Human Reproduction Update, pp. 1–29, 2017
doi:10.1093/humupd/dmx020

human reproduction update

Nitric oxide-heat shock protein axis in menopausal hot flushes: neglected metabolic issues of chronic inflammatory diseases associated with deranged heat shock response

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Submitted on March 24, 2016; resubmitted on June 11, 2017; editorial decision on June 27, 2017; accepted on June 28, 2017

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BACKGROUND: Although some unequivocal underlying mechanisms of menopausal hot flushes have been demonstrated in animal models, the paucity of similar approaches in humans impedes further mechanistic outcomes. Human studies might show some as yet unexpected physiological mechanisms of metabolic adaptation that permeate the phase of decreased oestrogen levels in both symptomatic and asymptomatic women. This is particularly relevant because both the severity and time span of hot flushes are associated with increased risk of chronic inflammatory disease. On the other hand, oestrogen induces the expression of heat shock proteins of the 70 kDa family (HSP70), which are anti-inflammatory and cytoprotective protein chaperones, whose expression is modulated by different types of physiologically stressful situations, including heat stress and exercise. Therefore, lower HSP70 expression secondary to oestrogen deficiency increases cardiovascular risk and predisposes the patient to senescence-associated secretory phenotype (SASP) that culminates in chronic inflammatory diseases, such as obesities, type 2 diabetes, neuromuscular and neurodegenerative diseases.

OBJECTIVE AND RATIONALE: This review focuses on HSP70 and its accompanying heat shock response (HSR), which is an anti-inflammatory and antisenescent pathway whose intracellular triggering is also oestrogen-dependent via nitric oxide (NO) production. The main goal of the manuscript was to show that the vasomotor symptoms that accompany hot flushes may be a disguised clue for important neuroendocrine alterations linking oestrogen deficiency to the anti-inflammatory HSR.

SEARCH METHODS: Results from our own group and recent evidence on hypothalamic control of central temperature guided a search on PubMed and Google Scholar websites.

OUTCOMES: Oestrogen elicits rapid production of the vasodilatory gas NO, a powerful activator of HSP70 expression. Whence, part of the protective effects of oestrogen over cardiovascular and neuroendocrine systems is tied to its capacity of inducing the NO-elicited HSR. The hypothalamic areas involved in thermoregulation (infundibular nucleus in humans and arcuate nucleus in other mammals) and whose neurons are known to have their function altered after long-term oestrogen ablation, particularly kisspeptin-neurokinin B-dynorphin neurons, (KNDy) are the same that drive neuroprotective expression of HSP70 and, in many cases, this response is via NO even in the absence of oestrogen. From thence, it is not illogical that hot flushes might be related to an evolutionary adaptation to re-equip the NO-HSP70 axis during the downfall of circulating oestrogen.

WIDER IMPLICATIONS: Understanding of HSR could shed light on yet uncovered mechanisms of menopause-associated diseases as well as on possible manipulation of HSR in menopausal women through physiological, pharmacological, nutraceutical and prebiotic interventions. Moreover, decreased HSR indices (that can be clinically determined with ease) in perimenopause could be of prognostic value in predicting the moment and appropriateness of starting a HRT.

Key words: menopause / hot flushes / heat shock response / chronic inflammatory diseases / cellular senescence / thermoregulation / heat therapy / obesity / insulin resistance / cardiovascular disease.

Introduction

Menopausal hot flushes (also known as hot flashes) alongside night sweats comprise a set of vasomotor symptoms (VMS) and other autonomic responses which lead to an uncomfortable trait of heat-defence responses, including skin vasodilation and sweating. These VMS are related to changes in hypothalamic processing of thermoregulation and are accompanied by an abrupt and huge feeling of heat, usually following a tiny rise in core temperature. Although hot flushes are the most common complaint that leads this group of women to seek clinical support, little has been added to our knowledge on their clinical approach in almost 20 years (Stearns *et al.*, 2002). The only irrefutable evidence is that oestrogen replacement therapy virtually eliminates hot flushes, while some non-hormonal treatments are currently under evaluation (Freedman, 2014). Even in relation to oestrogen, its involvement is not completely settled, as it is not the absolute level of oestrogen, but rather the pace of decrease in oestrogen plasma levels that determines the onset of the flushing time span (Kronenberg, 1990, 2010; Rance *et al.*, 2013). It is intriguing that all menopausal women have low circulating oestrogen levels, yet not all of them exhibit hot flushes. The appearance of hot flushes always coincides with oestrogen withdrawal, but this does not entirely explain the phenomenon because both natural (Freedman *et al.*, 1995) and surgical, (i.e. bilateral oophorectomised [Aksel *et al.*, 1976]) menopausal patients present oestrogen levels that do not differ between symptomatic and asymptomatic women (Freedman, 2005). Conflicting results, however, suggest that oestrogen levels may be indeed lower in symptomatic postmenopausal women (Erlik *et al.*, 1982) as well as in premenopausal patients presenting hot flushes (Kronenberg, 1990). Progesterone, which importantly drives sex-hormone elicited modulation of thermoregulation, has also shown to reduce VMS scores and frequencies in women within 10 years after last menstruation (Hitchcock and Prior, 2012).

Even though some animal studies have pointed out unequivocal mechanisms on which hot flushes rely (Dacks and Rance, 2010; Dacks *et al.*, 2011; Mittelman-Smith *et al.*, 2012; Rance *et al.*, 2013), the paucity of similar approaches in humans has been impeding further mechanistic outcomes. More specifically, oestrogen possesses the ability to induce the expression of HSP72 (Olazábal *et al.*, 1992a, 1992b; Knowlton and Sun, 2001; Hamilton *et al.*, 2004a, 2004b; Stice *et al.*, 2011), a member of the 70 kDa family of heat shock proteins (HSP70), which are anti-inflammatory and cytoprotective (i.e. cardioprotective, vasculoprotective, neuroprotective, antiatherosclerotic and antidiabetic) protein chaperones (detailed in 'The Heat Shock Response' section) whose expression is modulated by different types of physiologically stressful situations, including exercise and heat

stress (Newsholme and Homem de Bittencourt, 2014). Hence, it is not surprising that, during perimenopause, several homoeostatic functions based on oestrogen-dependent HSP70 expression start to collapse.

This is noteworthy because reduced production of HSP70, and its accompanying heat shock response (HSR), is a hallmark of chronic inflammatory diseases observed, in some degree and phases, in menopausal women. Depressed HSR is associated with insulin resistance (Kurucz *et al.*, 2002), with the worsening of diabetes in obese patients (Rodrigues-Krause *et al.*, 2012), with the severity of non-alcoholic fatty liver disease (NAFLD) in humans (Di Naso *et al.*, 2015) and with the spread of inflammation throughout the body (Newsholme and Homem de Bittencourt, 2014; Leite *et al.*, 2016). Furthermore, lower expression of HSP70 secondary to oestrogen deficiency may increase cardiovascular disease (CVD) risk (Tytell and Hooper, 2001), although ageing itself may lead to reduced HSP70 expression (Stice *et al.*, 2009; Leite *et al.*, 2016).

It is now clear that many (if not all) chronic degenerative diseases of low-grade inflammatory nature have, as their basis, a defective capacity of tissues to trigger an adequate HSR. This leads to a state of perpetual unresolved inflammation that disseminates throughout the tissues leading to a status of cellular senescence that positively feeds back inflammation through the induction of pro-inflammatory cytokines and potentiation of oxidative stress (Tchkonia *et al.*, 2010, 2013; Kim *et al.*, 2012; Newsholme and Homem de Bittencourt, 2014; Palmer *et al.*, 2015; Schafer *et al.*, 2017).

Interestingly, the antidiabetic drug metformin inhibits senescence patterns in cell models (Moiseeva *et al.*, 2013) and alleviates ischaemic retinopathy *in vivo* (Oubaha *et al.*, 2016) by blocking NF-κB downstream pathways (Moiseeva *et al.*, 2013; Saengboonmee *et al.*, 2017). Hence, part of beneficial effects of metformin in diabetes could be accounted for its ability to block cellular senescence, thus liberating HSR to start working again. Remarkably, 17α-oestradiol, which preferentially binds to nonclassical membrane oestrogen receptors (mER), does revert such the senescence-associated inflammatory profile (Stout *et al.*, 2017), whilst physiological inducers (e.g. exercise, heat treatment), pharmacological inducers (e.g. cyclopentenone prostaglandins (cyPGs)) and co-inducers (e.g. BGP-15, bimoclomol), as well as transgenic induction, of HSR have all proved to powerfully block chronic inflammatory states *in vivo* (Vígh *et al.*, 1997; Hargitai *et al.*, 2003; Ianaro *et al.*, 2003; Homem de Bittencourt *et al.*, 2007; Kokura *et al.*, 2007; Choi *et al.*, 2008; Chung *et al.*, 2008; Newsholme *et al.*, 2009; Gupte *et al.*, 2009, 2011; Bathaie *et al.*, 2010; Karpe and Tikoo, 2014; Sapra *et al.*, 2014). Unfortunately, however, there is as yet no published study targeting oestrogen effects (e.g. HRT) on HSR of menopausal women and its possible influence upon chronic inflammatory diseases as well as the possible masked or subclinical metabolic alterations that may be connected to hot flushes.

The interplay between hot flushes, menopause-related chronic inflammatory diseases and deranged HSR

The understanding of gradual alterations in HSR that may evolve from the perimenopausal to late postmenopausal phase is crucial to allow for the clinician to make the right decisions on the best timing for both pharmacological and non-pharmacological interventions in middle-aged women. In fact, hot flushes are strongly associated with low-grade inflammation. For instance, obesity is a major factor for the appearance of hot flushes (Thurston and Joffe, 2011), whereas hot flushes directly correlate with the risk of insulin resistance, Type 2 diabetes mellitus (T2DM) and metabolic syndrome (Thurston *et al.*, 2012a; Van Dijk *et al.*, 2015; Ryu *et al.*, 2015). Similar observations made by our group confirm these findings (Vincensi and Ludwig *et al.*, unpublished results). Furthermore, women with an early severe VMS profile are more prone to developing diabetes across a period of 15 years (Herber-Gast and Mishra, 2014). Indeed, the duration of the hot-flush time span is directly correlated with women's age at the time they firstly report hot flushes (Freeman *et al.*, 2011; Herber-Gast and Mishra, 2014; Ryu *et al.*, 2015; Avis *et al.*, 2015), so that the earlier women present frequent VMS (i.e. premenopausally or early perimenopausally), the longer the total VMS duration time is expected to be (median 11.6–11.8 years), whilst women who start having hot flushes postmenopausally tend to present the shortest (median 3.4–3.8 years) VMS duration (Freeman *et al.*, 2011; Avis *et al.*, 2015). Hence, precocious hot flushes may indicate prolonged periods during which women will be at enhanced risk of metabolic disease related to chronic low-grade inflammation.

