

# Interaction between heat shock protein 70 kDa and calcineurin in cardiovascular systems (Review)

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**Abstract.** Cells have the capability of defending themselves from various stressors by activating a genetic program with the production of substances known as heat shock proteins (Hsps) and their regulatory partners, the heat shock transcription factors. Hsps play a major role in systemic hypertension, coronary artery disease, carotid atherosclerosis, myocardial infarction and myocardial ischemia. In this review we discuss the interaction between Hsp70 and CaN which was carried out in our laboratory. We demonstrated that the cardiac Hsp70 stimulated a 2-fold increase in calcineurin (CaN) activity. In addition, the pull-down assay revealed that Hsp70 directly interacts with CaN. Furthermore, expressed cardiac specific Hsp70 was phosphorylated *in vitro* by cAMP-dependent protein kinase. The phosphorylated Hsp70 was unable to activate the phosphatase activity of CaN. For the first time we demonstrated that Hsp70 is phosphorylated by cAMP-dependent protein kinase and provides an on/off switch for the regulation of CaN signaling by Hsp70. This will lead to therapeutic benefit in human diseases such as atherosclerosis, cardiomyopathy, congestive heart failure, and ischemia.

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## 1. Introduction

Cells have the capability of defending themselves from various stressors by activating a genetic program with the production of substances known as heat shock proteins (Hsps) and their regulatory partners, the heat shock transcription factors (for review see refs. 1-7). Hsp(s) act as molecular chaperones by maintaining proper protein assembly, folding, and transport (for review see refs. 1-7). Hsp(s) have also been shown to increase in the presence of cardiovascular stress stimuli such as ischemia, hypoxia, oxidative injury, and endotoxemia (2). It has been proposed that Hsp(s) and heat shock transcription factors would be attractive pharmacologic targets for stimulating endogenous protective mechanisms in various cardiovascular diseases.

In this article we discuss the involvement of Hsp70 and calcineurin (CaN) including their possible roles in pathophysiology which has been carried out in our laboratory.

## 2. Structure and function of Hsp70

Classification of various Hsp(s) in families is based on their related function, size and the cellular compartment in which they reside (for review see refs. 3-7). The Hsp70 multi-gene family consists of at least four members: Hsp70, Hsc70, Grp78 (BiP) and mitochondrial Hsp75 (mtHsp75). Hsp70, Hsc70 are found in the cytosol and nucleus, mtHsp75 and Grp78 are found in the matrix of mitochondria and endoplasmic reticulum, respectively (8). The Hsp70 family of proteins has been implicated in a variety of processes including protein folding, the disassembly of oligomeric protein complexes and the translocation of polypeptides across intracellular membranes (9). The human Hsp70 chaperone is a 640 amino acid protein composed of two major functional domains. The NH<sub>2</sub>-terminal domain is highly conserved and contains an ATPase domain that binds ADP and ATP very tightly (in the presence of Mg<sup>2+</sup> and K<sup>+</sup>) and hydrolyzes ATP. The COOH-terminal domain on the other hand is required for polypeptide binding. This latter domain is divided into functionally relevant sub-domains, an 18 kDa peptide-binding domain and a 10 kDa C-terminal domain that contains the Glu-Glu-Val-Asp (EEVD) regulatory motif (9). Co-operation of both N-terminal and C-terminal domains is needed for protein folding (9).

### 3. Calcineurin

$\text{Ca}^{2+}$  is central to the control of cardiac growth and contractile function. Thus, it is not surprising that abnormalities in  $\text{Ca}^{2+}$  handling have been implicated in many forms of cardiac disease. CaN is a  $\text{Ca}^{2+}$ /CaM-dependent serine/threonine-specific phosphatase (10). CaN signaling affects the functions of a wide range of cell types and although many of its effects are ubiquitous, others are restricted to cardiac (and skeletal) muscle, providing muscle specificity to CaN signaling. CaN was discovered and purified as an inhibitor of CaM-dependent cyclic nucleotide phosphodiesterase (11,12). CaN has been characterized in numerous tissues including brain, heart, kidney, liver, eye, muscle and T-lymphocytes (13,14). CaN is comprised of 57-59 catalytic subunit and is referred to as CaN A, 19 kDa  $\text{Ca}^{2+}$  binding protein is referred to as CaN B (12,14). CaN A has four functional domains. These include a catalytic site, a CaN B binding domain, a CaM binding domain and an autoinhibitory domain near the carboxy terminus (15). The CaN A subunit has low endogenous phosphatase activity and requires  $\text{Ca}^{2+}$ , CaM and CaN B for full activity. The activity of CaN A can be further stimulated by divalent metal ions such as  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$  (16,17). Three mammalian isoforms of CaN A ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and two B subunit regulatory genes (B1, B2) have been identified (18-20). The CaN A $\alpha$ , A $\beta$  and B1 are ubiquitously distributed, while CaN A $\gamma$  and B2 expression are restricted to brain and testis (21). The most clinically significant feature of CaN is its inhibition by the immunosuppressant drug cyclosporin A (CsA)-cyclophilin and tacrolimus A. The role of CaN in T cell activation was discovered when these immunosuppressive compounds were found to inhibit  $\text{Ca}^{2+}$ -dependent activation of T cells (22).

