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Heat shock protein 60 involvement in vascular smooth muscle cell proliferation

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ABSTRACT

Aim: Heat shock protein 60 (Hsp60) is a mediator of stress-induced vascular smooth muscle cell (VSMC) proliferation. This study will determine, first, if the mitochondrial or cytoplasmic localization of Hsp60 is critical to VSMC proliferation and, second, the mechanism of Hsp60 induction of VSMC proliferation with a focus on modification of nucleocytoplasmic trafficking.

Methods and results: Hsp60 was overexpressed in primary rabbit VSMCs with or without a mitochondrial targeting sequence (AdHsp60^{mito-}). Both interventions induced an increase in VSMC PCNA expression and proliferation. The increase in VSMC PCNA expression and growth was not observed after siRNA-mediated knockdown of Hsp60 expression. Nuclear protein import in VSMC was measured by fluorescent microscopy using a microinjected fluorescent import substrate. Nuclear protein import was stimulated by both AdHsp60 and AdHsp60^{mito-} treatments. AdHsp60 treatment also induced increases in nucleoporin (Nup) 62, Nup153, importin-α, importin-β and Ran expression as well as cellular ATP levels compared to control. AdHsp60^{mito-} treatment induced an up-regulation in importin-α, importin-β and Ran expression compared to control. Hsp60 knockdown did not change nuclear protein import nor the expression of any nuclear transport receptors or nucleoporins. Both heat shock treatment and Hsp60 overexpression promoted the interaction of Ran with Hsp60.

Conclusions: VSMC proliferation can be modulated via an Hsp60 dependent, cytosol localized mechanism that in part involves a stimulation of nuclear protein import through an interaction with Ran. This novel cellular signaling role for Hsp60 may be important in growth-based vascular pathologies like atherosclerosis and hypertension.

1. Introduction

Heat shock protein (Hsp) 60 is a member of a diverse family of molecular chaperones that are induced under conditions of cellular stress and injury. During stress, Hsp60 can be secreted via exocytosis [1,2] and then activates cells via toll-like receptor signaling [3,4]. Intracellular Hsp60 is located in both the mitochondria and cytosol [5]. Within the mitochondria, Hsp60 works in concert with its co-chaperone Hsp10 to ensure proper protein folding [6]. In VSMCs, both exogenous and endogenous forms of Hsp60 have been shown to induce proliferation [7,8]. Consistent with this action, circulating levels of Hsp60 and anti-Hsp60 antibodies have been associated with atherosclerosis [9–11] and have been identified in both human and mouse atherosclerotic plaques [12,13]. On the basis of these findings and others [14–16], Hsp60 has been suggested to induce cell proliferation in the vasculature. Cytosolic Hsp60 also appears to have both anti-apoptotic and pro-apoptotic functions depending on the cell type [17,18]. However, the mechanism whereby Hsp60 may be involved in apoptosis, cell proliferation and atherosclerotic plaque development remains unclear.

One mechanism by which mitogenic agents exert cell fate decisions is through modulation of nuclear protein import. Nuclear protein import is an integral cellular process mediating the translocation of cytoplasmic proteins (e.g. transcription factors) into the nucleus to turn gene expression on or off. Nuclear protein import is altered during conditions of cell growth [19]. Stress stimuli can alter the rate of nuclear protein import in VSMCs [20,21]. The possibility that Hsp60 may

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induce VSMC proliferation through a modulation of nuclear protein import is, therefore, an attractive option. Hsp60 expression levels in VMSCs have recently been correlated with changes in nuclear protein import and nuclear pore complex (NPC) expression [16]. However, the direct determination of the capacity of Hsp60 to modulate nuclear protein import has not been studied. The following study used adenovirus transfection techniques to establish if cytosolic or mitochondrial Hsp60 is critical to the induction of cell proliferation and if intracellular Hsp60 can directly induce alterations in nuclear protein import in VSMCs.

2. Methods

2.1. VSMC culture

Male New Zealand White (NZW) rabbits weighing between 2.7 and 3.0 kg (Spilak Farms) upon arrival were housed in individual cages in rooms with controlled temperatures, humidity, and a 12-h light cycle. The animal use was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (2011). The experimental protocols were reviewed and approved by the University of Manitoba Protocol Management Review committee. Rabbits were anesthetized with isofluorane (5%, in oxygen, 2 L/min) and heparinized before excision of tissue. Primary VSMCs were isolated from the aorta of New Zealand White rabbits using an explant technique as previously described [21]. To induce synchronization, VSMCs were placed in serum-free DMEM (Invitrogen, Burlington, ON) supplemented with transferrin (5μg/mL), selenium (1 nmol/L), ascorbate (200 μmol/L), and insulin (10 nmol/L) for 72 h prior to the start of experiments. Multiple batches of VSMCs were isolated from different animals and the cells from the different animals were used separately to generate sample size.

