The Molecular Design of Thermo tolerance: MAPK Signalling pathway involved in Heat stress

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ABSTRACT
Global warming is causing drastic changes in the average weather conditions. With more frequent and severe heat waves in summers, the stakes are high to adapt to survive in changing environment. In this review we discuss the Mitogen Activated Protein Kinase Kinase (MAPK Kinase) pathway engaged in thermo tolerance leading to Heat Shock Proteins (HSPs) mediated cell survival in response to hyperthermia. HSPs mediate cytoprotective response by maintaining proper protein folding, releasing survival signals and preserving cytoskeletal integrity. Role of dietary supplements and specific small molecule inhibitors are also considered which target MAPK Kinase signalling pathway and can enhance the body’s ability to adapt to temperature changes. It is important to study the cellular, molecular and physiological changes that takes place in body in response to heat stress leading to activation of Heat Shock Proteins (HSPs). HSPs in turn mediate anti-apoptotic function and lead to cell survival. We summarise the molecular pathway of HSP activation and potential drugs and dietary supplements that can help protect the cells from hyperthermia.

INTRODUCTION
Heat stroke is defined as an illness in which rise in body temperature above hypothalamus set points due to external or internal heat is life threatening as it leads to central nervous system dysfunction and multi-organ failure [1]. It is mainly caused due to environmental conditions such as increase in temperature due to global warming, microbial infection or hyperthyroidism thereby leading to thermoregulatory failure, acute phase response and expression of heat stroke proteins [1]. This heat illness occurs in a series of events which increases in severity with time. The most benign condition is occurrence of heat cramps which are involuntary muscle spasms that results due to loss of large amounts of salt and water from body [2]. Followed by heat exhaustion or heat collapse which is associated with decrease in cardiac output due to water depletion and salt deprivation resulting in weakness, headache and loss of consciousness [3]. Heat exhaustion gives way to heat stroke which is the most serious condition resulting in rise in core body temperature above 41˚C, CNS dysfunction and multi-organ failure [2]. In this review we discuss the cellular changes that takes place in body in response to heat stress leading to activation of Heat Shock Proteins (HSPs). HSPs in turn mediate anti-apoptotic function and lead to cell survival. We summarise the molecular pathway of HSP activation and potential drugs and dietary supplements that can help protect the cells from hyperthermia.

KEYWORDS
ASK-1 - Apoptosis signal regulating kinase 1
ATF3 - Activating Transcription Factor
CHIP - C-Terminus of HSP70 Interacting Protein
GSTM1-1 - Glutathione S-Transferase Mu1-1
HSE - Heat shock Elements
HSF1 - Heat Shock Factor-1
HSP- Heat Shock Protein
IL-1 - Interleukin-1 (IL-1)
MAPK - Mitogen Activated Protein Kinase
MAPKAP Kinase 2 - Mitogen Activated Protein Kinase- Activated Protein Kinase
MAPKKs - MAPK Kinases
MAPKKs - MAPKK Kinases
Thr- Threonine
TNF-α - tumour Necrosis Factor α
TPR- tetra tricopeptide repeat

Body’s response to hyperthermia
There are individuals of particular age groups, professionals, individuals belonging to certain social-economic status’s and health conditions that are more prone to heat stress. It has been observed that people above the age of 75 years have 20 fold higher rate of heat related illness. Similarly, individuals living alone, unable or unwilling to leave one’s house, residing on the top floor of the
building where it’s hot are majorly affected by hyperthermia. Mental illness, alcoholism and drug use also results in increased heat production with compromised immune system and are also pre-disposed to heat stress. There are two types of heat stroke namely the classical heat stroke also called as the non-exertional heat stroke which arises due to exposure to high external temperature and exertional heat stroke which occurs due to strenuous physical exercises as seen in athletes and soldiers [2].

Genetic factors also make individuals more susceptible to hyperthermia such as over expression of genes related to cytokine production, coagulation proteins and many regulatory factors that are involved in adaptation to heat stress [1]. But when body’s core temperature is raised in response to environmental heat, many regulatory systems are activated to maintain its temperature within normal limits. This intrinsic property of the body to maintain its temperature within normal limits called as thermoregulation [4].

In humans the thermoregulatory centre is present in hypothalamus which receives inputs from thermo receptors present in skin and mucous surfaces, spinal cord and abdominal and visceral organs. These receptors survey the temperature of the blood as it passes through the body and lead to delivery of heated blood to the body surface. This physiological response called as skin peripheral vasodilation is very pronounced and leads to increase in skin blood flow to 8L/min and approximately 50%-70% increase in resting cardiac output. The increase in body temperature also increases the amount of thermal sweating in non-humid conditions as seen in figure 1 which leads to cooling down of body surface and dissipation of about 600kcal of heat per hour [1].

