Genetic Deletion of the \( p66^\text{Shc} \) Adaptor Protein Protects From Angiotensin II–Induced Myocardial Damage

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Abstract—Angiotensin II (Ang II), acting through its G protein–coupled AT\(_1\) receptor (AT\(_1\)), contributes to the precocious heart senescence typical of patients with hypertension, atherosclerosis, and diabetes. AT\(_1\) was suggested to transactivate an intracellular signaling controlled by growth factors and their tyrosin-kinase receptors. In cultured vascular smooth muscle cells, this downstream mechanism comprises the \( p66^\text{Shc} \) adaptor protein, previously recognized to play a role in vascular cell senescence and death. The aim of the present study was 2-fold: (1) to characterize the cardiovascular phenotype of \( p66^\text{Shc} \) knockout mice (\( p66^\text{Shc}^{-/-} \)), and (2) to test the novel hypothesis that disrupting the \( p66^\text{Shc} \) might protect the heart from the damaging action of elevated Ang II levels. Compared with wild-type littermates (\( p66^\text{Shc}^{+/+} \)), \( p66^\text{Shc}^{-/-} \) showed similar blood pressure, heart rate, and left ventricular wall thickness. However, cardiomyocyte number was increased in mutant animals, indicating a condition of myocardial hyperplasia. In \( p66^\text{Shc}^{+/+} \), infusion of a sub-pressor dose of Ang II (300 nmol/kg body weight [BW] daily for 28 days) caused left ventricular hypertrophy and apoptotic death of cardiomyocytes and endothelial cells. In contrast, \( p66^\text{Shc}^{-/-} \) were resistant to the proapoptotic/hypertrophic action of Ang II. Consistently, in vitro experiments showed that Ang II causes apoptotic death of cardiomyocytes isolated from \( p66^\text{Shc}^{+/+} \) hearts to a greater extent as compared with \( p66^\text{Shc}^{-/-} \) cardiomyocytes. Our results indicate a fundamental role of \( p66^\text{Shc} \) in Ang II–mediated myocardial remodeling. In perspective, \( p66^\text{Shc} \) inhibition may be envisioned as a novel way to prevent the deleterious effects of Ang II on the heart. 

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Key Words: angiotensin II ■ cardiomyocytes ■ endothelial growth factors ■ apoptosis
served as controls. In addition, an in vitro apoptosis test was performed on adult p66Shc−/− and p66Shc+/− cardiomyocytes that were stimulated with escalating concentrations of Ang II.

Our results document for the first time that p66Shc disruption combats Ang II–induced myocardial hypertrophy and protects cardiomyocytes and endothelial cells from apoptosis. Moreover, in p66Shc−/−, the number of cycling cardiomyocytes was higher under basal conditions and after Ang II.

Altogether, our findings open new avenues for mechanistic interventions targeting specific molecular pathways of Ang II–induced myocardial damage.

Materials and Methods

p66Shc−/− were generated from a 129 Sv background.7 p66Shc littermates (p66Shc+/+) served as wild-type controls. Experiments were performed on male mice aged 3 to 5 months.

Details of experimental procedures are provided in the Expanded Methods Online Supplement.

In Vivo Experimental Protocol

The body weight (BW), systolic blood pressure (SBP), and heart rate (HR) of p66Shc−/− (n = 21) and p66Shc+/+ (n = 18) were determined under basal conditions.

After completion of basal measurements, p66Shc−/− and p66Shc+/+ were randomized to receive a chronic infusion of Ang II at 10−11 M (n = 5 hearts from each strain), 10−9 M (p66Shc+/+: n = 14, p66Shc−/−: n = 17), or 10−7 M (n = 8 for each strain), or with saline (p66Shc+/+: n = 12, p66Shc−/−: n = 18). The percentage of apoptotic cardiomyocytes was then evaluated.

Expression of Shc Isoforms in the Hearts

p66Shc−/− or p66Shc+/+ received saline or Ang II for 22 days (n = 5 mice per each group). The contents of the 3 shc isoforms in heart homogenates were determined by Western blot. P46Shc and p52Shc levels were also determined in cardiomyocytes harvested from untreated mice of both strains.

