Changes in Hsp90 expression determine the effects of cyclosporine A on the NO pathway in rat myocardium

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Abstract Cyclosporine A (CsA) is associated with the development of cardiovascular toxicity in transplant patients but can exert myocardial protection against ischemia/reperfusion damages. We examined in a rat model of chronic CsA administration whether subtle variations in the NO pathway could account for these opposite effects. CsA treatment rapidly led to an increase in myocardial Hsp90 expression promoting the recruitment of Akt and calcineurin, thereby promoting eNOS activation through Ser1177 phosphorylation and Thr495 dephosphorylation, respectively. This was associated with an increase in myocardial VEGF expression and led to anti-apoptotic effects in isolated cardiac myocytes. Upon longer CsA exposure, cardiac toxicity developed, as documented by the infiltration of connective tissue and the increase in iNOS expression. These later effects were associated with a dramatic decrease in the abundance and scaffold function of Hsp90, thereby unraveling the key role of Hsp90 in governing CsA effects.

Key words: Cyclosporine; Nitric oxide; Akt; Hsp90; Calcineurin

1. Introduction

Cyclosporine A (CsA) is currently the most widely used immunosuppressive drug for preventing graft rejection and autoimmune diseases. The clinical use of CsA and other agents such as tacrolimus, however, is limited because of the induced development of nephrotoxicity [1] and hypertension [2]. Among the mechanisms leading to these side effects, various alterations in the nitric oxide (NO) signaling pathway have been identified as key determinants for the development of renal and coronary vasculopathies [3–11]. In these studies, the inhibition by CsA of the activity of a specific NOS isoform, e.g. eNOS or iNOS, was identified as a major trigger leading to vasodilation impairment. These reports, however, contrast with studies documenting that CsA might exert beneficial effects in the myocardium exposed to ischemia/reperfusion protocols through an eNOS-mediated pathway [12,13]. Other authors have also suggested that the cardiac damages associated with ischemia/reperfusion and the development of allograft arteriosclerosis were both prevented by the CsA-induced up-regulation of iNOS [14,15]. Thus, in a given pathophysiological context, the down- or up-regulation of either eNOS or iNOS activity seems to determine the deleterious or beneficial impact of immunosuppressive compounds on the cardiovascular tissues, respectively. Although changes in iNOS abundance can help to reconcile the above-mentioned conflicting reports about the role of the mostly transcriptionally regulated enzyme iNOS, other factors need to be considered to evaluate the effects of CsA on the eNOS activity.

Activation of the endothelial NOS isoform is, indeed, not only dependent on the enzyme abundance [10,11] but is also exquisitely regulated through post-translational interactions with proteins such as caveolin and the heat shock protein Hsp90 [16] as well as via site-specific phosphorylations (see [17] for references). Accordingly, the phosphorylation of eNOS on the serine 1177 has been extensively documented as a major kinase-dependent mode of eNOS activation, whereas two other sites within the eNOS sequence, e.g. Thr 495 and Ser 116, have been reported to participate, when phosphorylated, in the tonic inhibition of eNOS activity (see [17] for references). We previously reported that these different modes of regulation could interplay with each other. For instance, Hsp90 acts as a scaffold to recruit Akt in the eNOS complex and thereby promotes the phosphorylation of eNOS on serine 1177 [18]. Interestingly, Hsp90 was also reported to interact with calcineurin [19], a phosphatase thought to regulate eNOS dephosphorylation [20,21], although the demonstration of a direct relationship between these three proteins has never been given.

In this study, we therefore set up a model of chronic CsA administration in rats in order to evaluate the regulatory role of Hsp90 in the myocardium and of the changes in the phosphorylation status of eNOS. We also looked for modifications in VEGF and iNOS expressions as hallmarks of the potential protective and deleterious pathways induced by CsA treatment, respectively. The VEGF induction seems to be a common pro-survival pathway activated in response to a large variety of cellular insults and its protective effects are mediated through several of the eNOS modulators including Akt and calcineurin [18,22]. A direct link between all these different actors, however, has never been established in the context of CsA treatment. Conversely, iNOS is usually associated with late stages of cardiac diseases (when eNOS is down-regulated) and its expression level was therefore examined here in the light of morphological cardiac alterations; direct measure-

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ments of apoptosis were also carried out in an in vitro model of CsA-exposed cardiac myocytes.