Hot flushes are also associated with increased risk for CVD related to atherosclerosis and abnormal endothelial function (Rossouw *et al.*, 2007; Gast *et al.*, 2008, 2010; Thurston *et al.*, 2008, 2010, 2012a, 2012b; Huang *et al.*, 2009; Tuomikoski *et al.*, 2009; Bechlioulis *et al.*, 2010; Herber-Gast *et al.*, 2015; Sassarini and Lumsden, 2015). Although natural menopausal women present an unfavourable plasma lipid milieu that could itself justify an increased in CVD risk, women presenting VMS have augmented risk for CVD that can not be whole explained by classical CVD risk factors (Gast *et al.*, 2011). Vascular responses of women with severe VMS are abnormal when compared with non-flushing women (Sassarini *et al.*, 2011). Importantly, as in the case of metabolic disease, the severity and duration of the hot-flush time span is of relevance to the impact on CVD, particularly when women are undergoing HRT. For example, oestradiol-based HRT is effective in reducing the progression of subclinical atherosclerosis only if the treatment is initiated within 6 years after menopause, but not if HRT starts 10 years or more afterwards (Hodis *et al.*, 2016). Additionally, in older postmenopausal women (>20 years after last menstruation) with pre-existing coronary artery disease, oestrogen-progestin combined HRT may increase the risk of coronary events in the first year of treatment only in women with clinically significant hot flushes but not amongst those without (Huang *et al.*, 2009)!

Menopause-related CVD has multiple facets that concur with those of metabolic disease, particularly in relation to depressed HSR, which is always evident. As detailed in the next section, metabolic alterations due to preadipocyte over-utilization leads to obesity paralleled by a state of cellular senescence that virtually impairs an eloquent HSR when demanded (Kim *et al.*, 2012; Tchkonia *et al.*, 2013). Pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8 and IL-18) produced as the result of senescence-associated secretory phenotype (SASP) spread out to all the tissues, promoting insulin resistance (Palmer *et al.*, 2015), negatively affecting blood vessels and the heart, impairing both tissue repair and the efficiency of antioxidant systems (Minamino and Komuro, 2007; Wang and Bennett, 2012; Oubaha

et al., 2016) and, eventually, imposing incurable low-grade sterile inflammation (Newsholme and Homem de Bittencourt, 2014). As expected, besides inducing insulin resistance and abrogating HSR in blood vessels (Karpe and Tikoo, 2014), obesity itself increases vascular senescence and susceptibility to ischaemic injury, enhancing the likelihood of peripheral and cerebral ischaemia (Wang *et al.*, 2009). Of note, cellular senescence of vascular smooth muscle cells (VSMC) promotes atherosclerosis and plaque vulnerability (Wang and Bennett, 2012; Wang *et al.*, 2015).

Senescent cells produce an array of growth factors and SASP-related pro-inflammatory cytokines (Minamino and Komuro, 2007; Ritschka *et al.*, 2017) and, in the long-term, SASP instigates a state of chronic oxidative stress (Lee *et al.*, 2009) and low-grade inflammation within the vascular wall, which are hallmarks of atherosclerosis (Hopkins, 2013). Consequently, vascular HSR, which may indeed increase during the early phases of atherogenesis (Xu *et al.*, 2012; Akoumianakis and Antoniades, 2016), finally collapses at late stages leading to loss of proteostasis and deregulated nutrient sensing and contributing to plaque instability (Uryga and Bennett, 2016). Therefore, cellular senescence parallels the development of dysfunction in different tissues, having a central role in the aetiology and progression of common chronic inflammatory diseases (Zhu *et al.*, 2014), all of which are observable in menopause.

Oestrogen, on the other hand, increases re-endothelialisation and endothelial function after vascular injury (Krasinski *et al.*, 1997) and delays endothelial senescence (Imanishi *et al.*, 2005). As predicted, by blunting vascular senescence, oestrogen is anti-inflammatory. However, oestrogen-dependent vasoprotective and anti-inflammatory effects in ovariectomised rats are effective only in young ovariectomised animals (Miller *et al.*, 2007) and the same applies in relation to the oestrogen-dependent antisenescent effects on VSMC (Zhu *et al.*, 2011) and cardioprotection (Stice *et al.*, 2011). Furthermore, neuroprotective effects of oestrogen-HRT against cognitive impairment is observed only in middle-aged women, but not if HRT is initiated in late life (Whitmer *et al.*, 2011). This may explain why, in cohort clinical trials, the relative benefits or risks of HRT are strictly dependent on the women's age (Rossouw *et al.*, 2002; Hodis *et al.*, 2016). Just to illustrate this point, the mean age of women at the enrolment in Heart and Oestrogen/progestin Replacement Study (HERS) was 67 years (Hulley *et al.*, 2002; Huang *et al.*, 2009) and in the Women's Health Initiative (WHI) it was 63 years (Rossouw *et al.*, 2002).

Altogether, the above findings strongly suggest an intricate mechanistic and temporal relation of hot flushes with chronic inflammatory diseases observed in perimenopausal and menopausal women, that involves cellular senescence and impaired HSR. Noteworthy, the lack of oestrogen alone is not the whole reason for the reduced HSR observed in menopause (Miragem *et al.*, 2015). Rather, chronic deterioration of hypothalamic structures involved in HSP70 production also seem to be responsible.

The NO-HSP70 axis, thermoregulation and hot flushes

It is well established that at least some of the cytoprotective and anti-inflammatory effects of oestrogen-mediated HSR (Hamilton *et al.*, 2004a, 2014b; Takao *et al.*, 2005; Stice and Knowlton, 2008) is due to oestrogen-elicited production of the vasodilatory gaseous free radical nitric oxide (NO) in different tissues. This is because NO is a powerful activator of HSP70 expression (Malyshev *et al.*, 1995, 1996, 2000; Xu *et al.*, 1997) and its accompanying HSR. This also explains why NO is neuroprotective albeit inducing oxidative/nitrosative stress, because the mild NO-elicited redox imbalance triggers HSR (Calabrese *et al.*, 2007). Consistent with this notion, oestrogen protection against cerebral ischaemia (Lu *et al.*, 2002) and other types of injury (Mendelsohn and Karas, 1999) is attributable to its effects on HSP70 expression. Moreover, morphine (which is appreciated for being neuroprotective in misfolded-protein aggregatory diseases) protects neurons against intracellular amyloid-β toxicity by inducing oestrogen release and an enhanced HSR (Cui *et al.*, 2011). On the other hand, oestrogen elicits rapid production (in seconds) of NO through the activation of endothelial NO synthase (eNOS, encoded by the *NOS3* gene) (Prevot *et al.*, 1999), which occurs non-genomically via G_q-coupled membrane mER receptors (Chambliss and Shaul, 2002; Stice and Knowlton, 2008; Monteiro *et al.*, 2014). Local NO production is responsible for oestrogen-induced vasorelaxation (Scott *et al.*, 2007) whilst NO mediates the protective effects of oestrogen on the cardiovascular system in premenopausal women, and this transcends the simple effects of oestrogen on plasma lipids (Mendelsohn and Karas, 1999).

Hot flushes, in turn, may be a manifestation that follows on from chronic inflammatory diseases in which HSR is jeopardized, whilst these VMS are related to perturbed processing of thermoregulatory information at the level of hypothalamus. Notably, the hypothalamic areas involved in thermoregulation (infundibular nucleus in humans and arcuate nucleus in other mammals) are the same areas whose neurons are known to have their function altered after long-term oestrogen ablation, particularly the kisspeptin-neurokinin B (NKB)-dynorphin (KNDy) neurons (Nakamura and Morrison, 2010; Nakamura, 2011; Rance *et al.*, 2013). Interestingly, KNDy neurons also drive neuroprotective expression of HSP70 (Kim *et al.*, 2010; Wyon *et al.*, 2000; Chilumuri and Milton, 2013).

Besides acting upstream of GnRH neurons to control LH secretion patterns (Rance *et al.*, 2013; Skorupskaite *et al.*, 2014), KNDy neurons convey information to and receive projections from multiple nutrient/metabolic sensing hypothalamic nuclei to prompt appropriate autonomic responses. This may explain many metabolic dysfunctions related to sex hormones, such as hypogonadotropic hypogonadism observed in obesity and diabetes, in which KNDy function is depressed, as well as polycystic ovary syndrome (PCOS), in which KNDy neuron activity is exacerbated, as excellently reviewed (Skorupskaite *et al.*, 2014). Indeed, there is a coincidence of obesity and occurrence/severity of VMS in PCOS patients, who develop obesity and insulin resistance (Dupont and Scaramuzzi, 2016) as an intrinsic feature of the syndrome, not just a consequence (Stepto *et al.*, 2013). Still in line with the involvement of NO deficiency in such dysfunctions, insulin levels and the homoeostatic model assessment (HOMA) of PCOS patients negatively correlate with NO production (Nácul *et al.*, 2007).

Taking the above considerations as a whole, it is not illogical that hot flushes might be related to an evolutionary response attempting to re-equip the NO-HSP70 axis during the downfall of circulating oestrogen. Consequently, menopausal VMS may be a disguised clue

to neuroendocrine alterations linking oestrogen loss to defiencies in the anti-inflammatory and antisenescent HSR.

Methods

Results from our own group and recent evidence on hypothalamic control of central temperature guided a search on PubMed and Google Scholar websites. Boolean searches were performed, with no date- or species-limiting filters, by using different combinations of keywords.

The Heat Shock Response

The physiological performance of any protein depends on the integrity of the spatial conformation of the polypeptides. Therefore, during an ~2-billion-year gap, cells evolved a special set of proteins assigned to assist in non-covalent protein folding (Kampinga *et al.*, 2009; Kampinga and Craig, 2010; Zuiderweg *et al.*, 2017). These 'anti-misfolding' proteins, that are also able to correcting unfolded proteins, avoiding the formation of protein aggregates, are collectively referred to as chaperones, as examplified by HSP70.

Intracellular HSP70 and the antisenescent resolution of inflammation

Although HSPs were named after the serendipitous observation that heat-shocked *Drosophila busckii* fruit-fly cells strongly expressed this class of proteins (Ritossa, 1962), HSP70 expression is associated with other homeostatically stressful situations, not only heat (Heck *et al.*, 2011; Ludwig *et al.*, 2014; Newsholme and Homem de Bittencourt, 2014). The HSR pathway, i.e. the biosynthetic route that leads to HSP70 expression through the activation of its main transcription factor, heat shock factor-1 (HSF1) (Lindquist, 1986; Anckar and Sistonen, 2011), has general protective functions and involves confluent pathways that couple exercise, energy balance and protein homoeostasis (proteostasis) to inflammatory responses via HSP70 (Fig. 1). In addition, protein misfolding and accumulation of misfolded-protein aggregates is at the core of several degenerative conditions, such as Alzheimer's, Huntington's and Parkinson's diseases (Leite *et al.*, 2016). We shall focus on inflammation because chronic inflammatory diseases (many, if not all, of them encountered in menopausal women at some phase) share a defective HSR as the common underlying aetiology.

