Insights into the role of heat shock protein 72 to whole-body heat acclimation in humans

Fabiano Trigueiro Amorim[1],*, Ivana T Fonseca[1], Christiano A Machado-Moreira[2], and Flávio de Castro Magalhães[1]

[1]Laboratório de Biologia do Exercício; Centro Integrado de Pesquisa em Saúde; Universidade Federal dos Vales do Jequitinhonha e Mucuri; Diamantina, Brazil; [2]Departamento de Educação Física; Universidade Federal de Juiz de Fora; Governador Valadares, Brazil

Heat acclimation results in systemic and cellular adaptions that reduce the negative effect of heat and, consequently, the risk of heat illness. Although the classical changes observed with heat acclimation lead to increased tolerance to exercise in the heat by reducing heat storage (reflected in reduced core and skin temperatures) and increasing whole-body capacity for heat dissipation (greater plasma volume, sweat output, and skin blood flow), it appears that heat acclimation also induces changes at the cellular level that might increase tolerance of the whole organism to a higher core temperature for the development of fatigue. Thermotolerance is a process that involves increased resilience to an otherwise lethal heat stress that follows a sublethal exposure to heat. Thermotolerance is believed to be the result of increased content of heat shock proteins (Hsp), specially a member of the 70 kDa family, Hsp72 kDa. In humans, we and others have reported that heat acclimation increases intracellular Hsp72 levels. This increase in intracellular Hsp72 could improve whole-body organism thermotolerance by maintaining intestinal epithelial tight junction barriers, by increasing resistance to gut-associated endotoxin translocation, or by reducing the inflammatory response. In this review, we will initially provide an overview of the physiological adaptations induced by heat acclimation and emphasize the main cellular changes that occur with heat acclimation associated with intracellular accumulation of Hsp72. Finally, we will present an argument for a role of whole-body heat acclimation in augmenting cellular thermotolerance, which may protect vital organs from deleterious effects of heat stress in humans.

Introduction

Heat acclimation or acclimatization results from chronic exposure to heat stress, generally resulting in physiological and functional changes that reduce the negative effect of heat and, consequently, the risk of heat illness (for a review see ref. 1 and 2). The classical changes observed with heat acclimation includes a series of physiological adaptions such as higher and earlier sweating and skin blood flow (for a given core temperature), lowered body temperatures (resting and exercise), improved body fluid and electrolyte balance.[3] Although heat acclimation is known to reduce heat storage by increasing body heat dissipation capacity and tolerance to exercise in the heat with less strain, it is not well investigated the impact of heat acclimation on cellular adaptation and thermotolerance in humans.[4] In isolated cells[5] and animal studies,[6] thermotolerance (also called heat

preconditioning) is characterized as a process that involves increased resilience to an otherwise lethal heat stress that follows a sublethal exposure to heat. Thermotolerance is believed to be the result of increased content of heat shock proteins (Hsp), specially a member of the 70 kDa family, Hsp72 kDa.[5] In humans, we[7-9] and others[10-16] have reported that heat acclimation increases intracellular Hsp72 levels. This increase in intracellular Hsp72 could improve whole-body organism tolerance to exercise in hot environment (mirroring thermotolerance) by maintaining intestinal epithelial tight junction barriers, by increasing resistance to gut-associated endotoxin translocation, or by reducing the inflammatory response. This review will initially provide a brief overview of the physiological adaptations induced by heat acclimation, and subsequently emphasize the cellular changes that occur with heat acclimation associated with intracellular accumulation of Hsp72. Finally, this review will present an

argument for a role of whole-body heat acclimation in augmenting cellular thermotolerance, which may protect vital organs from deleterious effects of heat stress in humans.