Figure 1- Flow chart depicting the sequence of events that takes place when heat stress causes an increase in core body temperature. The thermoregulatory centres activation in hypothalamus causes many characteristic changes in body leading to vasodilation, decreased metabolic rate and increased sweating. Sweating activates the sweat glands thereby increasing evaporation. Vasodilation and reduced metabolic rate dissipates heat ultimately decreasing the core body temperature [5].
Exposure to excessive heat over a long period of time adapts the body in response to high external temperature. This phenomena is called as acclimatization which causes increase in cardiac output, activation of renin-angiotensin system and escalated glomerular filtration rate. These mechanisms increases salt conservation and water reabsorption through kidney thereby maintaining hydration [4].

Heat stress also leads to activation of a cellular reaction called as Acute Phase response which increases the translation of pro-inflammatory cytokines leading to fever production (Leon, 2003) that persists for at least 7-14 days. Instead of being the initial response to heat insult, fever occurs as a physiological response to stress due to complications that ensues after heat exposure i.e. due to systemic inflammatory response [2]. The inflammatory response increases the levels of pro-inflammatory cytokines such as IL-6 and TNF-α which causes high intra-cranial pressure, low cerebral blood flow and neuronal injury [1].

Prolonged heat exposure also leads to activation of thrombin-anti thrombin III complexes and fibrin monomers which are involved in coagulation. At the same time, increased levels of von Willebrand factor antigen, thrombomodulin, E-selectin and intra-cellular adhesion molecule 1 are also observed with heat stress [1].

At the same time, plasma glucose homeostasis gets disturbed, leading to hyper or hypo-glycemia due to thermal injury in liver [2]. But the most dangerous effect of hyperthermia is ischemia i.e. abrupt discontinuation of blood flow which causes the production of Reactive Oxygen Species (ROS). ROS can damage proteins, RNA and DNA which can lead to loss of K⁺ and Ca⁺ and ATPase activity that perturbs the cellular metabolism. Loss of blood flow also activate vascular endothelial growth factors in brain which can disturb the blood brain barrier. The inflammatory cytokines also lead to release of bacterial endotoxins that causes sepsis [6]. The excessive cytokines, ROS, bacterial endotoxins and production of enzymes involved in normal homoestasis can lead to multi-organ failure. Key organs that are affected in heat stroke are liver, kidney, spleen, heart, lung, small-intestine, brain and skeletal muscles. Analysis of serum levels of creatine phosphokinase, uric acid and alanine aminotransferase and aspartate aminotransferase can help determine the extent of damage in peripheral organs. Complications in G.I barrier function also takes place as large amount of blood flow is shunted to skin for heat dissipation.

resulting decrease in splanchnic blood flow causes G.I barrier dysfunction which is observed as dilation of central lacteals of intestinal villi [2].

**Gastrointestinal barrier dysfunction leading to inflammatory response**

The Gastrointestinal barrier is formed by enterocyte membranes and tight junctions in the intestinal epithelium where absorption of solutes occur by active transportation and facilitated diffusion. Mucous and tissue macrophages also contribute to providing restriction against unwanted substances from entering the intestine. These unwanted molecules such as food antigens, bile, hydrolytic enzymes and endotoxins are usually more than 150 kDa and are impermeable into intestine.

But during heat stress, GI barrier dysfunction takes place leading to increased permeability of the toxic substances inside the intestine. The major cause of this dysfunction is decrease in blood supply due to splanchnic vasoconstriction and produces hypoxia, oxidative and nitrosaline stress leading to local acidosis and ATP depletion by altering ion pump activity, opening the tight junctions, damaging enterocyte membranes and causes necrosis. The leakage of endotoxins due to hyperthermia and reduced intestinal blood flow, secrete pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α). Activated T cells and Natural Killer cells also release Interferone α. These substances affect the actin cytoskeleton that regulate tight junctions’ permeability [7] as shown in Figure 2.
Disseminated intravascular coagulation and haemorrhage are observed just hours after the heat stroke followed by respiratory distress syndrome, rhabdomyolysis in muscle cells, hepatic failure and acute renal failure thereby causing multi organ dysfunction. It has been observed that the end stage organ failure is initiated by GI barrier breakdown and endotoxemia [7] as seen in figure 3. This observation makes the GI barrier dysfunction a major site of regulation and therapeutic action.