Whatever the evolutionary reason, inflammation mounts a HSR, which, in turn, acts as a negative feedback, being anti-inflammatory. When an acute inflammatory response is activated somewhere in the body, there is a massive production of pro-inflammatory arachidonic acid-derived prostaglandins (PGs) as well as other lipid mediators and vasoactive compounds. Such signalling molecules warn immune cells and sensory pathways about the presence of an invader or tissue injury, besides increasing vascular permeability that allows the arrival and activation of additional inflammatory cells and tissue repair (Medzhitov, 2008). This occurs due to the triggering of a finely orchestrated expression of inducible proteins centred at nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family (NF-κB), which drive inflammation during the initial phase (Oeckinghaus and Ghosh, 2009) but also allow its resolution (Gilroy *et al.*, 1999; Serhan, 2011; Newsholme and Homem de Bittencourt, 2014).

As early as within 2 h after the start of an inflammatory response, there is always a pronounced NF-κB-induced expression of cyclo-oxygenase-2 (COX-2), which synthesizes conspicuous amounts of PGE_2 from arachidonic acid. At this point, both selective COX-2 inhibitors (COXIBs) as well as the traditional dual COX-1/COX-2 nonsteroidal anti-inflammatory drugs are able to inhibit the early phase. However, such inhibitors strongly exacerbate inflammation at late stages (48 h), thus preventing the resolution phase of inflammation (Serhan, 2011); this tends to perpetuate the inflammatory state.

PGE_2 induces fever by blocking the processing of thermosensory information at the preoptic area (POA) of the hypothalamus (Nakamura *et al.*, 2004; Nakamura, 2011), which consequently activates autonomic heat-sparing mechanisms triggered at rostral medullary raphe pallidus premotor nuclei. Following core temperature elevation, the highly evolutionarily conserved HSR turns on a transcriptional program based on the activation of HSF1, which is heat-sensitive (Singh and Hasday, 2013). Structural changes in the plasma membrane during the establishment of fever participates in HSF1 activation as well (Török *et al.*, 2014). The prime impact of HSF1 activation is the elevated production of HSP70, a molecular chaperone that reverses the formation of protein aggregates, reducing protein denaturation that could emerge during the rise of temperature. Therefore, heat-stress stimulates HSF1-induced HSP70 expression to protect cells against proteotoxic stress, so that HSR supports proteostasis and cytoprotection (Anckar and Sistonen, 2011). Strikingly, HSP70 associates with the complex formed by NF-κB with its inhibitor (IκB) thus impeding NF-κB translocation into the nucleus (Chen *et al.*, 2005).

Besides inducing fever, PGE_2 undergoes dehydration into PGA_2 (Gutierrez *et al.*, 2008), which is a highly electrophilic α,β-unsaturated cyPG capable of shutting off NF-κB, both directly and via the activation of HSF1 (Rossi *et al.*, 2000). Heat itself induces HSF1-dependent expression of COX-2 (Rossi *et al.*, 2012) leading to huge PG production. Altogether, these mechanisms result in an explosive production of HSP70 which, *per se*, inhibits NF-κB-dependent pro-inflammatory pathways (Fig. 1). Finally, inducers of HSR, such as cyPGs, potently inhibit viral replication in a myriad of DNA and RNA viruses (Santoro *et al.*, 1980, 1982, 1987; Santoro, 1994), including human immunodeficiency virus type-1 (HIV-1) (Rozera *et al.*, 1996), this being mediated by HSF1-dependent inhibition of NF-κB activation and concurrent activation of HSR for full antiviral activity (Santoro *et al.*, 1989; D'Onofrio *et al.*, 1990; Amici *et al.*, 1992; Rossi *et al.*, 1997). Blocking of virus replication by cyPGs depends on HSP70 synthesis (Amici and Santoro, 1991; Amici *et al.*, 1992) and this explains why hyperthermic treatment (including fever-like range) is antiviral (De Marco and Santoro, 1993). This is remarkable because menopausal women present imbalanced immune function (Ghosh *et al.*, 2014) whilst virus infection is independently associated with VMS in natural menopause (Cieloszyk *et al.*, 2009). Although, there is still much contradictory information to dissociate virus infection from menopausal symptoms (Imai *et al.*, 2013), there is a trend toward the reporting of more sleep disturbances and elevated anxiety symptoms associated with learning deficits amongst HIV-infected women compared to uninfected women (Rubin *et al.*, 2014). Also, age at menopause is ~2 years earlier in HIV-infected women (Boonyanurak *et al.*, 2012).

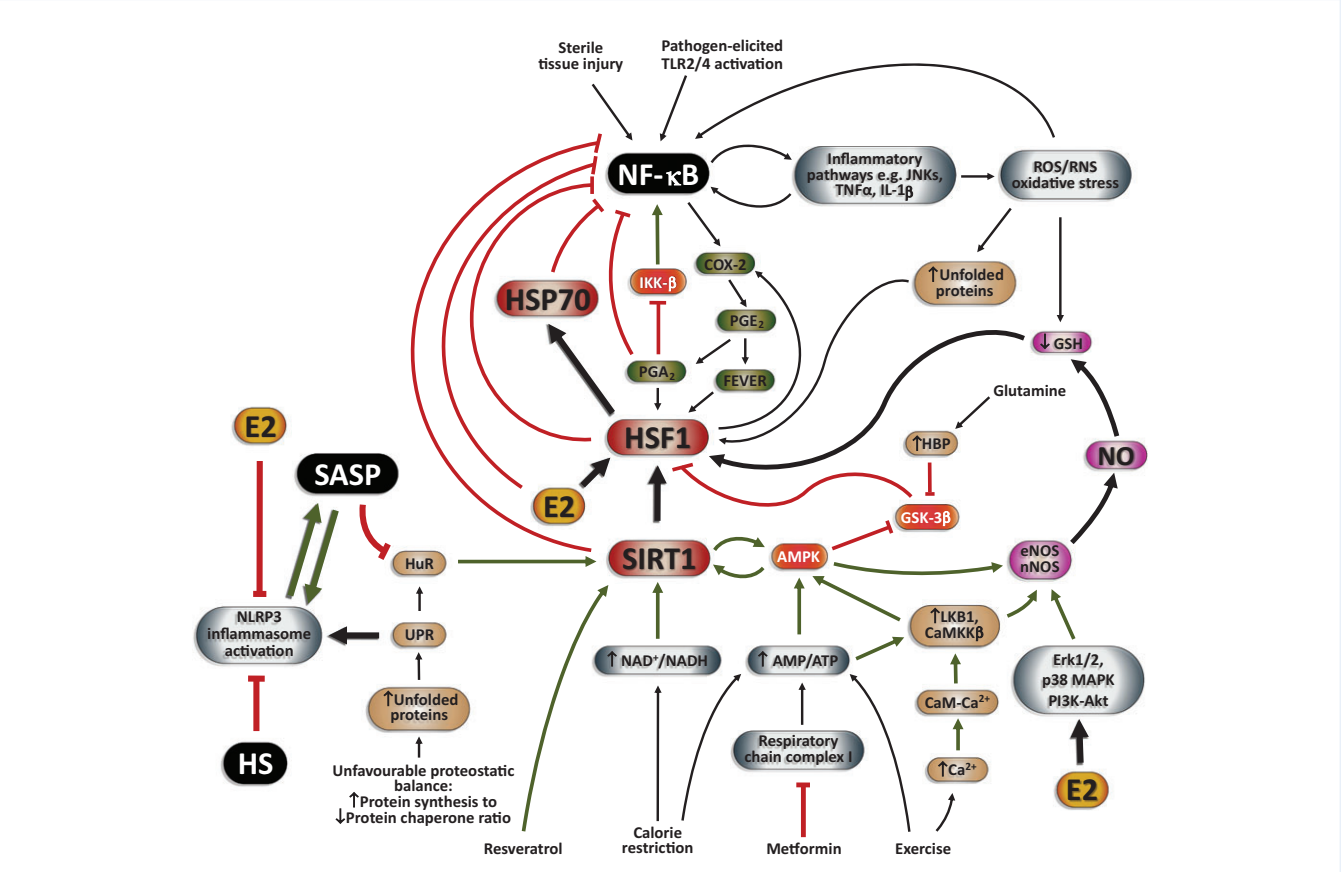

Figure 1 The HSR pathway. Stress-activation of the biosynthetic pathway that leads to HSP70 expression from HSF1 couples oestrogen, exercise, energy balance and proteostasis to anti-inflammation via HSP70. During either sterile tissue injury or pathogen-elicited Toll-like receptor-2 and -4 (TLR-2/4)-triggered inflammatory responses, nuclear factors of κB family (NF-κB) are activated and signal to pro-inflammatory gene expression. On the other hand, a physiological negative feedback system that 'resolves' inflammation is also enabled via the HSR pathway. HSF1 may be directly activated by PGE_2-induced rise in temperature (fever) during nuclear factor NF-κB-elicited cyclo-oxygenase-2 (COX-2) induction. PGA_2, the dehydration product of PGE_2, is also able of counteracting NF-κB downstream effects by directly blocking IκB kinase-β (IKKβ). HSP70, *per se*, blocks NF-κB activation and transcribing activity. Following oxidative stress and the formation of reactive oxygen and nitrogen species, ROS/RNS, conformational changes in unfolded proteins can be relayed to HSF1 either directly or via changes in GSH/protein sulphydryl redox status or, finally, after the activation of the UPR protocol that activates HSF1 through the SIRT1 pathway. This happens through UPR-mediated activation of HuR, an RNA-binding protein that stabilizes SIRT1 mRNA and, consequently, its expression. SIRT1 downstream signals can also be transmitted to the HSR pathway via metabolic alterations, such as those that increase nicotinamide dinucleotide redox status (↑NAD +/NADH ratio) or adenosine monophosphate to triphosphate (↑AMP/ATP) ratio and the consequent activation of 5′-AMPK. AMPK, the master fuel sensing kinase, may also be turned on by calorie restriction, physical exercise or the antidiabetic drug metformin, which is also the gold standard for treating metabolic disorders associated with PCOS. Activated AMPK phosphorylates and blocks GSK-3β, which constitutively inhibits HSF1, so that activated AMPK causes disinhibition of HSF1, leading to an anti-inflammatory HSR. Glutamine metabolism, by increasing metabolic flux through the HBP, also blocks GSK-3β thereby enhancing HSR. Oestrogen (E2) can activate HSR machinery both directly acting over HSF1 activation and by membrane surface oestrogen receptors that signals through Erk1/2, p38 MAPK and PI3K/Akt pathways leading to endothelial and neuronal NO synthase (eNOS/nNOS) activity and NO production which, in turn, unveils a discrete redox imbalance that activates HSF1. Additionally, oestrogen blocks SASP that emanates from continuous activation of NLRP3 (nucleotide binding and oligomerisation domain-like receptor family pyrin domain-containing-3) inflammasome which, in turn, impairs HuR-dependent activation of HSR through the SIRT1-HSF1 pathway. SASP results from long-term ER stress and its consequent unremitted activation of UPR. Similarly, HS treatment, even in the fever-like range, blocks NLRP3 inflammasome-dependent SASP, re-establishing HuR-SIRT1-HSF1 downstream pathways, so that, HS itself can, paradoxically, re-establish the HSR. SASP, senescence-associated secretory phenotype. HSR, heat shock response; PCOS, polycystic ovary syndrome; HSF1, heat shock factor; GSH, glutathione; ER, endoplasmic reticulum; AMPK, adenosine monophosphate kinase; HBP, hexosamine biosynthetic pathway; UPR, unfolded protein response; SIRT1, sirtuin; GSK-3β, glycogen synthase kinase-3β.

