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REVIEW

Resolution of inflammation in chronic disease *via* restoration of the heat shock response (HSR)

Helena Trevisan Schroeder[a,1] · **Carlos Henrique De Lemos Muller**[b,2] · **Thiago Gomes Heck**[c,3] · **Mauricio Krause**[b,4] · **Paulo Ivo Homem de Bittencourt Jr**[a,*,5]

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Abstract

Effective resolution of inflammation *via* the heat shock response (HSR) is pivotal in averting the transition to chronic inflammatory states. This transition characterizes a spectrum of debilitating conditions, including insulin resistance, obesity, type 2 diabetes, nonalcoholic fatty liver disease, and cardiovascular ailments. This manuscript explores a range of physiological, pharmacological, and nutraceutical interventions aimed at reinstating the HSR in the context of chronic low-grade inflammation, as well as protocols to assess the HSR. Monitoring the progression or suppression of the HSR in patients and laboratory animals offers predictive insights into the organism's capacity to combat chronic inflammation, as well as the impact of exercise and hyperthermic treatments (e.g., sauna or hot tub baths) on the HSR. Interestingly, a reciprocal correlation exists between the expression of HSR components in peripheral blood leukocytes (PBL) and the extent of local tissue proinflammatory activity in individuals afflicted by chronic inflammatory disorders. Therefore, the Heck index, contrasting extracellular 70 kDa family of heat shock proteins (HSP70) (proinflammatory) and intracellular HSP70 (anti-inflammatory) in PBL, serves as a valuable metric

Abbreviations: **AMPK**, 5'-adenosine monophosphate-activated protein kinase; **COVID-19**, coronavirus disease-2019; **COXIBs**, inhibitors of cyclooxygenase (prostaglandin endoperoxide H synthase); **CRP**, C-reactive protein; **CVD**, cardiovascular disease(s); **ER**, endoplasmic reticulum; **GSK-3β**, glycogen synthase kinase-3β; **HbA1c**, % of glycated hemoglobin $A1_C$; **HBP**, hexosamine biosynthetic pathway; **HFD**, high-fat diet; **HOMA-IR**, homeostasis model assessment-insulin resistance index; **HSF1**, heat shock transcription factor-1; **HSP**, heat shock protein; **HSP70**, the 70 kDa family of heat shock proteins; **iHSP70**, intracellular HSP70; **eHSP70**, extracellular HSP70; **HSR**, heat shock response; **HuR**, human antigen R, a.k.a. ELAV-1, for *E*mbryonic *L*ethal, *A*bnormal *V*ision, Drosophila, Homolog-Like protein-1; **IRE1**, Inositol Requiring Enzyme-1, also known as Endoplasmic Reticulum-to-Nucleus Signaling-1, ERN1; **JNK**, c-Jun *N*-terminal kinase; **NAFLD**, non-alcoholic fatty liver disease; **NF-κB**, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; **NLRP3**, NOD-like receptor pyrin domain-containing protein-3 inflammasome; **NSAIDs**, non-steroid anti-inflammatory drugs; **PBMC**, peripheral blood mononuclear cells; **PGs**, prostaglandins; **cyPGs**, cyclopentenone prostaglandins; **PERK**, *P*rotein kinase-like *ER K*inase; **SASP**, Senescence-Associated Secretory Phenotype; **SIRT1**, NAD^+-dependent deacetylase sirtuin-1; **T1DM**, type 1 diabetes mellitus; **T2DM**, type 2 diabetes mellitus; **TLR**, Toll-like receptor; **TNFα**, tumor necrosis factor-α; **UPR**, unfolded protein response

[1]0000-0003-4000-3671
[2]0000-0003-0205-7775
[3]0000-0002-1242-5423
[4]0000-0001-9814-742X
[5]0000-0003-1907-3341

* Paulo Ivo Homem de BittencourtJr
pauloivo@ufrgs.br

[a] Laboratory of Cellular Physiology (FisCel), Department of Physiology, Institute of Basic Health Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil

[b] Laboratory of Inflammation, Metabolism and Exercise Research (LAPIMEX), Department of Physiology, ICBS, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil

[c] Post Graduate Program in Integral Health Care (PPGAIS-UNIJUÍ/UNICRUZ/URI), Regional University of Northwestern Rio Grande Do Sul State (UNIJUI) and Post Graduate Program in Mathematical and Computational Modeling (PPGMMC), UNIJUI, Ijuí, Rio Grande do Sul, Brazil.

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for HSR assessment. Our laboratory has also developed straightforward protocols for evaluating HSR by subjecting whole blood samples from both rodents and human volunteers to *ex vivo* heat challenges. Collectively, this discussion underscores the critical role of HSR disruption in the pathogenesis of chronic inflammatory states and emphasizes the significance of simple, cost-effective tools for clinical HSR assessment. This understanding is instrumental in the development of innovative strategies for preventing and managing chronic inflammatory diseases, which continue to exert a substantial global burden on morbidity and mortality.

Keywords HSP70 · Heat shock response · Low-grade inflammation · Heck index of HSP70 · Type 2 diabetes mellitus

Introduction

Inflammation is a highly conserved physiological response that evolved to eliminate microorganisms or facilitate tissue recovery during an aseptic injury. While these responses are typically acute, a Western lifestyle based on insufficient physical activity and surplus energy consumption predisposes to progressive suppression of the physiological resolution of inflammation through the heat shock response (HSR). This is mainly because lifestyle-elicited energy imbalances overwhelm the endoplasmic reticulum (ER) of adipose tissue leading to ER stress and eventually to the unfolded protein response (UPR). However, the UPR has an inflammatory branch through the protein kinase-like ER kinase-elF2α-ATF4-CHOP and inositol requiring enzyme-1–c-Jun *N*-terminal kinase (JNK) pathways that relay inflammatory signals throughout the body.[1] As discussed by Schroeder and colleagues,[2,3] continuous activation of UPR downstream pathways culminates in uninterrupted activation of NOD-like receptor pyrin domain-containing protein-3 (NLRP3) inflammasome in adipose tissue, which leads to the production of activated caspase-1. Caspase-1, in turn, progressively degrades human antigen R (also known as embryonic lethal, abnormal vision, Drosophila, homolog-like protein-1), an mRNA-binding protein that is indispensable for the expression and activation of the heat shock transcription factor-1 (HSF1). Therefore, chronic ER stress of adipose tissue gradually impedes the resolution of inflammation *via* the HSR in several tissues, not just in the adipose tissue. This leads to the establishment of chronic low-grade inflammation that accompanies chronic degenerative conditions such as insulin resistance, obesity, type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD), cardiovascular diseases (CVDs), and neurodegenerative diseases (e.g., Alzheimer's, Parkinson's, Huntington's).

Although chronic degenerative diseases of an inflammatory nature take place precisely because the HSR becomes jeopardized as long as noxious stimuli (e.g., Western lifestyle) do not cease to activate NLRP3 inflammasomes, the HSR can be nonetheless re-established through strategies that are known to trigger the activation of HSF1 and 70 kDa family of heat shock proteins (HSP70) expression. If this could appear paradoxical at first glance, heat shock (HS) (i.e., fever, thermotherapy) or even exercise, by different mechanisms, are able to dismantle NLRP3 inflammasome activation, resuming the HSR and thereby relieving the above chronic inflammatory conditions.

In the present manuscript, we review current physiological, pharmacological, and nutraceutical approaches aimed at rearming the HSR in chronic inflammatory conditions. We also discuss simple and cost-effective tools used to clinically evaluate the progression or suppression of the HSR in humans and laboratory animals.