Statistics

Results were expressed as mean±SEM. Multivariate repeated-measures ANOVA was performed. In multiple comparisons in which ANOVA indicated significant differences, the statistical value was determined according to Bonferroni. Differences within and between groups were determined using paired or unpaired Student t test, respectively. A P value <0.05 was interpreted to denote statistical significance.

Results

Basal Cardiovascular Phenotype of p66Shc−/−

As shown in Table 1, under basal conditions, no difference was recorded between strains in terms of BW, SBP, or HR.

The impact of p66Shc gene deletion on cardiac phenotype was evaluated in more detail at the end of a 4-week infusion with saline or Ang II.

As shown in Table 2, no differences were observed between saline-treated p66Shc−/− and p66Shc+/+ with regard to the weight of LV and RV and linear LV parameters. However, the LV chamber volume was smaller in p66Shc−/−.

Myocardial capillary density was similar in both strains.

We then evaluated whether p66Shc deletion alters cardiomyocyte composition and volume. No difference between strains was observed as far as the percentage of mononucleated and binucleated cells is concerned (data not shown). Similarly, no difference was detected between p66Shc−/− and p66Shc+/+ in terms of mononucleated cell length (85.0±5.7 μm versus 80.8±4.9 μm) or volume (15201±1245 μm³ versus 14136±1014 μm³), and of binucleated cell length (106.7±3.5 μm versus 106.7±5.9 μm) or volume (19210±919 μm³ versus 17754±1412 μm³) (P=N.S. for all comparisons). The myocardial capillary volume occupied by cardiomyocytes was higher in p66Shc−/− ([22.56±10.00 mm³ versus 105.14±2.64 mm³ in p66Shc+/+, P<0.01]. As a consequence, the mathematical calculation of myocyte number per heart indicates higher numbers of mononucleated (1.6×10⁶±9.2×10⁴) and binucleated cardiomyocytes in p66Shc−/− (5.1×10⁶±2.9×10⁵) than in p66Shc+/+

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Measurements were obtained on untreated mice of both strains.
Effects of Ang II on the Cardiovascular Phenotype of p66Shc+/+ and p66Shc−/−

The cardiac phenotype of the two strains was differentially modified by chronic infusion of Ang II. As shown in Table 2, Ang II did not change tail-cuff SBP or HR or intraaortal MBP in either strain. However, profound differences were observed in heart histomorphology. As shown in Table 2 and Figure 1, Ang II–infused p66Shc+/+ displayed LV hypertrophic remodeling (as denoted by the 1.11-fold increase in cardiomyocyte transverse diameter, 1.23-fold increase in LVW/BW, and 1.49-fold reduced cavitary volume, P<0.05 versus saline-infused for all comparisons). In contrast, in p66Shc−/−, Ang II neither produced myocyte hypertrophy nor altered the LVW/BW and LV cavitary volume (P=N.S. versus saline infusion for all comparisons).

After Ang II, myocardial capillary density decreased without difference between strains. Thus, deletion of p66Shc preserves the heart from Ang II–induced hypertrophic remodeling, but it does not avoid cardiac capillary rarefaction.

Resistance of p66Shc−/− Hearts to Ang II–Induced Apoptosis

As shown in Figure 2A, the number of TUNEL+ cardiomyocytes was similar in saline-infused p66Shc+/+ or p66Shc−/−. Apoptosis was strikingly enhanced by Ang II in p66Shc+/+, but it remained at low levels in p66Shc−/−. Microphotographs in Figure 2B show greater abundance of apoptotic cardiomyocytes (indicated by arrows) in LV sections from Ang II–infused p66Shc+/+ as compared with p66Shc−/−.
Cycling Cardiomyocytes Are More Abundant in \( p66^{shc/-} \)

In the LV of saline-infused \( p66^{shc+/+} \), a small fraction of cardiomyocytes was MCM-2+ (\( \text{circum} \) 0.2% of total), and this figure was doubled in \( p66^{shc/+/+} \). At variance, in saline-infused \( p66^{shc/-/-} \), MCM-2+ cardiomyocytes numerically exceeded TUNEL+ cardiomyocytes. Ang II increased the number of cycling cardiomyocytes in both strains. Nevertheless, in \( p66^{shc+/+} \), apoptosis exceeded proliferation, whereas the opposite figure was observed in \( p66^{shc/-/-} \). Figure 2D shows 2 cells in active cell cycling, as revealed by the nuclear expression of MCM-2 (green fluorescent dots) in the LV of Ang II–infused \( p66^{shc/+/+} \) and \( p66^{shc/-/-} \). Cardiomyocytes are positive for \( \alpha \)-sarcomeric actin (purple), TUNEL+ nuclei are stained in dark brown. Nuclei are counterstained by hematoxylin. (Optical microscopy. Original magnification 200×).