**Heat Shock Proteins to the rescue**

Free radicals and inflammatory cytokines damage the macromolecules such as lipids, DNA and
proteins due to which it is paramount for the cells to initiate a defence mechanism to protect its cellular function. Heat Shock Proteins (HSPs) are the strategic defence proteins that are positioned in the cytoplasm, mitochondria, peroxisomes and endoplasmic reticulum to provide an organised network of quality control. Heat shock proteins are classified according to their molecular weight such as HSP 27, HSP 47, HSP 60, HSP70, HSP 90 and HSP110. They are under the transcriptional control of Heat Shock Factor-1 (HSF-1) and play an important function to ensure quality of cell [9].

One of the most eminent roles of chaperones is to assist in proper folding of synthesised proteins and prevent protein misfolding and aggregation under normal and stressful conditions. The partially folded or misfolded proteins tend to aggregate due to exposed hydrophobic amino acid residues in the polypeptide backbone thereby forming a less ordered, oligomeric intermediates which can be toxic for the cells leading to diseases. Together with chaperones, ubiquitin proteasome system and sumoylation machinery help to clear the unfolded or misfolded proteins to prevent aggregate formation and ultimately death [10]. Along with proper protein folding, HSPs prevent apoptosis and release survival signals. Different HSPs have been seen to regulate apoptosis by inhibiting various key factors involved in programmed cell death. HSPs can interfere with the extrinsic and intrinsic signalling pathways by preventing the activation of pro-caspases 9 and physically blocking the cytochrome c apoptotic signalling [11, 12].

One of the important families of HSPs that are involved in maintaining the cellular function is HSP70 family of proteins which interact with the hydrophobic region of extended polypeptide segment of unfolded protein for proper folding. This way they protect against myocardial ischemic damage and ameliorate organ dysfunction. At the same time it suppresses apoptotic function of c-Jun-N-Terminal kinase activity [13] and along with HSP 90 binds to Apaf 1, a component of Apoptosome to prevent caspase 9 activation and thereby cell death [11].

The activity of all HSPs is under the transcriptional control of HSF-1. HSF-1 is a transcription factor that oligomerises and binds to heat shock elements (HSE) sequence and control the expression of HSF genes [14]. It was first identified in cellular extracts from S. cerevisiae and Drosophila melanogaster and in stress conditions forms an active homotrimer by getting released from the inactive complex consisting of HSP 90, HSP 40 and HSP70 [15]. After activation it migrates into the nucleus to promote the transcription of desired genes. Studies in HSF mutant mice have also shown the importance of HSF-1 in thermo tolerance. HSF-1 plays an important role in maintaining cellular function by supressing pro inflammatory cytokines and controlling the transcription of various genes involved in cell regulation [6].

HSPs also require the help of co-chaperones to regulate their function. Co-chaperones assist in proper protein folding and HSP activation. C-Terminus of HSP70 Interacting Protein(CHIP) is one such co-chaperone. It acts as E-ubiquitin ligase to promote ubiquitylation and degradation of various substrate proteins and checks if the synthesised protein should be folded by HSPs in the folding pathway or be ubiquitylated in the degradation pathway. It’s a ubiquitin ligase activity is present in the RING finger domain at the C-terminus and helps to regulate the cellular balance [16].

**The molecular signalling in heat stress**

The Mitogen Activated Protein Kinase (MAPK) cascade are signalling pathways that convey their signal to downstream kinases by phosphorylation. Activation of three sequential protein kinases takes place which are MAPKs, MAPK Kinases (MAPKKs) and MAPKK Kinases (MAPKKKs) [17].

The signalling is initiated in response to environmental stresses such as oxidative stress, U.V. radiation, Heat stress and extracellular stresses such as osmolarity changes and inflammation. These stresses activate MAPKKKs such as ASK-1 (Apoptosis signal regulating kinase 1). ASK-1 in turn activates MAPKK 3 and MAPKK 6 or MAPKK 4 and MAPKK 7. These MAP2Ks act downstream and activate p38 and JNK respectively [18]. ASK-1 is a major regulator of signalling in response to heat stress and is regulated by self dimerisation, phosphorylation and protein-protein interaction (Hwang et al, 2004). The activation of ASK-1 is highly regulated. Phosphorylation of Threonine residue (Thr 38) within the activation loop of the kinase domain is required for its activation. At the same time homo oligomerisation of ASK-1 is also required which takes place by the coiled-coil domain in the C-terminus [19].

Downstream to ASK-1, the p38 MAPK pathway can be activated in response to Reactive oxygen species, inflammatory cytokines such as TNF-α and IL-1 which are released in response to heat stress.
This in turn initiates a regulatory signal through activation of MAP-KAP Kinase 2 (Mitogen Activated Protein Kinase- Activated Protein Kinase) present downstream. MAP-KAP Kinase 2 stimulate the phosphorylation of Heat Shock Protein 27 at the serine residue [20] thereby activating it (Figure 4).

