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The cardioprotective effects elicited by p66^{Shc} ablation demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury

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ABSTRACT

Although a major contribution to myocardial ischemia–reperfusion (I/R) injury is suggested to be provided by formation of reactive oxygen species (ROS) within mitochondria, sites and mechanisms are far from being elucidated. Besides a dysfunctional respiratory chain, other mitochondrial components, such as monoamine oxidase and p66^{Shc}, might be involved in oxidative stress. In particular, p66^{Shc} has been shown to catalyze the formation of H_2O_2. The relationship among p66^{Shc}, ROS production and cardiac damage was investigated by comparing hearts from p66^{Shc} knockout mice (p66$^{Shc-/-}$) and wild-type (WT) littermates. Perfused hearts were subjected to 40 min of global ischemia followed by 15 min of reperfusion. Hearts devoid of p66^{Shc} were significantly protected from I/R insult as shown by (i) reduced release of lactate dehydrogenase in the coronary effluent (25.7 ± 7.49% in p66$^{Shc-/-}$ vs. 39.58 ± 5.17% in WT); (ii) decreased oxidative stress as shown by a 63% decrease in malondialdehyde formation and 40 ± 8% decrease in tropomyosin oxidation. The degree of protection was independent of aging. The cardioprotective efficacy associated with p66^{Shc} ablation was comparable with that afforded by other antioxidant interventions and could not be increased by antioxidant co-administration suggesting that p66^{Shc} is downstream of other pathways involved in ROS formation. In addition, the absence of p66^{Shc} did not affect the protection afforded by ischemic preconditioning. In conclusion, the absence of p66^{Shc} reduces the susceptibility to reperfusion injury by preventing oxidative stress. The present findings provide solid and direct evidence that mitochondrial ROS formation catalyzed by p66^{Shc} is causally related to reperfusion damage.

1. Introduction

Formation of reactive oxygen species (ROS) contributes to many pathological processes accelerating, or even determining, the evolution of cell injury toward necrosis or apoptosis [1–6]. Although ROS formation is also involved in physiological processes [7–9], the imbalance between their generation and removal that is termed oxidative stress plays a major role in cardiac diseases, especially in myocardial injury caused by ischemia and reperfusion (I/R) [10–12].

A large body of evidence supports the concept that ROS are formed within mitochondria under physiological and pathological conditions [3,7,13,14]. Although mitochondria are considered the most relevant site for the formation of reactive oxygen species (ROS) in cardiac myocytes, a major and unsolved issue is where ROS are generated in mitochondria.

Respiratory chain is generally indicated as a main site for ROS formation [3,13]. However, other mitochondrial components are likely to contribute to ROS generation [5]. Recent reports highlight the relevance of p66^{Shc} (reviewed in [6,15]). This protein is a splice variant of p52^{Shc}/p46^{Shc}, two cytoplasmic adaptor proteins involved in the propagation of intracellular signals from activated tyrosine kinases to Ras [16]. p66^{Shc} has the same modular structure of p52^{Shc}/p46^{Shc} (SH2-CH1-PTB) and contains a unique N-terminal region (CH2); however, it is not involved in Ras regulation, but rather functions in the intracellular pathway(s) that regulates ROS metabolism and apoptosis [17–19]. A fraction of p66^{Shc} localizes within the mitochondrial inter-membrane space where it oxidizes reduced cytochrome *c*. As a consequence of the p66^{Shc}-mediated

Abbreviations: DCB, disulfide cross-bridges; TBARS, thiobarbituric acid reactive substances; Tm, Tropomyosin; MPG, mercaptopropionylglycine; MAO, monoamine oxidase; LDH, lactate dehydrogenase; ROS, reactive oxygen species; IPC, ischemic preconditioning

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oxidation of cytochrome *c*, molecular oxygen is partially reduced to peroxide. In mitochondria the H_2O_2 generated by $p66^{Shc}$/cytochrome *c* reaction contributes to the opening of the mitochondrial permeability transition pore and the triggering of apoptosis [20]. This explains why the genetic ablation of $p66^{Shc}$ results in a marked reduction of oxidative stress. In fact, reduced ROS levels have invariably been detected in cells derived from $p66^{Shc}$ knock out ($p66^{Shc-/-}$) mice [19–24]. In vivo, $p66^{Shc-/-}$ tissues show less intracellular and systemic oxidative damage [23,25–27]. In addition, $p66^{Shc-/-}$ cells are resistant to apoptosis induced by a variety of different signals, including hydrogen peroxide [19,21], ultraviolet radiation [18,19], staurosporine [20], growth factor deprivation [19], calcium ionophore and CD3–CD4 cross-linking [24]. Similarly, $p66^{Shc-/-}$ mice are resistant to apoptosis induced by paraquat, hypercholesterolemia and ischemia [18,23,27]. Notably, $p66^{Shc-/-}$ mice live longer [18] and show signs of retarded aging [23,27].

