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REGULATION OF INFLAMMATORY TRANSCRIPTION FACTORS BY HEAT SHOCK PROTEIN 70 IN PRIMARY CULTURED ASTROCYTES EXPOSED TO OXYGEN–GLUCOSE DEPRIVATION

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Abstract—Inflammation is an important event in ischemic injury. These immune responses begin with the expression of pro-inflammatory genes modulating transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and signal transducers and activator of transcription-1 (STAT-1). The 70-kDa heat shock protein (Hsp70) can both induce and arrest inflammatory reactions and lead to improved neurological outcome in experimental brain injury and ischemia. Since Hsp70 are induced under heat stress, we investigated the link between Hsp70 neuroprotection and phosphorylation of inhibitor of κB (IκB), c-Jun N-terminal kinases (JNK) and p38 through co-immunoprecipitation and enzyme-linked immunosorbent assay (ELISA) assay. Transcription factors and pro-inflammatory genes were quantified by immunoblotting, electrophoretic-mobility shift assay and reverse transcription-polymerase chain reaction assays. The results showed that heat stress led to Hsp70 overexpression which rendered neuroprotection after ischemia-like injury. Overexpression Hsp70 also interrupts the phosphorylation of IκB, JNK and p38 and blunts DNA binding of their transcription factors (NF-κB, AP-1 and STAT-1), effectively downregulating the expression of pro-inflammatory genes in heat-pretreated astrocytes. Taken together, these results suggest that overexpression of Hsp70 may protect against brain ischemia via an anti-inflammatory mechanism by interrupting the phosphorylation of upstream of transcription factors.

Key words: ischemic injury, inflammation, 70-kDa heat shock protein, transcription factors, phosphorylation.

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Abbreviations: AP-1, activator protein-1; BSS, balanced salt solution; ELISA, enzyme-linked immunosorbent assay; EMSA, Electrophoretic-Mobility Shift Assay; Hsp70, 70-kDa heat shock protein; IκB, inhibitor of κB; IL-1β, interleukin-1 beta; JAKs, Janus kinases; JNK, c-Jun N-terminal kinases; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor-κB; OGD, oxygen and glucose deprivation; RT-PCR, reverse transcription-polymerase chain reaction; STAT-1, signal transducers and activator of transcription-1; TNF-α, tumor necrosis factor alpha.

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INTRODUCTION

Cerebral ischemia results in a number of hemodynamic, biochemical, and neurophysiologic alterations that can be clinically linked to behavioral and pathologic disturbances. With declining blood flow, neuronal activity is affected first, and as ischemia progresses, metabolic activity is suppressed in order to maintain the structural integrity of the brain cells (Hossmann, 1998). These events lead to glutamate-mediated excitotoxicity, calcium overload, oxidative stress, stress signaling, inflammation and cell death (Mehta et al., 2007).

Inflammatory events initiated at the blood-microvessel interface a few hours after the onset of ischemia mark the transition from ischemic to inflammatory injury. In the inflammatory response, major players are cytokines, as well as transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and signal transducers and activator of transcription-1 (STAT-1). The activation of NF-κB, AP-1 and STAT-1 is mediated through phosphorylation of their regulatory proteins and the activation of other kinases. These transcription factors regulate the transcription of many genes involved in immunity, inflammation, and protection from programed cell death (Ghosh et al., 1998).

NF-κB plays an important physiological and pathological role in a variety of tissues and cells, including brain cells (O'Neill and Kaltschmidt, 1997). In astrocytes, NF-κB activity is required for the inducible expression of various genes involved in post-ischemic inflammation. NF-κB complexes are mainly composed of p65 and p50 subunits (Karin, 1999; Stasiolek et al., 2000). These remain sequestered in the cytoplasm of resting cells by association with a family of inhibitor of κB (IκB) proteins. Following the appropriate stimuli, the IκB proteins are rapidly phosphorylated by the IκB kinase complex (IKK), ubiquitinaed, and degraded by the 26 S proteasome (Sun et al., 1993; Chen et al., 1995). As a result, NF-κB translocates to the nucleus where it binds specific transcription sites and promotes expression of target genes (Sun et al., 1993).

AP-1 also takes part in the regulation of several genes expressed in the brain in response to ischemic injury, including cytoskeletal proteins and growth factors that support regeneration and repair the destroyed brain tissues (Pennypacker et al., 2000; Akaji et al., 2003). AP-1 is a heterodimer consisting of proteins in the Fos and Jun families (i.e. c-Fos and c-Jun). Upon binding to

specific AP-1 site in the promoter region of target genes, this associated c-Fos/c-Jun complex enhances gene transcription including expression of diverse inflammatory proteins (Shaulian and Karin, 2001). AP-1 is thus a key player in post-ischemic events that are mediated through phosphorylation of c-Jun N-terminal kinases (JNK) signaling pathways. JNK activity leads to immediate early gene AP-1 activation in RBA-1 cells (Wang et al., 2009).

Lastly, STAT proteins are latent cytoplasmic transcription factors that become activated by tyrosine phosphorylation. Phosphorylated STAT proteins dimerize and translocate to the nucleus, where they interact with DNA-binding elements and induce transcription (Jacobson et al., 1995; Takeda et al., 1996; Monteleone et al., 2003). Prior data suggest that STAT-1 also regulates early phases of T-cell differentiation in immune cells (Afkarian et al., 2002; Neurath et al., 2002). STAT-1 is induced by activation of p38 MAP kinase under hypoxic conditions (Bode et al., 1999).

