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A Study To Find Out The Relationship Of Hsp70 Activation And Lowered [Ca2+]i

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Abstract

Preconditioning cardioprotection is mediated by heat shock protein 70 (HSP70). Increased intracellular calcium ([Ca2+]i) causes damage, and lowering it is thought to be responsible for cardioprotection. Preconditioning of HSP70 may restore [Ca2+]i homeostasis damaged by ischemic insult by lessening the defective handling of Ca2+ at various sites, as we expected. HSP70 activation was found to be effective.

When testosterone levels and the expression of HSP70, a stress response gene, decline with age, the effects of preconditioning are reduced or eliminated. To explain the delayed cardio protection, it was hypothesized that testosterone at physiological quantities is essential for HSP70 activation during preconditioning.

This hypothesis was tested in three different ways in male SD rats: the causal link between HSP70 activity and lower [Ca2+]i overload, the effects of HSP70 activation on Ca2+ handling at diverse sites, and (iii) the involvement of testosterone in HSP70 activation and cardio protection.

Keywords: cardio protection, HSP70, ischemic, homeostasis

Introduction

A sub-lethal ischemic shock increases the myocardium's tolerance to a subsequent ischemia stress during ischemic preconditioning (IP) (Bolli, 2000). An acute phase (acute IP), lasting only a few hours, and a delayed phase (late IP), reappearing 12 to 24 hours later and lasting up to three days, are the two distinct windows of cardio protection (Bolli, 2000). A lot of research has gone into figuring out what, if anything, mediates the protective effects of IP. Present paradigm has three linear elements, including triggers (such as opioids), protein kinases (such as protein kinase C) and mediators/effectors, such as mitogen-activated protein kinases (MAPK) (e.g. heat shock proteins and KATP channels). The enhanced transcription and synthesis of various cardio protective genes and proteins in late IP serve as co-mediators for the recurrent protection 1 to 3 days later, in contrast to acute IP, which activates the existing messagers. A lot of attention should be paid to the second window's extended cardio protection, although the exact signalling pathways are still a mystery.

There is speculation that the heart "remembers" the preconditioning experience by phosphorylating an unknown protein through the protein kinase C (PKC) pathway, which keeps the KATP channel open (Downey et al., 1994). An early candidate for IP was heat shock proteins (HSPs), a collection of cardio protective proteins that are activated by various stressful stimuli (such as heat, ischemia and metabolic inhibition). One of the best-understood HSP families is the 70-kDa family, which includes HSC70 (a constitutive form) and HSP70 (a highly inducible form). Donnelly et al., 1992; Yellon et al., 1992) and heat shock (Marber et al., 1993a) protect hearts from further ischaemia/reperfusion while also inducing HSP70 production in isolated perfused hearts or intact hearts within entire animals. A few other studies have revealed a link between ischemia and reperfusion-induced HSP70 levels and reduced infarct size (Hutter et al., 1994). To show that induction of HSP70 can give protection even in the absence of any other effect, overexpression of HSP70 in transfected cardiac myocytes (Cumming and colleagues, 1996) or transgenic

mice (Plumier and associates, 1995; Trosta et al., 1998) results in cardiac protection. The results of a previous study in our lab showed that activation of HSP70 in ventricular myocytes is directly associated with preconditioning's cardio protection against simulated ischemia, and that cardio protection is abolished when HSP70 synthesis is blocked using selective antisense oligonucleotides (AS) (Zhou et al., 2001). As a result, preconditioning's delayed cardio protection is mediated by HSP70.

Heat shock transcriptional factors control the production of HSPs (HSFs). HSF1 is the most important member of the HSF family when it comes to stressor-induced HSP expression (Pirkkala et al., 2001). Heat shock element (HSE) in the promoter region of HSPs gene mediates its transcription and leads to accumulation of HSPs when exposed to stress by HSF1. Since HSF1's DNA binding is independent of its transcriptional activity (Jurivich et al., 1992), this suggests that HSF1's transactivating potency in mammalian model systems is influenced by phosphorylation.

HSPs have long been known to serve as "molecular chaperones," helping to assemble and/or repair freshly generated or damaged proteins (Benjamin and McMillan, 1998). Recent investigations have also discovered specific biological processes targeted by HSPs, including as apoptosis suppression, cytoskeleton protection, nitric oxide generation enhancement, and calcium mobilisation reduction. Though HSP70 activation delays cardioprotection during preconditioning, the signalling transduction pathways are yet unclear.