Numerous studies relate $p66^{Shc}$ to cardiovascular pathophysiology (reviewed in [5,28]. Oddly, with the single exception of a study on hindlimb ischemia [23], ischemic injury has so far attracted scarce attention and no information is available on the possible role of $p66^{Shc}$ in the wide spectrum of alterations associated with myocardial I/R. Here, we show that the susceptibility to I/R injury is greatly decreased in hearts devoid of $p66^{Shc}$ along with a marked reduction in oxidative alterations of proteins and lipids.

2. Materials and methods

2.1. Treatment protocols for ischemia studies

All aspects of animal care and experimentation were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Italian regulation concerning the care and use of laboratory animals and were approved by the Ethical Committee of the University of Padova.

Adult (aged 4 months) male $p66^{Shc-/-}$ [18] and C57BL/6 WT mice (Charles River, Milan, Italy) were anaesthetized with an intraperitoneal injection of Zoletil 100 (30 mg/kg). Hearts were perfused with bicarbonate buffer gassed with 95% O_2–5% CO_2 at 37 °C (pH 7.4) at a constant flux of 5 ml/min. Perfusion was performed in the nonrecirculating Langendorff mode, as previously described [29]. The perfusion buffer contained (in mM) 118.5 NaCl, 3.1 KCl, 1.18 KH_2PO_4, 25.0 $NaHCO_3$, 1.2 $MgCl_2$, 1.4 $CaCl_2$ and 5.6 glucose. Hearts were treated as follows ($n \geq 6$/group): after 10 min of stabilization, hearts were subjected to 40 min of global ischemia (I-40) by stopping the coronary flow and 15 min of reperfusion (R-15) with or without preceding IPC, which consisted of three episodes of 5 min of ischemia and 5 min of reperfusion. After reperfusion hearts ($n=8$) were quickly immersed into PBS containing 0.5% Triton X100 and homogenized for measurement of lactate dehydrogenase (LDH), and hearts ($n=6$) were frozen in liquid nitrogen and stored at −80 °C for future analysis. Treatments were performed adding the MAO inhibitor pargyline (0.5 mM) or MPG (1 mM) to the perfusion buffer.

2.2. Measurement of lactate dehydrogenase activity

To determine the amount of lactate dehydrogenase (LDH) released from the hearts exposed to I/R, coronary effluent was collected at 1 min intervals during the 15 min of reperfusion as previously described [30]. At the end of reperfusion hearts were collected and homogenized for assessing the residual activity of LDH in the whole tissue. LDH activity was determined by means of a classic procedure [31]. Since all values were normalized to heart weight, the amount of LDH released was expressed as % of total (i.e., effluent + homogenate) to rule out possible changes due to variations in heart size [32].

2.3. Measurement of lipid peroxidation

Lipid peroxide level was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids [33]. Heart tissues were homogenized in 1 ml of 0.15 M KCl and 0.024% butylated hydroxytoluene (BHT) using motor driven Teflon pestle followed by centrifugation of tissue homogenate at 10,000 g. The supernatant obtained after centrifugation was added with 0.5 ml of 30% trichloroacetic acid (TCA) followed by 0.5 ml of 0.8% TBA. After incubation for 1 h at 100 °C, samples were centrifuged at 800 g for 10 min. The absorbance of supernatant was measured at 535 nm. Results were expressed as picomoles of TBA reactive substances (TBARS), i.e., essentially malondialdehyde, per mg of tissue.

2.4. Protein extraction and immunoelectrophoresis

Tropomyosin oxidation was assessed as previously described [34,35]. Briefly, hearts were stored in liquid nitrogen and then homogenized in ice-cold PBS, pH 7.2 containing an antiprotease mixture. Protein samples were loaded on 12% SDS-PAGE separating gels and then transferred to 0.45 μm pore-size nitrocellulose membranes (Bio-Rad) The membranes were blocked by incubation with 3% BSA and then incubated at room temperature with an anti-tropomyosin monoclonal antibody (CH1 clone, Sigma Chemical Co.) and revealed by anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Cardiac interstitial and cardiomyocytes were lysed in RIPA buffer and clarified by centrifugation at 4 °C for 12,000 g for 10 min and boiled with SDS sample buffer. Western blot analysis was performed as above. Blots were probed with anti-SHC (BD Transduction Laboratories) and anti-desmin (Oncogene) antibodies. Protein bands were detected using anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Signals were visualized by a chemiluminescence detection system checking that exposures were within the linear range of detection.