CaN has attracted great attention as a critical mediator for cardiac hypertrophy (23,24). Divergent reports were available on CaN concentration and pressure-overload hypertrophy. Increased (25,26), no change (27) or a decrease (28) of CaN activity have been reported in response to pressure overload hypertrophy. The discovery of CaN-NFAT signaling in the heart helps to elucidate the molecular mechanism underlying cardiac hypertrophy (29,30). Apoptotic cell death plays a critical role in a variety of cardiovascular diseases including myocardial infarction, heart failure and atherosclerosis. Myocardial infarction, as well as non ischemic forms of heart failure, are thought to involve an irreversible loss of cardiac myocytes through apoptosis or programmed cell death (31). Various signaling molecules that enhance cardiomyocyte apoptosis include tumor necrosis factor- $\alpha$ , c-jun N-terminal kinase, p53,  $\beta$ -adrenergic receptors and nitric oxide (32). CaN was identified as an important regulator of cardiomyocyte apoptosis (33). Both pro- and anti-apoptotic modulatory roles of CaN have been reported in cardiomyocytes (34-36). Ischemia is associated with multiple alterations in the extracellular and intracellular milieu of cardiomyocytes that may act as inducers of apoptosis (37). We reported the activation of CaN expression in ischemia-reperfused rat heart and in human ischemic myocardium (38). Transgenic mice expressing an activated form of calcineurin in the heart are largely protected from ischemia-reperfusion-induced DNA laddering, further suggesting that calcineurin activation antagonizes cardiomyocyte apoptosis *in vivo* (36). More

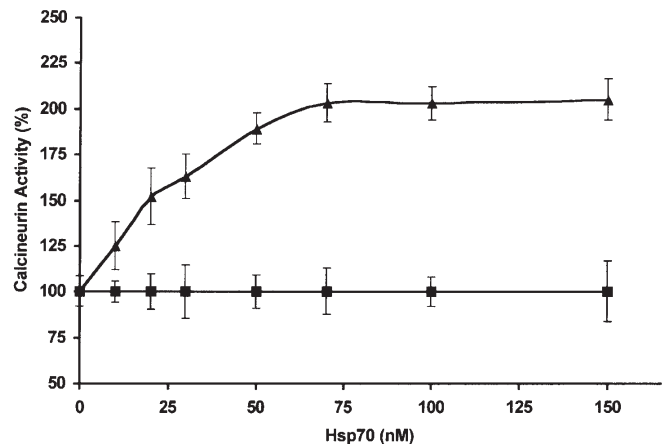


Figure 1. Effect of cardiac Hsp70 on CaN phosphatase activity. The CaN phosphatase assay was carried out in presence (▲) or in absence (■) of Hsp70. For details see ref. 47.

knowledge on the cellular signaling network of CaN is needed to elucidate its exact role in cardiomyocyte apoptosis.

### 4. Interaction between Hsp70 and CaN

The molecular relationships between Hsp(s) and various signaling proteins appear to be critical for the normal function of signal transduction pathways (39). The involvement of Hsp(s) in stabilizing the conformational transitions of newly synthesized protein is well documented (40). Most notable is the interaction of Hsp90 with a number of signaling proteins, including Ras, Raf and pp60<sup>v-src</sup> kinase (41,42). Overexpressed Hsp70 significantly inhibits the enzymatic activities of protein kinase A and protein kinase C, but it stimulates the activity of protein serine/threonine phosphatases, protein phosphatase-1 and protein phosphatase-2A (43). A recent observation suggested that Hsp70 binds the dephosphorylated carboxyl terminus of mature protein kinase C, thus stabilizing the protein and allowing the rephosphorylation of the enzyme. Disruption of this interaction prevents rephosphorylation and targets the enzyme for down-regulation (44). Hsp90 and Hsp70 family proteins interact with various signaling molecules, including nuclear hormone receptors, tyrosine and serine/threonine kinases, cell cycle regulators and cell death regulators (1). Various reports demonstrated the direct interaction of Hsp(s) with calcineurin (CaN) (45,46). CaN, a calmodulin (CaM) dependent protein phosphatase, is activated by Hsp90 and Hsp70 in CaM-independent and -dependent mechanisms, respectively (45). Hsc82, an Hsp90 homologue in yeast, binds to the catalytic subunit of CaN and stabilizes this CaM-dependent phosphatase (46). In our study, the recombinant Ni-NTA purified cardiac Hsp70 activated brain CaN phosphatase activity 2-fold at a concentration of 70 nmol Hsp70 (Fig. 1). Furthermore, pull-down assay was carried out using His<sub>6</sub>-Hsp70 to examine the direct interaction between Hsp70 and CaN. His<sub>6</sub>-Hsp70 was immobilized on Ni-NTA and incubated with CaN (Fig. 2). CaN was bound to His<sub>6</sub>-Hsp70 (Fig. 2A, lanes 2-4) (47). However, no CaN binding was detected using an irrelevant control protein His<sub>6</sub>-p85 immobilized similarly (Fig. 2A, lanes 6-8). The His<sub>6</sub>-p85