Altogether, the above results indicate that, in \( p66^{shc+/+} \), Ang II triggers cardiomyocyte hypertrophy and apoptosis over-
Isolated Cardiomyocytes

In vitro experiments were conducted to exclude the interference of hemodynamic factors, completely. As shown by Figure 3A, the rate of spontaneous cardiomyocyte apoptosis was similar in the 2 strains. Ang II at 10⁻⁵ to 10⁻⁷ mol/L increased apoptosis of p66Shc+/− cardiomyocytes, whereas Ang II 10⁻¹¹ mol/L was not effective. The apoptotic response to Ang II was abrogated in p66Shc−/−. These results suggest that p66Shc is essential for Ang II to induce cardiomyocyte apoptosis. Figure 3B and 3C show representative images of the experiments performed incubating p66Shc+/− and p66Shc−/− cardiomyocytes with 10⁻⁹ mol/L Ang II.

Cardiac Progenitor Cells

CPCs might be implicated in myocardial plasticity. Therefore, we explored whether p66Shc deletion influences the relative abundance and cycling rate of CPCs in the adult heart.

As shown in Figure 4A and 4B, after saline infusion, the relative abundance of c-kit+/GATA-4+ putative CPCs was similar in p66Shc+/− and p66Shc−/−. However, a strain difference was observed with regard to CPC response to Ang II. In fact, Ang II augmented CPC density in p66Shc+/−, but not in p66Shc−/−. The difference is compatible either with a stimulatory action of Ang II on CPCs through p66Shc or with a compensatory activation of CPCs aimed at counteracting the Ang II-induced myocyte loss.

Figure 4C shows a LV section from Ang II–infused p66Shc+/−, where a CPC (white arrow) is identified by its double positivity for c-kit (green fluorescence) and GATA-4 (magenta fluorescent dots in the nucleus) and cardiac myocytes are recognized by their expression of α-sarcomeric actin (red fluorescence). Because of CPC scarcity in the heart, this picture is not representative of the actual CPC density.

As shown by Figure 4D, the percentage of MCM-2+ CPCs was higher in saline-treated p66Shc−/− than in p66Shc+/−. Ang II did not affect CPC cycling in any strain. Assuming the CPC proliferation rate to be constant through all the infusion period, the latter finding indirectly suggests that CPC numerical increment in p66Shc+/+ may be attributable to differentiation from more immature cardiac stem cells rather than to CPC proliferation.

Myocardium Capillary Density and Turnover of Cardiac ECs

As shown in Figure 5A, p66Shc−/− hearts were also protected from Ang II–induced EC apoptosis. Figure 5B shows images of LV sections from Ang II–infused p66Shc+/− and p66Shc−/−. Apoptotic ECs (indicated by the arrows) can be recognized by concomitant positivity for TUNEL (dark brown) and Factor VIII (purple).

As shown in Figure 5C, under saline infusion, the number of MCM-2+ ECs was higher in p66Shc−/− than in p66Shc+/−.
However, capillary density was similar in the 2 strains (Figure 5D).

As shown in Table 2 and Figure 5D, Ang II similarly reduced cardiac capillary density in p66Shc/H11001/H11001 and p66Shc/H11002/H11002. We hypothesize that p66Shc controls EC proliferation under physiological levels of Ang II, while facilitating EC death as triggered by exaggerated Ang II.

Shc Isoforms
We then evaluated the possibility that p46 Shc and p52 Shc expression may be affected by p66 Shc knock-out. As shown in the representative Western blot bands of Figure 6, the cardiac expression levels of p46 Shc or p52 Shc were similar in saline-infused p66 Shc+/+ and p66 Shc−/−. Similar findings were obtained in cardiomyocyte lysates (data not shown). Chronic Ang II did not affect the myocardial expression of p46 Shc or p52 Shc in any strain. As expected, p66 Shc was not detected in p66 Shc−/−.