2.5. Immunohistochemistry

The immunohistochemistry analysis was performed as previously described [35]. Briefly, cryosections were incubated with anti-tropomyosin monoclonal antibodies (CH1 clone) (1:25 in PBS containing 0.3% BSA). After several rinses with PBS, sections were incubated with fluorescein-conjugated secondary antibodies (Dako). The fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon lamp and a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments, Monmouth Junction, NJ, USA) as previously described. For detection of the fluorescein fluorescence, 488 ± 25 nm excitation and 522 nm longpass emission filter settings were used. Data were acquired and analyzed using Metamorph software (Universal Imaging, West Chester, PA, USA).

2.6. Cardiac interstitial cells and cardiomyocytes isolation

Myocytes were separated from cardiac interstitial cells (i.e., non-myocytes) as previously described [36]. Briefly, cell dissociation was obtained by perfusion with collagenase B (1 mg/ml) (Roche, Milan, Italy) in the absence of added Ca^{2+} at 37 °C for 10 min. After replacing the enzymatic solution with a 0.25 μM Ca^{2+} solution the ventricles were minced. Cardiomyocytes were collected by centrifugation at 200 rpm for 2 min. and the supernatant was filtered sequentially with 40, 20 and 15 μm nylon mesh. Cardiac interstitial cells were collected by centrifugation. Contamination of interstitial cells in myocyte samples was ruled out by assessing desmin content.

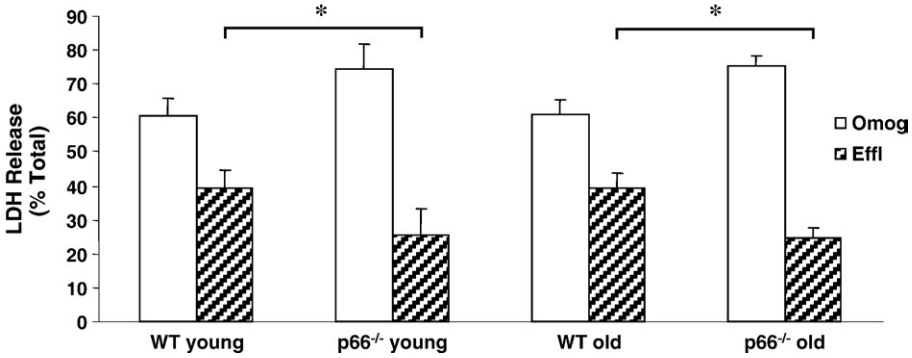

Fig. 1. Myocardial loss of viability induced by post-ischemic reperfusion is reduced in hearts lacking $p66^{Shc}$. Loss of viability was evaluated as the percentage of total LDH activity released in the coronary effluent during post-ischemic reperfusion. Hearts isolated from young (4 months) and old (24 months) WT or $p66^{Shc-/-}$ mice were subjected to 40 min of global ischemia followed by 15 min of reperfusion. To avoid changes due to variations in heart size, LDH activity was assessed in both coronary effluents collected at 1 min intervals during reperfusion (dashed bars) and tissue homogenates obtained at the end of the perfusion protocols (white bars). The cardioprotective effect associated with $p66^{Shc}$ ablation was independent of aging. Values are means ± S.E. *$p<0.05$.

2.7. Statistics

Results are presented as means ± SE. Statistical analysis was performed utilizing the unpaired Student's *t*-test.

3. Results and discussion

3.1. Hearts isolated from $p66^{Shc-/-}$ mice are protected against loss of viability induced by post-ischemic reperfusion

Cardioprotective effects elicited by the genetic deletion of $p66^{Shc}$ were investigated in isolated hearts under conditions of ischemia and reperfusion. According to well established notion, in hearts isolated from wild-type (WT) mice the readmission of coronary flow after 40 min of no flow global ischemia caused a significant loss of viability. Indeed, the release of LDH in the coronary effluent was paralleled by a decreased activity of this enzyme in the hearts collected at the end of the perfusion protocol (Fig. 1). Notably, in hearts isolated from $p66^{Shc-/-}$ mice the degree of tissue necrosis was largely attenuated. In fact the amount of LDH released during post-ischemic reperfusion was decreased by 35% (39.58 ± 5.17% in WT vs. 25.70 ± 7.49% in $p66^{Shc-/-}$; $p<0.05$). The cardioprotective effect associated with the absence of $p66^{Shc}$ was no longer present when the ischemia duration was longer than 60 min. However, under these extreme conditions we could never detect protection from any substance or intervention.

Due to life span prolongation induced by $p66^{Shc}$ deletion, the effects of ischemia were compared between hearts from young (4-month old) and senescent (24-month old) mice. Aging did not modify the degree of injury in hearts from wt mice as well as the degree of protection in hearts from $p66^{Shc-/-}$ littermates.