HSP 25 is a small heat shock protein which is also termed as HSP 27 in human cells. It plays an important role in cytoskeletal remodelling by binding to cytoskeletal elements and inhibits actin polymorphism [21].

At the same time HSP 27 is anti-apoptotic in nature and directly inhibits caspase 3 and 9 to prevent programmed cell death. It also prevents the translocation of pro-apoptotic Bcl-2 family members to the mitochondria for mitochondria-mediated cell death. Being a chaperone, it promotes cell-survival functions by inducing proper folding of proteins and can inhibit the proteins present upstream of MAPK signalling [22] such as ASK-1 to inhibit the activation of pro-apoptotic proteins by ASK-1 signalling. ASK-1 can also be regulated by heat sensitive repressor Glutathione S-Transferase Mu1-1 (GSTM1-1) which binds to ASK-1 and prevent its oligomerisation [18]. Similarly CHIP which is a co-chaperone also regulates ASK-1 either by directly binding to it or indirectly by regulating HSF-1 transcription factor and HSP70 protein function [19].

HSF-1 activates and suppress the heat shock protein pathway at the transcriptional level. But its own activation is regulated by HSP 70, HSP 90 and HSP 40. HSP 70 is known to form a stable complex with the transactivation domain of HSF-1 to inhibit HSF-1 activity by preventing the recruitment of transcriptional machinery.

HSF-1 activity is itself regulated by CHIP’s tetra tricopeptide repeat (TPR) motif which regulate HSP 70 ATPase activity and substrate affinity. It has been found that CHIP regulates HSF-1-chaperone complex by inducing conformational changes leading to exposure of the region masked by HSP 70 that is required for HSF-1 activation [20].

HSF 70 and HSF-1 then inhibit ASK-1 dependent apoptosis by preventing ASK-1 dimerisation (Hwang et al, 2005). HSF-1 can also inhibit the cytokines that activate ASK-1 pathway. It binds directly to TNF α promoter and physically binds to the activator of IL-1β to suppress the expression of cytokines. HSF-1 can also activate the expression of Activating Transcription Factor (ATF3) protein which is a member of ATF/CREB family of transcription factor to prevent cytokine release to heat stress [23].
Future Prospective

With the elucidation of the pathway that is responsible for HSP mediated thermo tolerance by inhibition of ROS production, inflammatory cytokines and endotoxemia, the molecular footprints leading to evolution can be tracked. It can help us understand the role of various genes and proteins in heat stress and how they can be manipulated to inhibit heat stress related signalling. In order to prevent multi-organ damage initiated by GI barrier dysfunction, anti-oxidants and scavengers of free radicals are useful to decrease the free radicals generated during G. I. permeability leading to local and systemic response. For example, an inhibitor of Xanthine oxidase called as Allopurinol regulates the production of super oxides and hydrogen peroxide [24].

Dietary supplements with bovine colostrum and goat milk powder significantly reduce GI barrier dysfunction as they contain nutrients, antibodies, growth factors, anti-viral and anti-bacterial substances leading to protection against G.I. damage. Supplementation in the diet of rats has shown decrease in GI permeability after 7 days when the core body temperature was raised to 41.5°C as observed by [7]. But further study is required to see the effect and mechanism of these dietary supplements in humans.

It has also been suggested that glutamine can act as a fuel for enterocytes as it has been shown in rats that oral administration of 0.65 g/kg of glutamine twice a day helps to reduce GI permeability in just 5 days as it increases HSP 70 and HSF-1 expression [8].

Therapeutic targets of ASK-1 are also being screened and it has been identified that 3H-naptho(1, 2, 3-de) quinoline-2, 7-diones and its derivative ethyl 2, 7-dioxo-2, 7-dihydro 3H-naptho (1, 2, 3-de) quinolone-1-carboxylate (NQDI-1) show the strongest inhibitory effect against ASK-1. But one of the critical factors to remember is that ASK-1 regulates multiple signalling pathways. It is involved in not just activating HSPs for stress prevention but also induce cellular apoptosis, cytokine production and pathophysiological conditions such as neurodegenerative diseases. Due to which inhibition of ASK-1 may have many side effects leading to infection and pre-cancerous conditions [20].

ASK-1 is also regulated by GSTM1-1 which modulates ASK-1 activity in cells specifically in heat stress conditions. GSTM1-1 analogs may play an important role in regulating ASK-1 mediated cell survival. But further research is required in this area as little is known about the mechanism of repression by GSTM1-1 [19].

Further analysis of mechanism of action of these supplements and inhibitors is required to get a complete understanding of the pathway they might regulate to induce tolerance against heat stress. These drugs may hold a promise for novel strategies for therapeutic action against heat shock.

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