Physiological approaches

The physiological activation of the HSR primarily hinges on two key factors: proteostasis-threatening conditions and metabolic stress. These factors can be mutually exclusive, with the prevalence of one over the other dependent on a delicate metabolic balance referred to as "*caloristasis,*"[4] as elaborated upon in.[2] For instance, an increase in the bodily core temperature represents a proteostasis-threatening situation, as heat can alter protein conformation, potentially leading to the formation of toxic protein aggregates that trigger an inflammatory response. Therefore, hyperthermic treatments such as saunas or hot tub baths can robustly activate the HSR throughout the body. Additionally, metabolic signals directed toward energy-conserving pathways, such as 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) or NAD^+-dependent deacetylase sirtuin-1 (SIRT1), can also induce a genuine HSR. This is evident in the case of physical exercise, where AMPK activation may result from decreased intracellular glycogen levels, directly correlating with elevated mRNA and protein levels of HSP72.[5] AMPK activation in such cases inhibits glycogen synthase kinase-3β, which consistently and constitutively represses HSF1 activity,[6] thereby mitigating a bona fide HSR. Notably, HSR activation during

exercise is not solely reliant on increased body or muscle temperature.[7] However, if cellular metabolic sensors perceive that metabolic stress poses a greater threat to overall bodily homeostasis than the activation of the HSR to correct misfolded proteins (an energy-intensive process, as core chaperones like HSP70 or HSP90 utilize ATP to preserve protein function), AMPK may impede HSF1 activation.[8,9] In essence, whether the proteostasis-preserving HSR is triggered or not hinges on the delicate balance governing caloristasis.

With these reservations duly noted, (re)activating the HSR can often be achieved through fever, or rather, by deliberately not suppressing fever in many situations may prove beneficial.[2] Remarkably, fever adeptly orchestrates antimicrobial defenses and contributes to the regulation of inflammation and tissue healing, a phenomenon observed even in cold-blooded vertebrates, where there exists a discernible selectivity in the immune responses induced by fever. In essence, fever not only suppresses inflammation but also substantially amplifies the process of wound repair, as detailed in a recent study by Haddad *et al.,*[10] extending its effects across the entire metazoan kingdom.

Fever, in its broad sense, is thought to have evolved in modern animals approximately 600 million years ago.[11] It is not limited to homeothermic mammals and birds; many poikilothermic animals, including lower vertebrates, arthropods, and annelids, can also increase their core temperature in response to infection or injury through a variety of behaviors.[10,12] However, generating a fever is a complex response that is very metabolically costly.[13] In humans, producing fever may require a six-fold increase in metabolic rate, while maintaining core temperature at febrile levels may demand a 12% increase in metabolic rate per 1 °C increase.[11] In essence, despite the significant energy expenditure it demands from an organism, evolution has consistently favored fever as an indispensable mechanism for maintaining homeostasis. Indeed, fever, or the sustained elevation of body temperature, emerges as a last resort and a natural therapeutic tool with the potential to curtail excessive cytokine production in critically septic patients and those afflicted with chronic inflammatory metabolic disorders, as highlighted by Heck *et al.*[13] Consequently, indiscriminately suppressing fever through the use of antipyretic medications seems, to say the least, unwise. This consideration becomes even more crucial when considering the disruption caused by inhibitors of cyclooxygenase-2 (prostaglandin endoperoxide H synthase-2; COXIBs) and traditional nonsteroid anti-inflammatory drugs (NSAIDs) in the physiological resolution of inflammation orchestrated by the HSR, as expounded upon in the works of Schroeder *et al.*[2,3]

Fever works as an integrative response regulating the induction and resolution phases of acute inflammation.[10,11] A rise in core temperature of about 2–3 °C initiates the HSR,[11] which provides physiological resolution of inflammation at the same time that it impedes intracellular protein aggregation because the HSR produces anti-aggregative protein chaperones, such as HSP70.[14,15] Structural changes in the plasma membrane during the establishment of fever also participate in HSF1 activation.[16]

Because HSP70s are stress-inducible proteins activated by both hyperthermia and hypothermia,[17,18] physical approaches that can elevate local or central temperature may be employed to stimulate the HSR. As stated above, exercise is a powerful activator of HSR through mechanisms that include intracellular metabolic challenges (e.g., increase in AMP/ATP and NAD^+/NADH ratios), elevated sympathetic tonus to striated muscle, adipose tissue, and pancreatic islets, as well as slight increases in core temperature itself. This explains at least partially the well-known beneficial impacts of exercise.[19–28] Nonetheless, besides exercise, hyperthermic treatment itself has demonstrated innumerable beneficial effects for the body by reducing fat deposition, alleviating insulin resistance, and decreasing total body mass.[25]

Although hyperthermic treatment was well-known to the ancient Romans and by Hippocrates himself, the first indication for the therapeutic use of thermal therapy occurred, incredible, only in 1999, when Professor Philip L. Hooper investigated the ability of hot-tub therapy (37.8–41.0 °C water temperature with average oral temperature rise of 0.8 °C) to reduce blood glucose in diabetic humans.[29] Interestingly, Professor Hooper's primary curiosity was not the HSR itself. Instead, he and his colleagues conjectured that the effects of partial immersion in a hot tub could mimic the known beneficial effects of exercise by increasing blood flow to skeletal muscle. Actually, most of the beneficial effects of thermotherapy in the muscle are a consequence of sympathetic stimulation of the muscle vasculature (so, vasoconstriction; not vasodilation) in order to prevent a dangerous fall in cardiac output due to cutaneous vasodilation that follows immersion in hot water.[25,30] Thus, thermotherapy could in fact be of value in treating diabetic people, as one of the features of insulin resistance is the decreased expression of HSP72 in the skeletal muscle of T2DM patients.[31]

Hot tub treatment (40 °C, 30-min sessions) has been shown to improve life quality and hemodynamic function in chronic heart failure patients, including postmenopausal women.[32] In our laboratory, for instance, by using a mouse model of atherosclerosis (a typical inflammatory disease), we applied HS treatment with

Table 1
Characteristics of studies in humans employing heat therapy to increase HSP70.

Study	Sample size	Condition	Treatment	Duration (week)	Frequency (times/week)	HSP70 result	Main findings
Ref.[36]	20	Healthy young	Heat therapy	8	4–5	↑ PBMC	↓ NF-κB in PBMC, superoxide production and MnSOD
Ref.[37]	20	Healthy young	Heat therapy leg	1	6	↑ Muscle	↑ PGC-1α and mitochondrial respiratory capacity
Ref.[38]	18	Overweight adults	Hot water immersion	2	5	↓ Plasma ↔ monocytes	↓ FBG and insulin
Ref.[42]	40	Metabolic syndrome or T2DM middle-aged	MES + HS	12	4	↑Expression in monocytes	↓ Visceral adiposity, wc, BP, FPG, HOMA-IR, TNF-α and CRP. ↑ adiponectin.
Ref.[43]	60	Obese with T2DM middle-aged/elderly	MES + HS	12	2, 4, or 7	↑Expression in monocytes	↓ Visceral adiposity, wc, BMI, FPG, HOMA-IR, HbA1c, TNF-α and CRP. ↑ adiponectin.

Abbreviations used: BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; FPG, fasting plasma glucose; HbA1c, % of glycated hemoglobin A1C; HOMA-IR, homeostasis model assessment-insulin resistance; HS, heat shock; HSP70, 70 kDa family of heat shock proteins; MES, mild electrical stimulation; MnSOD, manganese superoxide dismutase; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; PBMC, peripheral blood mononuclear cells; T2DM, type 2 diabetes mellitus; TNF-α, tumor necrosis factor-alpha; wc, waist circumference.

hot water immersion (41.5 °C for 15 min) once a week over 8 weeks and observed an impressive decrease in the animal mortality rate, significant improvement of the established vascular disease, increased blood flow, amelioration of ultrasonographic parameters, along with reversal of the depressed HSR.[33] Heat treatment, even in the fever-like range, blocks the NLRP3 inflammasome-dependent senescence-associated secretory phenotype (SASP),[34] re-establishing the human antigen R-SIRT1-HSF-1 downstream pathways. Therefore, HSR, through HS may, paradoxically *a priori*, reactivate its major biochemical pathway, namely, the HSR.