3.2. The absence of $p66^{Shc}$ reduces the degree of oxidative stress caused by post-ischemic reperfusion

Since excess ROS production is a major cause of injury in ischemic hearts, oxidative stress was analyzed in WT and $p66^{Shc-/-}$ hearts at the end of post-ischemic reperfusion. To this aim TBARS formation was assessed as a marker of lipid peroxidation, whereas the oxidation of tropomyosin (Tm) was measured as a reliable probe of the oxidative damage to contractile proteins [34,35].

Fig. 2 illustrates that TBARS formation was increased almost three-fold in hearts from WT mice subjected to ischemia and reperfusion independently from the age of the animals. This large increase in lipoperoxidation was totally abrogated by $p66^{Shc}$ deletion. In fact, hearts from $p66^{Shc-/-}$ mice displayed TBARS levels similar to those detected in freshly excised hearts or in hearts perfused under normoxic conditions. Similarly to what we

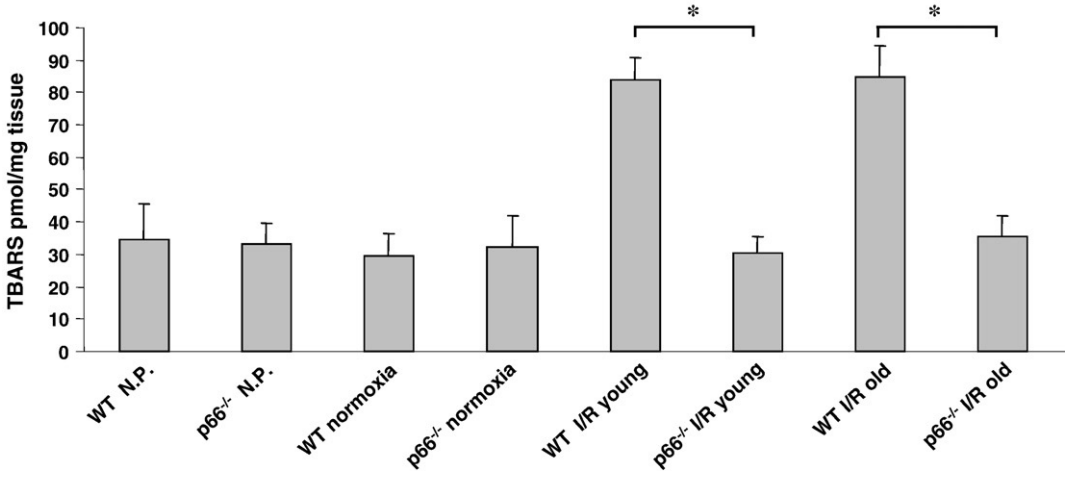

Fig. 2. Reperfusion-induced lipoperoxidation is blunted in $p66^{Shc-/-}$ hearts. Lipid peroxidation was evaluated as TBARS formation in hearts isolated from young and old WT or $p66^{Shc-/-}$ mice and perfused under conditions of ischemia and reperfusion. Lipid peroxidation was greatly enhanced by post-ischemic reperfusion in WT hearts independently of aging. This increase was not detected in hearts lacking $p66^{Shc}$. WT, wild-type; N.P., freshly excised (i.e., non-perfused) hearts; I/R, ischemia (40 min) followed by 15 min of reperfusion. Values are means ± S.E. *$p<0.01$.

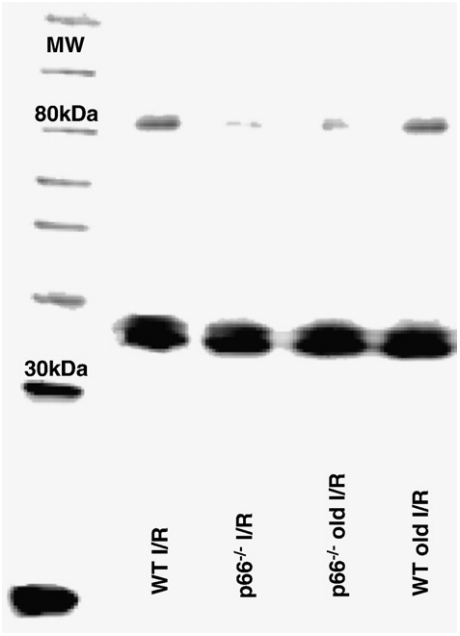

Fig. 3. Tropomyosin oxidation caused by post-ischemic reperfusion is largely decreased in hearts isolated from $p66^{Shc-/-}$ mice. In hearts from both young and old WT mice, post-ischemic reperfusion resulted in tropomyosin oxidation detected as the appearance of higher molecular weight bands due to the formation of disulfide cross-bridges (see Materials and methods and [35]). The degree of tropomyosin oxidation was largely reduced in hearts from young and old $p66^{Shc-/-}$ mice. In fact, the density of the higher molecular weight peptides was not different from those detected in samples from normoxic hearts (not shown). Molecular weight markers are shown in the first lane.