As HSR is a multifaceted anti-inflammatory and antiviral pathway that naturally resolves inflammation, selective manipulation of the HSR has the potential to control and obviate multifactorial diseases (Morimoto and Santoro, 1998). Because of this, it is not surprising that the long-term use of COXIBs, which seriously compromises physiological triggering of HSR, has proven to be so harmful to human

health (FitzGerald, 2004; Grosser *et al.*, 2010), leading to cardiovascular complications that resulted in the withdrawal of rofecoxib and valdecoxib from the market (Grosser *et al.*, 2010).

Proteotoxic stimuli, senescence and HSR

Not only fever or PGE_2 metabolism can activate HSR, but other potentially proteotoxic signals also converge to enhance HSP70 expression. As depicted in Fig. 1, NF-κB-elicited inflammatory pathways trigger the production of reactive oxygen and nitrogen species (ROS/RNS), which tend to deplete intracellular glutathione (GSH) contents, thus increasing oxidation of protein thiols and augmenting the amount of unfolded proteins; all the aforementioned challenges are inducers of HSF1 activation and HSR (Anckar and Sistonen, 2011; Ahn and Thiele, 2003; Liu *et al.*, 1996). HSF1 is redox-regulated due to highly conserved cysteines C35 and C105 (Ahn and Thiele, 2003). In the case of NO, protective redox-mediated induction of HSP70 by this free radical was first demonstrated in rat hepatocytes treated with tumour necrosis factor-α (TNF-α) (Kim *et al.*, 1997), in which NO-induced HSP70 expression followed oxidation of cysteine-containing GSH molecules, leading to a moderate redox imbalance. NF-κB-elicited expression of inducible NO synthase (iNOS, encoded by the *NOS2* gene) also produces copious amounts of NO which, in turn, strongly activates HSR (Xu *et al.*, 1997), being responsible for NO-based cytoprotection (Kim *et al.*, 1997). NO results in accumulation of HSP70 in tissues (Malyshev *et al.*, 1996), and whole-body HS in conscious animals induces marked NO-dependent expression of HSP70 in different organs (Malyshev *et al.*, 1995). Indeed, concerted NO production and HSP70 expression in the rat brain mediates the adaptation of blood pressure to heat treatment-induced hypotension (Malyshev *et al.*, 2000), which explains why some can benefit from chronic heat treatment (e.g. sauna or hot tub) without any appreciable perturbation of blood pressure regulation (Krause *et al.*, 2015c).

As summarized in Fig. 1, HSR is crucial for the resolution of inflammation. Unfortunately, however, HSR is grievously impaired in metabolic tissues (e.g. skeletal muscle, liver and adipose tissue) during low-grade chronic inflammatory diseases (Leite *et al.*, 2016). T2DM patients, for example, present reduced intramuscular expression of the chaperones haem oxygenase-1 (HSP32) and HSP70 (Bruce *et al.*, 2003), leading to a state of exacerbated inflammation at the muscle level, with out-of-control NOS2-dependent NO production and impaired insulin receptor downstream signalling (Carvalho-Filho *et al.*, 2005). Moreover, we also observed that the HSF1-HSP70 axis is progressively suppressed in adipose tissue and liver of insulin resistant obese patients, as NAFLD evolves from steatosis, towards more inflammatory manifestations of the disease (Di Naso *et al.*, 2015). In this case, a blunted HSR is correlated with enhancement of activated c-Jun N-terminal Kinases (JNK1 and JNK2) in adipose tissue, which tends to exponentially enhance inflammation.

In addition, and most importantly, reduced HSP70 expression in obesity and diabetes is always accompanied by reduced HSF1 expression (Rodrigues-Krause *et al.*, 2012; Di Naso *et al.*, 2015), so that it is impossible for the tissues to resolve inflammation through HSR because its principal trigger (HSF1) is practically absent. This raises the question as to why chronic inflammation suppresses HSF1 expression thus impeding its physiological resolution? The answer is related to cellular senescence that is activated by a series of concatenated events. First, positive energy balance and/or continuous lipid input and turbulent shear stress overburdens endoplasmic reticulum (ER) of local tissues leading to ER stress (Hotamisligil, 2010; Gregor and Hotamisligil, 2011; Cnop *et al.*, 2012; Arruda *et al.*, 2014). As ER stress is not resolved because the noxious stimuli do not disappear (i.e. inappropriate diet, low-physical activity and hypertension), ER stress evolves to the unfolded protein response (UPR), which is an evolutionarily conserved strategy to stop cell function, thus avoiding the accumulation of protein aggregates and the consequent apoptotic cell death and tissue injury (Hetz, 2012). However, UPR activates several cascades of potentially pro-inflammatory pathways so that, if the ER stress-initiating factors are not removed, the UPR becomes indefinitely inflammatory.

Persistent UPR leads to continuous activation of NLRP3 (nucleotide binding and oligomerisation domain-like receptor family pyrin domain-containing-3) inflammasome, which mediates the cleavage of inactive procaspase-1 and pro-interleukins into their active forms (Chalkiadaki and Guarente, 2012), giving rise to SASP and cellular senescence (with the clinical implications for menopausal women, as discussed in the 'Introduction' section). SASP-related production of inflammatory cytokines strengthens a persistent DNA damage-like response in which NLRP3-dependent caspase-1 may promote apoptosis. In this process, NLRP3-dependent caspase-1 mediates the cleavage of an RNA-binding protein (HuR) (Talwar *et al.*, 2011) that is responsible for maintaining high-intracellular expression and activity of HSF1 (Kim *et al.*, 2012), via the sirtuin-1 (SIRT1) route, as illustrated in Fig. 1. Caspase-1 switches HuR function from pro-survival to pro-apoptotic (Von Roretz *et al.*, 2013). However, long-term NLRP3 inflammasome activation and disseminated SASP eventually lead to cellular senescence instead of apoptosis. Indeed, senescence would be an alternative mechanism to UPR for the cell to avoid apoptotic death as a result of an inoperative anti-inflammatory HSR. In fact, senescent cells resist apoptosis, so that chronically inflamed cells are likely to persist in tissues (Burton, 2009), thus eternalizing chronic inflammatory diseases.

Strikingly, oestrogen depletion is implicated in a variety of inflammatory events in the central nervous system (CNS) that are associated with NLRP3 inflammasome activation. Oestrogen replacement reverts this scenario by shutting off NLRP3 inflammasome activation in a type-β oestrogen receptor (ERβ)-dependent way (Xu *et al.*, 2016). The same has been observed in reproductively senescent female rats (De Rivero Vaccari *et al.*, 2016). Similarly, after global cerebral ischaemia (a condition known to produce intense oxidative stress and inflammation), NLRP3 inflammasome activation is completely inhibited by local oestrogen administration (Thakkar *et al.*, 2016). Today, it is known that both oestrogen and progesterone mediate anti-inflammation in the CNS by downregulating inflammasome activation (Slowik and Beyer, 2015). All of these observations confirm the anti-inflammatory effects of oestrogen that parallel its antisenescent actions, as discussed above. This corroborates the paradigm that at least some of the protective effects of oestrogen are related to its ability to sustain a strong HSR by interrupting the vicious cycle that decreases HSF1 availability due to cellular senescence. Importantly, HS treatment hampers the activation of NLRP3 inflammasome (Levin *et al.*, 2008), thus overcoming the destruction of HuR and tissue senescence associated with depressed HSF1 expression

and low HSR. Hence, HS alone can, paradoxically, re-establish the HSR. These observations alongside the findings described in the following sections persuasively indicate that heat treatment should be evaluated in menopausal women.

Metabolic activation of HSR

HSR is considered the most highly conserved genetic system known, existing in every organism in which it has been sought, from archaea to bacteria and eukarya, from plants to animals (Lindquist and Craig, 1988). As impressive as it may seem, HSR is also recruited from other branches of metabolism very far from proteostasis and inflammation, at least *a priori*. HSF1 expression (Kim *et al.*, 2012) and transcribing activity (Westerheide *et al.*, 2009) are strongly enhanced by the nicotinamide adenosine dinucleotide (NAD^+)-dependent histone deacetylase of class III family SIRT1, because SIRT1 prolongs HSF1 binding to the promoters of HS genes by maintaining HSF1 in a deacetylated, DNA-binding competent state (Westerheide *et al.*, 2009). This links calorie restriction (which enhances the NAD^+ to NADH ratio) and the consumption of resveratrol-rich natural products (e.g. red wine) to HSR because both quoted situations activate SIRT1 (Fig. 1). Metformin, which attenuates hyperglycaemia-induced cellular senescence and apoptosis, reverts such unhealthy conditions by restoring SIRT1 expression and activity (Arunachalam *et al.*, 2014). Although, human clinical trials of resveratrol have been inconclusive, its metabolically advantageous effects in postmenopausal (Chow *et al.*, 2014) and PCOS women (Banaszewska *et al.*, 2016) are evident and involve SIRT1-dependent arming of HSR.

HSF1 activation via the SIRT1 route can also be attained by metabolic changes that promote 5′-adenosine monophosphate kinase (AMPK) activity, such as physical exercise and metformin (Fig. 1) (Hardie *et al.*, 2012; Kauppinen *et al.*, 2013; Hooper *et al.*, 2014). Notably, metformin activates AMPK after enhancing AMP to ATP ratios by inducing a transient inhibition of mitochondrial respiratory chain complex I (Viollet *et al.*, 2012). However, metformin- and exercise-induced activation of AMPK provides also NO production via both endothelial (NOS3) and neuronal (NOS1) NO synthases because AMPK activates both enzymes by direct phosphorylation (Chen *et al.*, 2009; Kar *et al.*, 2015). As metformin can bypass the SIRT1 step of HSR by activating a collateral pathway (LKB1/CaMKKβ/NOS/NO), this drug shows antisenescent and anti-inflammatory effects (Moiseeva *et al.*, 2013; Arunachalam *et al.*, 2014; Oubaha *et al.*, 2016) that inhibit NF-κB downstream pathways (Moiseeva *et al.*, 2013; Saengboonmee *et al.*, 2017), also independently of AMPK activation (Moiseeva *et al.*, 2013). It is of note that oestrogen, via mER (Prevot *et al.*, 1999; Chambliss and Shaul, 2002; Stice and Knowlton, 2008), also enhances this branch of HSR through NO production (Fig. 1).