Literature Review

HSPs in Drosophila salivary glands have been studied extensively since the discovery of the first HSPs in the salivary glands of these flies in 1962. Depending on their function and size, HSPs are divided into families ranging in size from 10 to 170 kDa. Grps are a unique family of HSPs whose synthesis is boosted when glucose is depleted. Normally, HSPs are restricted to a single compartment of the cell, but during stress, they can spread to different parts of the cell. When a cell is under stress, Hsp27 and Hsp110 move to the nucleus from the cytoplasm.

The HSP70 family consists of constitutive or cognate members (Hsc70, mitochondrial Hsp75, GRP78) as well as the inducible member, Hsp72, and is the most well-studied class of HSPs (i.e. so-called HSP70 in the present study). Because they depend on ATP to function, members of this family can't regulate their own conformation or ATP binding (Parcellier et al., 2003).

Heat shock transcriptional factors (HSFs) attach to HSEs in the upstream promoter site of HSP70's gene to start its production. The number of 5'-nGAAn-3' alternating-oriented arrays is varied in the HSEs (n stands for less conserved nucleotides). HSF binding to HSE requires at least two nGAAn units organised head-to-to-head or tail-to-to-tail (Xiao et al., 1991).

The functions of testosterone in adult men are well-documented. Post-pubertal men need this to maintain sexual function, muscle strength, bone density, and mental well-being beyond puberty (Wu, 1997). In the morning and in the spring, the testosterone level in the blood is at its highest (Malkin et al., 2004). Sex-hormone binding globulin binds to testosterone in a way that prevents free testosterone from circulating in the body. The remaining minority is biologically active and referred to as the "bio-available" part, with 30% loosely attached to albumin and 3% free (Tremblay and Dube, 1974).

On average, males create 5-7 milligrammes of testosterone per day, mostly as a result of LH activation. Although only 5% of testosterone output is converted to DHT (5-reduced metabolite) or estradiol, the conversion both amplifies and diversifies the action of testosterone by activating oestrogen receptors, despite the fact that DHT has a higher molar potency.

This gender disparity narrows after the menopause, which is well-known for its effect on age-adjusted

mortality and morbidity rates due to coronary artery disease (CAD) (Lerner and Kannel, 1986). Aside from that, the gender gap persists despite substantial variations in absolute CAD mortality rates and dietary habits among 52 different nations (Kalin and Zumoff, 1990). Recently, this male preponderance in CAD was mostly seen as an absence of estrogen-mediated cardioprotection, and little thought was given to testosterone's role in the aetiology of CAD.

There has been no evidence that combined estrogen/progestin therapy in menopausal women improves cardiovascular health in large, randomised clinical trials (Hulley et al., 1998; Rossouw et al., 2002). Furthermore, two large, well-conducted trials have demonstrated that oestrogen medication in men increases the risk of cardiovascular disease and death (1967; 1973). In fact, the oestrogen protection concept is dubious because there is no break-point at the projected age of menopause, in contrast with biological markers like breast cancer or bone density that are clearly estrogen-dependent (Liu et al., 2003). As an added bonus, the gender-based disparity is consistently 2.5-4.5:1, whereas ethnic/geographic disparity is much greater, with rates of CAD 5-10 times higher in eastern/northern Europe than in southern/southern Europe and Japan, indicating that other risk factors acting on both men and women are more important than the gender effect (Wu and von Eckardstein, 2003). The fact that the gender disparity narrows after middle age backs up this claim (Barrett-Connor, 1997). As a result, we must reexamine the significance of sex hormones, particularly testosterone, in the development of coronary artery disease.

Research Gap

Researchers found that preconditioning ventricular myocytes with metabolic inhibition or U50,488H delayed cardio protection and reduced [Ca2+]i overload in the presence of MeI/A. This cardio protection was accompanied by an increase in HSP70 expression, and blocking HSP70 synthesis eliminated the preconditioning effect completely. However, the findings show for the first time that HSP70 triggered by preconditioning is responsible for restoring the [Ca2+]i level after ischemic shock, which confirms the involvement of HSP70 in delayed cardio protection. HSP70 may be involved in the delayed cardio protection of preconditioning by restoring calcium homeostasis, as [Ca2+]i excess is widely considered a precursor to cell death.

Hyperthermia-induced infarction in intact rat hearts was found to be reduced by left coronary artery closure, and this was found to be associated with high levels of HSP70 expression (Donnelly et al., 1992). In awake rabbits, IP reduced infarction and arrhythmias while increasing HSP70 expression (Yang et al., 1996). HSP70 activation may provide cardioprotection, according to these findings. HSP70 preconditioning is controversial due to the lack of increased protein expression in rabbits preconditioned with adenosine (Bernardo et al., 1999) or the time delay between HSP70 expression and cardio protection (Bernardo et al., 1999). (Yamashita et al., 1997). We found that preconditioning with metabolic inhibition or U50, 488H delayed cardio protection and increased HSP70 expression, and that blocking the production of HSP70 with a specific AS eliminated the protection in a prior work in our lab (Zhou et al., 2001). The findings show without a doubt that preconditioning cardio protection is mediated by HSP70.