Whatever the mechanisms involved, hyperthermic manipulations have been employed therapeutically in complications related to obesity and T2DM,[24–26,29,30] especially for people to whom access to physical exercise is initially difficult.[35]

While the existing body of research on HSP70 levels in individuals undergoing heat therapy is limited, these studies, as summarized in Table 1, yield promising results. In one study involving young sedentary adults who underwent 4–5 sessions of hot water immersion per week for 8 weeks, an increase in intracellular HSP70 (iHSP70) coincided with a reduction in nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family (NF-κB) activation in peripheral blood mononuclear cells (PBMCs).[36] Similarly, in a comparable group of volunteers exposed to six consecutive sessions of pulsed short-wave diathermy-induced heat stress, a significant rise in muscular HSP70 levels was observed, which correlated with enhanced mitochondrial function.[37] However, sedentary overweight adults undergoing 2 weeks of hot water immersion (a total of 10 sessions) did not experience changes in HSP70 content within monocytes. Nevertheless, they exhibited a decrease in plasma HSP70 levels concurrent with improvements in fasting glucose and insulin.[38] This observation holds significance because elevated levels of extracellular HSP70 (eHSP70) are linked to low-grade inflammation and the exacerbation of T2DM.[25,39,40] Furthermore, a study involving both older and younger vervet monkeys, subjected to biweekly thermal hydrotherapy sessions over 5 weeks, revealed a notable correlation between the elevation of HSP70 levels in skeletal muscle and a reduction in fasting glucose levels.[41] These findings raise the possibility that the beneficial effects observed in thermotherapy may extend to individuals with various chronic low-grade inflammatory conditions.

Another series of studies examined the effects of mild electrical stimulation (MES) combined with HS (MES + HS) on individuals with chronic low-grade inflammation, including those with metabolic syndrome and T2DM.[42,43] The MES + HS treatment involved placing a device with electrodes on both the front and back of the abdomen, delivering a direct current at 1.4 ± 0.1 V/cm (55 pulses/s) and heat (42 °C), each pulse lasting 0.1 ms. After 4 weeks of MES + HS applied four times a week for 60 min, the studies revealed an increase in HSP70 expression in monocytes. Furthermore, following 12 weeks of treatment, significant improvements were observed in body composition, blood pressure, fasting blood glucose levels, the homeostasis model assessment-insulin resistance (HOMA-IR) index, and inflammatory markers.[42] In another study by the same research group, they investigated the effects of MES + HS in obese individuals with T2DM over 12 weeks.[43] Participants were divided into three groups, each receiving the treatment at different frequencies: two times a week (group 1), four times a week (group 2), and seven times a week (group 3), all for 60 min per session. Following the treatment, an increase in HSP70 expression in monocytes was observed in all groups. Moreover, when comparing all groups with their baseline measurements, significant reductions were noted in visceral adiposity, waist circumference, body mass index, fasting blood glucose, HOMA-IR, % of glycated hemoglobin A1C (HbA1c), and proinflammatory markers. Conversely, adiponectin levels increased. Notably, higher treatment frequencies were associated with more pronounced reductions in HOMA-IR, HbA1c, and diastolic blood pressure, indicating an improvement in the parasympathetic to sympathetic tone.[43] In summary, these studies consistently demonstrated favorable outcomes in terms of body composition, blood glucose regulation, and inflammatory markers when applying HS to specific body regions in conjunction with MES. Additionally, this treatment led to enhanced basal expression of HSP70 in monocytes among individuals with metabolic disorders.

In addition to hot tubs, saunas represent another form of hyperthermic treatment with a proven safety profile across various clinical conditions. Beyond its metabolic benefits, sauna heat therapy has been shown to induce an anti-senescent effect on blood vessels.[30] This effect is mediated by the HSR, which leads to the disinhibition of key regulators such as AMPK, SIRT1, and endothelial nitric oxide synthase (eNOS) expressions.[44] These findings strongly imply that the decreased risk of sudden cardiac death, fatal coronary heart disease, fatal CVD, and overall mortality associated with increased sauna bathing frequency in humans[45] may be attributed to the heightened HSR achieved through heat therapy. Notably, sauna therapy is now recognized as beneficial for CVD patients by improving endothelial function[46] and overall cardiovascular health,[47,48] even among those with heart failure.[30]

While sauna is contraindicated *a priori* for patients with unstable angina pectoris and recent myocardial infarction, it is generally considered safe for most coronary heart disease patients, particularly those with a history of stable angina pectoris and a remote history of myocardial infarction.[49] Furthermore, sauna therapy has been demonstrated as safe during uncomplicated pregnancies among healthy women, as affirmed by numerous studies.[49]

Table 1 provides an overview of the outcomes from various studies investigating the effects of hyperthermic treatment on the HSR in obese, T2DM, and healthy individuals.

While sympathetic tonus to the musculature may be a principal mechanism for triggering the HSR,[25,30] many of the health-benefiting effects of heat treatment, especially when applied locally, rely on the localized production of the vasodilatory and gaseous free radical nitric oxide (NO). NO, in fact, serves as a potent activator of HSP70 expression[50–53] and the accompanying HSR because it induces mild oxidative stress, ultimately activating HSF1 through disulfide bond formation.[15,30] Consequently, this slight redox imbalance induced by NO acts as a catalyst for the HSR.[54] Furthermore, a portion of the cytoprotective and anti-inflammatory effects attributed to estrogen-mediated HSR[55–58] can be linked to estrogen's capacity to stimulate NO production in various tissues. In alignment with this concept, estrogen's protective effects against cerebral ischemia[59] and various other types of injuries[60] are primarily ascribed to its influence on HSP70 expression. An inherent deduction drawn from these observations is that enhancing the production of NO within the vascular wall may furnish a cytoprotective HSR in the context of CVD induced by diabetes, as emphasized by Hooper *et al.*[22]

Rearming the HSR through the manipulation of gut microbiota

The HSR is influenced by the gut microbiota, with a direct relationship to the production of NO, a powerful HSR inducer, as stated above. The gut microbiota has the capacity to generate NO from nitrate,[61,62] enhancing the HSR in the intestinal epithelium. However, excessive NO production in the gut can be detrimental, leading to enterocyte apoptosis and hindering epithelial restitution processes.[63] While the precise balance of NO remains uncertain, understanding the regulation of HSR at the intestinal level is crucial. This is because the gut microbiota ecosystem impacts low-grade inflammation and related chronic inflammatory diseases,[64,65] and obesity induces alterations in gut microbial metabolism linked to the proinflammatory senescence-associated secretory phenotype (SASP) and tissue senescence.[66] Furthermore, the fermentation of carbohydrate prebiotics by the gut microbiota modulates NOS/NO pathways and NO-producing bacteria, simultaneously mitigating systemic endothelial dysfunction.[67,68] Additionally, evidence suggests that the enteric microbiota is a key determinant of immunity through the modulation of HSP production in intestinal epithelial cells.[69]

Gut microbiota plays a pivotal role in regulating the immune, metabolic, and even eating-behavioral aspects of mammals,[70–74] and the *diversity* of commensal microbiota is closely tied to an organism's health.[75] For instance, in conditions such as obesity and T2DM, the interaction between gut microbiota and the host plays a pivotal role in the development of metabolic disorders. Excessive dietary fat intake, for example, heightens systemic exposure to potentially proinflammatory free fatty acids and their derivatives, elevating plasma lipopolysaccharide (LPS) levels, a phenomenon termed "*metabolic endotoxemia*."[64,65,76] Imbalances in the distribution of gut microbiota taxa further contribute to the disruption of gut barrier integrity.[76] Conversely, adherence to a Mediterranean diet has been linked to various health benefits, including reduced mortality, lower rates of obesity, T2DM, low-grade inflammation, cancer, Alzheimer's disease, and depression. Additionally, recent studies suggest that following a Mediterranean diet may delay the onset of Crohn's disease.[77]