HSR-dependent anti-inflammation intertwines with metabolic regulation also via glycogen (i.e. energy storage) metabolism. As illustrated in Fig. 1, AMPK activates HSR because AMPK blocks glycogen synthase kinase-3β (GSK-3β), which constitutively inhibits HSF1 (He *et al.*, 1998; Xavier *et al.*, 2000). GSK-3β, a negative regulator of glycogen synthase, is inhibited under 'energy plentiful' situations, eventually directing glucose storage into glycogen (Newsholme *et al.*, 2016). This makes sense physiologically since AMPK is a major 'energy sensing' kinase for 'fuel shortage' stress. Curiously, AMPK-dependent blockade of the inhibitory GSK-3β occurs physiologically via the hexosamine biosynthetic pathway (HBP) (Kazemi *et al.*, 2010), which is also a nutrient-sensing pathway (Wang *et al.*, 1998) that presents multiple connections with energy metabolism and HSR, not only with glycogen synthesis (Newsholme *et al.*, 2013; Curi *et al.*, 2016, 2017; Leite *et al.*, 2016). Conversely, glutamine (an HSP70 co-inducer) potentialises the HSR after being metabolized through the HBP, which hampers GSK-3β inhibition over HSF1 activation (Leite *et al.*, 2016). In total, activated AMPK causes disinhibition of HSF1, leading to an anti-inflammatory HSR.

Energy sensing and the proteostasis-protecting HSR are also connected to anti-inflammation through SIRT1 activation that may take place after UPR, as stated above. Cellular insults, including genotoxic stress and UPR, activate the mRNA-binding protein HuR (Gorospe and De Cabo, 2008), which, in turn, associates to the 3′-untranslated region of SIRT1 mRNA thus enhancing its stability and increasing SIRT1 protein expression levels. SIRT1 itself blocks NF-κB transcriptional activity (Yeung *et al.*, 2004) by physically interacting with the RelA/p65 subunit of NF-κB. Moreover, SIRT1 directly inhibits NLRP3 inflammasome activation (Lee *et al.*, 2015). As a result, SIRT1 is anti-inflammatory *per se*. The HSR can also be activated via the UPR-SIRT1 route (Fig. 1). This firmly tightens any imbalance in proteostasis with anti-inflammation through HSR. On the contrary, chronic activation of NLRP3 inflammasome and persistent oxidative stress, as seen in low-grade inflammatory diseases, depress HuR expression (Talwar *et al.*, 2011) and consequently, ablates the production of SIRT1 molecules, leading to a dramatic decrease in HSF1 expression (Kim *et al.*, 2012).

The above relationships between the anti-inflammatory HSR and metabolic regulation helps us to understand why the hypothalamic areas involved in dictating GnRH and oestrogen availability are, at the same time, connected to and under influence of metabolic signals (e.g. insulin, leptin and glucose levels) that integrate energy metabolism and reproduction (Rance *et al.*, 2013; Skorupskaite *et al.*, 2014). Apropos, these nuclei are the same as those involved in oestrogen actions via HSR (Olazábal *et al.*, 1992a,1992b), as detailed next.

HSP70, thermoregulation and cytoprotective thermotolerance

Dissipation of excess heat is crucial for life because minimal increments in core or brain temperature can shut down cellular functions due to protein denaturation (Nakamura and Morrison, 2010). Therefore, animals incorporated physiological mechanisms devoted to heat dissipation whilst avoiding protein aggregation. For instance, warming signals from the peripheral warm sensors reach the POA of hypothalamus resulting in autonomic heat-defence responses, such as cutaneous vasodilation and inhibited thermogenesis. Medial preoptic (MPO) and median preoptic (MnPO) nuclei of POA are highly responsive to the febrile effects of PGE_2 (Nakamura, 2011). Conversely, PGE_2-induced fever as well as PGA_2 produced from PGE_2 dehydration are strong inducers of HSF1 activation and of the HSP70-centred HSR which, in turn, promotes neuroprotection (Lindquist, 1986; Anckar and Sistonen, 2011).

Animals can obtain protection against overheating also by acquired thermotolerance, which comprises a set of physiological accommodations that permit the organism to still survive, despite the presence of

further heat challenges. Indeed, HSP70 synthesis is the capital element for such a preventive response (Lindquist, 1986), including during whole-body hyperthermia-induced thermotolerance (King *et al.*, 2002). Remarkably, PGE-derived cyPGs of A-type powerfully induce HSP70-based thermotolerance in human cells (Elia *et al.*, 1996). Contrarily, PGs of the B, E or F type (which are not α,β-unsaturated cyPGs) are not effective (Amici *et al.*, 1993).

Since various stressful routes converge to HSR, there exists a protective 'cross-reactive thermotolerance', whether the previous defiance had been heat or any other HSP70 inducer. Therefore, strong HSR inducers, either physiological [e.g. physical exercise and hot tub therapy (Hooper *et al.*, 2014; Krause *et al.*, 2015a, 2015b, 2015c)] or pharmacological [e.g. cyPG formulations (Homem de Bittencourt *et al.*, 2007)], are also cytoprotective, leading to amelioration of unhealthy conditions, such as diabetes (Hooper, 1999; Chung *et al.*, 2008) and atherosclerosis (Gutierrez *et al.*, 2008). Whether menopausal women lose oestrogen-induced cytoprotection via thermotolerance is a matter that deserves to be addressed urgently. However, it is envisaged that chronic heat treatment, which is the most powerful physiological inducer of thermotolerance, should improve the HSR of women especially in the absence of oestrogen.

Extracellular HSP70 (eHSP70) as a danger signal and HSR indices

The same stressful stimuli that promote intracellular HSP70 (iHSP70) expression can trigger its export towards the outside space. Detection of extracellular HSP70 (eHSP70) has been reported in a variety of cell types (Chirico *et al.*, 1988; Hightower and Guidon, 1989; Hunter-Lavin *et al.*, 2004; Calderwood *et al.*, 2007; Ireland *et al.*, 2007; De Maio, 2011; De Maio and Vazquez, 2013; Heck *et al.*, 2017), both *in vitro* and *in vivo*, in response to heat treatment, exercise and α_1-adrenergic stimulation (Chin *et al.*, 1996; Walsh *et al.*, 2001; Lacoste *et al.*, 2001a, 2001b; Febbraio *et al.*, 2002; Johnson *et al.*, 2005; Johnson and Fleshner, 2006; Giraldo *et al.*, 2010; Henstridge *et al.*, 2016; Schöler *et al.*, 2016). Although cells may pour out their iHSP70 contents after cell disruption (e.g. during necrotic cell lysis), eHSP70 is mainly exported through a specialized non-canonical secretory mechanism via exosomes (Lancaster and Febbraio, 2005; Mambula *et al.*, 2007).

Once secreted, eHSP70 works as a pro-inflammatory cytokine and signals to physiological systems for the presence of homoeostatic challenges (Heck *et al.*, 2011; De Maio and Vazquez, 2013) after eHSP70 binding to Toll-like receptors (TLR-2, -4 and -7) in a variety of cells. This activates NF-κB-centred pro-inflammatory pathways (Asea *et al.*, 2000, 2002; De Maio, 2011; De Maio and Vazquez, 2013; Ott *et al.*, 2014; Grunwald *et al.*, 2014, 2017), thereby stimulating the immune system at different key points and eventually triggering pro-inflammatory responses that elevate immunosurveillance (Whitham and Fortes, 2008). eHSP70 pro-inflammatory behaviour is similar to that observed for eHSP60, which exerts its pro-inflammatory actions by interacting with CD14/TLR4 complexes, and is regarded as a risk factor for atherosclerosis (Xu *et al.*, 2012). In so far as, eHSP70 is secreted upon the stressful environment of inflamed tissues, serum eHSP72 concentrations positively correlate with markers of chronic inflammation in humans, e.g. C-reactive protein, monocyte count and TNF-α (Krause *et al.*, 2014, 2015b; Leite *et al.*, 2016). As per these characteristics, eHSP70 is reputed to be a danger signal that enhances the state of awareness of all the physiological systems for the presence of homeostatically threatening conditions (Matzinger, 1994; Todryk *et al.*, 2000; Welch, 2001; Heck *et al.*, 2011). This is reinforced by the fact that overall HSP70 metabolism (including its intra- and extracellular actions) works in perfect parallel with the tonic status of sympathetic nervous system (SNS), which is the master sentinel of homoeostasis.

Because iHSP70 and eHSP70 tend to show opposed behaviours and patterns of expression, it has been conjectured that there should exist an optimal physiological balance between eHSP70 and iHSP70, so that the immunoinflammatory equilibrium is attained (Heck *et al.*, 2011; Krause *et al.*, 2015b). This supposition led us to compare the evolution of eHSP70-to-iHSP70 ratios (H-index of HSR) in different situations and, in fact, the H-index, measured in different tissues and cell types in relation to plasma or culture media, has started to be ascribed as a novel and overall index of immunoinflammatory status of an individual (Krause *et al.*, 2015a, 2015b; Keane *et al.*, 2015; Schöler *et al.*, 2016; Leite *et al.*, 2016; Goettems-Fiorin *et al.*, 2016; Heck *et al.*, 2011, 2017, see technical details in Heck *et al.*, 2017).

The assessment of the H-index in menopausal women is envisaged to become invaluable in estimating patients' HSR and its associations with hot flushes. This specific utilization is currently under investigation in our laboratory.

Adrenergic stimulation

Once the SNS developed as the master physiological avenue for the activation of protective responses against aggressions to homoeostasis, integration between the SNS, energy sensing and HSR naturally evolved. In fact, HSP70 expression is induced by α-adrenergic stimulation in many tissues (Matz *et al.*, 1996a; Lacoste *et al.*, 2001a, 2001b; Giraldo *et al.*, 2010). Conversely, α_1-adrenergic blockade impedes HSR (Matz *et al.*, 1996b; Cox *et al.*, 2014). Acute stress, via α_1-drenoceptors, also increases the secretion of eHSP70-containing exosomes (Beninson *et al.*, 2014) and circulating levels of eHSP70 (Johnson *et al.*, 2005), consistent with a general protective role of both SNS and eHSP70 in warning the physiological systems about the presence of homoeostasis-threatening situations (Campisi *et al.*, 2003; Heck *et al.*, 2011). Exercise, which is one of the most powerful inducers of iHSP70, strongly induces eHSP70 export into the circulation (Febbraio *et al.*, 2002; Speaker *et al.*, 2014) and culture media of cells obtained from exercised animals (Schöler *et al.*, 2016); this is associated with enhanced immunosurveillance (Ortega *et al.*, 2006, 2009; Rossato *et al.*, 2014) that is known to be mediated by α_1-adrenergic signalling (Johnson *et al.*, 2005). Strikingly, however, if a volunteer ingests glucose during the exercise sessions, the expected rise in circulating eHSP70 is abolished (Febbraio *et al.*, 2004).