Discussion
p66 Shc regulates life span in mammals and is a critical component of the apoptotic response to oxidative stress acting as a downstream target of the tumor suppressor p53.12 Here, we show that p66 Shc−/− display a normal basal phenotype with regards to BW, systemic hemodynamics, and left ventricular wall thickness. CPC density was also similar in the two strains. At variance with what reported for the limb skeletal muscle,13 myocardial capillary density was normal in p66 Shc−/−.

However, there were also peculiar differences that could be related to the mutation. In particular, the number of cardiomyocytes and the cycling index of both cardiomyocytes and CPCs were all increased in p66 Shc−/−. This was associated with a reduced LV chamber volume in mutant animals. The

Figure 5. Bar graphs show the effects of Ang II or vehicle (V) on apoptosis (recognized by TUNEL staining; A) or cycling (recognized by MCM-2 staining; C) of endothelial cells (ECs). Pictures of B are representative of apoptotic ECs (pointed by the arrows) in the hearts of Ang II–infused p66 Shc+/+ and p66 Shc−/−. Factor VIII staining (purple) identifies ECs. TUNEL staining (dark brown) identifies the apoptotic nuclei. Nuclei are counterstained by hematoxilin (Optical microscopy. Original magnification 200×). Bar graph of D shows the capillary density of the endomyocardium in the 4 groups. Values are mean±SEM. *P<0.05 and **P<0.01 vs vehicle, §P<0.05 and §§P<0.01 vs p66 Shc+/+.

Figure 6. Western blot bands of p66 Shc, p52 Shc, and p46 Shc in lysates from total hearts of saline- or Ang II–infused p66 Shc+/+ and p66 Shc−/−. β-tubulin was used as a reference protein.
combination of these characteristics is compatible with a condition of myocardial hyperplasia. We hypothesize that these features may derive from increased cell proliferation together with reduced apoptosis during the development of p66Shc−/− hearts. We know from the literature that apoptosis participates in the intratrus and early postgestational heart development14,15 and that all the components of the renin–angiotensin system are expressed by the fetal heart and participate in its normal development.16,17 Interestingly, a recent study from a coauthor of this article showed that the 3 Shc isoforms are activated in the human fetal heart, in a period that is characterized by apoptotic and proliferative processes.15 In our opinion, the p66Shc knock-out may interfere with the heart development by inhibiting the apoptotic and hypertrophic signaling emanating from AT1 or other factors as well as enhancing the proliferation of cardiomyocyte and CPCs.

The major objective of this study was to ascertain whether p66Shc modulates the cardiac effects of moderate increments of circulating Ang II. A large body of evidence supports the notion that Ang II, independently of its blood pressure-elevating effect, plays a central role in the pathophysiology of heart remodeling and failure. The underlying molecular mechanisms are complex and not yet completely clarified. Here, we report for the first time that Ang II produces damaging effects on the heart through a p66Shc-comprising pathway. Circumstantial evidence suggests that activation of the p66Shc, which lays downstream to tyrosin kinase receptors, may be implicated in Ang II–induced cardiovascular alterations. For instance, 2 studies showed increased p66Shc expression in Ang II–stimulated VSMCs18,19. Using genetically modified mice, we specifically challenged the hypothesis that p66Shc participates in the cascade leading to Ang II–induced ventricular remodeling. To eliminate the confounding effects of hypertension, Ang II was chronically infused at a dose known to be devoid of pressor activity. Accordingly, systemic hemodynamics remained unchanged in both strains. Nevertheless, relevant differences were observed with regard to the cardiac effects of the agent: chronic Ang II caused myocardial hypertrophy and apoptosis in p66Shc−/− but not in p66Shc−/−, thus suggesting a fundamental role of p66Shc in Ang II–induced cardiac damage. Consistently, p66Shc−/− were protected from apoptosis caused by limb ischemia,13 high-fat diet,20 and free radicals generating poisons.7 However, p66Shc−/− were not spared from Ang II–induced rarefaction of myocardial microcirculation. This fact is apparently difficult to reconcile with our own findings of reduced EC apoptosis in p66Shc−/−. However, we also show that Ang II reduced the rate of spontaneous proliferation of myocardial ECs, particularly in p66Shc−/−. Therefore, the capillary rarefaction in response to Ang II may derive from differential effects on ECs in the 2 strains.