A reduction in gut microbiota species *diversity* has been observed in obesity and T2DM,[78,79] along with an imbalance between beneficial and harmful species.[80] Such alterations can also result from pathogenic infections, as seen in COVID-19.[81] Notably, diabetic individuals exhibit an increased presence of Proteobacteria,[82–84] a pattern also associated with conditions like Crohn's disease and colitis.[85] Although Firmicutes and Bacteroidetes represent 90% of gut microbiota, the dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia.[86] In addition, the relative proportion of Bacteroidetes genera has long been known to be decreased in obese people in comparison with lean people, while this proportion increases with low-calorie diet-induced weight loss.[87]

Gut microbiota has a preponderant role in insulin resistance.[88] The composition of the microbiota is even proposed as a marker for diseases or disease stages, such as fibrosis in NAFLD patients.[89] Furthermore, probiotics, live microorganisms used to promote health,[90] have demonstrated potential benefits as adjunctive treatment of chronic inflammatory diseases. For instance, the yeast probiotic *Saccharomyces boulardii* changes gut microbiota

and reduces hepatic steatosis, low-grade inflammation, and fat mass in obese and T2DM db/db mice.[91] Probiotics are promising also for the treatment of humans with T2DM.[92]

Nutritional imbalances and shifts in gut microbiota are key contributors to obesity and insulin resistance. Dysregulation of gut microbiota due to the Western lifestyle results in defects in intestinal tight junctions, leading to chronic endotoxemia and exacerbating systemic inflammation.[76] This mechanism is intricately linked to an increase in circulating LPS, as demonstrated in the seminal work by Cani and colleagues, which revealed that a high-fat diet alters gut microbiota and leads to elevated LPS levels and bacterial translocation into the bloodstream in mice.[64,65] In addition to the presence of plasma LPS and bacteremia, this situation is unfortunate as the increased prevalence of bacterial LPS-producing microbes results in LPS-induced metabolic endotoxemia. This condition triggers obesity, insulin resistance, and diabetes by disrupting insulin sensitivity and weight control through the LPS/CD14 system.[64] Subsequent studies have consistently reaffirmed these findings.[91,93,94]

Alterations in intestinal barrier permeability and heightened nutrient availability[95,96] are also involved. Dietary fat directly diminishes the distribution and expression levels of tight junction components, including occludin, E-cadherin, claudin, and junctional adherens molecule, leading to the disruption of intestinal permeability.[97] Furthermore, high intake of γ-linolenic (ω-6) and docosahexaenoic (ω-3) acids, typical in the Western diet, may also modulate intestinal permeability by stimulating intracellular signaling pathways *via* protein kinase C pathways.[97] Altogether, these factors contribute to low-grade inflammation, fostering insulin resistance independently of weight gain.[98] LPS activates toll-like receptor 4 (TLR4), triggering the JNK pathway, which can obstruct insulin activation downstream to insulin receptor phosphorylation.[99] Gut microbiota imbalances may also induce insulin receptor *S*-nitrosation by boosting inducible NOS activity, which is NF-κB-dependent.[100,101]

To address this inflammatory condition, the preservation of the intestinal barrier integrity is paramount, and the HSR appears to play a pivotal role. HSF1 directly promotes the expression of the tight junction protein occludin, ensuring a secure seal and integrity of the intestinal epithelial barrier. This occurs through direct binding to its motif in the occludin promoter region.[102] Consequently, activation of HSF1 during thermal stress within the fever-like range serves as a protective measure against heat-induced disruption of the intestinal tight junction barrier. Conversely, compromised HSF1 activity in T2DM contributes to gut-derived endotoxemia.

Strategies aimed at elevating intestinal HSR and HSP production have been proposed as therapeutic interventions against gastrointestinal toxicity.[103] Alterations in the normal gut microbiota influence mucosal HSP72 expression and may render the organism more susceptible to harmful agents, such as *Clostridium difficile* toxin A.[104] Perturbations in microbiota composition or immune status can contribute to pathogenic processes causing localized intestinal injury.[105,106] Notably, an increase in HSP70 within intestinal barrier cells has demonstrated protective effects in cases of colitis.[107] Additionally, HSP70 polymorphisms appear to influence the severity of intestinal barrier disruptions.[108] Furthermore, small HSPs, such as HSP27, play a role in the cytoskeletal activity of intestinal epithelial cells, crucial for preserving their integrity.[109,110]

Exercise stands as a cost-effective means to combat chronic inflammatory diseases, as extensively documented.[24,25] Notably, exercise also exerts a profound influence on the host-gut microbiota axis.[111] A study involving overweight adolescents who underwent a 10-week program of low-calorie intake and increased physical activity demonstrated beneficial effects on gut microbiota composition. This intervention reduced Firmicutes genera while elevating Bacteroidetes.[111] Exercise alone can impact gut microbiota composition, although taking into account its influence on gut motility and transit time.[112]

The intertwined relationship among diet, metabolism, and exercise has become increasingly apparent, with well-controlled studies on elite athletes shedding light on this synergy (see, for instance,[113] for review). Within this context, whey protein (WP) supplements have gained prominence. Renowned for their postexercise recovery and muscle hypertrophy benefits, WP and whey protein hydrolysates (WPH) have the potential to influence gut microbiota composition and, by extension, lipid metabolism. This notion is supported by metagenomic analyses in relevant samples.[113] Furthermore, WPH is emerging as a novel antidiabetic agent, exhibiting favorable effects on glycemia in animal models and humans. It has been demonstrated to enhance blood glucose clearance, reduce hyperinsulinemia, and restore pancreatic islet insulin secretion in response to glucose in ob/ob mice.[114]

While there is not a "*one-size-fits-all*" optimal gut microbiota composition, given its substantial interindividual variation,[86] manipulations aimed at promoting a more physiologically balanced bacterial phyla (high diversity of genera) composition in the intestines hold promise for enhancing the HSR capacity in the intestines and, consequently, overall health. As exercise mimics many effects of heat therapy on the body, heat

therapy may similarly influence HSR in intestinal barrier cells. Currently, no study has explored the impact of heat therapy on the gut microbiota of individuals with obesity or diabetes. However, we hypothesize that akin to exercise,[26,27] elevating core temperature may induce favorable adaptations in the gut microbiota, potentially enhancing gut barrier function (possibly through increased HSP70 expression in enterocytes), elevating butyrate production, and mitigating chronic LPS/bacterial infiltration into the bloodstream. Our laboratory is actively investigating the effects of heat therapy on gut microbiota to shed light on this intriguing possibility.

Nutraceutical and pharmacological approaches

Various molecules, obtained from both natural sources and synthetic means, have demonstrated their potential as inducers or co-inducers of the HSR, offering benefits in the context of chronic inflammatory diseases. These compounds have been administered as nutraceutical supplements or pharmacological agents, opening promising avenues for therapeutic intervention. Here, we explore some of these approaches.