Glucose concentrations, energy sensing, HSR and sympathetic activity are interconnected at the level of ventromedial hypothalamus (VMH), which integrates feeding, fear, thermoregulation and sexual activity. On the other hand, the VMH is where a cogent oestrogen-induced HSP70 takes place (Olazábal *et al.*, 1992b). In addition, hypothalamic mechanisms for triggering fever (and HSR) in the POA can be modulated by norepinephrine (Nakamura, 2011). Notably, as in the case of eHSP70 secretion (Febbraio *et al.*, 2004), glucose ingestion abrogates hypoglycaemia-induced counterregulatory actions of

the SNS (Sprague and Arbeláez, 2011), which depend on VMH neuronal circuitry (Garfield *et al.*, 2014). Therefore, it is not surprising that the incidence of hot flushes, which are precipitated due to abnormal activity of hypothalamic KNDy-POA neuron network (Rance *et al.*, 2013), may be significantly reduced during experimental elevation of glycaemia in healthy menopausal women (Dormire and Reame, 2003). Although the exact mechanisms are still under debate (Dormire, 2009), this response is remarkably similar to premenstrual syndrome (PMS)-related hot flushes and other VMS, which are thought to occur owing to monthly oestrogen withdrawal (Casper *et al.*, 1987; Hahn *et al.*, 1998). This is also in line with the observation that women who experience PMS are more likely to eat sweets and carbohydrates in the luteal phase (Cross *et al.*, 2001; Bryant *et al.*, 2006), whilst there is an association between the severity of PMS and metabolic markers such as glycaemia, insulinaemia and HOMA-IR (Zarei *et al.*, 2013). This is similar to menopausal VMS that are associated with the risk of metabolic and CVD (discussed above) while being associated with defective HSR and elevated central sympathetic activation (Freedman, 2014). Remarkably, it is thought that high incidence of CVD and heart failure in clinical trials employing α_1-antagonists and Ca^{2+}-channel blockers are due to the fact that both strategies block HSR (Hooper, 2001).

Thence, abnormal autonomic responses observed in menopause, which are related to deranged HSR, could be resumed after a rise in SNS activity. As a corollary, it is predicted that, during hot flushes, eHSP70 plasma levels should be increased, in a glucose-sensitive fashion, whilst iHSP70 should be enhanced in a NO-dependent way. Therefore, it is plausible that, during such VMS episodes, a certain degree of cytoprotection is somehow attained via the HSR, irrespective of the fact that hot flushes are associated with increased risk for chronic inflammatory diseases.

Oestrogen-induced HSR and the NO-HSP70 axis

Oestrogen reduces ER stress by decreasing nitrosative and oxidative stress (Kooptiwut *et al.*, 2014) and by activating ER chaperone machinery that obviates ER stress (Luvsandagva *et al.*, 2012; Guo *et al.*, 2014). Therefore, the anti-ER stress and anti-NLRP3 inflammasome effects of oestrogen allows for oestrogen-elicited combat to UPR, leading to an antisenescent-associated anti-inflammatory effect, as stated in the 'Introduction' section.

Anti-inflammatory and cytoprotective effects of oestrogen may be mediated by both ERα and ERβ receptors (Stice and Knowlton, 2008; Xing *et al.*, 2012), but ERβ is the major route for oestrogen-mediated HSR (Yu *et al.*, 2006a, 2006b, 2006c) and oestrogen-elicited blockade of NLRP3 inflammasome activation, which results in the known antisenescent effect of oestrogen (De Rivero Vaccari *et al.*, 2016; Xu *et al.*, 2016). Beside of this, oestrogen-mediated HSR and cytoprotection are also attained by NO production after oestrogen binding to caveolae-located mER that trigger downstream signals (Erk1/2, p38 MAPK and PI3K-Akt) thus providing cytoprotection against atherosclerosis and vasomotor dysfunctions (Chambliss and Shaul, 2002; Stirone *et al.*, 2005). mER is encoded by G protein-coupled oestrogen receptor-1 (GPER1) gene in humans and plays an array of important roles in physiology and disease in women (Gaudet *et al.*, 2015; Feldman, 2016; Kasap *et al.*, 2016; Méndez-Luna *et al.*, 2016; Wei *et al.*, 2016; Weissenborn *et al.*, 2017). Although, mER and nuclear ER originate from a single transcript (Razandi *et al.*, 1999), oestrogen-binding affinity for GPER1 is very low, $K_d \approx 0.5$ nM, which is ~10-fold lower than that measured for nuclear ER (Revankar *et al.*, 2005). Moreover, GPER1 may localize to plasma membrane but can also undergo retrograde trafficking (Gaudet *et al.*, 2015). This has raised some doubts about the existence of other intracellular oestrogen receptors or cytosolic adaptors that could mediate rapid oestrogen effects (Stice and Knowlton, 2008; Monteiro *et al.*, 2014). Nevertheless, it is now clear that GPER1 is a bona fide mER (Gaudet *et al.*, 2015) that mediates rapid oestrogen-dependent mobilization of intracellular Ca^{2+} and synthesis of phosphatidylinositol 3,4,5-trisphosphate (IP_3) that precedes the activation of NOS and NO synthesis. The common gene transcript origin of mER and nuclear oestrogen receptors also explain why rapid mER-dependent vasodilation (Guo *et al.*, 2005) and cytoprotection (Cordeau *et al.*, 2016) cannot be observed in either ERα or ERβ knockout mice.

Altogether, the above findings reinforce that at least part of the NO-dependent protective effects of oestrogen should be credited to its ability to induce NO-elicited HSP70 expression, particularly via mER (Fig. 1).

Temperature Control, Thermoneutral Zone, Oestrogen and Flushes: Clues for a Hidden HSR

In parallel with spinothalamic relay of temperature information from peripheral warm sensors for cortical temperature perception, warm signals are also transmitted to warm-sensitive neurons of the POA of the hypothalamus where brain, spinal cord, visceral and skin temperature signals are integrated at the level of MPO and MnPO nuclei, thus driving autonomic thermoregulation (Nakamura, 2011). In other words, warming signals stimulate GABAergic inhibitory projections to rostral raphe pallidus premotor nuclei that are responsible for triggering sympathetic vasomotor responses and brown adipose tissue thermogenesis (Nakamura *et al.*, 2004; Nakamura, 2011). Thence, heat results in decreased thermogenesis, cutaneous vasodilation and evaporative sweating for heat loss, whereas cooling or pyrogenic signals (via PGE_2) cause disinhibition of such sympathetic responses leading to heat preservation or fever (and, consequently, HSR). Coincidently, these POA nuclei are governed by neurons located in the infundibular nucleus in humans (corresponding to the arcuate nucleus in other mammals), which are the same as those regulated by oestrogen and whose functions are altered in long-term oestrogen ablation: KNDy neurons. Remarkably, KNDy neurons also operate neuroprotective HSR (Wyon *et al.*, 2000; Kim *et al.*, 2010; Chilumuri and Milton, 2013) via NO, whether in the presence or absence of oestrogen.

In order to dissect neural mechanisms involved in the triggering of hot flushes and their associations with HSR, we shall discuss the feedback systems that regulate, both negatively and positively, the pulsatile GnRH secretion and midcycle surge of LH. This is because the way steroid hormone feedback systems behave after ovary failure

explains much of the alterations in hypothalamic POA that regulates body temperature and HSR in menopause.

KNDy neurons, GnRH pulsatility and sexual steroid hormone feedback systems

During each menstrual cycle throughout the reproductive-age of women, the normal pattern of negative feedback of low sex-steroid concentrations over GnRH neurons is interrupted and suddenly switched to a positive feedback response to the sustained elevation of oestradiol at the end of the follicular phase. This positive feedback triggers the surge of GnRH release, which, alongside oestradiol effects at the level of pituitary (increasing GnRH sensitivity), activates the LH surge thus triggering ovulation (Moenter *et al.*, 2009). However, as GnRH neurons do not express oestrogen receptors (Herbison and Theodosis, 1992), sex-hormone feedback systems must obligatorily be mediated by an intermediate relaying system. In fact, neurons of POA and infundibular nucleus of the human forebrain [corresponding, respectively, to the anteroventral periventricular (AVPV) and arcuate nuclei in other mammals] possessing steroid receptors do directly project to POA neurons in the median eminence, close to GnRH neurons (Simerly, 2002).

Dual-labelling studies have revealed that virtually all KNDy neurons colocalise gonadal steroid receptors, namely ERα (Goubillon *et al.*, 2000; Smith *et al.*, 2005a; Burke *et al.*, 2006; Franceschini *et al.*, 2006; Goodman *et al.*, 2007) and progesterone (Foradori *et al.*, 2002) receptors in females, and androgen receptors in males (Ciofi *et al.*, 1994; Smith *et al.*, 2005b). In addition, it is now firmly established that kisspeptin, a major KNDy neuron neurotransmitter, directly target GnRH neurons, thus directing GnRH secretion and gonadal function (Skorupskaite *et al.*, 2014; Han *et al.*, 2015; Meczekalski *et al.*, 2016). GnRH pulsatility originating in KNDy neurons of the infundibular/arcuate nucleus provides a rhythmic drive to GnRH neurons that culminates in pulsatile (each 30–40 min) gonadotropin secretion in midfollicular/preovulatory phase healthy women (Meczekalski *et al.*, 2016). Indeed, kisspeptin pulsatility is essential for sustaining normal gonadotropin secretion and fertility in mammals of both sexes (Alreja, 2013).

Kisspeptins comprise a family of closely related peptides of varied amino acid lengths (e.g. kisspeptin-54 and kisspeptin-10) that are encoded by the *KISS1* gene and operate through the G protein-coupled receptor GPR54, also termed kisspeptin receptor or KISS1R (Roa *et al.*, 2008; Oakley *et al.*, 2009; Pinilla *et al.*, 2012; Navarro and Tena-Sempere, 2012). Kisspeptin(s), whose expression is epigenetically suppressed until puberty (Lomniczi *et al.*, 2013), are now considered as the most potent inducers of GnRH/gonadotropin release (Roa *et al.*, 2009) and the master regulator(s) of pulsatile GnRH secretion. Therefore, diminished kisspeptin secretion is implicated in hypogonadotropic hypogonadism in patients with obesity and diabetes, in whom KNDy activity is affected (George *et al.*, 2010, 2011, 2013).

Autoexcitatory signals coming from KNDy neurons convey autorhythmicity for GnRH secretion along with metabolic and limbic clues from other parts of the encephalon (Skorupskaite *et al.*, 2014). Therefore, the kisspeptin-GnRH axis works to adjust reproduction to metabolic and environmental signals, including thermoregulation (Rance *et al.*, 2013). Autorhythmicity is possible because KNDy neurons not only co-express NKB, encoded by the tachykinin *TAC3* gene in humans and *Tac2* in rodents, and dynorphin A (Dyn) genes/proteins (Burke *et al.*, 2006; Goodman *et al.*, 2007), but also the primary NKB receptor (NK_3R) and the κ-opioid receptor (κ-OR) for Dyn (Navarro *et al.*, 2009), so that they autoregulate their rhythmicity and, consequently, GnRH pulsatile patterns and the secretion of both LH and FSH (Merkley *et al.*, 2012; Navarro and Tena-Sempere, 2012; Goodman *et al.*, 2013; Lehman *et al.*, 2013; Ruiz-Pino *et al.*, 2015; Narayanaswamy *et al.*, 2016; Fergani and Navarro, 2017). KNDy neurons modulate GnRH secretory patterns also through substance P (via neurokinin 1 receptor, NK_1R) and NKA (via neurokinin 2 receptor, NK_2R), in addition to the already classical NK_3R (Navarro *et al.*, 2015).