Methods

A literature search was done employing the PubMed and MEDLINE database using a combination of the following keywords: "heat acclimation," "cellular adaptations," "heat shock protein," "hsp72," and "thermotolerance." Papers from the authors personal collections were included if they suited the search parameters. Inclusion and exclusion criteria for manuscripts were defined before the search was conducted. This review will address only human heat acclimation studies.
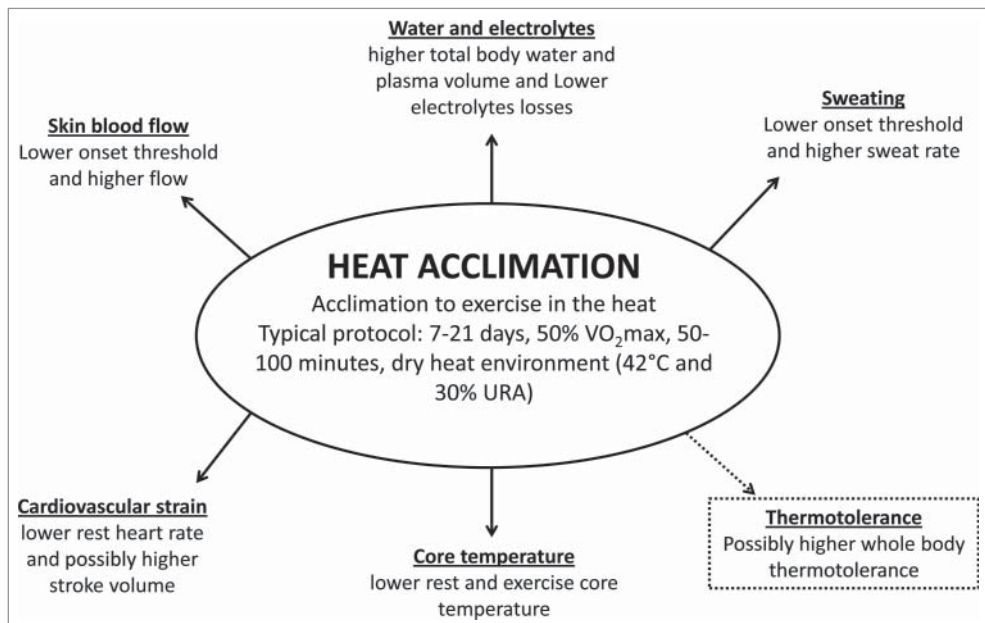
Heat acclimation

Heat acclimation or acclimatization is a process of adaptation resulting from frequent, continuous or intermittent heat exposures, in natural or artificial environments (e.g., controlled laboratory setting), respectively, which cause transient increases in core and skin temperatures and promote moderate to profuse sweating.[17] Heat acclimation can be induced by passive exposure to hot environments, or by exercise in hot environments.[18] The magnitude of heat acclimation adaptations depends on environmental characteristics (dry versus wet heat), intensity, duration, frequency, and number of heat exposures.[3] Protocols involving repeated exposure to moderate intensity exercise in the heat are often used[1] and thus in this review we will refer to this process as acclimation to exercise in the heat.

Heat acclimated humans have a higher tolerance to exercise in the heat usually expressed by a series of physiological changes: 1) lower cardiovascular strain, indicated by reduced heart rate for a given workload[17,19,20]; 2) cutaneous vasodilatation begins at a lower core temperature threshold, and skin blood flow is higher for a given core temperature, which may enhance core-to-skin heat transfer[21]; 3) sweating begins at a lower core temperature threshold and there is an increase in sweat rate per degree rise in core temperature, increasing the potential for evaporative heat loss and reducing core and skin temperatures[17,21]; 4) less accumulation of body heat during exercise performed at the same relative intensity and environment before acclimation[1,17,20]; 5) a possible increase in stroke volume[17,23]; 6) an increase in plasma volume[24]; 7) a decrease in sweat sodium concentration loss[17,20] (**Figure 1**). Together all these adjustments increase exercise tolerance in the heat. For a detailed review on the effects of heat acclimation on exercise performance in the heat as well as on the physiological mechanisms involved (for a review see refs. 1, 2, and 25).

However, less investigated is the change induced by whole-body heat acclimation to tolerate higher core temperature during exercise, postponing heat-induced fatigue. The only study[23] that investigated the effects of heat acclimation on final core temperature observed that heat acclimation did not change the final core temperature (~39.8°C) when endurance trained athletes reached voluntary fatigue. The authors justified that high core temperature was the critical factor for fatigue in heat stress, both before and after heat acclimation. However, it is worth observing that there was no reduction in muscle or skin blood flow, lactate concentration or ability to recruit motor units measured at the point of fatigue during a maximal voluntary contraction test. Additionally, the final heart rate was lower after (153 ± 6 bpm) than before (164 ± 6 bpm) heat acclimation. It might be speculated that the lack of motivation associated with heat stress could have induced a prematurely exercise termination and not core temperature.