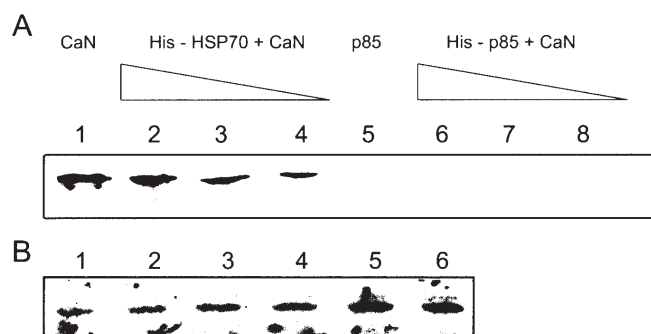


Figure 2. Interaction of Hsp70 with CaN *in vitro*. (A) Pull-down assays with His<sub>6</sub>-Hsp70 or His<sub>6</sub>-p85 (negative control) were performed using CaN. Lane 1, CaN alone (2 µg); lanes 2-4, His<sub>6</sub>-Hsp70 Ni-NTA beads with CaN; lane 5, p85 alone; lanes 6-8, His<sub>6</sub>-p85 Ni-NTA beads with CaN. (B) Pull-down assays with His<sub>6</sub>-Hsp70 were performed using CaN in presence of ATP, ADP, K<sup>+</sup> and K<sup>+</sup> with ATP. Lane 1, CaN alone; His<sub>6</sub>-Hsp70 Ni-NTA beads with CaN: unbound nucleotides (lane 2), ATP (lane 3), ADP (lane 4), K<sup>+</sup> (lane 5), K<sup>+</sup> with ATP (lane 6). For details see ref. 47.

control protein showed no pull-down signal indicating that the CaN pulled-down by the His<sub>6</sub>-Hsp70 fusion protein was specific. This result indicates the direct protein-protein interaction between Hsp70 and CaN *in vitro*. Hsp70 is known to bind nucleotide very tightly. To analyze the effect of nucleotide(s) on the interaction of Hsp70 and CaN, pull-down assay was carried out in presence of ATP, ADP, K<sup>+</sup> and K<sup>+</sup> with ATP. His<sub>6</sub>-Hsp70 was immobilized on Ni-NTA and incubated with CaN in presence of ATP, ADP, K<sup>+</sup> and K<sup>+</sup> with ATP (Fig. 2B). We observed that CaN was bound to His<sub>6</sub>-Hsp70 in presence of ATP, ADP, K<sup>+</sup> and K<sup>+</sup> with ATP (Fig. 2B, lanes 2-5) (47). This result indicates that there was no influence of nucleotides or K<sup>+</sup> on the direct protein-protein interaction between Hsp70 and CaN *in vitro*. Various reports suggest that COOH-terminal side from the CaM-binding domain of the CaN A is structurally unstable (48,49). The interaction of CaN with a variety of proteins (perhaps Hsp70) may be important to stabilize this region of the CaN A subunit.

## 5. Phosphorylation of Hsp70

There are several types of modifications that may cause progressive charge shift of Hsp70 proteins in the cell. Post-translational modifications, including methylation (50), ADP-ribosylation (51), and phosphorylation (52) have been reported for in Hsp70 proteins. Phosphorylation and dephosphorylation of proteins are essential for cellular homeostasis. Ca<sup>2+</sup>-dependent autophosphorylation *in vitro* has been reported for proteins in the Hsp70 family (53). The possibility that Hsp70 family proteins may be phosphorylated by various protein kinases has not been explored and the potential regulatory function of this protein after phosphorylation is also unclear. For the first time we demonstrated that the cAMP-dependent protein kinase catalyzes the phosphorylation of cardiac Hsp70 with incorporation of 0.1 mol of phosphate/mol Hsp70 (47). Increased phosphate incorporation was not observed even with the use of higher concentration