Capillary destabilization in response to chronic Ang II is apparently discrepant with neo-angiogenesis which reportedly occurs in ischemic limb muscles of Ang II–infused animals.21 One major difference, however, consists of the presence or absence of the hypoxic environment as a determinant of microvascular responses to Ang II. Another possible explanation deals with expressive and functional differential of vascular endothelium from different organs.22 Whatever is the interpretation, microvascular factors are not accountable for the cardiac protection ensured by p66Shc gene deletion.

In vitro experiments on adult cardiomyocytes definitively documented that Ang II–induced apoptosis is abrogated in the absence of p66Shc. Similarly, primary cells (hematopoietic precursors, fibroblasts, and ECs) isolated from p66Shc−/− are resistant to oxidative stress–induced apoptosis, whereas p66Shc overexpression induces apoptosis in the same cells. Ang II, via AT1 receptors, upregulates the expression of a variety of redox-sensitive factors and NADPH oxidase, thus increasing the generation of reactive oxygen species (ROS) and cellular damage.24 p66Shc was initially shown to be involved in tyrosin-kinase receptor–initiated apoptosis.25 Recent studies indicate that p66Shc regulates apoptosis by controlling mitochondrial transmembrane potential and ROS production/accumulation.12 Accordingly, p66Shc−/− cells have reduced ROS under basal concentrations and after p53-induced apoptosis. Thus, it is possible that p66Shc modulates Ang II–induced apoptosis via regulation of ROS. Furthermore, Ang II induces the expression of heat shock protein 70,26 whose anti-apoptotic action is constitutively inhibited when complexed with mitochondrial p66Shc. Thus, another possibility is that the p66Shc−/− may unveil the protective action of counter-regulatory mechanisms activated by Ang II.

Recent evidence suggests that the heart is not a postmitotic organ and that a continuous turnover of cardiac cells may result in a heterogeneous population of immature, adult, and senescent cardiomyocytes.11 The contribution of resident cardiac stem cells and CPCs in physiological and pathological turnover of adult cardiomyocyte is also a matter of intense debate. Here, we report that Ang II stimulates the cycling of adult cardiomyocytes and increases the number of putative CPCs. These compensatory responses might not be sufficient to counteract ongoing cell loss by apoptosis in the p66Shc−/− hearts. p66Shc deficiency did not affect the cycling of adult cardiomyocytes under Ang II stimulation, but it prevented the increase in c-kit+/GATA-4+ putative CPCs. These results may reflect the fact that in the absence of significant cardiomyocyte apoptosis there is no need for activation of compensatory responses. Alternatively, we might hypothesize a direct effect of p66Shc in the control of cardiac cell maturation and turnover.

**Perspectives**

Our study newly demonstrates the involvement of p66Shc in the deleterious cardiac responses to increased Ang II, namely hypertrophy of cardiomyocytes and LV and apoptotic death of cardiomyocytes and ECs. These results may have important clinical and therapeutic implications. It is possible that p66Shc expression and activity differ between individuals, thus accounting for inter-individual variation in the susceptibility to Ang II–induced organ damage.

The present study also points to p66Shc as a potential target to combat myocardial remodeling and senescence triggered by exaggerated Ang II. In perspective, p66Shc inhibitors might protect from excessive apoptotic loss of cardiomyocytes, thereby delaying the onset of cardiac decompensation.
Acknowledgments

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References

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Animal procedures complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, 1996).

**Basal Hemodynamics.**

After 3 training sessions, the systolic blood pressure (SBP) and heart rate (HR) of conscious mice were measured by plethysmography (Visitech System, USA) for 3 consecutive times at 72-hour intervals and then averaged.¹

**Angiotensin II Infusion.**

Infusion of Angiotensin II (Ang II, Sigma, Italy) or its vehicle saline was made by means of osmotic minipumps (mod. 2004 Alzet) implanted into the abdominal cavity under 2,2,2-tribromoethanol anesthesia (880mmol/kg IP).