Commercial WP and WPH represent a notable example of nutraceutical supplementation that has gained popularity, particularly due to their observed positive influence on gut microbiota, as stated in the previous section. WPH, in particular, has demonstrated intriguing anti-inflammatory properties and the ability to augment the HSR. Research on rats subjected to WPH treatment revealed increased HSP70 expression following a single acute treadmill session.[115] Therefore, WPH effects depend on a previous HSR arming stress (exercise). Similarly, a study involving healthy older individuals reported an elevation in HSP70 and HSF-1 levels in response to a combination of resistance training and WPH.[116]

Notably, camel WPH, despite sharing a similar composition with bovine WPH, exhibits superior antioxidant activities due to its higher content of antioxidant amino acids. Studies involving camel WPH have indicated a reduction in NF-κB activity and associated proinflammatory pathways in lymphocytes and hepatocytes in the context of heat stress-induced damage. Importantly, these effects were accompanied by the maintenance of baseline HSP70 levels in both models.[117,118] Indeed, WPH's beneficial effects can be attributed to its amino acid composition, as the effects observed with WPH supplementation were not replicated when casein or nonhydrolyzed WP were administered. This underscores the potential significance of the amino acid profile in hydrolysates.[115]

Furthermore, research has highlighted the benefits of supplementing with nonessential amino acids in various conditions, including exercise and diabetes. In the context of the HSR signaling, these amino acids appear to play a crucial role, as evidenced by the reduction in HSF1 activity observed during amino acid deprivation.[119] Importantly, studies conducted *in vitro* and *in vivo* have demonstrated that the supplementation of L-glutamine or L-alanyl-L-glutamine dipeptide can enhance HSP70 expression in skeletal muscle, liver, and immune cells and tissues of mice. This effect extends to scenarios such as endotoxemia and contributes to improvements in metabolic status and antioxidant profiles.[120–123] Conversely, exercise, a potent trigger of systemic HSR (second only to fever or heat itself), concurrently serves as a significant supplier of glutamine to the circulation.[124] Of note, exercise is linked to increased gut permeability and elevated endotoxin levels in human subjects, particularly in hot environments. A single bout of exercise induces gut damage and heightened permeability in healthy individuals, with exacerbated damage observed in hot conditions.[125] Conversely, oral glutamine supplementation mitigates this impairment.[126] In the same study, it was observed that glutamine enhances the HS-induced expression of HSP70, HSF1, and occludin in cell cultures of the intestinal epithelial line Caco-2.[126] These findings align with previous research (Rearming the HSR through the manipulation of gut microbiota) indicating that HSF1 directly promotes occludin expression, and the HSR improves the intestinal epithelial barrier, thereby preventing LPS-mediated metabolic endotoxemia.[64,65,76,102]

While several amino acids, including glycine, alanine,[127] arginine,[128] and taurine,[129] have demonstrated the capacity to boost HSP70 expression, glutamine stands out as the primary co-inducer within this category. Importantly, in cellular or animal models lacking HSP induction mechanisms, the protective effects of glutamine are not evident,[130,131] thus reinforcing that this amino acid acts by potentiating an existing HSR.

A proposed mechanism for the enhanced HSR elicited by glutamine involves the hexosamine biosynthetic pathway (HBP),[132] as outlined by Leite *et al.*[23] Glutamine serves as a substrate for HBP,[133,134] which, in turn, triggers HSF-1 *N*-acetylglycosylation, ultimately leading to increased HSP70 expression.[132,135] Consequently, glutamine actually operates as a co-inducer of the HSR, necessitating prior activation of HSF1 before the enhancement of HSF1 activity facilitated by glutamine (a concept elaborated upon in the referenced publication).[3]

Glutamine and its derivatives, known enhancers of the HSR, have demonstrated the capacity to ameliorate metabolic status[121,122] and enhance pancreatic β-cell

function both *in vivo* and *in vitro* by bolstering the HSR biochemical pathway.[123,136–138] Studies have also shown that combining glutamine supplementation with exercise provides cytoprotective benefits by augmenting the HSR in animal models.[120,139] However, there are significant unresolved aspects in this context. Notably, plasma glutamine levels have exhibited a positive correlation with coronary artery disease in both male and premenopausal female individuals.[140] A meta-analysis of 10,083 women further revealed that menopausal status is associated with elevated serum glutamine levels.[141] However, plasma glutamine concentrations may not necessarily reflect the organism's capacity for glutamine utilization and its associated effects.[124,142] Moreover, the mechanistic and clinical implications of these findings remain to be fully elucidated, especially given that both chronological age and menopausal status are independently linked to CVD risk factors.[143] Furthermore, conflicting results have emerged from animal models.[144] Currently, there is a dearth of studies specifically investigating the effects of glutamine supplementation, whether alone or in conjunction with HSR inducers like exercise or heat treatment, on menopausal CVD risk factors or hot flushes.[30]

In addition to the natural regulation of the HSR through methods such as heat treatment, either independently or in conjunction with physical exercise or prebiotics, pharmacological intervention offers an alternative approach. An intriguing example of unexplored and potentially valuable molecules lies in the α,β-unsaturated cyclopentenone prostaglandins (cyPGs) of the A-type (but not other *non*-α,β-unsaturated PGs), with PGA_2 being a prominent representative. cyPGs are highly electrophilic compounds naturally produced during the resolution phase of inflammation and exhibit potent anti-inflammatory and antiproliferative properties. They exert their effects by interrupting the entire NF-κB activation pathway.[145] Notably, cyPGs have demonstrated the capability to entirely reverse atherosclerotic lesions both *in vivo* and *in vitro*.[146] A-type cyPGs also engage in physical interactions with 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting step in cholesterol synthesis.[147] This interaction may contribute to the antiproliferative effects of cyPGs, given the indispensable role of cholesterogenesis (not only cholesterol synthesis) in cell proliferation. Consequently, PGA_2, an inducer of the HSR, holds promise as a novel nonstatin inhibitor of (3-hydroxy-3-methylglutaryl-coenzyme A) reductase with potential therapeutic applications in CVD. However, PGA_2's efficacy in chronic inflammatory conditions beyond atherosclerosis[146,148] remains unexplored.

Pharmacological interventions utilizing hydroxylamine derivatives such as bimoclomol and BGP-15 have emerged as promising strategies for addressing a spectrum of metabolic disorders characterized by inflammation. These disorders encompass diabetes, obesity, and related conditions such as CVD, NAFLD, as well as neuromuscular and neurodegenerative ailments.[149] Much like the observed effects of glutamine, which, upon metabolism through the hexosamine biosynthetic pathway (HBP), extends the activation and transcriptional activity of HSF1,[23] bimoclomol and BGP-15 also serve as HSR co-inducers. This implies that these drugs do not instigate a genuine HSR by themselves but rather necessitate a triggering event, such as exercise, heat exposure, or oxidative stress, to potentiate it.

As anticipated, bimoclomol has been demonstrated to accumulate HSP70 in tissues affected by chronic diseases, including diabetes, heart disease, and kidney dysfunction. Importantly, it has exhibited clinical safety in human trials.[150] Animal studies have further substantiated the effectiveness of bimoclomol as an insulin sensitizer, ameliorating peripheral neuropathy in diabetic rats[151] and affording protection to rat cardiomyocytes from severe heat stress (47 °C for 2 h) *ex vivo* and myocardial infarction *in vivo*, with these effects being contingent on HSP70.[152]

Several natural compounds have demonstrated their ability to induce the HSR, leading to an increase in HSP expression in both *in vivo* and *in vitro* settings. Celastrol, a triterpenoid derived from plant root extracts, exhibits anti-inflammatory properties by inhibiting NF-κB and increasing HSP70 levels in cultured cells.[153] Leucinostatin, a fungal peptide mycotoxin, has been shown to enhance HSP70 expression in stressed cells.[154] Carvacrol, also known as cymophenol, a phenolic monoterpenoid found in plant oils, can co-induce HSP70 expression, both *in vitro* and in models of arthritis.[155,156] Geranylgeranylacetone is another HSP70 inducer that demonstrates protective effects in rat models of ischemia-reperfusion injury.[157] Geranylgeranylacetone has also shown promise in improving glucose tolerance in diabetic monkeys through an increase in HSP70 levels[158] and has been considered for use in liver surgery.[159] Similarly, various nontoxic hydroxylamine derivatives known to act as HSP inducers have been investigated. For example, arimoclomol[160,161] besides bimoclomol[162] has been shown to prolong the activity of HSF1, thereby increasing HSP70 induction.[152]