2. Materials and methods

2.1. Animals, treatment and sample preparation

The study included 48 Wistar rats with a weight of 200–250 g. Each procedure was approved by the local authorities according to national animal care regulations. The animals were divided into four groups (of 12 animals each). The control animals were daily injected subcutaneously with olive oil for 21, 42 and 63 days (four animals for each period). The animals of the other groups were daily injected (s.c.) with CsA (Sandimmun, Sandoz; 15 mg kg^{-1} in olive oil) for 21, 42 and 63 days, as previously described [23]. The animals were killed at the end of the treatment periods. The hearts were removed, washed in PBS and cut transversely into small pieces. Some samples were fixed according to standard procedures, embedded in paraffin and serially sliced for immunohistochemical study. In some experiments, neonatal mouse cardiac myocytes were isolated and cultured as previously described [24].

2.2. Immunohistochemical study

Heart sections were probed with antibodies against Hsp90, iNOS, eNOS, and VEGF (BD Transduction Labs) and with Sirius Red (to stain collagen). Briefly, 5 μm thick sections were first deparaffinized and rehydrated, and then immersed in 3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxidase activity. Sections were then incubated with goat or rabbit serum (diluted 1:5) for 60 min, then serially treated with HSP90 (goat polyclonal, diluted 1:10), iNOS, eNOS (rabbit polyclonal, diluted 1:50), and VEGF (VEGF121: goat polyclonal, diluted 1:10) at 4°C overnight. The sections were washed in TBS buffer and sequentially incubated with biotinylated secondary antibodies, and avidin–biotin horseradish peroxidase complex according to the manufacturer's instructions (ABC kit, Dakopatts, Milan, Italy). The sections were stained by immersion in a solution of 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide. All slides were counterstained with hematoxylin, dehydrated and mounted. Control reactions were performed in the absence of the primary antibodies.

2.3. Immunoprecipitation and immunoblotting

Liquid nitrogen-frozen rat heart pieces or cultured neonatal myocytes were homogenized by using an Ultra-Turrax and a sonicator. The lysis buffer contained 60 mM octylglucoside, 50 mM Tris–HCl (pH 7.4), 125 mM NaCl, 2 mM dithiothreitol, 100 μM EGTA as well as protease and phosphatase inhibitor cocktails (Sigma). Lysates were centrifuged at $1000 \times g$ and the supernatants processed for immunoblotting (IB) or immunoprecipitation (IP), as described previously [16,18]. In the IP experiments, we used the lysis buffer for the incubations with the antibodies and the protein G–Sepharose beads (as well as for the washing steps). The eNOS, iNOS and Hsp90 antibodies were from BD Transduction Labs; Akt, calcineurin, phospho-caspase-3 and caspase-3 antibodies from BD PharMingen, and phospho-eNOS antibodies from NEB Cell Signaling Technology or Upstate Biotechnology Inc.

2.4. Statistical methods

The results are given as mean ± S.E.M.; for the IB experiments, data were normalized for the amounts of protein in the dish. Statistical analyses of results between experimental groups were performed with one-way analysis of variance (ANOVA) and the Student–Newman–Keul post-test.

3. Results

We first performed a series of immunoblot experiments using lysates from myocardium of CsA-treated and untreated rats. As shown in the upper panel of Fig. 1A (see also bar graph, Fig. 1B), the abundance of Hsp90 was found to be increased by almost three-fold as soon as 21 days after initiation of CsA administration. Longer exposure to CsA led to a progressive decrease in Hsp90 expression to reach a value