have previously reported for isolated and perfused rat and microembolized pig hearts [34,35], post-ischemic reperfusion-induced tropomyosin oxidation in WT mouse hearts. This was detected as the appearance of disulfide cross-bridges (DCB) in immunoblots with anti-tropomyosin antibody carried out after electrophoresis under non-reducing conditions (Fig. 3). Further supporting the decrease of oxidative stress in $p66^{Shc-/-}$ hearts, DCB formation was largely prevented in hearts devoid of $p66^{Shc}$. Also in this case the increased oxidation in the WTgroup as well as the reduced DCB formation in $p66^{Shc-/-}$ hearts were not affected by aging.In pig and rat hearts, Tm oxidation is reflected by an increase in its immunoreactivity. Therefore, it is possible to visualize and quantitate the extent of Tm oxidation in cryosections. Fig. 4 shows that Tm immunoreactivity was significantly increased in reperfused wt hearts as compared to normoxic samples. On the other hand, the absence of $p66^{Shc}$ prevented the increase in immunoreactivity occurred during reperfusion. The changes in immunoreactivity were independent of animal age.

Cardioprotective and antioxidant effects in hearts devoid of $p66^{Shc}$ could be ascribed to processes occurring in cardiac myocytes or in non-myocyte elements. This issue cannot be addressed by measuring ROS formation since the available methodologies do not allow identifying in which cells of an intact heart these toxic metabolites are generated. Therefore we analyzed in which heart cells $p66^{Shc}$ is preferentially expressed. Fig. 5 documents that while other members of the Shc family are expressed in both myocytes and interstitial cells of mouse heart, $p66^{Shc}$ is clearly and only expressed in cardiac myocytes suggesting that ischemia–reperfusion injury is largely contributed by $p66^{Shc}$-induced ROS formation within cardiac myocytes.

3.3. Cardioprotection and antioxidant effects of $p66^{Shc}$ deletion are comparable to and not additive with those afforded by other antioxidant or protective interventions

Having demonstrated that the absence of $p66^{Shc}$ protects against ischemia/reperfusion injury by preventing oxidative stress, the questions were (i) whether these protective effects are similar to those elicited by other antioxidant interventions and (ii) whether the effects of $p66^{Shc}$ deletion can be modified by the co-administration of other antioxidants. These issues have never been evaluated so far in studies characterizing $p66^{Shc}$. To this aim the results obtained in $p66^{Shc-/-}$ hearts were compared with those afforded by (i) perfusion with 1 mM mercaptopropionylglycine (MPG), a well known antioxidant [34]; (ii) perfusion with 0.5 mM pargyline, a MAO inhibitor [37]. Fig. 6 shows that the degree of protection detected as a decrease in release of LDH in $p66^{Shc-/-}$ hearts was not significantly different from