Although it is not clear, heat acclimated subjects may tolerate higher core temperature, delaying heat-induced fatigue development and thermal injury.[3] Whereas untrained subjects seem to tolerate lower levels of exercise-induced hyperthermia and develop fatigue symptoms with an average core temperature around 38–39°C,[31] highly trained individuals (supposedly also heat acclimated) may tolerate core temperature around 40°C, with individual values over 41°C being reported in the literature.[28,32] Our speculation is

Figure 1. Summary of physiological adaptations induced by heat acclimation.

based on previous reports, in which individuals who frequently perform exercise in the heat (assumed as heat acclimated), for example marathon runners and cyclists athletes, can achieve and tolerate higher core temperatures ($>41°C$) with no symptoms of heat illness.[26-30] This capacity is not only related to high heat dissipation and lower heat storage, but it appears to result from additional adaptations that allow cells and tissues to function with high internal temperatures, resembling the experimental thermotolerance observed in cell studies.

Intracellular heat shock proteins

On a cellular level, temporary modifications in gene expression to cope with stress (heat, oxidative stress and hypoxia) have been attributed to HSP. The HSP are a highly conserved group of proteins expressed in both prokaryote and eukaryote organisms. These proteins are classified by their molecular weight (ranging from 27 to 110 kDa) and grouped into families (for a review see ref. 33). They are found in different cellular compartments and play key roles in physiological conditions and cellular stress, involving both individual cells[4] and the whole-body organism.[34] In response to cellular stressors, such as direct effect of heat, HSP gene transcription is activated and intracellular levels of HSP accumulate. Although the precise function of each HSP family member is still being uncovered, some studies have shown that HSPs are involved in many regulatory pathways (protein trafficking across cell membrane, cell apoptosis prevention, and immune function) and behave as molecular chaperones for other cellular proteins.[33]

The 70-kDa-family (HSP70) consists of proteins with a molecular mass of 72, 73, 75, and 78-kDa. The 72-kDa family member (Hsp72) is the most heat sensitive and highly inducible HSP. In unstressed conditions, heat shock transcription factor (HSF-1) is maintained in a monomeric state, attached to Hsp72 in the cytoplasm. In stressful conditions, such as heat shock, thermally denatured and malfolded proteins can accumulate in the cytoplasm, inducing the dissociation of the HSF-Hsp72 complex. The free Hsp72 then binds to the denatured proteins and facilitates refolding to restore cellular homeostasis by maintaining the cytoskeletal structure and ensuring cell function and survivability. The unbound HSF-1 monomer will trimerize, be phosphorylated, and migrate to the nucleus where it will attach to the Heat Shock Element (HSE) located in the promoter region of heat shock protein genes, leading to increase in transcription and expression of mRNA of heat shock genes. Protein translation will increase intracellular levels of Hsp72.[35] This increase in intracellular concentration of Hsp72 can result in a state of thermotolerance, which has been described as the ability to induce transient resistance to an otherwise lethal heat stress. The thermotolerance process consists of first exposing a cell or whole organism to a sublethal heat shock, which makes it tolerant to a second otherwise lethal heat exposure. This thermotolerant state correlates with the level of intracellular Hsp72.[5] Interestingly, the induction of Hsp72 is associated with an increased resistance to other

stressors as well, including hypoxia, acidosis, ischemia-reperfusion, and reactive oxygen species.[33]

During extreme heat stress it is proposed that heat exposure inhibits gene expression, altering transcriptional and translational kinetics in the cell.[36] Also proteins are partially denatured, exposing hydrophobic sites, which interact to form insoluble aggregates.[37] It is suggested that thermotolerant cells present an accelerated translational activity, increased Hsp72 synthesis, and a more rapid recovery to the normal $37°C$ translational pattern, as compared to non-heat tolerant cells.[35] Liu et al.[38] demonstrated that the expression of human Hsp72 in Rat-1 fibroblast cells facilitates the ability of these cells to recover from heat-induced inhibition in protein and RNA synthesis. After heating the cells to $45°C$ for 25 min, the time required for the recovery of normal RNA and protein synthesis was considerably shorter in cells with higher levels of Hsp72 compared to control cells.