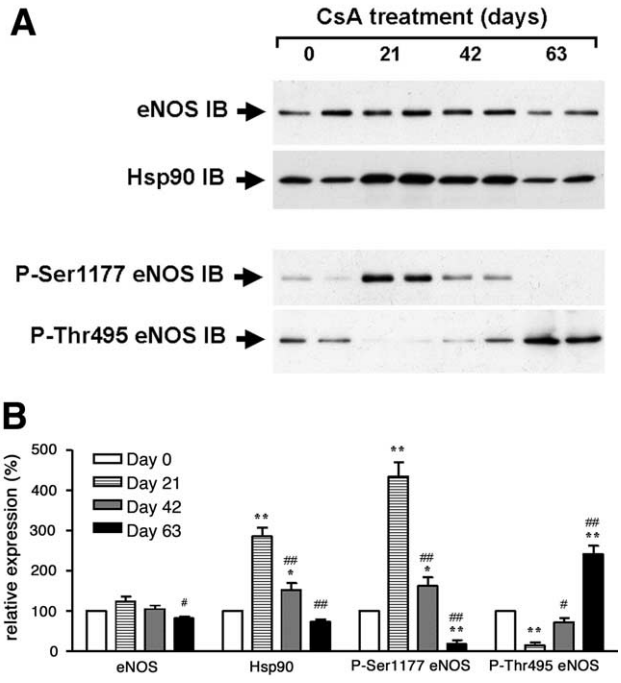

Fig. 1. Effects of CsA exposure on eNOS and Hsp90 expression and on the phosphorylation of eNOS on Ser 1177 and Thr 495. Hearts from rats treated, or not, with CsA for 0, 21, 42 and 63 days were collected. A: Lysates from CsA-treated heart were immunoblotted (IB) with antibodies directed against eNOS, Hsp90 and eNOS phosphorylated on the indicated residues. B: Bar graph represents the relative expression of eNOS, Hsp90, phospho-Ser 1177 and -Thr 495 eNOS determined by densitometric analyses of different IB as the ratio between rats treated or not with CsA; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. day 0, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. day 21 ($n = 4$).

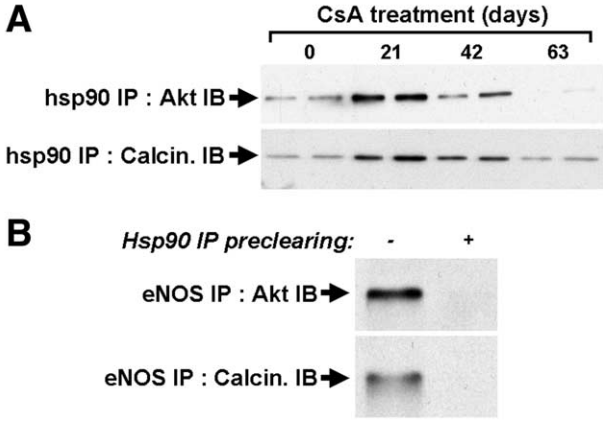

Fig. 2. Effects of CsA exposure on the Hsp90-mediated formation of a multiprotein complex between eNOS, Akt and calcineurin. Hearts from rats treated, or not, with CsA for 0, 21, 42 and 63 days were collected. A: Lysates from CsA-treated heart were immunoprecipitated (IP) with Hsp90 antibodies and then immunoblotted (IB) with antibodies directed against Akt and calcineurin. B: Lysates from 21-day CsA-treated heart were precleared, or not, by immunoprecipitation with Hsp90 antibodies, and then a new immunoprecipitation was carried out with eNOS antibodies before immunoblotting with Akt and calcineurin antibodies. These experiments were consistently reproduced with lysates isolated from different rats ($n = 4$; max. values at day 21 are given in the text).