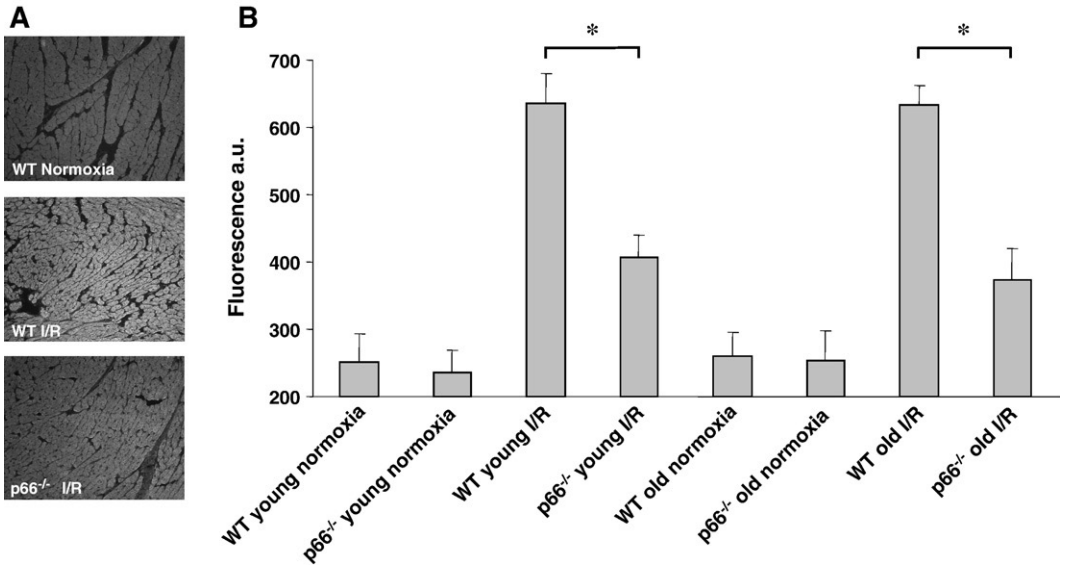

Fig. 4. Reperfusion-induced increase in tropomyosin immunoreactivity is significantly reduced in hearts isolated from $p66^{Shc-/-}$ mice. The oxidation of tropomyosin is reflected by an increase in its immunoreactivity against an anti-tropomyosin antibody (CH1 clone). This antibody was used to stain cryosections from young and old wild-type and $p66^{-/-}$ mouse hearts subjected to the I/R protocols described in the previous legends. Panel A illustrates typical examples of the variations in tropomyosin immunoreactivity. Panel B shows the quantitative analysis of fluorescence carried out on digital images as described in the Materials and methods section. Deletion of $p66^{Shc}$ prevented the increase in tropomyosin immunoreactivity caused by post-ischemic reperfusion. Also in this case the observed variations were not affected by aging. Values expressed as arbitrary units are means ± S.E. $*p<0.05$.

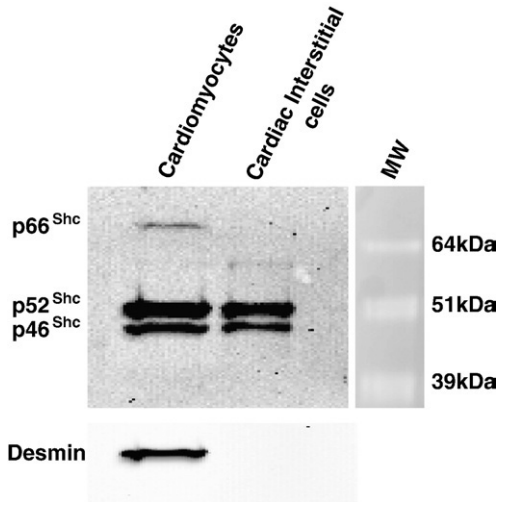

Fig. 5. p66^{Shc} is preferentially expressed in cardiac myocytes. Following collagenase perfusion (as described in the Materials and methods section), cardiac myocytes were isolated and separated from interstitial (i.e., non myocyte) cells. Immunoblots were stained with an anti-Shc antibody that detects the three isoforms of the protein. p66^{Shc} was only detected in cardiac myocytes. The presence of contaminating cardiomyocytes in the cardiac interstitial cell fraction by staining with an anti-desmin antibody.

that resulting from the other antioxidant interventions. In addition, the cardioprotection associated with p66^{Shc} absence appeared to be maximal, since it was not increased by co-administering other antioxidants. As shown in Fig. 7, also the reduction of oxidative stress observed in p66$^{Shc-/-}$ was not increased by antioxidant treatments. In fact, in p66$^{Shc-/-}$ hearts administration of pargyline or MPG did not modify the decrease in reperfusion-induced Tm oxidation (Fig. 7) or TBARS formation (results not shown). Finally, the possible involvement of p66^{Shc} in self-endogenous mechanisms of protection was investigated. In particular, we considered ischemic preconditioning (IPC), since the powerful protection resulting from this protocol has been attributed to a slight accumulation of ROS during the

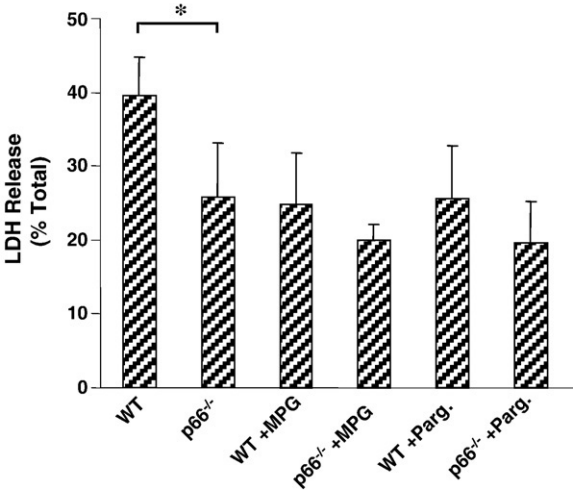

Fig. 6. Cardioprotection associated with p66^{Shc} ablation is comparable to that afforded by other antioxidant interventions. Loss of viability was evaluated by measuring LDH release during reperfusion after 40 min of global ischemia (see also legend to Fig. 1 and Materials and methods section). For the sake of simplicity only the values related to coronary effluents are reported. The cardioprotective effect observed in p66$^{Shc-/-}$ hearts was compared with those afforded by the following interventions: (i) perfusion with 1 mM mercaptopropionylglycine (MPG); (ii) perfusion with 0.5 mM pargyline that inhibits monoamine oxidase. The effect elicited by p66^{Shc} ablation was mimicked by the other antioxidant interventions. In addition, the degree of protection observed in hearts devoid of p66^{Shc} was not increased by co-treatment with any other antioxidant. Values are means ± S.E. *$p<0.05$.

