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Original article

Disruption of the cyclic AMP phosphodiesterase-4 (PDE4)–HSP20 complex attenuates the β-agonist induced hypertrophic response in cardiac myocytes

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ABSTRACT

The small heat shock protein HSP20 is known to be cardioprotective during times of stress and the mechanism underlying its protective abilities depends on its phosphorylation on Ser16 by PKA (protein kinase A). Although the external stimuli that trigger Ser16 phosphorylation have been well studied, the events that modulate spatial and temporal control of this modification remain to be clarified. Here, we report that inhibition of cAMP phosphodiesterase-4 (PDE4) induces the phosphorylation of HSP20 in resting cardiac myocytes and augments its phosphorylation by PKA following β-adrenergic stimulation. Moreover, using peptide array technology, *in vitro* binding studies, co-immunoprecipitation techniques and immunocytochemistry, we show that HSP20 binds directly to PDE4 within a region of the conserved catalytic domain. We also show that FRET-based, genetically-encoded cAMP reporters anchored to HSP20 exhibit a larger response to PDE4 inhibition compared to free cytosolic cAMP reporters, suggesting that the interaction with PDE4 is crucial in modulating the highly localised pool of cAMP to which HSP20 is exposed. Using information gleaned from peptide array analyses, we developed a cell-permeable peptide that serves to inhibit the interaction of PDE4 with HSP20. Disruption of the HSP20–PDE4 complex, using this peptide, suffices to induce phosphorylation of HSP20 by PKA and to protect against the hypertrophic response measured in neonatal cardiac myocytes following chronic β-adrenergic stimulation.

1. Introduction

The small heat shock proteins (sHSPs) are a highly conserved family of molecular chaperones that are ubiquitously expressed throughout nature [1]. The expression of sHSPs [2] depends on factors such as changes in physical/chemical stress, development and pathological status [3]. Increased expression of sHSPs results in cellular protection and often allows cells to better tolerate changes to their local environment triggered by physiological stresses during disease. Of the 10 known sHSPs [4], HSP20 (also known as HspB6) has recently been described as a protective agent against a number of diseases of the brain including cerebral amyloid angiopathy [5], Alzheimer disease [6] and forebrain ischaemia [7]. A functional role for HSP20 is also well established in the context of cardiac disease, where it is regarded as acting as an innate protector during cardiac ischaemia/reperfusion [8], chronic β-adrenergic stimulation [9,10], pharmacological treatment by doxorubicin [11], vasospasm and platelet aggregation [12], endotoxin induced myocardial dysfunction [13] and congestive heart failure [14]. Functionally, HSP20 confers cardiac protection by a number of diverse mechanisms including the suppression of NF-k-B signalling [13], inhibition of caspase activity [13], promotion of autophagy [2], stabilisation of the cytoskeleton [15], reduction of myocardial necrosis and apoptosis [8], increase in myocyte shortening rate *via* increases in calcium uptake [16] and enhancement of Akt/PKB signalling [11]. Intriguingly, the cardio-protective potential of HSP20 is only realised after it has been phosphorylated on a PKA/PKG consensus motif within the N-terminal of the protein following a stimulus that increases cyclic nucleotide concentration within cells [17]. Diminished phosphorylation at Ser16 abrogates the protective effect of HSP20 [18] and overexpression of a constitutively non-phosphorylated mutant (S16A), increases susceptibility to *ex vivo* ischaemia/reperfusion injury [2], induces cell necrosis [2] and increases infarct areas [2]. Conversely, a constitutively phosphorylated mutant form of HSP20 (S16D) confers full protection from apoptosis *via* inhibition of caspase 3 [10].

PKA and PKG can, potentially, effect a myriad of processes in heart [19,20], including the cardio-protective phosphorylation of HSP20 at

Abbreviations: sHSP, Small heat shock protein; PDE4, cAMP phosphodiesterase-4; PKA, Protein kinase A; PKG, Protein kinase G; cAMP, cyclic AMP; β1-Ar, β1-adrenergic receptor; β2-Ar, β2-adrenergic receptor; ELISA, Enzyme-linked immunosorbent assay; FRET, Fluorescence resonance energy transfer; ANF, Atrial natriuretic factor.

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Ser16. This residue occurs within a classical and robust consensus site (13-RRASA-17) for PKA phosphorylation, which is of the form RRxS-hydrophobic amino acid. Indeed, there is comprehensive evidence to suggest that, in response to β-agonists, PKA is the sole kinase responsible for phosphorylation at this site. Thus, pre-incubation with either peptide [21] or pharmacological inhibitors of PKA [22,23] clearly ablate Ser16 phosphorylation of HSP20. Indeed, cell-permeable, phospho-peptide analogues that encompass either the PKA phospho-site of HSP20 (Ser16) [24] or the entire phosphorylated protein sequence [25], can mimic some of the protective functions of this chaperone. However, the functional use of these peptides is limited by the difficulties associated with their entry into cells. Treatment with the adenylate cyclase activator, forskolin, has provided a commonly employed means to induce the phosphorylation of HSP20 by PKA in order to allow evaluation of the mechanics behind this modification [21]. However, these studies have not provided detailed functional information due to the nature of the non-specific, global increases in cAMP triggered by the ensuing activation of the complete adenylate cyclase pool in cells.