Research Objective & Methodology

Collagenase perfusion was used to isolate cardiac ventricular myocytes (Wu et al., 1999). Trypan Blue was unable to pass through the majority of the cells because they were rod-shaped and impenetrable. They were kept alone for at least 30 minutes to allow for stabilization before tests. For 30 minutes, the cells were cultured in a 100 mm culture dish. In 5 ml of MEM, the suspended cells were then suspended in Ca2+-free MEM containing 1.25 mM Ca2+, 5 percent FBS and insulin at 5 g/mL, apo-transferrin at 5 g/mL, and streptomycin at 100 g/mL, before being plated at a density of 3x10-5 cells per well on six-well plates.

A glucose-free Krebs solution was used to simulate ischaemia, and ventricular myocytes were treated for 10 minutes with an inhibitor of glycolysis, 10-mM 2-deoxy-D-glucose (2-DOG), and 10-mM sodium dithionite (NaS2O4), an oxygen scavenger that induces MeI/A. (Ho et al., 2002). After that, the myocytes were perfused for ten minutes with regular Krebs solution (RE).

ventricular myocytes were pretreated for 30 minutes with either a selective agonist for the OR receptor, 30 M U50,488H (UP), or metabolic inhibition with a glucose-free Krebs buffer at pH 6.5, containing lactate and 2-DOG, to induce preconditioning before being subjected to MeI/A for 20 or 23.5 hours. During the pre-treatment phase, 5 M nor-binaltorphimine (BNI), a selective -OR antagonist, was delivered at 5 min intervals to determine whether or not the effects were -OR mediated. The doses were selected based on findings from earlier research (Wu et al., 1999; Zhou et al., 2001).

CellTiter-Blue Reagent (Promega, Madison, WI) was also used to test for viability because it contains highly purified resazurin (dark blue). Resazurin can be converted into the pink and extremely luminous resorufin by living cells. The indicator dye is not produced by nonviable cells, which soon lose their metabolic capacity. Sample absorbance was determined at 570 nm using a reference wavelength of 600 nm. There was no difference in absorbance between the culture medium without medications and the culture medium that did include pharmaceuticals. There were three copies of each sample analysed.

Injured cells secreted LDH, which was employed as a marker for cell injury. Using a UV-Rate Test Kit, we ran an enzyme activity spectrophotometric test (Stanbio Lab, Boerne, Texas). LDH is responsible for catalysing the oxidation of lactate to pyruvate, which results in the reduction of NAD to NADH as a byproduct. As LDH activity increases, so does the rate at which NADH develops. The increase in the supernatant's NADH absorbance at 340 nm (A/min) was measured at the conclusion of the experiments. Three copies of each sample were used in the testing process. LDH activity was then computed using the method (A/min*3.376) to arrive at a value of U/ml.

The Ca2+ indicator Fura-2/AM was utilised as the spectrofluorometric spectrofluorometer to measure [Ca2+]i. After 30 minutes of incubation with 5 M Fura-2/AM, the unincorporated Fura-2/AM was washed three times from the ventricle myocytes. When they had stabilized for 30 minutes, mycoytes were transferred to a bathing chamber perfused with a Krebs-bicarbonate buffer containing (in mM): 118 sodium chloride, 5 potassium chloride, 1.2 magnesiosodioxane, 1.25 calcium chloride, 25 sodium hydroxide and 11% glucose, and a gas phase of 95% oxygen-50% carbon dioxide, pH 7.4. Once the experiment had stabilized for 15 minutes, one rod-shaped, quiescent myocyte with distinct striations was chosen. A dual-wavelength excitation spectrofluorometer (Photo Technical International) recorded fluorescent signals at 340 nm (F340) and 380 nm (F380) excitation wavelengths during MeI/A and reperfusion. Cellular [Ca2+]i was quantified by looking at the F340/F380 ratio in a ventricular myocyte sample. Caffeine-induced [Ca2+]i transients (C[Ca2+]i) were measured by administering 10 mM caffeine directly to the ventricular myocyte to measure electrically induced [Ca2+]i transients (E[Ca2+]i). As the difference between the resting and peak [Ca2+]i levels was utilized to calculate the amplitude of E[Ca2+]i or C[Ca2+]i, the decay of both transients could be quantified using t-50.