During ischemia, the 70-kDa inducible heat shock protein (Hsp70) is thought to enhance cell survival by its chaperone functions: preventing protein aggregation and facilitating the refolding of partially denatured proteins (Giffard et al., 2004; Xu et al., 2006). The neuroprotective mechanism of Hsp70 is still not completely understood, particularly in the central nervous system. Prior studies from our group have established that overexpressing Hsp70 is protective against focal and global cerebral ischemia and neurotoxicity (Yenari et al., 1998; Lee et al., 2001). One of the earliest reports also described Hsps as capable of modulating immune responses either by potentiating or inhibiting them in brain ischemia or injury (Srivastava, 2002). To better understand the mechanisms by which Hsp70 interacts with inflammatory transcription factors after ischemia, we and others have investigated how Hsp70 inhibits NF-κB's transcriptional activity by directly binding NF-kB, or how it may interfere with its inhibitory kinase (Ran et al., 2004; Zheng et al., 2008; Sheppard et al., 2014). These observations demonstrate that Hsp70 overexpression impacts inflammatory transcription factors in ischemic injury. Here we explored whether heat stress is related to the observed neuroprotection by Hsp70 overexpression, and how Hsp70 expression modulates transcription factors in an *in vitro* model of ischemic injury.

EXPERIMENTAL PROCEDURES

Animals and Primary astrocyte culture

Experiments were performed according to a protocol approved by the Yonsei University Animal Care and Use Committee in accordance with NIH guidelines. Primary cortical astrocytes were cultured from 1- to 3-day-old postnatal ICR mice and maintained in minimum essential medium (MEM, Gibco, USA) containing 10% fetal bovine serum and 10% equine serum (Hyclone, USA).

Heat Pretreatment and oxygen and glucose deprivation (OGD)

Primary astrocyte cultures were washed three times with balanced salt solution ($BSS_{5.5}$) containing 5.5 mM glucose, 116 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1 mM NaH_2PO_4, 14.7 mM $NaHCO_3$, and HEPES at pH7.4. The culture medium was then exchanged with $BSS_{5.5}$ and incubation continued at 43 °C for 30 min. The culture medium was then changed to BSS containing no glucose ($BSS_{0.0}$). Astrocytes cultures were kept in an oxygen-free chamber at 37 °C for 6 h, thus depriving them of glucose and oxygen. Cultures were then transferred to a 37 °C incubator with 5% CO_2 and reperfused with glucose at a concentration of 5.5 mmol/l ($BSS_{5.5}$) at normoxia for 24 h. All experiments were performed in triplicate.

Hoeschst-PI nuclear staining

Cell death was evaluated by staining non-viable cells with propidium iodide (Sigma, St. Louis, Missouri, USA) and living cells with Hoechst 33,258 dye (Sigma, USA). Hoeschst dye (2–5 μg/ml) was added to the culture medium, and cells were kept at 37 °C for 30 min. Propidium iodide (2–5 μg/ml) was added immediately prior to observation in an Olympus microscope equipped for epifluorescence with UV filter block. PI-positive cells were counted as dead cells (Bokara et al., 2011).

Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation was performed by following a protocol from Stressgen Biotechnologies with minor modifications. Astrocyte cell lysates were pre-cleared by adding 50-ml Protein A/G PLUS-Agarose (Santa Cruz, Dallas, Texas, USA), 2 mg of tissue lysate in 1 mL of complete RIPA buffer. Precleared lysates (200 ml) were then incubated with 2.5 mg of mouse monoclonal Anti-Hsp70 antibody (Stressgen, San Diego, California, USA) or an IgG isotype control (2.5-mg normal mouse IgG, Santa Cruz) at 4 °C overnight. The Protein A/G PLUS-Agarose was then collected and the supernatant was aspirated off by microcentrifuging the mixture for 2 min at 4300*g*. After washing all reactions five times, samples were boiled for 5 min and then microcentrifuged briefly to pellet Protein A/G PLUS-Agarose. For the transcription factor immunoblots, cytoplasmic and nuclear protein subfractions were prepared as described previously (Zheng et al., 2008). 10–20-μg aliquots of protein were run in 10% SDS–PAGE electrophoresis, then transferred to PVDR membrane (Millipore, Billerica, Massachusetts, USA), and probed for the protein of interest by incubating in mouse anti-Hsp70 (1:1000, Stressgen) and phospho-IκB (1:1000, Santa Cruz) or rabbit anti-NF-κB (1:1000, Millipore), phospho-p38 mitogen-activated protein kinase (MAPK) (1:1000, Santa Cruz), phospho-STAT-1 (1:1000, Cell Signaling, Danvers, Massachusetts, USA), phospho-SAPK/JNK (1:1000, Cell Signaling), phospho-c-Jun (1:1000, Cell Signaling), β-actin (1:1000, Sigma), and anti-histone H1 antibodies (1:1,000, Santa Cruz). The membrane was then incubated with the secondary antibody and thoroughly washed. Immunoreactive bands were visualized using SuperSignal (Thermo, Waltham, Massachusetts, USA).