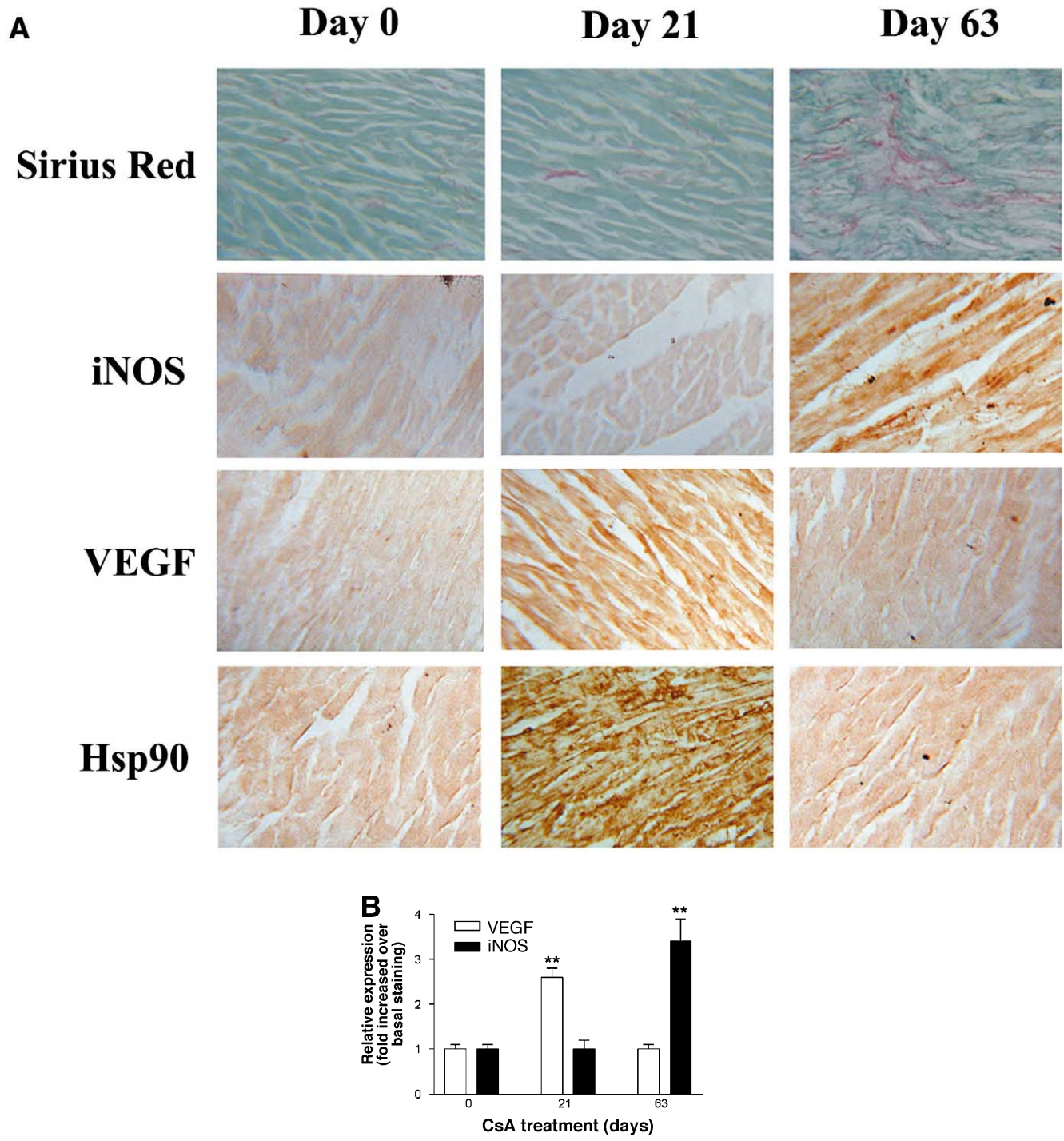

Fig. 3. Morphological and immunohistochemical analyses of rat myocardium exposed to CsA. A: Slices from ventricles of rats treated with CsA for 0, 21 and 63 days were colored with Sirius red to detect the presence of connective tissues or immunostained with antibodies directed against iNOS, VEGF and Hsp90, as indicated. B: Quantitative analyses of the expression of VEGF and iNOS (as detected by IH) are presented in bar graphs; $^{**}P < 0.01$ vs. day 0 ($n = 4$).

below the control level after 63 weeks ($73 \pm 5\%$ vs. control) (Fig. 1B). Although we did not observe any significant change in eNOS expression when compared with the values at day 0, the difference in eNOS expression between day 21 and 63 of CsA treatment was statistically significant ($P < 0.05$). These changes were not attributable to aging, since in control rats (sham-treated or untreated) sacrificed at the same age, we did not identify a difference in eNOS abundance between day 0, 21, 42 and 63. We also studied the impact of CsA exposure on the phosphorylation status of eNOS. Fig. 1A (lower panel) shows that the phosphorylation of eNOS on Ser 1177 was maximal at day 21 and then rapidly decreased to be undetectable after 63 days of CsA treatment. A mirror image was obtained for the extent of eNOS phosphorylation on Thr

495, e.g. minimal after 21 days and maximal at day 63 (see Fig. 1A,B).