KNDy neurons also express leptin receptors thereby coupling reproduction to metabolic regulation (Smith *et al.*, 2006a, 2006b; Farooqi and O'Rahilly, 2009). Because of this, low levels of kisspeptin coincides with low gonadotropin and sex-steroid concentrations in diabetes, which is overcome by kisspeptin treatment (Castellano *et al.*, 2006, 2009).

In non-human mammals, KNDy neurons of the AVPV hypothalamus are inhibited by oestrogen, thus playing a role in negative feedback regulation of GnRH secretion, whilst KNDy neurons located in the arcuate nucleus (infundibular nucleus in humans) provide positive feedback of oestrogen on to GnRH neurons (Smith *et al.*, 2005a, 2005b; Gottsch *et al.*, 2009; Moenter *et al.*, 2009; Lehman *et al.*, 2010, 2013; Skorupskaite *et al.*, 2014). In humans, however, there is no evidence of diverse KNDy nuclei located elsewhere, other than in the infundibular nucleus, to perform both negative and positive preovulatory feedbacks of oestrogen over GnRH secretion (Ottowitz *et al.*, 2008; Skorupskaite *et al.*, 2014; Fergani and Navarro, 2017). This very intriguing observation is partially because kisspeptin release by KNDy neurons present an exquisite autosynaptic regulation through the secretion of NKB (stimulatory, via NK_3R) and Dyn (inhibitory, via κ-OR), both regulated by ERα and mER, which, in turn, possesses different affinities to oestrogen and different timing for operation (Fig. 2).

This raises the question as to how could the same KNDy neuron differently mediate the expression of identical gene sets under the command of oestrogen, depending on the phase of menstrual cycle. Evidence converges to a single KNDy-type neuron responding differently to low- and high-oestrogen concentrations in humans (Lehman *et al.*, 2010). In this context, Dyn has a critical role. Dyn is an endogenous opioid peptide that also mediates gonadal negative feedback over GnRH secretion via the κ-OR (Goodman *et al.*, 2004), whereas multiple pieces of evidence suggest that Dyn pathways are the missing link between sex steroids and KNDy autoregulation (Foradori *et al.*, 2002; Goodman *et al.*, 2004,2007). For example, the stimulating effect of naloxone on GnRH/LH secretion is not observed in postmenopausal and oophorectomised young women, whereas replacement of oestrogen or progesterone can restore the ability of naloxone to release LH (Melis *et al.*, 1984; Casper and Alapin-Rubillovitz, 1985; Shoupe *et al.*, 1985). On the other hand, KNDy nuclei in the infundibular nucleus of postmenopausal women exhibit hypertrophy, but the number of neurons expressing Dyn mRNA is rather markedly reduced, compared to premenopausal women (Rometo and Rance, 2008). But if this is so, how could one account for the differential responses to oestrogen during the course

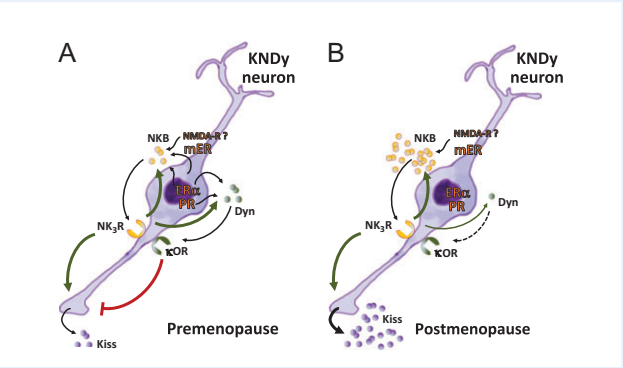

Figure 2 Model of the KNDy neuron receptor hierarchy and its relation with sexual steroid receptors during pre- and postmenopausal periods. (**A**) In reproductive-age women, KNDy neurons present an auto-pulsatile pattern of KNB secretion that is increased by oestrogen via intracellular type-α (ERα) receptors. NKB acts autosynaptically onto KNDy neurons to enhance NK_3R expression, which boosts both KNB and kisspeptin (Kiss) neurotransmission. At the same time, NKB stimulates dynorphin (Dyn) secretion, which negatively feeds back kisspeptin secretion through κ-opioid receptors (κOR). Dyn-dependent feedback is attained via both ERα and progesterone receptors (PR) during normal cycles. The NKB pulsatile pattern is probably adjusted by glutamatergic stimulation (NMDA-R) that occurs in parallel. During the preovulatory period (late-follicular phase), enhanced oestrogen plasma concentrations stimulates nonclassical (and lower affinity) mER, which rapidly and preferentially stimulate NKB secretion. This leads to oestrogen-dependent positive feedback on kisspeptin pulses and the consequent GnRH/LH surge at midcycle. (**B**) After gradual decrease in oestrogen levels due to the ovarian failure observed peri- and postmenopausally, ERα-dependent secretion of Dyn is practically abolished so that only the auto-pulsatile pattern of NKB subsists. This leads to exacerbated NKB autostimulation and enhanced kisspeptin neurotransmission that provokes increased liberation of GnRH and higher LH levels during early menopause. KNDy, kisspeptin-neurokinin B-dynorphin; mER, membrane oestrogen receptors.

of follicular phase thus allowing both negative and positive feedback within the same KNDy neuron?

Classical nuclear (ERα) and membrane (mER) oestrogen receptors not only respond through diverse mechanisms, but also with different timing. mER responds to oestrogen much faster (seconds) than do ERα (Glidewel-Kenney *et al.*, 2007), whose responses may elapse from minutes to hours. Also, the secretion of NKB (kisspeptin-stimulating) and Dyn (kisspeptin-inhibiting) in response to oestrogen (Ruiz-Pino *et al.*, 2015) has also a very subtle temporal difference: whilst NKB secretion is very rapid, Dyn is stored in large (80–120 nm diameter) dense-core vesicles whose release differs considerably from that of small synaptic vesicles (e.g. NKB-containing vesicles), so that a more intense and prolonged stimulus is needed to cause the large vesicles to release their contents into the synaptic cleft. Whence, differential secretory mechanisms may account for the delayed response of Dyn to oestrogen permitting fluctuations (pulses) of kisspeptin secretion.

The other and mostly crucial point is the differential affinity presented by ERα (much higher, K_d ~ 10 pM) and mER (up to K_d ~ 2000 pM!) (Salomonsson *et al.*, 1994; Lin *et al.*, 2013). Considering that oestradiol concentrations in women rise almost 10-fold from ~60 pg/ml (45 pM in CSF) in early follicular phase to up to 400 pg/ml (300 pM in CSF) in late-follicular phase (Bäckström *et al.*, 1976; Thiery *et al.*, 2006), it is clear that the intracellular ERα should be saturated respective to oestrogen during the transition from mid- to late-follicular phase. At this point, therefore, the higher levels of oestrogen provoked by increasing activity of the dominant follicle may dictate a change from negative towards a positive feedback of oestrogen on KNDy neurons by preferentially activating mER, which can be physiologically activated only at high-oestrogen concentrations. As mER responds almost instantaneously feedforwarding NKB-NK_3R route (Lehman *et al.*, 2010), there is no time for Dyn secretion and Dyn-dependent negative feedback over kisspeptin secretion.

These observations extend the current concept of autoregulation of KNDy neurons (Navarro *et al.*, 2009; Navarro, 2013) and explain the negative feedback of oestrogen during the early-to-midfollicular phase of human menstrual cycle (Fig. 2A), whilst the only 'required' difference to divert negative feedback to the late-follicular positive feedback is the concentration of oestrogen. Accordingly, when the dominant follicle commences to produce oestrogen in amounts that suffice to activate mER, its rapid responses may overcome the inhibitory actions of Dyn, so that kisspeptin (and, consequently, GnRH/LH) pulses are no longer observed; instead, a conspicuous and increasing surge of kisspeptin takes place thus leading to ovulation. Curiously, however, this is almost the same as observed in the absence of oestrogen in that the lack of Dyn leads to exaggerated NKB-NK_3R signalling to kisspeptin secretion, as illustrated in Fig. 2B.

KNDy neuron activity may also be regulated by glutamatergic afferents (Pompolo *et al.*, 2003; Ciofi *et al.*, 2006; Cravo *et al.*, 2011; García-Galiano *et al.*, 2012), which confirms the known role of glutamate to relay the positive feedback of oestrogen during the preovulatory GnRH surge (Mahesh and Brann, 2005; Lehman *et al.*, 2010; Navarro, 2013; Skorupskaite *et al.*, 2014). Whence, it is likely that, after menopause, only glutamatergic signalling to KNDy neurons persist as an external stimulus to them. If this is so, kisspeptin must indeed be expected to be overproduced in menopause, being accompanied by the known hypertrophy observed in KNDy neurons (Rance *et al.*, 1990, 2013; Rance and Young, 1991; Guida *et al.*, 2012), as suggested in Fig. 2B.

Beyond any pragmatic application emerging from the understanding of the mechanisms that integrate reproduction with internal milieu and the external environment, it is fascinating that evolution naturally and painstakingly selected a so gracious and delicate mechanism utilizing the very same neuron to perform a double-feedback task, depending on changes in circulating sex steroids.

KNDy nuclei in thermoregulation and the shift of thermoneutral zone in menopause

A remarkable alteration observed in the hypothalamus of postmenopausal women is the hypertrophy (not hyperplasia) of neurons located in the POA. This is accompanied by a conspicuous increase in the activity and gene expression of KNDy neurons, with increased production of NKB and kisspeptin, but not Dyn (Rance *et al.*, 2013). As expected, this causes disruption of KNDy neuron autoregulation thereby inducing increased GnRH secretion and the neuroendocrine alterations observed after long-term oestrogen deprivation. As a

corollary, the lack of Dyn in the face of oestrogen withdrawal (a situation that determines an open-cycle regulation of KNDy cells) culminate in exaggerated kisspeptin (and, consequently, GnRH/LH) liberation. In the absence of oestrogen, KNDy neurons remain solely under the influence of the positive autofeedback of NKB and, perhaps, affected by glutamatergic stimulation (Fig. 2B).

Concurrently, KNDy neurons are upwardly connected to MnPO and MPO nuclei of the POA (Fig. 3), which link the hypothalamic-pituitary-gonadal axis to thermoregulation (Nakamura *et al.*, 2004; Krajewski *et al.*, 2010; Dacks *et al.*, 2011; Nakamura, 2011; Mittelman-Smith *et al.*, 2012; Rance *et al.*, 2013). Indeed, there is a very close relation between oestrogen and temperature regulation (Laudenslager *et al.*, 1980; Clarke *et al.*, 2013). Consequently, this circuitry also couples KNDy hyperfunction to altered processing of thermosensory information at the POA that may explain menopausal hot flushes. Importantly, the selective NK_3R agonist, senktide, induces a hypothermic response which is virtually identical in ovariectomised rats with or without oestrogen replacement, whilst NKB remarkably induces hot flushes in follicular phase young women (Jayasena *et al.*, 2015). In total, as nicely approached by Rance *et al.*, (2013) and confirmed by many other studies (Skorupskaite *et al.*, 2014), the gradual changes in KNDy neurons that accompany decreasing oestrogen levels in the hypothalamus of postmenopausal women do contribute to the generation of flushes.

