenic mice. Interestingly, the number of TUNEL positive cells was significantly higher in the right ventricle in both groups. n= three independent experiments. * $p<0.05$.

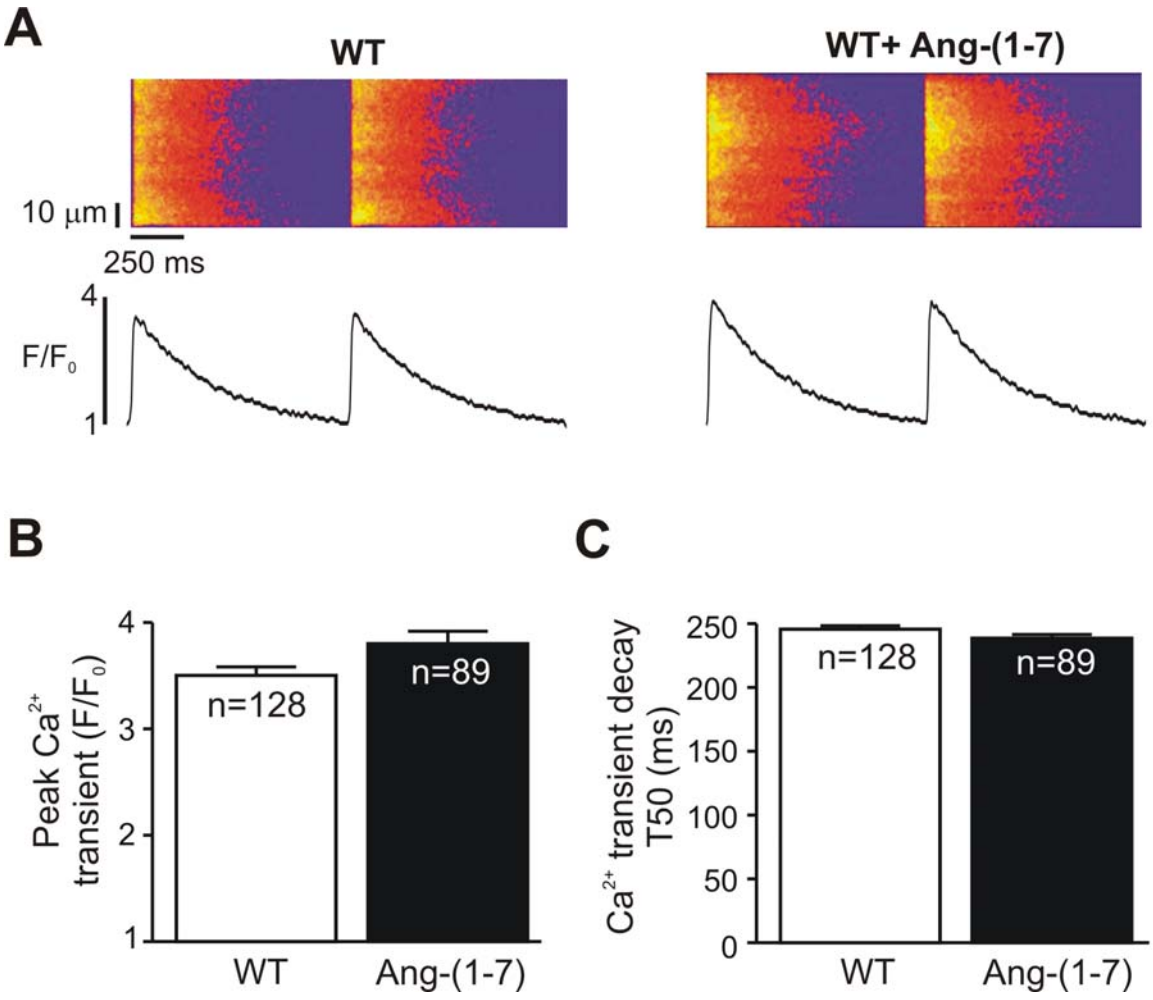

Figure S3: Acute Ang-(1-7) treatment failed to change Ca^{2+} transient parameters in cardiomyocytes. **A.** Sample line scan-images (top) and Ca^{2+} transient profile (bottom) from WT cardiomyocytes treated or not with 10 nmol/L Ang-(1-7) for 15 min. **B.** Bar graph shows that control averaged-peak Ca^{2+} transient is not altered by Ang-(1-7) treatment. **C.** In a similar way, Ca^{2+} transient kinetics of decay were not altered by Ang-(1-7) treatment in WT cardiomyocytes. T50 represents the time from peak Ca^{2+} transient to 50% decay. n= number of cardiomyocytes.