**Measurement of Intra-Carotid Blood Pressure.**

A polyethylene catheter (PE-10, Clay Adams) was inserted into the left carotid artery of anaesthetized mice, tunneled under the skin, and finally exteriorized at the back of the neck. Mice were then maintained unrestrained in their individual cage. Four hours after animals had regained consciousness, carotid mean blood pressure (MBP) was recorded for 30 min via a pressure transducer connected to a polygraph
(Mac Lab System). Calibrations of instruments to measure tail-cuff or intra-arterial BP were performed with a mercury sphygmomanometer.\(^1\)

**Heart Histology.**

Mice were anesthetized and their chest was opened to stop the heart in diastole by intraventricular injection of cadmium chloride (100 nmol). Heart was excised and weighted after removal of the atria and external vessels. After 48-hour fixation in 4% formalin, the left ventricle (LV), inclusive of the septum, was separated from the right ventricle (RV). LV and RV were weighted and the length of the LV chamber was measured from the apex to the aortic valve. Transverse sections of the mid parts of the LV and RV were cut perpendicularly at the major axis. Morphometric analyses (Image Pro-plus) of sections permitted evaluating the following parameters: LV transverse diameter, LV free wall thickness, RV wall thickness. The LV chamber volume was calculated according to the Dodge equation.\(^2,3\)

**Evaluation of Adult Cardiomyocyte Dimensions following their Dispersion.**

Anaesthetized adult mice were heparinezed (100 UI/kg BW, IP) and their hearts were rapidly excised and hanged in a Laghendorff apparatus. Isolated cells were obtained by collagenase perfusion of the heart.\(^4\) The procedure allows for a low degree of contamination from non-myocytes, that ranged from 1% to 3%.\(^5\) Cardiomyocytes were plated at a density of
2x10⁴/cm² on laminin coated two-well chamber slides, cultured at 37°C in an atmosphere containing 5% CO₂ for 48h in serum free medium, and then fixed in 4% PFA. Myocytes were stained with fluorescein isothiocyanate (1 µg/mL, for 30 min) to visualize the cell cytoplasm and with acridine orange (20 µM, for 1h) to label the nuclei. One hundred myocytes per heart were examined by confocal microscopy, considering the two cellular populations (mononucleated and binucleated myocytes). The percentage and the dimension of mononucleated and binucleated myocytes were determined. Myocytes dimensions were calculated by measuring the major and minor axis. In addition, myocyte thickness was determined by optical sectioning of each cell in the Z plane. Cell volume was then calculated assuming an elliptical cross-section with a major axis that is equivalent to cell width and a minor diameter computed from the axis ratios, as previously reported. Cross-sectional area was multiplied by myocyte length to calculate myocyte volume.

**Calculation of the Total Number of Adult Cardiomyocytes.**

The total volume of the myocardium was first determined by dividing its weight by the specific gravity of muscle tissue (1.06). Then, the volume fraction of myocytes and interstitium in each heart was determined in H&E-stained heart cross sections. The total volume of myocytes in the heart was then calculated from the product of heart volume and the volume fraction of myocytes. From the volume fraction of myocytes in the myocardium and the proportion of mononucleated
and binucleated cells, determined as described above, the volume percent of each cell population in the tissue was obtained. This information, combined with the total volume of myocytes in the heart, allowed the estimation of the aggregate volume of mononucleated and binucleated cells in the myocardium. The numbers of mononucleated and binucleated cells in the heart were computed from the quotient of their aggregate volumes and their corresponding average cell volume.

Measurement of Cardiomyocyte Transverse Diameter in LV Sections.

In H&E-stained sections of the LV, the myocyte transverse diameter was determined (at 250X magnification) in the region of the nucleus of longitudinally oriented myocytes. Fifty measurements from each LV were averaged.

Identification of Cardiomyocyte Progenitor Cells (CPCs) and Identification of Cycling CPCs, Cardiomyocytes, and Endothelial Cells (ECs).

To evaluate the presence of cardiac progenitor cells (CPCs), heart sections were first incubated with an antibody for the stem cell receptor factor c-kit (rabbit polyclonal antibody, 1: 20, Santa Cruz, pre MW treatment), which was revealed using a FITC-conjugated anti-rabbit
secondary antibody. Then sections were incubated with an antibody for GATA-4 (rabbit polyclonal antibody, 1:20, o.n., Santa Cruz) followed by a TRITC-conjugated anti-rabbit secondary antibody.