preconditioning phase [38]. Therefore, a modest formation of ROS has been proposed to trigger defense mechanisms that eventually confer protection against the large burst of ROS formation at the onset of reperfusion [39]. This concept is supported by the ability of antioxidants to abrogate IPC protection [40]. Accordingly, also the decrease in oxidative stress associated with p66^{Shc} deletion might be expected to counteract the beneficial effects of IPC. Fig. 8 shows that this was not the case. IPC reduced markedly reperfusion-induced LDH release, yet this protection was not significantly different in p66$^{Shc-/-}$ hearts.

4. Discussion

The present results provide clear evidence that myocardial injury caused by post-ischemic reperfusion was greatly reduced in hearts devoid of p66^{Shc}. In particular the absence of this protein involved in mitochondrial ROS formation prevented the oxidative attack of structural components of cardiomyocytes, such as lipids and proteins. The reduced oxidative stress is likely to underlie the maintenance of tissue viability that was observed in hearts isolated from p66$^{Shc-/-}$ mice. Indeed, among the many factors contributing to the ischemic injury p66^{Shc} directly affects only ROS formation.

The present results demonstrate the crucial role of mitochondrial ROS formation in myocardial I/R injury. In this respect, our findings support and complement recent evidence highlighting the role of MAO [41] and the protection afforded by its inhibition. Interestingly, in the present study the cardioprotective efficacy due to p66^{Shc} ablation

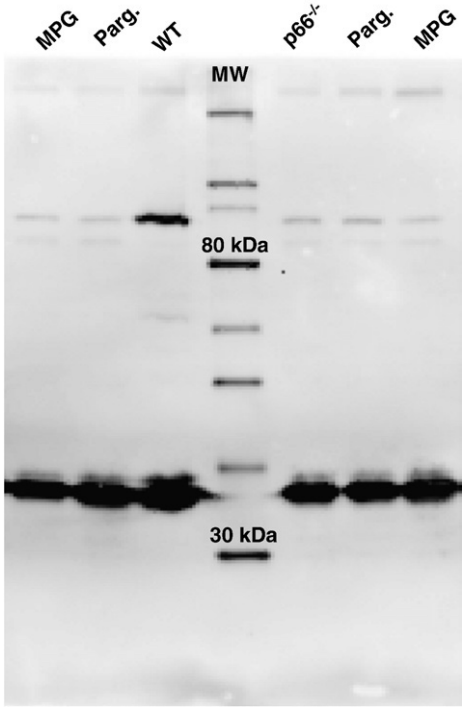

Fig. 7. The decrease in reperfusion-induced oxidation of tropomyosin observed in p66$^{Shc-/-}$ hearts is similar to that afforded by other antioxidant treatments. Tropomyosin oxidation was evaluated as the appearance of higher molecular weight bands due to the formation of disulfide cross-bridges (see Materials and methods and legend to Fig. 3). The lane with molecular weight markers separates the left panel showing typical examples from WT hearts, whereas samples from p66$^{Shc-/-}$ hearts are illustrated in the right panel. The protection against tropomyosin oxidation observed in p66$^{Shc-/-}$ hearts was compared with that afforded by the following interventions: (i) perfusion with 1 mM mercaptopropionylglycine (MPG); (ii) perfusion with 0.5 mM pargyline that inhibits monoamine oxidase. The effect elicited by p66^{Shc} ablation was mimicked by the other antioxidant interventions. In addition, the degree of protection observed in hearts devoid of p66^{Shc} was not increased by co-treatment with any other antioxidant.