Although Hsp72 is known to function as a protein chaperone, studies have shown that Hsp72 also can inhibit cellular apoptosis.[39,40] In this regard, Hsp72 inhibits caspase-dependent and caspase-independent apoptotic stimuli. Hsp72 has been reported to block stress kinases, including JNK, the formation and activation of the Apaf-1 complex and, the subsequent activation of caspase-9.[40] Li and colleagues[41] investigated the effect of Hsp72 on apoptotic processes using heat shocked cells ($42°C$ for 30 min) and transfected cells over-expressing Hsp72 during in vitro experiments. After a lethal heat shock treatment ($45°C$ for 60-80 min), the authors observed that Caspase-3 cleavage and DNA fragmentation were detected in cytosolic fractions from control cells, but not from thermotolerant cells or gene-transfected cells. Moreover, the addition of purified recombinant Hsp72 to normal cytosolic fractions prevented caspase-3 cleavage and DNA fragmentation, suggesting that Hsp72 prevents apoptosis upstream of caspase-3 processing. The authors concluded that Hsp72 acts as a strong suppressor of apoptosis acting downstream of cytochrome c release and upstream of caspase-3 activation.

Induction of Hsp72 may also alter proteins and genes recognized to be involved in inflammatory responses. The nuclear factor kappa B (NF-κB) transcription factor is involved in immune and inflammatory responses, altering the expression of cytokines, chemokines, cell adhesion molecules, growth factors, and immunoreceptors.[42] Inactive NF-κB is normally found in the cytoplasm bound to its inhibitory protein, IκB (Inhibitor kappa B). NF-κB is activated by a number of incoming signals from the cell surface, including ischemia oxidative stress, and endotoxin exposure.[40] These signals lead to activation of IκB kinase, which phosphorylates IκB, allowing NF-κB to translocate into the nucleus and bind to its target genes. The targeted genes include those that activate the inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), chemokines, and inducible nitric oxide synthase. It has been speculated that Hsp72 could interact with NF-κB inhibitor protein, IκB, and prevent NF-κB dissociation.[40] Jo and colleagues,[43] using a model of acute renal failure in rats, demonstrated that ischemia/

reperfusion–induced NFκB activation was suppressed by Hsp72 accumulation (42°C for 15 min), with a subsequent decrease in inflammatory mediators. Heat preconditioning also suppressed the accumulation of phosphorylated inhibitory IκB, indicating that Hsp72 blocked the activation of the IκB kinase complex. Induction of Hsp72 by heat shock or Hsp72 over-expression can be an important factor reducing mortality in experimental models of septic shock and heat stroke, by down-regulating expression of inflammatory genes, such as TNF-α, IL-1, interleukin 12 (IL-12), and interleukin 18 (IL-18).[44]

Cellular adaptation and heat acclimation

As noted above, intracellular Hsp72 plays a critical role in the ability of cells, tissues and whole organisms in becoming thermotolerant. The process of heat acclimation is known for reducing the risk of complications caused by heat exposure.[1] Therefore, it has been speculated that Hsp72 might be involved in the adaptations caused by heat acclimation.[4] Thus, it is surprising that although heat acclimation reduces thermal stress and avoids heat complications, and is recommended to athletes and workers from occupational activities in the heat such as miners, rural and construction workers, soldiers and firefighters, relatively few studies have investigated intracellular alterations regarding Hsp72 in response to heat acclimation in humans.[7-16] An even less studied and perhaps more relevant issue is what are the functional roles that these cellular adaptations play in response to heat acclimation.[9,13,14]

The results of the studies involving the effects of heat acclimation on intracellular Hsp72 in humans are not consistent (**Table 1**). Some studies did not observe alterations in intracellular Hsp72 after a short period of heat acclimation. Marshall et al.,[10] for example, showed no increase in intracellular Hsp72 in peripheral blood mononuclear cells (PBMC) after 2 days of heat acclimation, and Watkins et al.[12] did not observe any difference in intracellular Hsp72 in the *vastus lateralis* muscle after 7 days of 30 minutes/day of heat acclimation. However, the short length protocols, as well as the session duration, might not have been enough to induce adaptations at the cellular level. On the other hand, results from our group[7-9] and from others [11-16] have repeatedly shown that longer and more stressful heat acclimations protocols increase intracellular Hsp72 content in humans. For example, McClung et al.[11] and Yamada et al.[7] used a 10-day, fixed-intensity exercise heat acclimation protocol, and Magalhaes et al.[8] used an 11-day, controlled-hyperthermia technique heat acclimation and showed increases in intracellular Hsp72 content in PBMC and total leukocytes. Recently, Gibson et al.[45] demonstrated that 3 different heat acclimation protocols (fixed-intensity – 50% maximum oxygen consumption (VO$_2$max), continuous isothermic – targeted 38.5°C, and progressive isothermic targeted ~39.0°C) during short (5 days) and long term (10 days) heat acclimation showed similar changes in leukocyte Hsp72 mRNA expression. Therefore, it appears that to induce cellular changes related to the increased content of Hsp72 in response to