Among the above-mentioned HSR co-inducers, BGP-15, apart from other cellular effects that confer cytoprotection,[163] stands out as a well-studied compound with excellent tolerability and a promising insulin-sensitizing effect.[164–167] Comparative studies in animal models strongly support its potential application in a range of conditions, including CVD, diabetes, metabolic

syndrome, and muscular dystrophies.[168–171] BGP-15 is currently being investigated as a therapeutic option for metabolic diseases.[149,164,172,173] BGP-15, which is a small molecule co-inducer of HSP72, mimics the beneficial effects seen with genetic overexpression of HSP72 and exercise training. These effects include an increase in mitochondrial area and enhanced insulin sensitivity.[149] The interest in BGP-15 as a potential therapeutic agent for inducing the HSR stemmed from its ability to act as a pharmacological mimic of exercise. During exercise, contracting muscle cells significantly increase the flow of glutamine into the circulation and through the HBP. This heightened HBP flow inhibits glycogen synthase kinase-3β (GSK-3β), which usually hampers HSF1 binding to the HS gene promoters. Additionally, the enhanced HBP flow promotes increased AMPK activity, leading to heightened SIRT1 expression and its binding activity to the HSF-1 promoter of HS genes under circumstances of predominantly proteotoxic stress.[2] These combined effects enhance the HSR.[23]

While exercise remains the most potent physiological inducer of the HSR, comparable only to fever, BGP-15, as an HSR co-inducer, not only reduces the production of reactive oxygen species but also possesses the ability to remodel cholesterol-rich membrane domains. Furthermore, it can block the activity of JNKs through direct binding.[173]

In summary, a myriad of molecules, ranging from amino acids to dietary compounds and pharmacological agents, have demonstrated their potential to co-induce the HSR, offering promising avenues for therapeutic intervention in chronic inflammatory diseases and metabolic disorders. Further research is needed to unravel the intricacies of these approaches and translate them into effective clinical strategies.

The integrity of the HSR can be assessed by the Heck index and whole blood heat challenge tests

HSP70 members present some peculiarities regarding their site of responses, stressful situations to those each member may be recruited, and compartmentalization of the products.[174] Elevated iHSP70 expression is closely related to insulin signaling.[31] Insulin itself is a signal for increased expression of iHSP[70175] and regulation of its induction.[176] The intracellular location of HSP70 is related to the preservation and improvement of insulin sensitivity in both skeletal muscle and vascular endothelium.[44,177] Consequently, HSP70 ($HSP72^{-/-}$) knockout mice show poor insulin signaling,[172,178] while transgenic mice overexpressing HSP70 ($HSP72^{+/+}$) present improvement in insulin signal.[149,172] Strikingly, when insulin downstream pathways are turned on, they inhibit the activation of HSF1. There is also a coupling between reduced insulin levels (e.g., restricted nutrient intake) and increased lifespan in a variety of animals, ranging from worms and flies to mice.[179] Actually, we presume that, maybe, HSF1 suppression by insulin is also involved in insulin-mediated insulin resistance[180] and perpetuation of low-grade inflammation in obesity and T2DM! On the other hand, this metabolic behavior is intertwined with the caloristasis equilibrium.[2]

The release of the same HSP70 into the plasma (eHSP70), on the other hand, acts as a danger signal, potentially stimulating the innate and adaptive immune system[181–183] and works as an active mediator of inflammatory pathways, including *via* TLR4 priming.[184] Furthermore, eHSP70 (e.g., eHSP72) has been associated with the danger-associated molecular pattern in activating caspase-1[185] while it has been suggested that a major part of eHSP70 content in the blood is released by circulating immune cells.[186,187] *Acutely*, eHSP70 can be an important defense signal for the maintenance of homeostasis in stressful conditions, being also implicated in motoneuron protection[21] and even in providing anti-inflammatory resources.[188] *Chronic exposure* to high plasma levels of eHSP70, however, is firmly accepted to be deleterious to an array of tissues (including the pancreas) from obese patients and those bearing T2DM, CVD, NAFLD, and menopause-associated metabolic imbalances.[21,30,39,40,189]

The ability to produce and release appropriate amounts of eHSP70 is also associated with the anti-inflammatory HSR,[27,188,190] while the adequate balance between eHSP70 and iHSP70 is now assumed to be directly correlated to the immunoinflammatory status of individuals.[187,191–194] Indeed, increased eHSP70-to-iHSP70 ratios are elevated in different acute and chronic challenges to health[187] but are virtually always associated with low-grade chronic inflammation thus permitting the use of these ratios to follow up patients and laboratory animals.[25] eHSP70 is also linked to arterial hypertension.[195] Besides, elevated eHSP70-to-iHSP70 ratios were found to disrupt vascular responses to calcium and to activate the TLR4/MD2 complex in type 1 diabetes mellitus (T1DM), as reported by De Oliveira and colleagues.[194] This can be partially explained by the rise of a pro-inflammatory cytokine (eHSP70) that mobilizes Ca^{2+} in vascular cells and the reduction of a powerful anti-inflammatory (iHSP70), thus leading to vascular cell dysregulation. Additionally, iHSP70 was found to regulate Ca^{2+} mobilization participating in the contraction of vascular smooth muscle.[194] On the other hand, intracellular Ca^{2+} mobilization within the cytoplasm is a

powerful inducer of HSF1 activation and HSR.[15] Finally, eHSP70 present in the plasma of T1DM rats increases α-adrenergic-induced contraction of aorta rings in a TLR4/MD2-dependent way, collaborating to disturb vascular reactivity in T1DM.[194]

Chronically, the elevation of plasma HSP70 (eHSP72) correlates with tumor necrosis factor-α increase, insulin resistance, and pancreatic β-cell failure.[40] As expected, there is also a negative correlation of eHSP70 with insulin levels and HOMA-IR in overweight young men.[196] Increased eHSP72 levels are associated with sarcopenia[197] and duration of T2DM.[198] On the other hand, conditions that stimulate the tonus of the sympathetic nervous system (as probably occurring during saunas and hot tubs) can augment HSP70 release from immune cells.[24,25] Additionally, adrenergic sympathetic activation occurs following overfeeding.[199] Therefore, in states of continuous surplus energy imbalance,[200,201] such as in obesity, adrenergic stimulus to many tissues may take place. Contrariwise, adipose tissue itself acts as an endocrine organ releasing adipocytokines (e.g. leptin, resistin, adiponectin) that, along with insulin resistance-derived hyperinsulinemia, increase sympathetic activity *via* signaling at the central level, that is, in the nucleus tractus solitarius.[202] Adrenergic stimulus can increase the expression and release of HSP70 by circulating immune cells.[203] Together with the fact that immune cells are reputed to be the main exporters of eHSP70 in stressful situations, measuring the release of HSP70 by this "circulating tissue" became a good tool to assess organismal capacity to activate the HSR under stressful conditions.

The antagonistic relation between intra and eHSP70 signals can be used to assess the inflammatory profile. PBMCs are widely used to access HSP70 production in laboratory animals and humans,[190,204–206] because the expression of HSP70 in PBMC is inversely correlated with the degree of proinflammatory status in tissues from chronic disease patients.[187,190,207] As PBMC evaluation represents a less invasive method that perfectly matches the muscle content of HSP[70,208] we have started to use this approach as a representative of bodily HSP70 intracellular content.

This approach is possible because alterations in proinflammatory parameters are directly correlated with an increased eHSP70-to-iHSP70 ratio, so such ratio can be used as a predictor of the proinflammatory or anti-inflammatory response and to the development of insulin resistance, even before alterations in classical parameters, such as HbA1c, can be noticed.[24,25,209] As previously mentioned, the rationale behind this can be partly explained by the increase in the proinflammatory cytokine eHSP70 and the decrease in the potent anti-inflammatory iHSP70 that correlates with eHSP70-to-iHSP70 ratio. Furthermore, our laboratory's studies have provided clear evidence to support that this postulation is correct.[187] Therefore, taking PBMC and plasmas from patients or laboratory animals allows for the assessment of the time course of the evolution of plasma-to-leukocyte ratios with ease. These ratios can be obtained by a simple division between the absolute values of eHSP70 and iHSP70 in different times or situations, irrespective of the method used to assess these HSP70 contents.