To determine the cycling fraction of CPCs, we investigated the expression of minichromosome maintenance protein-2 (MCM-2) by using a goat polyclonal antibody (Santa Cruz) revealed by a cyanine secondary antibody. Nuclei were visualized by bisbenzimide blue. Slides were evaluated at 1000X magnification.

To calculate the proliferating fractions of myocytes and ECs, a set of LV sections were first incubated with an antibody for MCM-2 (1:50, 2 h at 37°, pre MW treatment) followed by a FITC-conjugated anti-goat secondary antibody. The same sections were then stained with an antibody for $\alpha$-sarcomeric actin (mouse monoclonal antibody, DAKO, Glostrup, Denmark, 1:30), which was revealed by a TRITC-conjugated anti-mouse secondary antibody. A different set of LV sections was stained for MCM-2 and then incubated with an antibody for the EC antigen Factor VIII (rabbit antibody polyclonal antibody, DAKO, Glostrup, 1:20), followed by a TRITC-conjugated anti-rabbit secondary antibody. Nuclei were visualized by bisbenzimide blue.

**Evaluation of Apoptotic Cardiomyocytes and Apoptotic ECs in LV Section.**

LV sections (3 µm in thickness) were prepared onto poly-lysin coated slides for evaluation of DNA fragmentation by terminal
deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunostaining assay (TdT-FragEL, Oncogene, Italy).\textsuperscript{11} ECs were identified by staining for Factor VIII (rabbit polyclonal antibody, DAKO, Denmark, 1:20), and revealed by VIP kit (VECTOR Laboratories, CA, USA). Cardiomyocytes were recognized by the cardiac marker $\alpha$-sarcomeric actin (mouse monoclonal antibody, DAKO, Denmark, 1:30), and revealed by VIP kit. Slides were evaluated at 1000X magnification.

**Analysis of Capillary Density.**

The analysis of capillary density was performed in sections stained with silver methenamine, which defines basal membranes. Morphometric sampling at x1000 magnification consisted of counting the number of capillary profiles in a measured area of tissue sections of both the epicardium and endocardium in which myocytes are transversally oriented. A square uncompressed tissue area of 9.8 $\mu$m$^2$ was delineated in the microscopic field by an ocular reticle containing 42 sampling points (Wild Heerbrugg Instruments). By counting the fraction of points lying over myocytes, the percentage of the area occupied by muscle cells was determined, and the number of capillaries per unit area of myocytes was computed. This approach was followed to eliminate the effects of variations caused by changes in the interstitial compartment. Sampling of capillary measurements involved a minimum of 20 and a maximum of 30 microscopic fields for each LV of each animal.\textsuperscript{12}
Evaluation of Cardiomyocyte Apoptosis in Vitro.

Adult cardiomyocytes were seeded at a density of $2 \times 10^4$/cm$^2$ on laminin coated two-well chamber slides and cultured (37°C, 5% CO$_2$) for 24h in serum free medium. Cardiomyocytes were then stimulated for additional 24 h with Ang II or vehicle. Apoptosis was recognized in fixed cardiomyocytes by in situ staining with the Cell Death Detection kit (POD, Boeringher). Nuclei were visualized with bisbenzimide blue. The percentage of apoptotic cardiomyocytes was determined under a confocal microscope by counting TUNEL positive and negative nuclei.$^{13}$

Western blot for Shc isoforms.

Hearts or cardiomyocytes were homogenized in homogenization buffer [Tris-HCl 500 mmol/L (pH 7.5), NaCl 150 mmol/L, 1 % Triton-X 100, 1 % sodium deoxycholate, 0.1 % SDS]. After sonication and centrifugation at 4 °C (10 min, 10000×$g$), the supernatant was used for determination of protein concentrations, and equal amounts of total solubilized proteins were eluted by heating with SDS-PAGE sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. The membrane was blocked at room temperature with 5 % non-fat dry milk in Tris-buffered saline with 0.1 % (v/v) Tween-20 (TBST) and incubated with a rabbit polyclonal anti-SHC antibody (anti-SH2, BD Transduction Laboratories). HRP-conjugated anti-rabbit was used as a secondary antibody. Membranes
were then reprobed with a rabbit polyclonal anti-tubulin (Boheringer). Antibodies were used at the concentration suggested by the manufacturers. The ratio of each shc isoforms to β-tubulin was quantified by densitometry analysis using Image Processing and Analysis in Java Software (Image J).
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