heat acclimation, longer (>7 days) heat acclimation protocols should be used.

To address the question as to whether the increase in intracellular Hsp72 content induced by heat acclimation replicate the thermotolerant state induced *in vitro* in cells, McClung et al. [11] and Amorim et al.[9] exposed PBMC before and after heat acclimation to an *in vitro* heat shock treatment (>42°C) or control temperature (37°C). Both studies showed similar results and described that Hsp72 content in the non-acclimated cells increased more after the heat shock treatment than in the acclimated ones. Similarly, Magalhaes et al.[7] described that after 11 days of heat acclimation the basal levels of Hsp72 in total leukocytes was increased and also that the increase in Hsp72 induced by a 90 minutes heat stress test was blunted after the heat acclimation protocol. Altogether, these results suggest that heat acclimation induces a state of thermotolerance involving an increase in baseline Hsp72 content, similar to that observed in cells or experimental animals.

In a more recent study, Gibson et al.[16] observed that heat acclimation increased basal Hsp72 mRNA and Hsp90 mRNA expressions and blunted the Hsp72 mRNA increase to a 30 minute hypoxic tolerance test (FiO$_2$ = 0.12; 10 min rest, 10 min of exercise at 40% VO$_2$max, 10 min of exercise at 65% VO$_2$max). Interestingly, Hsp90 mRNA increase was not different comparing pre- and post-heat acclimation to the hypoxic tolerance test. Reductions in Hsp72 mRNA in post-heat acclimation hypoxic test are compatible with the majority of studies showing that heat acclimation leads to accumulation of Hsp72 protein[7,9,11,13-15] and reduced requirements for further gene transcription and mRNA translation. Although it has been demonstrated that heat acclimation also leads to the accumulation of Hsp90 in PBMC,[11] and, as stated above, *in vitro* heat shock led to no further increase in the protein content of Hsp9010, the results from Gibson et al.[16] seem to contradict these previous results. It has been demonstrated that Hsp90 basal content was lower than Hsp72,[11] and therefore, it can be suggested that basal Hsp72 might have been sufficient to cope with the hypoxic stress post heat acclimation, while further increase in Hsp90 may have been necessary. However, differences in the methods (mRNA expression vs. protein content) also make comparisons between these 2 studies difficult. mRNA content might not necessarily reflect increased translation and augmented protein content,[46] and therefore, one cannot exclude the possibility of post-transcriptional regulation of Hsp90 mRNA affecting its translation and protein content. Another explanation for these contradictory results might stem from the difference in the stimuli used for the induction of the heat shock response. While McClung et al.[11] applied an *in vitro* heat shock to PBMC from heat acclimated subjects, Gibson et al.[16] used an *in vivo* hypoxic tolerance test. Therefore, the different nature of these stimuli (whole body hypoxia exposure versus *in vitro* heat stress) might induce different cellular responses regarding the expression of Hsp72 and Hsp90. Although it has been shown that heat shock and hypoxia/ischemia induce similar changes in expression of various genes,[47] including increases in Hsp72 and erythopoietin,[48,49] leading to

Table 1. Summary of heat acclimation studies that measured heat shock protein 72 in humans.