2.2. Adenoviral vectors and infection protocol

Recombinant adenovirus expressing human HSP60 (Ad-HSP60) was constructed using AdEasy Vector System (Qbiogen, Carlsbad, CA) [8]. Human HSP60 cDNA (kind gift of Dr. Radhey S. Gupta, McMaster University, Hamilton, ON) was tagged with three tandem copies of the 9-residue *HA1* epitope [22]. In order to obtain Ad-HSP60 mito$^-$, adenovirus expressing human HSP60 lacking the putative mitochondrial targeting sequence [23], the N-terminal presequence of 26 amino acids was removed and a new initiation codon as well as three tandem copies of the 9-residue *HA1* epitope was introduced by PCR reaction. The recombinant adenoviral constructs were then transfected into QBI-293A cells to produce viral particles. The titer of the viral stock was determined using the tissue culture infectious dose 50 ($TCID_{50}$) method. A successful transfer of a reporter gene into 100% of VSMCs was achieved with a MOI (multiplicity of infection) of 100 to 200 viral particles/cell after 48 h of incubation in 0.5% FBS supplemented DMEM. VSMCs were seeded at 1.5×10^5 cells/well in 6-well plates for western blot analysis, cellular fractionation and the determination of ATP levels. For the nuclear protein import assay and immunocytochemistry, VSMCs were seeded at 4×10^4 cells/well on glass coverslips in 6-well plates. Cells were transfected with AdHsp60, AdHsp60^{mito-} or AdQBI (infection control) at an MOI of 100–150 in 0.5% FBS supplemented DMEM for 48 h as previously described [16]. Increasing the MOI did increase the levels of Hsp60, however, we attempted to use doses that mimicked levels previously reported in response to infectious stimuli [8,15]. We have another adenoviral preparation that further increases Hsp60 expression (4 fold vs 2 fold) which increases factors such as PCNA and some of the other NPI proteins, however the maximal expression levels that we obtained for the AdHsp60^{mito-} construct was 2-fold [15]. Therefore, in order to insure our comparisons with the non-truncated form were close to the truncated form, as well as close to physiological changes, we limited the expression levels to a 2 fold increase.

siRNA against Hsp60 was used to reduce Hsp60 protein expression in VSMCs. Hsp60 siRNA and non-targeting siRNA was obtained commercially through Thermo Scientific. siRNA transfection of VSMCs was performed using Thermo Scientific DharmaFECT transfection reagents and protocol. In brief, siRNA was combined with liposomes in FBS-free DMEM, resulting in the formation of siRNA-containing liposome carriers. This mixture was added to 0.5% FBS in DMEM without antibiotics to become the transfection media. The transfection media was then added to VSMCs in 6 well plates, such that the final concentration of siRNA in each well was 25 nM. An siRNA concentration of 25 nM was chosen as it yielded the highest transfection efficiency in VSMCs. VSMCs were treated with Hsp60 siRNA, non-targeting siRNA (negative control), or no siRNA (control) for 48 h.

2.3. Measurement of nuclear protein import by confocal microscopy

Assessment of nuclear protein import in VSMCs was performed as previously described [20,21]. In brief, VMSCs on coverslips were transferred to a Leiden dish and maintained in a perfusate buffer at 37 °C using a microscope mounted microperfusion chamber. A micropipette was loaded with an Alexa-BSA-nuclear localizing signal (NLS) fluorescent substrate [24], and subsequently inserted into the VSMC cytoplasm using a micromanipulator. Following injection of the substrate into the cell, the micropipette was removed and images of the cell after microinjection were acquired on either a Bio-Rad MRC600 CLSM or a Zeiss Axiovert 200 scope. Images were taken with an Axiocam camera and Axiovision software of the pre- and post-injected cells to observe the rate of nuclear import of the Alexa-BSA NLS fluorescent protein for each cell over time (up to 30 min). The ratio of nuclear fluorescence to cytoplasmic fluorescence was assessed using ImageJ software.

2.4. Western blot analysis

VSMCs cells were harvested and lysed in sample buffer at 48 h. For cellular fractionation, VSMCs pooled from a 6-well plate were processed using a mitochondria isolation kit (Pierce, Rockford, IL). Proteins were separated on 9%, 12%, or 15% polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with anti-mammalian Hsp60, anti-α-Tubulin, anti-COX4, anti-Hsp70, anti-Hsp90, anti-Hsp27, anti-Hsp10, anti-proliferating cell nuclear antigen (PCNA), anti-Mab414 (Nup62), anti-Nup153, anti- importin-α, anti-importin-β, anti-Ran, anti-NTF2, and anti- total actin primary antibodies. HRP conjugated anti-mouse and anti-rabbit IgGs were used as secondary antibodies. Bands were visualized with Supersignal West Pico Chemiluminescent Substrate or Luminata Forte Western HRP Substrate and subsequently quantified by densitometry using Quantity One software (Bio-Rad). Tubulin and COX4 were used as loading controls for cytoplasmic and mitochondrial fractions, respectively.

2.5. Immunocytochemistry

For subcellular localization of Hsp60 and HA tag (adenoviral expressed Hsp60) at 48 h, VSMCs on coverslips were washed 2× with 1xPBS and incubated with a MitoTracker Red CMXRos (80 nM) (Molecular Probes, Burlington, ON) for 20 min at 37 °C and subsequently processed as previously described [21]. Anti-mammalian Hsp60 and anti-HA were utilized along with an Alexa 488-conjugated goat anti-mouse IgG secondary. Nucleus was stained with DAPI and coverslips were mounted on a microscope slide using Prolong Gold antifade reagent (Molecular Probes, Burlington, ON). Stained cells were visualized using a 100× oil immersion objective on a Nikon TE 2000s microscope and images captured using NIS Elements software (Nikon).