Recent studies using genetically encoded cAMP FRET reporter constructs in cardiac myocytes have suggested that certain cAMP phosphodiesterase (PDE) inhibitors can raise localised cAMP concentrations in defined cellular locations, in response to different agonists [26] to affect distinct complements of cellular functions [27]. Specifically, these studies suggest a robust functional coupling of one family of PDEs, namely the cAMP specific PDE4, with the catecholamine-induced cAMP response associated with β-adrenergic signalling [28]. PDE4 is of much interest as selective inhibitors provide potential therapeutics for various inflammatory diseases [29]. Each PDE4 isoform is characterised by a unique N-terminal region that contains a targeting sequence of which one critical function is to direct them to specific intracellular locations/signalling complexes [30]. Such targeting has functional relevance in the heart where PDE4 isoforms form complexes to underpin signal specific responses. This, for example, is evident for the association of PDE4D5 with the signal scaffold, βarrestin [31], with the βarrestin–PDE4D5 complex co-ordinating the β_2-adrenergic receptor desensitisation [32] *via* simultaneous dampening of both Gs and Gi signalling [33]. PDE4D also plays a crucial regulatory role in regulating cAMP fluctuations at the sarcoplasmic reticulum, thereby directly controlling PKA phosphorylation of the ryanodine receptor [34]. Hyper-phosphorylation of this channel is known to be a predisposing factor in certain heart diseases and PDE4D3 association with this channel may provide a novel point of therapeutic intervention.

Here, we show that HSP20 sequesters PDE4 isoforms and that targeted disruption of this complex results in a hyper-phosphorylated, more cardio-protective form of HSP20, which attenuates apoptosis and negates the hypertrophic response triggered by chronic β-agonist administration.

2. Methods

Neonatal cardiac myocytes were cultured as described before [35].

2.1. Materials

PolyFect Transfection Reagent was from Qiagen. Protease inhibitor cocktail tablets were from Roche. Anti-HSP20 antibody was from Millipore (07–490), Anti-VSV antibody was from Abcam (ab1874), and Anti-phospho-HSP20 antibody was from Abcam (ab58522). Anti-tubulin antibody was from Abcam (ab18251) and anti-ANF antibody was from Santa Cruz (sc-20158). Isoprenaline was from Sigma. Protein G beads were from Amersham. ECL was from Pierce. Peptide 906 and peptide control were synthesised by GenScript, dissolved in DMSO to a stock concentration of 10 mM and used at a final concentration of 10 μM.

2.2. Generation of Hsp20 expression constructs

Ultimate™ ORF clone IOH57317 (Invitrogen), containing the open reading frame of Hsp20 in pENTR221 vector, was used to generate pcDNA6.2-Hsp20, by Gateway cloning into pcDNA6.2/V5-DEST (Invitrogen).

2.3. Generation of Hsp20-targeted cAMP FRET sensor

The Hsp20-targetted cAMP FRET probe was generated by cloning the Hsp20 open reading frame, excluding the stop codon, immediately 5′ to the EPAC1-based FRET sensor [36]. Hsp20 open reading frame was amplified from the Ultimate™ ORF clone IOH57317 (Invitrogen) using the following primers to incorporate HindIII restriction sites at both 5′ and 3′ ends: Forward 5′-gcacaagcttATGGAGATCCCTGTGCCT GTGC-3′Reverse 5′-gcacaagcttCTTGGCTGCGGCTGGCGGTGG-3′. The resulting fragment was cloned, in frame, into the HindIII site 5′ to the EPAC1-based sensor, clones were screened for correct orientation of insert and correct clones were sequenced.

2.4. Site directed mutagenesis

HSP20 mutants were constructed using QuickChange (Stratagene) and verified by DNA sequencing.

2.5. Cell culture, transfection, immunoprecipitation and Western blotting

HEK293B2 cells are a stable cell line overexpressing the GFP-tagged β_2AR were cultured as previously described [37]. HEK293B2 cells were transiently transfected with wild type HSP20 or a phosphorylation defective HSP20 (S16A) or a phosphorylation mimic HSP20 (S16D). Cell lysis, immunopurification and western blotting were done as described before by us in some detail [37]. Briefly, HEK 293 cells were transiently transfected with wild-type HSP20 by using Polyfect Transfection Reagent (Qiagen). Transfected HEK 293 cells were treated with 10 μM peptide 906 or 10 μM control peptide for 0, 15, 30, 60 and 120 min. Control and treated transfected HEK 293 cells were then washed thrice with ice cold PBS (phosphate buffered saline) before cellular lysates were prepared. Cellular lysates were prepared in lysis buffer containing 25 mM Hepes, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and Complete™ EDTA-free protease inhibitor cocktail tablets (Roche). Protein concentration of lysates was determined using the Bradford assay and all samples were equalised for protein concentration. Proteins were separated by SDS/PAGE (4–12% Bis-Tris gels) and transferred onto nitrocellulose membranes for Western blotting. Antibodies were used to detect phospho-proteins and native proteins in lysates from control and treated cells.

2.6. Phosphodiesterase assays

PDE activity was measured using a radioactive cAMP hydrolysis assay that has been described previously [38]. [8-^{3}H] adenosine cyclic-3′, 5′-mono-phosphate was sourced from Amersham Biosciences (Little Chalfont, U.K.) and cyclic-3′, 5′-mono-phosphate from Sigma.

2.7. Pull-down assays

GST-PDE4 fusions proteins (500 μl of 2 μM) were mixed with 100 μl slurry of 50% (v/v) PBS-washed Glutathione beads for 2 h at 4 °C. The beads were pelleted by centrifugation (14,000 *g*, 1 min) and washed twice with PBS containing 1% Triton X-100 before the addition of equal molar amounts of HSP20-His fusion protein in a 1-ml solution of PBS and Triton X-100 containing 5 mM dithiothreitol. After a 2 h incubation at 4 °C, the beads were collected and washed