Study	n	Heat acclimation protocol	Tissue	Hsp72 Outcomes	Heat tolerance
Yamada et al. 2007	12	10-days of HA - 100 min walking at 56% of VO$_2$max in an environmental chamber (42.5°C, 27.9% RH)	Peripheral blood mononuclear cells	↑	Not tested
Marshall et al. 2007	7	3 days of HA – 120 min cycling at 38% of VO$_2$max in an environmental chamber (38°C, 60% RH)	Peripheral blood mononuclear cells	↔	Not tested
McClung et al. 2008	8	10 days of HA – 90 min walking (3.5 mph, 4% grade), in an environmental chamber (49°C, 30% RH)	Peripheral blood mononuclear cells	↑	Not tested
Watkins et al. 2008	10	7 days of HA –cycling at 75% of VO$_2$max in an environmental chamber (39.5°C, 27% RH)	Skeletal muscle	↔	Not tested
Magalhães et al. 2010	9	11-days of HA – 60 min walking/running (controlled hyperthermia technique) in an environmental chamber (40.0 ± 0°C, 45 ± 0 % RH)	leukocyte (mononuclear and granulocytes cells)	↑	Not tested
Amorim et al. 2011	9	10-days of HA - 100 min walking at 56% of VO$_2$max in an environmental chamber (42.5 ± 0.18°C dry bulb, 25.9 ± 0.4°C wet bulb)	Peripheral blood mononuclear cells	↑	↔
Kuennen et al. 2011	8	7 days of HA – 100 min walking (controlled hyperthermia technique), in an environmental chamber (46.5°C, 20% RH)	Peripheral blood mononuclear cells	↑	↔
Hom et al. 2012	11	11 days of HA – 90 min walking (3.5 mph, 5% grade), in an environmental chamber (33°C, 30–50% RH)	T lymphocyte cell (CD3$^+$CD4$^+$)	↔	↔
Lee et al. 2015	16	3 days of HA – 60 min cycling at 50% of VO$_2$max in an environmental chamber (40°C, 20% RH)	Monocyte cells (CD14$^+$)	↑	Not tested
Gibson et al. 2015a	24	10 days of HA – 90 min cycling at 50 and 65% of VO$_2$max in an environmental chamber (40.2°C, 39% RH)	leukocyte	↑	Not tested
Gibson et al. 2015b	16	10 days of HA – 90 min cycling at 65% of VO$_2$max in an environmental chamber (40.2°C, 41% RH)	leukocyte	↑	Not tested

the proposal of the cross-tolerance hypothesis.[50] Taylor et al.,[51] for example, showed that 10 consecutive days of hypoxic exposures (75 min at 2,980 m) increase monocyte Hsp72 expression. For a detailed review on hypoxia-heat cross-tolerance, please see Ely et al.[52]

A response unknown until recently was whether the increase in Hsp72 content could be somehow related to the whole-body systemic adaptations observed in response to heat acclimation. Kuennen et al.[13] tackled this question using a drug that inhibited the increase in Hsp72 during the heat acclimation protocol (quercetin). In that study, while in the placebo group all classical systemic adaptations were observed after the heat acclimation protocol, in the subjects treated with quercetin internal temperature during exercise was not reduced, suggesting that the inhibition of Hsp72 increase is related to this classical systemic heat acclimation adaptation. Furthermore, the authors observed that the gastrointestinal barrier permeability remained elevated and that circulating cytokines IL-6 and IL-10 levels were not altered in the quercetin group after heat acclimation. These results and the ones described previously show that heat acclimation and the

thermotolerant state share a common mechanism: the increase in Hsp72 content.

Conclusions and future directions

The studies above indicate that having a greater supply of intracellular Hsp72 available presumably allows the cell or whole-body to cope with greater stress. However, it remains to be seen whether cellular changes with heat acclimation are able to change the core temperature threshold for cellular injury observed during heat stroke in humans.

Further research is necessary to address issues related to heat acclimation, and cellular changes including Hsp72 regulation. Some key questions include: a) Does heat acclimation increase cellular resistance to stresses commonly described during heat stroke, including heat, hypoxia, cytokines, and combined effects? b) Does heat acclimation increase tolerance to higher core temperature during exercise? c) Is there a dose-response relationship for increased Hsp72 to augment benefits at the cellular, systemic

or whole body level? d) Are sex differences in temporal patterning to heat acclimation also evident at the cellular level? e) Do other heat shock proteins participate in these processes? f) Are there other cellular system related to thermotolerance affected by heat acclimation? Answers to these questions may lead to a better understanding of how internal and/or external environmental stressors might provoke cellular adaptations to improve whole-body tolerance.

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