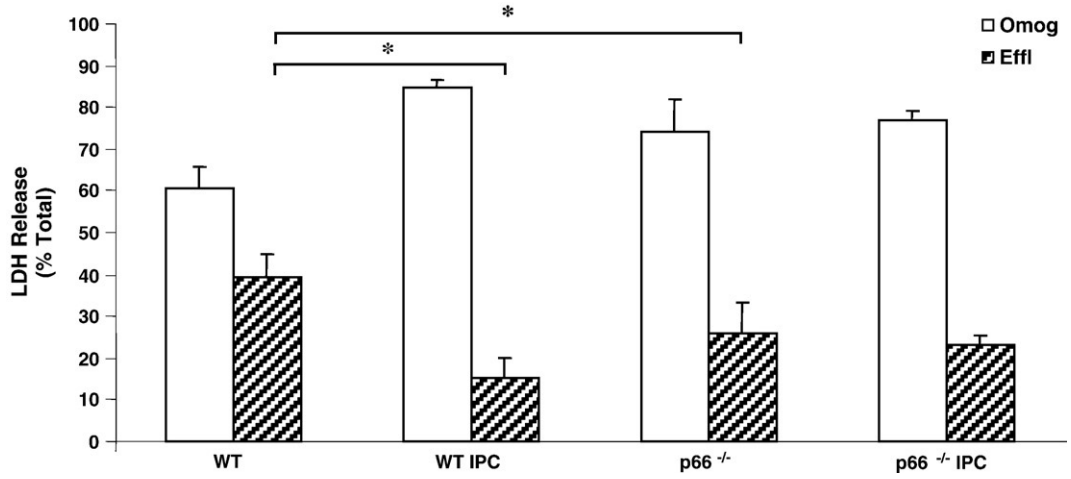

Fig. 8. p66^{Shc} ablation does not affect cardioprotection by ischemic preconditioning. The protection afforded by ischemic preconditioning (3 cycles of 5 min of ischemia followed by 5 min of reperfusion) was evaluated as a decrease in the release of LDH during post-ischemic reperfusion. The degree of protection was similar to that observed in hearts devoid of p66^{Shc} in the absence or the presence of ischemic preconditioning. Values are means ± S.E. *$p<0.05$.

was similar to that associated with MAO inhibition and these effects were not additive. Similarly, MAO inhibition does not afford any additional protection against oxidative stress in fibroblasts devoid of p66^{Shc} (data not shown). However, at present it is not clear whether MAO and p66^{Shc} interact to generate ROS or their final ROS products cumulate. On the other hand, it is worth pointing out that MAO involvement in cardiomyocyte injury has been exclusively referred to an increased availability of serotonin, an optimal substrate for MAO-A, due to its massive release from platelets [37,41]. However, in our model of crystalloid perfused heart MAO inhibition elicited a significant degree of protection in the absence of any increase in serotonin supply. It is likely that MAO substrates, such as serotonin or catecholamines are released from endogenous stores under conditions of ischemia and reperfusion.

The degree of protection afforded by p66^{Shc} was also similar to that obtained with treatment with MPG or IPC. This latter finding implies that mitochondrial ROS formation hardly contributes to IPC protection. However, we wonder whether the involvement of ROS in the cardioprotective signaling of IPC that has been documented in other species [40] applies also to mouse heart.

At the end of reperfusion p66$^{Shc-/-}$ hearts displayed a reduced oxidation of tropomyosin. This finding might extend the relevance of p66^{Shc} beyond the maintenance of viability. In fact, we have previously demonstrated that in microembolized pigs the degree of contractile impairment is directly correlated to the extent of tropomyosin oxidation [35]. Therefore, during reperfusion also in cardiomyocytes that remain viable oxidation of tropomyosin and other myofibrillar proteins might hamper contractile function. It is tempting to hypothesize that a reduced oxidation of myofibrillar proteins might have contributed to the maintained function in p66$^{Shc-/-}$ subjected to protocols of heart failure [42,43]. In any case, our findings establish a direct link between mitochondrial dysfunction and myofilament oxidation that is likely to contribute significantly to several forms of contractile impairment.

Since p66^{Shc} ablation protects against I/R injury as well as from other insults, the question might be why cells contain such a dangerous weapon. Although the physiological roles of p66^{Shc} are far from being conclusively elucidated, recent studies relate p66^{Shc}-induced ROS formation to both adipogenesis and immune response. In particular, p66^{Shc} has been recognized a downstream target of the insulin signaling pathway, and a critical mediator of insulin-dependent ROS-upregulation in adipocytes [44]. In its absence, nutrient storage in the form of triglyceride accumulation along with thermoinsulation and thermogenesis are likely to be hampered possibly jeopardizing the growth of young individuals under adverse conditions, such as food restriction or exposure to low temperatures. On the other hand p66^{Shc} has been shown to prevent excessive lymphocyte activation that has been observed in p66$^{Shc-/-}$ mice resulting in the occurrence of a lupus-like autoimmune disease. Therefore, the loss of this protein appears to hamper immunologic tolerance favoring systemic autoimmunity [45].

In conclusion, the findings obtained in mice lacking p66^{Shc} prove beyond any doubt that mitochondrial ROS formation is not just an accidental by-product of the respiratory chain, and that most intracellular oxidative stress originates in mitochondria. However, and unfortunately, at present the translation of these concepts into clinical practice is limited by the lack of drugs that prevent ROS formation by p66^{Shc}.

Acknowledgments

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