We then evaluated whether the transient CsA-induced overexpression of Hsp90 could account for changes in the ability of the kinase Akt and the phosphatase calcineurin to alter the phosphorylation status of eNOS (see Section 1). As shown in Fig. 2A, the patterns of interaction with Akt and calcineurin exactly reflected the bell-shaped changes in Hsp90 with a maximum at day 21 (3.6- and 2.1-fold increase versus day 0, respectively; $P < 0.01$; $n = 3$) and then a progressive decrease with time of exposure to CsA. We also examined whether, in our model, Hsp90 could act as an adaptor recruiting Akt and calcineurin in the eNOS complex. Accordingly, we performed immunoprecipitation with eNOS antibodies either from total lysates or from Hsp90-precleared lysates, e.g. depleted of Hsp90 by immunoprecipitation. Fig. 2B reveals that, while Akt and calcineurin were found to be associated with eNOS in control lysates, Hsp90 preclearing led to the disappearance of both regulatory proteins from the eNOS complex.

We also performed morphological and immunohistochemical (IH) analyses of rat myocardium at different times of CsA treatment. Accordingly, hearts from control rats or rats treated for 21 days with CsA revealed a normal morphology, consisting of striated muscle fibers and scarce connective tissue (see Fig. 3A). By contrast, hearts from rats treated for 63 days with CsA showed clear degenerative changes with muscle fiber disorganization and abundant Sirius red-stained connective tissues (+280%, $P < 0.01$, $n = 4$) (Fig. 3A). Importantly, CsA treatment and not aging accounted for these changes, as validated by the lack of myocardium degeneration when comparing hearts from sham rats at day 0 and 63 (data not shown). The morphological pattern after 42 days of CsA treatment was intermediary, although closer from the 21-day condition, suggesting that the cytotoxic potential of this drug needed a certain delay to exert its effects or that cytoprotective effects of CsA could fade with time.

To address these non-exclusive hypotheses, we examined whether CsA could induce changes in the myocardial expression of the inducible NOS isoform (iNOS) and of the VEGF/eNOS pathway. We observed a dramatic up-regulation of iNOS at day 63 of CsA exposure, whereas VEGF (121-kDa isoform) expression was up-regulated at day 21 of CsA treatment and then decreased to basal levels (Fig. 3A,B). By contrast, in the limits of our quantitative immunohistochemical analysis, we did not observe significant changes in the expression of eNOS: its abundance was found unaltered by CsA treatment in cardiac myocyte and microvascular endothelial cells (although present at much higher levels in the latter). Data presented in Fig. 3A also confirmed that a transient, although dramatic, increase in the myocardial expression of Hsp90 was detectable upon 21 days of CsA exposure.

To more definitely establish the link between the increase in Hsp90 and the cytoprotective effects of CsA, we pre-treated mouse neonatal cardiac myocytes with CsA (1 μM) and examined the level of apoptosis induced by serum deprivation. Accordingly, the detection of the active 17-kDa form of caspase-3 was performed in parallel to the immunoblotting of the 32-kDa (uncleaved) pro-caspase-3. Fig. 4 shows that the CsA-induced increase in Hsp90 expression (+193%, $P < 0.05$, $n = 3$) was associated with lesser amounts of active caspase-3 (−97%, $P < 0.05$, $n = 3$). Importantly, the treatments with geldanamycin or L-NAME did not significantly alter the relative CsA-induced increase in Hsp90 (+180% and +146%, respectively; $P < 0.05$, $n = 3$) but completely abrogated the anti-apoptotic effects of CsA exposure (i.e. they did not prevent the cleavage of pro-caspase-3) ($P > 0.1$ vs untreated myocytes, $n = 3$).