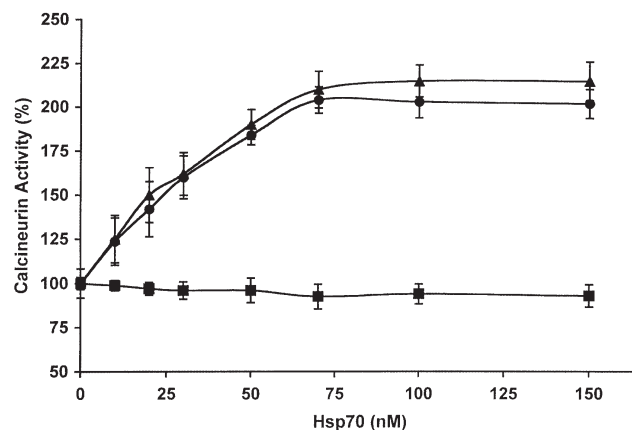


Figure 3. Effect of phosphorylation of cardiac Hsp70 on CaN phosphatase activity. Phosphorylated Hsp70 (■), non-phosphorylated Hsp70 was treated identically to the phosphorylated sample except the buffer was substituted for [γ-<sup>32</sup>P]ATP (▲) or protein kinase (●). For details see ref. 47.

of cAMP-dependent protein kinase. Furthermore, no difference in phosphate incorporation was observed either in presence of Ca<sup>2+</sup>/CaM or EGTA. A low level of Hsp70 autophosphorylation, measured in the absence of cAMP-dependent protein kinase, is shown for comparison. Phosphorylation of Hsp70 by cAMP-dependent protein kinase was further confirmed by autoradiogram studies. These results suggest that Hsp70 could be a substrate for cAMP-dependent protein kinase. Equal amount of phosphate incorporation by Hsp70 in presence of either Ca<sup>2+</sup>/CaM or EGTA indicate that phosphorylation of cardiac Hsp70 by cAMP-dependent protein kinase is not dependent on Ca<sup>2+</sup>/CaM. Furthermore, ATP/ADP bound Hsp70 was used for the phosphorylation studies. The result indicated that nucleotide additions did not alter the stoichiometry of phosphate incorporation in Hsp70.

Activation of CaN using phosphorylated Hsp70 was carried out to determine whether phosphorylated Hsp70 has any effect on CaN (Fig. 3). Surprisingly, the activation of CaN phosphatase activity was essentially eliminated by the phosphorylated cardiac Hsp70 (47). However, non-phosphorylated Hsp70 continued to show a 2-fold stimulation in CaN activity (Fig. 3). Furthermore, we also examined the interaction between phosphorylated Hsp70 with CaN. Pull-down analysis demonstrated that there is no interaction between phosphorylated Hsp70 with CaN. This result suggests that cAMP-dependent protein kinase phosphorylation provides an on/off switch for the regulation of CaN by Hsp70.

There are several types of modifications that may cause progressive charge shifts of Hsp70 proteins in the cell. Post-translational modifications, including methylation (50), ADP-ribosylation (51), phosphorylation (52) have been reported in Hsp70 proteins. Earlier, it has been reported that there is a low level of phosphorylation of Hsp22 (5 × 10<sup>-5</sup> mol of phosphate per mol of Hsp22) (54). Khan *et al* (55) reported that the stoichiometry of phosphorylation for recombinant Hsp60 was 0.5 mol of phosphate per mole of recombinant Hsp60 when phosphorylated by protein kinase A. We observed the cAMP-dependent protein kinase was found to catalyze the phosphorylation of bovine cardiac Hsp70 with an incorporation of

0.1 mol of phosphate per mol of recombinant cardiac Hsp70. The low phosphorylation of recombinant cardiac Hsp70 may have been due to the masking of the phosphorylation site during protein folding. In the presence of  $\text{Ca}^{2+}$ , *in vitro* autophosphorylation of Bip, a member of Hsp70 protein family was observed (56). However, a very low level of autophosphorylation of *E. coli* expressed bovine Hsp70 was observed. Furthermore, following phosphorylation by cAMP-dependent protein kinase, we found that Hsp70 was not able to activate CaN. We provided the first evidence that phosphorylation of Hsp70 by cAMP-dependent protein kinase can inhibit the ability of Hsp70 to stimulate CaN phosphatase activity (47). Further work will be needed to identify the cAMP-dependent protein kinase phosphorylation site on Hsp70 and the location of the domain of Hsp70 responsible for the activation of CaN.

## 6. Conclusion

Hsp(s) play an important role in various signaling process and act as central coordinators in deciding the fate of cells. Hsp(s) and CaN are now under investigation as potential targets for clinical application. Hsp70-CaN interaction and other signaling molecules which could lead to therapeutic benefit in human heart diseases such as atherosclerosis, ischemia, cardiomyopathy, and congestive heart failure.

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