Unlike simply estimating the evolution of eHSP70/iHSP70 ratios, which gives a static picture in different situations, the eHSP70-to-iHSP70 ratio *index* can also be tracked by the Heck index or H-index of organismal HSP70 status. Heck index consists of the comparison between the eHSP70/iHSP70 ratio in a situation with that of the eHSP70/iHSP70 ratio in a different condition, but using the *ratio of eHSP70/iHSP70 ratios*, instead of merely accompanying the linear progression of the eHSP70/iHSP70 ratio.

Heck index has been recently identified as a novel and comprehensive index of an individual's immunoinflammatory status. Similarly to that described above for simple eHSP70/iHSP70 ratios, the Heck index can also be taken by evaluating HSP70 contents in PBMC and plasma, but comparing *the ratio of ratios*. This is possible because eHSP70/iHSP70 ratios between *plasma and PBMC* reflect eHSP70/iHSP70 ratios between *plasma and (metabolic) tissues*.[24,25,181,187,191,207,209–216]

The reasoning for this is that higher eHSP70 levels signify an increase in inflammatory signals, as eHSP70 is inherently proinflammatory. Conversely, cells that respond to stressful stimuli by enhancing intracellular iHSP70 levels are more likely to be in a state of anti-inflammation or equilibrated HSR. To calculate the Heck index, one initially takes $R_c = (eHSP70)_c/(iHSP70)_c$ as the HSP70 ratio in a baseline (control) situation and $R_j = (eHSP70)_j/(iHSP70)_j$ as the HSP70 ratio in any other situation "j," irrespective of the techniques used to measure each eHSP70 and iHSP70. Heck index can then be calculated as the quotient of any R_j by the control R_c, which is considered to be unity ($R_c = 1$) and normalizes all other results in "j" situations allowing for easy comparisons between different conditions. Hence, the Heck index = R_j/R_c and can be used to compare any stressful situation "j" with the assumed control situation. This index can be used to estimate the immunoinflammatory status of an individual (or groups of individuals) in a variety of situations, including immune responses, diabetes, and the immunological impacts of exercise.

As previously shown,[25,187] assuming a Heck index of 1 ($R_c = 1$) for the controls (resting, unstimulated),

exercise can cause a shift in Heck indices of up to approximately 5, which is accompanied by an increase in inflammatory markers and cell proliferation. A Heck index value >5 indicates an exacerbated proinflammatory condition. Conversely, a Heck index value between 1 and 5 suggests a predominantly equilibrated HSR while values <1 indicate an anti-inflammatory status (for more details, see supplemental *Table S2* in Ref.[187]).

Heck index allows for the follow-up of the temporal evolution of immunoinflammatory status in patients and laboratory animals, that is, the occurrence of a background inflammation. High basal Heck indices have been associated with high HOMA-IR values,[39,40] visceral obesity, and insulin resistance,[211] as well as elevated levels of ultra-sensitive C-reactive protein.[217] Heck index is higher in streptozotocin-treated diabetic rats and correlates with vascular abnormalities observed in the animals[194] as well. Consequently, changes in the Heck index have emerged as a potentially novel biomarker for low-grade inflammation and a highly sensitive indicator of an individual's inflammatory status permitting an initial evaluation of the HSR in patients and laboratory animals. As such, maintaining an appropriate balance between extracellular and iHSP70 is now believed to be directly correlated with an individual's immunoinflammatory status.[191–194]

Although the Heck index is especially useful for giving initial clues about the inflammatory status of an individual, it does not furnish the capacity to trigger a pronounced HSR under stress. To supplement the Heck index, we developed a straightforward technique to evaluate HSR status in rodents and humans, using short-term heat challenges of whole-blood samples under different conditions. This method allows the monitoring of HSR integrity capacity,[27] particularly because the expression of HSR components in PBMC is inversely correlated with the degree of proinflammatory "tonus" in chronic disease tissues.[13,190,207] Notably, PBMC incubated at various temperatures for 2 h exhibit an iHSP70 peak at 42 °C, with lymphocytes as the primary producers under this condition.[186] However, after just 1 h of incubation at 40 °C or 43 °C, a significant increase in iHSP70 is observed only at the higher thermal stress level.[206] Similar results are observed upon incubations of circulating monocytes at 41 °C or 42 °C (Schöler *et al.* manuscript in preparation).

Basal iHSP70 contents and thermal stress sensitivity in human PBMC and polymorphonuclear leukocytes differ depending on the cell clusters.[218] Monocytes exhibit a more robust response between 39 °C and 41 °C after a 2-h incubation period, while lymphocytes respond better at 42 °C within the same duration.[219] At temperatures up to 41 °C, lymphocytes and polymorphonuclear leukocytes show only a modest increase in iHSP70, whereas monocytes display a strong induction at 39 °C, with iHSP70 expression at 41 °C being 10-fold higher than in control monocytes at 37 °C.[219] In healthy volunteers who had been exposed to a hot water bath to induce whole-body hyperthermia in a fever-like range (39 °C), iHSP70 induction was observed in all leukocytes, with cell type-specific variations comparable to those observed *in vitro*, albeit less pronounced.[219] Although there are disagreements regarding the temperatures and incubation times used for specific cell types in the literature, our current studies indicate that using a 2-h incubation at 42 °C is optimal for testing iHSP70 production.

To assess organismal HSR under different clinical conditions, we employ a whole-blood HS challenge *ex vivo*. Three approaches are in use in our laboratory, all involving 2-h incubations of whole blood at 42 °C, with control samples kept for the same period at 37 °C. In *protocol #1*, extracellular eHSP70 is promptly measured after a 2-h thermal (or control) challenge,[190] followed by the evaluation of iHSP70 in the PBMC fraction (e.g., Ficoll-Hypaque) after 6 h of rest at 37 °C in 5% CO_2 atmosphere.[220]

In *protocol #2*, we compare both HSP70 in PBMC (intracellular, iHSP70) and supernatant HSP70 (extracellular, eHSP70), after 6 h from the beginning of the 2-h incubation period.[216] We have used the same protocol to compare the HSR in the human ovarian cortex obtained by closed metal container vitrification or the slow-freezing technique of cryopreservation.[221]

Although we believe that the protocols explained above satisfactorily permit evaluation of organismal HSR (see details in Table 2), they have some limitations under specific conditions. A considerable sample volume (at least 400 μL) is required for the test, which for human samples is perfect but is limiting when studying small animals (e.g., mice). Also, the use of gradient-separating products, such as Histopaque or Ficoll-Hypaque (protocol #1 and protocol #2), makes the method more complex and expensive. This is why we set up a simpler and straightforward technique to assess the status of the HSR in rodents and humans by using the short-term heat challenge of whole-blood samples, without requiring prior or post-incubation cell separation. Moreover, changing the culture medium after the heat challenge does not affect the results (Schroeder *et al.* manuscript in preparation). Therefore, *protocol #3* involves diluting whole-blood samples and incubating them at 42 °C (or 37 °C for controls) for 2 h. The samples are then incubated for an additional 6 h at 37 °C, allowing for the accumulation of inducible HSP70 (HSP72), a marker of HSR capacity. By using this

Table 2
Characteristics of current HSR assessment approaches

Technique	Method	Characteristics	Interpretation
Basal measurement	eHSP70 by ELISA kit	Direct measurement with absolute values to compare	Reference values are not known
	iHSP70 by ELISA kit, WB, FCM, IHC	Possible visualization of cellular compartmentalization of the iHSP70	
	Heck index (eHSP70 to iHSP70 ratio index)	Allow quantitation of the ratios between intra and extracellular HSP70 forms as pro/anti-inflammatory markers	$R < 1$ = anti-inflammatory status; $1 \leq R \leq 5$ equilibrated immunoinflammatory surveillance status; $R > 5$ chronic proinflammatory status
Heat shock challenge	Protocol #1 Whole Blood (isolation *after* test)	Provides different combinations and evaluates the overall stimulation of cells	↑HSP70 = HSR preserved
	Protocol #2 Whole Blood (isolation *before* incubation)	Isolation responses from different cell types	
	Protocol #3 Whole Blood (*without* PBMC isolation)	Easy to perform, cost-effective	

Abbreviations used: eHSP70, extracellular HSP70; FCM, flow cytometry; HSP70, 70 kDa family of heat shock proteins; HSR, heat shock response; iHSP70, intracellular HSP70; IHC, immunohistochemistry; PBMC, peripheral blood mononuclear cells; WB, Western blotting.

technique, it is possible to establish the timeline of HSR suppression in animal models of high-fat diet-induced obesity, based on the differences in iHSP70 between the two test temperatures (ΔHSP70). The ΔHSP70 demonstrates a 5-parameter logistic correlation with fasting glycemia (negative), fast insulinemia (negative), HOMA-IR, negative, and Quantitative Insulin Sensitivity Check Index (positive) (Schroeder *et al.* manuscript in preparation). The illustration of *protocol #3* can be found in Figure 1.