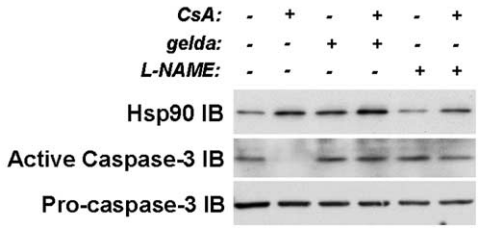

Fig. 4. Effects of CsA exposure on the Hsp90-mediated NO-dependent protection against serum deprivation-induced apoptosis. Cultured neonatal cardiac myocytes were treated or not with CsA for 48 h, then exposed to serum-deprived medium for the next 36 h. Lysates were immunoblotted (IB) with antibodies directed against Hsp90, active (cleaved) caspase-3 and pro-caspase-3. These experiments were repeated with lysates prepared from three different cultures of isolated myocytes (significant differences derived from densitometric analyses are given in the text).

4. Discussion

In the present study we found that CsA treatment could induce changes in the post-translational regulation of eNOS in rat myocardium. Accordingly, we have documented that 21-day exposure to CsA led to the activation of eNOS through Ser 1177 phosphorylation and Thr 495 dephosphorylation. These changes preceded the development of cardiac toxicity (max. at 63 days of treatment), as authenticated in our model by the down-regulation of VEGF, the induction of iNOS and the connective tissue infiltration. This late phase of CsA effects was also associated with a progressive decrease in the eNOS interaction with Akt and calcineurin, as revealed by the reduction in the extent of the Ser 1177 phosphorylation and the tonic phosphorylation of Thr 495, respectively. Importantly, we provided mechanistic insights on how changes in Hsp90 expression can orchestrate these changes in eNOS post-translational regulation during CsA treatment.

The expression of Hsp90 appeared, indeed, to be dramatically influenced by the time of exposure to CsA. Hsp90 expression peaked at 21 days after the initiation of the immunosuppressive treatment. This was paralleled by a strong up-regulation of VEGF, a well-characterized activator of the Hsp90–eNOS pathway [18,25]. Together with the observation that Hsp90 acts as an adaptor recruiting Akt and calcineurin in the eNOS complex, these data indicate that a strong activation of eNOS occurred at this early stage of CsA treatment. Indeed, eNOS phosphorylation on Ser 1177 and dephosphorylation on Thr 495 have been extensively documented to promote a sustained NO production by increasing the calmodulin affinity and improving the electron flux through the enzyme [26–28]. In our study, this Hsp90-driven eNOS-activation phase was associated with a myocardium devoid of any morphological alterations or fibrosis and can therefore be correlated with the previously reported NO-mediated protective effects of CsA after ischemia/reperfusion [12,13]. Furthermore, using cultured neonatal cardiac myocytes, we showed that CsA could rapidly induce an increase in Hsp90 abundance and thereby protect these cells against apoptosis induced by serum deprivation (see Fig. 4). Altogether, these data indicate

that Hsp90 acts as an important control point to determine the fate of (cardiac) tissues responding to a stress by inducing pro-survival pathways before being overwhelmed by toxicity.

It is also noteworthy that the well-known inhibitory effects of CsA on calcineurin [29] can apparently be counterbalanced by the myocardial up-regulation of Hsp90, leading to the recruitment and activation of this phosphatase in the eNOS complex. Recent reports [20,21] of the CsA-induced inactivation of eNOS through an inhibition of calcineurin, and the associated blockade of eNOS dephosphorylation, need therefore to be interpreted with caution. In fact, our study brings the concept of a compartmentation-like effect allowing calcineurin to exert its activity, whereas an overall inhibition of the phosphatase is driven by the CsA exposure.

In conclusion, the observation of the CsA-induced changes in Hsp90 expression, i.e. first increased three-fold then reduced below basal levels, brings some rationale to the very puzzling issue of the balance between the beneficial, tissue-protective effects of CsA and the cardiovascular toxicity of this immunosuppresive drug. Moreover, the identification of Hsp90 acting as a scaffold to recruit not only Akt [18] but also calcineurin (this study) in the eNOS complex allows us to understand how eNOS can *simultaneously* be phosphorylated and dephosphorylated at two very distinct sites (both modifications leading to the enzyme activation). Improving Hsp90 function therefore appears to be a very promising therapeutic strategy to prevent eNOS inactivation in various disease states characterized by endothelial dysfunction.

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