Single myocytes were photographed with a laser-scanning confocal microscope outfitted with the rhod-2 fluorescent probe to monitor [Ca2+]m (Fluoview FV300; Olympus). A single net positive charge on Rhod-2 increases the electrophoretically observed accumulation of Rhod-2/AM in mitochondria because the inner mitochondrial membrane is extremely negatively charged. Rhod-2/AM enters the mitochondrial matrix and is cleaved by mitochondrial esterases into rhod-2 free acid and free [Ca2+]m (Product information, Molecular Probes). After a 20-hour culture, cells were treated for 2.5 hours in a 37°C incubator with 10 M rhod-2/AM, which had been converted by sodium borohydride into nonfluorescent dihydrorhod-2/AM

beforehand, as previously described with a few changes (Murata et al., 2001). Dihydrorhod-2/AM easily oxidises back to Ca2+-dependent fluorescent rhod-2/AM in mitochondria due to mitochondria's strong oxidative capacity. As a result, this procedure makes it easier for rhod-2 to accumulate in the mitochondrial matrix.

With a laser excitation of 543 nm, the Ca2+-sensitive fluorescent indicator Rhod-2 was excited and 1 minutes worth of time-lapse confocal pictures (10241024 pixels) was taken. Fluoview software was used to analyses the results of the experiments (version 4.2, Olympus). [Ca2+]m was calculated as a ratio of the measured rhod-2 fluorescence intensity to the initial intensity.

LDH release was also measured with the use of a Cytotoxicity Detection Kit (Roche, Indianapolis, United States) technique. Formazan (red) formation in the test correlates directly with enzyme release. At a wavelength of 490 nm, the absorbance of the samples was measured. Percentages of total LDH release were calculated by adding 2% Triton X-100 to untreated cells. In either the culture media alone or the medium including medicines, no substantial LDH release was seen. There were three copies of each sample analyzed.

For the HSP70, HSC70, and HSF1 expression test, myocytes or left ventricle tissues were used. In this experiment, myocytes were first treated with 50 mg/ml of Tris-HCl (pH 7.4), 1 percent NP-40, 150 mg/ml of NaCl, 1 milligramme of EDTA, 1 milligramme of phenylmethylsulfonyl fluoride (PMSF), 1 milligramme of aprotinin, and 1 milligramme of leupeptin in 0.5 ml of lysis buffer before being sonic A tiny amount of left ventricle tissue was chopped and homogenised with a Polytron PT 35 homogenizer for 30 seconds in lysis buffer (PBS with 1% Nonidet P-40, 0.5 percent sodium deoxycholate, 0.1 percent SDS) before being used. A 12,000-g centrifuge was used for 10 minutes at 4°C to separate the samples, which were then chilled to -70°C for storage. The Bio-Rad protein assay, based on the Bradford dye-binding method and using BSA as the standard, was used to measure the protein concentration.

Data Analysis & Findings

End-diastolic pressure (LVEDP) was set at 4-8 mmHg after the balloon was placed into the left ventricle. A PowerLab/4SD analog-to-digital converter constantly monitored cardiac parameters such as heart rate (HR), left ventricular developed pressure (LVDP), and contraction and relaxation velocities (dp / dtmax) (AD Instruments, Australia). The amount of coronary effluent collected allowed researchers to calculate the coronary flow rate (CF).

Nine weeks following gonadectomy, blood samples were obtained according to the manufacturer's instructions for the measurement of serum total testosterone levels using a commercially available RIA kit (Diagnostic Products, Los Angeles, CA). Part of the experiment was carried out with the assistance of Ms. Tsang S.

All results were presented as mean standard error of measurement (SE). To see if the mean values from different studies differed, a one-way ANOVA was used, followed by Newman-Keuls multiple comparison tests. In order to be included in the study, a difference of P0.05 was required.

Conclusion

After preconditioning, the researchers discovered a new connection between increased HSP70 expression and decreased [Ca2+]i overload. This was a groundbreaking discovery. HSP70 may mediate delayed cardio protection by restoring calcium homeostasis that has been disrupted by ischemic shock, as [Ca2+]i excess is a triggering cause of cell damage and death.

Preconditioning recovers the [Ca2+]i homeostasis disrupted by ischemic insult by acting at various Ca2+

handling sites, thereby providing delayed cardio protection via activating HSP70. Furthermore, preconditioning of HSP70 in males necessitates physiological levels of testosterone.

To summarize, activation of HSP70 confers delayed cardio protection while increasing Ca2+ release by RyR and speeding up Ca2+ uptake via SERCA and Ca2+ elimination via NCX in rat cardiomyocytes, as shown in this work. Ca2+ homeostasis is restored and [Ca2+]i overload is lessened as a result of these activities. To our knowledge, this is the first time that activation of HSP70 has been shown to mitigate the effects of [Ca2+]m overload.

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