Blood samples collected in heparinized tubes are subjected to dispersion and then diluted in a 1:10 ratio with an appropriate culture medium. The resulting diluted blood samples are incubated in a temperature-controlled water bath at 42.00 ± 0.01 °C for a period of

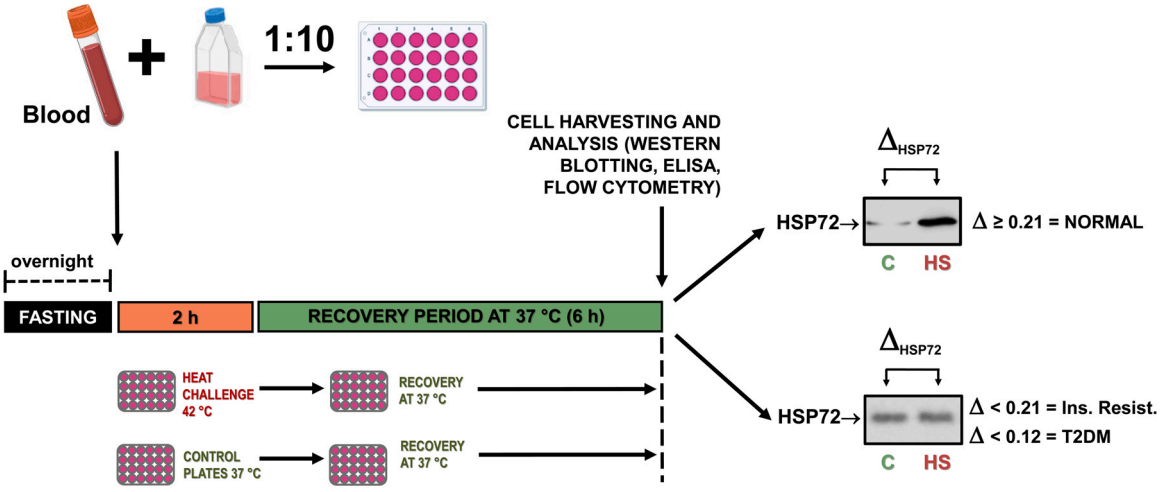


Fig. 1 Whole-blood heat shock challenge *ex vivo* to assess organismal heat shock response. Heparinized blood samples are collected from overnight-fasted individuals and diluted 1:10 with culture medium. Diluted blood samples are, then, incubated in a temperature-controlled water bath at 42.00 ± 0.01 °C for 2 h. Parallel control preparations are to be maintained in another water bath at 37 °C for the same time period. After incubations, cells from both groups are incubated for additional 6 h at 37 °C to allow for a robust accumulation of the inducible forms of HSP70 (HSP72), which serves as a marker of the HSR capacity. Total experimental time, since blood harvesting to the end of incubations, is 8 h. HSP70 accumulation can be assessed by Western blotting, ELISA, and flow cytometry. The *difference* (Δ) between HSP72 expressed after 42 °C compared with that after 37 °C is consistent with IR and T2DM. This illustration was prepared by using free icons from Biorender.com (available at https://app.biorender.com/). Abbreviations used: HS, heat shock; HSP70, 70 kDa family of heat shock proteins; HSR, heat shock response; T2DM, type 2 diabetes mellitus.

2 h. Parallel control preparations are maintained in another water bath at 37 °C for the same duration of time. Following the water bath incubations, cells from both the control and heat-treated groups are incubated for an additional 6 h at 37 °C to allow for the significant accumulation of the inducible forms of HSP70 (HSP72), which serve as markers of the HSR capacity. After the incubation period, cells can be processed for iHSP70 analysis using various techniques, including EIA kits, Western blotting, flow cytometry, or immunofluorescence. This technique is particularly useful when there is no significant difference in basal expressions of iHSP70 between control and test groups, such as in cases of diabetes, obesity, or CVD. In our experience, evaluation of the HSR by *ex vivo* whole-blood HSR protocol #3 is sensitive enough to detect insulin resistance in mice from week to week, even when glucose tolerance tests and basal glycemia are normal.

Concluding remarks

In summary, inflammation, a fundamental physiological response, has evolved as a crucial defense mechanism against microbial threats and tissue damage. However, in our modern Western lifestyle characterized by sedentary habits and excessive calorie consumption, the body's natural mechanisms for resolving inflammation, notably the HSR, become jeopardized. The relentless strain placed on adipose tissue's ER leads to chronic ER stress and the subsequent UPR. Unfortunately, the UPR possesses an inflammatory arm that propagates inflammatory signals throughout the body. This persistent activation of UPR pathways eventually triggers the continuous activation of the NLRP3 inflammasome, initially in adipose tissue, and then affecting the resolution of inflammation by the HSR in multiple tissues beyond just adipose tissue.

This domino effect leads to the establishment of chronic low-grade inflammation, a hallmark of various degenerative conditions including insulin resistance, obesity, T2DM, NFLD, CVDs, and neurodegenerative diseases. Remarkably, strategies do exist to re-establish the HSR, even in the face of persistent noxious stimuli of our Western lifestyle. Heat-based interventions, such as fever and thermotherapy, as well as exercise, operate through distinct mechanisms to dismantle NLRP3 inflammasome activation and reactivate the HSR. This offers hope for alleviating chronic inflammatory conditions.

In this manuscript, we have explored current physiological, pharmacological, and nutraceutical approaches designed to reawaken the HSR in chronic inflammatory conditions. Additionally, we have highlighted clinical tools that can evaluate HSP70 status, offering valuable insights into the HSR's progression or suppression in both patients and experimental animals. Overall, understanding and harnessing the power of the HSR holds promise for helping to combat the widespread burden of chronic inflammatory diseases in our society.

"Quæ medicamenta non sanat æ ferrum sanat. Quæ ferrum non sanat æ ignis sanat. Quæ vero ignis non sanat æ insanabilia existimare oportet.

That which drugs fail to cure the scalpel can cure. That which the scalpel fails to cure heat can cure. If heat cannot cure, it must be determined to be incurable."

(Aphorisms of Hippocrates, by Elias Marks, from the Latin version of Verhoofd, Collins & Co. New York, 1817).

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Author contribution HTS and PIHB conceptualized the paper while HTS prepared the first draft. All the authors were involved in co-writing this work. PIHB prepared the figures and supervised the finalization of the manuscript. All the authors have read and agreed to the submitted and published versions of the manuscript.

Data availability statement Data will be made available on request.

Declarations of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. On behalf of all the authors, Paulo Ivo Homem de Bittencourt declares that they have no potential conflict of interest related to the present manuscript, and no competing interests such as consultancies, financial involvement, patent ownership, etc. in relation to the work described. CNPq, FAPERGS, and CAPES (the funding organisms) had no involvement in the propositions presented in this manuscript.

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