The 'lack-of-Dyn' principle of menopausal KNDy hypertrophy (Fig. 2) and neuroendocrine alterations that accompany the flushes can be appreciated in rats after acute or chronic morphine treatment. Acutely, low doses of morphine evoke a marked and short-lasting rise in body temperature alongside vasoconstriction of tail skin blood vessels and warm-seeking behaviour (heat preservation), an effect mimicked by the direct injection of morphine into the preoptic hypothalamus (Cox *et al.*, 1976). On the other hand, long-term morphine administration causes opioid receptor desensitization (Allouche *et al.*, 2014), so that continuous use of morphine causes chemical dependence and the need for increasing doses to achieve the same opioid effects. Because of this, animals treated with morphine for a long time become addicted, whilst the administration of the opioid antagonist naloxone to morphine-dependent rats rapidly causes 'withdrawal symptoms' which parallel a significant tail skin vasodilation leading to subsequent fall in core temperature (Katovich *et al.*, 1986). These rat VMS in response to morphine withdrawal are unfailingly accompanied by accelerated heart rate and preceded by LH surge (Simpkins *et al.*, 1983), exactly as observed in humans following heroin or morphine withdrawal (Täschner, 1986; Kreek *et al.*, 2010; Wakim, 2012) and identical to menopausal hot flushes.

The above observations are explained as follows. Acutely administered at non-desensitizing low doses, morphine stimulates κ-OR in KNDy cells, taking the place of Dyn, the physiological ligand. This blocks KNDy autoexcitation, leading to decreased kisspeptin release (Fig. 2A). As kisspeptin stimulates MnPO and MPO neurons to drive GABAergic signals to rostral raphe pallidus premotor nuclei, low doses of morphine lead to vasoconstriction and energy preservation (Fig. 3A). Conversely, if the animal becomes morphine-dependent, κ-OR of KNDy neurons undergo desensitization, so that physiological production of Dyn (during KNDy neuron autoexcitatory cycles) is insufficient to robustly drive the negative autofeedback part of the KNDy neuron cycle via κ-OR. In other words, a new equilibrium of KNDy autoexcitation is attained that consists of higher NKB signals in relation to Dyn ones. Consequently, when the animal is administered with an opioid antagonist (e.g. naloxone), the already-weak negative feedback is completely abolished, leading to a massive kisspeptin secretion, as NKB-NK_3R pathway is still preserved and enhanced (Fig. 2B). This causes an almost instantaneous activation of MnPO and MPO neurons that cause a strong GABAergic-dependent blockade of sympathetic routes responsible for tonic vasoconstriction (Fig. 3B).

Therefore, in morphine-dependent individuals, naloxone triggers 'withdrawal symptoms' that include GnRH/LH surge and heat dissipation through skin vasodilation, as observed in menopausal hot flushes. This is why the 'morphine dependence/withdrawal' model mimics so trustworthily hot flushes in humans (Simpkins *et al.*, 1983; Katovich *et al.*, 1986). One proof of this is that naloxone effects on GnRH/LH secretion is not observed in postmenopausal and oophorectomised young women, whereas replacement of oestrogen or progesterone can restore the ability of naloxone to release LH (Melis *et al.*, 1984; Casper and Alapin-Rubillovitz, 1985; Shoupe *et al.*, 1985). Interestingly, the same neuronal circuitry and KNDy neuron behaviour is observed in men (Spetz *et al.*, 2003; Irani *et al.*, 2010; Ruka *et al.*, 2013).

The flushes are an exaggerated heat dissipation response (Roepke *et al.*, 2010) initiated at the preoptic warm-sensitive nuclei and characterized by VMS routinely associated with increased environmental temperature (Kelly and Rønnekleiv, 2015). Animal and human studies vehemently suggest that prolonged oestrogen withdrawal causes a shift in the thermoneutral zone to lower ambient temperatures in a mechanism dependent on defective Dyn-mediated negative autofeedback of the KNDy neuron cycle (Stearns *et al.*, 2002, Dacks and Rance, 2010; Dacks *et al.*, 2011; Mittelman-Smith *et al.*, 2012). With the narrowing of the thermoneutral zone, as oestrogen levels decrease in perimenopause, women are prone to cross both the upper and lower thresholds (Krause and Nakajima, 2015), being extremely vulnerable for developing flushes, night sweats and even chills (leftmost upper parts of Fig. 3A and B). Conversely, oestrogen can virtually remit hot flushes raising the sweating threshold in postmenopausal flushing women, which explains why long-term oestrogen-HRT alters thermoregulatory and vasomotor function in postmenopausal women (Freedman and Blacker, 2002).

As oestrogen levels decline, central norepinephrine levels increase, thus causing an increase in hypothalamic serotonin receptors, and further narrowing of the thermoneutral zone (Krause and Nakajima, 2015). In fact, higher sympathetic adrenergic status is also involved in the flushes and central adrenergic activation is one factor responsible for narrowing of the thermoneutral zone (Täschner, 1986; Spetz *et al.*, 2003; Thurston *et al.*, 2010; Freedman, 2014). However, as sympathetic stimulation is an inducer of HSR, it cannot be ruled out the existence of a hidden HSR during the flushes, albeit this possibility has never been tested. In any case, elevated central norepinephrine in conjunction with oestrogen withdrawal takes part in the aetiology of the flushes (Freedman, 2014).

Hot flushes, however, may be associated with much important physiological conditions that could also involve the protective HSR. For example, women who report tamoxifen-associated hot flushes at baseline breast cancer follow-up usually present less propensity to suffer relapse than those who do not report hot flushes, so that hot flushes constitute an independent predictor of reduced recurrence of breast cancer (Mortimer *et al.*, 2008). Additionally, hot flushes are

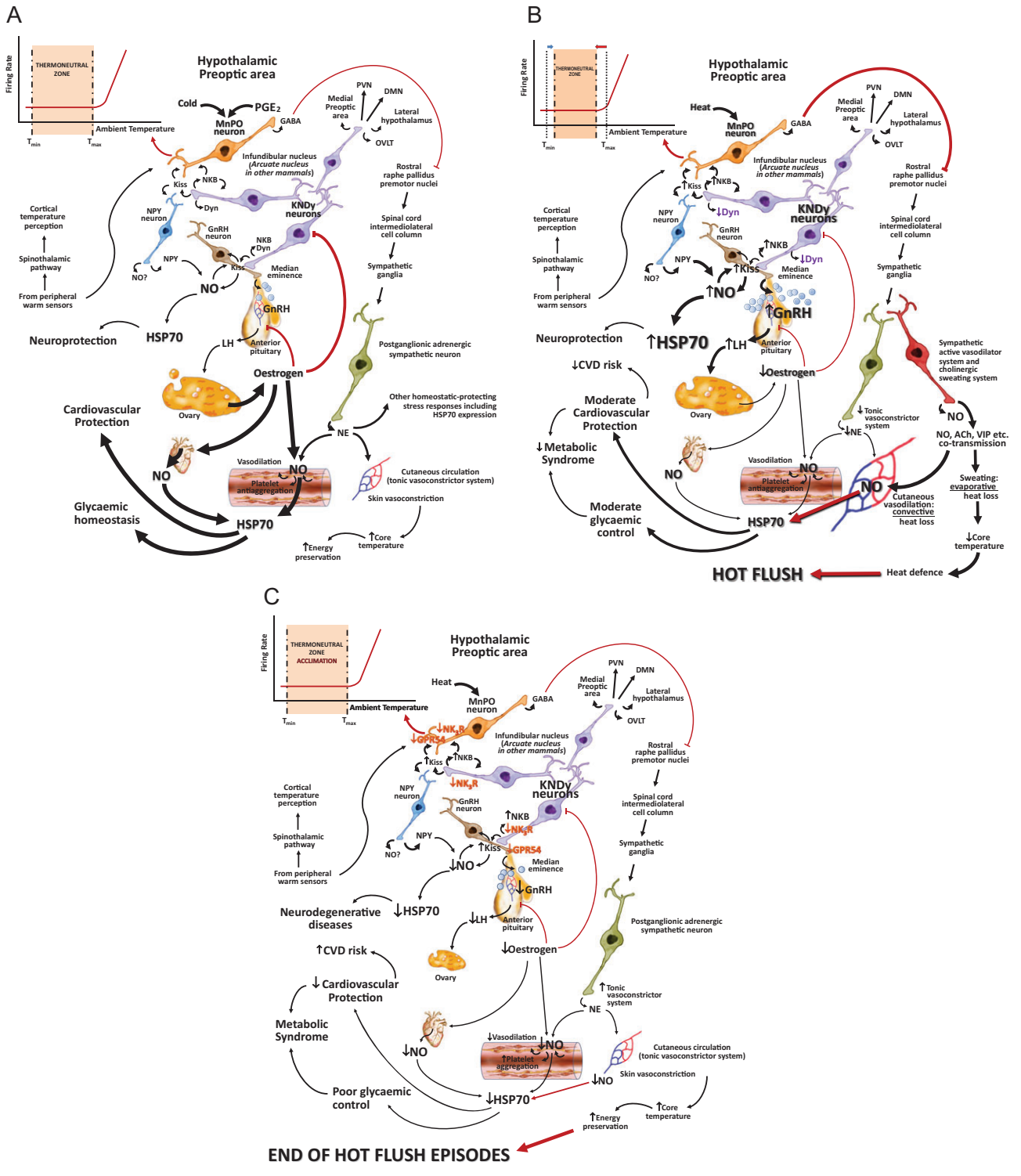

Figure 3 The oestrogen-NO-HSR axis and its consequences for thermoregulation and hot flushes. In premenopausal health women, ambient temperature information is relied to the somatosensory cortex for conscious temperature perception, via the classical spinothalamic pathway, but also to warm-sensitive neurons located at the hypothalamic POA, where peripheral and central warm sensor activity is translated into autonomic adjustments of energy balance and core temperature. This is attained by the activation of GABAergic projections to rostral raphe pallidus premotor nuclei that decrease the tone of noradrenergic postganglionic neuron activity leading to vasodilation and heat-defence responses. On the contrary, as occurs when tonically activated, the sympathetic adrenergic trunk is responsible for enhanced heat production, energy preservation and other homeostatically protecting stress responses, which include, norepinephrine-elicited HSR and cytoprotective HSP70 production. Particularly, MPO

directly correlated with risk of insulin resistance, T2DM and metabolic syndrome (Thurston *et al.*, 2012a; Van Dijk *et al.*, 2015; Ryu *et al.*, 2015), which are human conditions directly associated with depressed HSR (Newsholme and Homem de Bittencourt, 2014). Moreover, hyperfunction of KNDy neurons are frankly implicated in central and peripheral production of NO, a powerful inducer of HSR. This raises the question as to whether hot flushes could indeed ameliorate such menopause-related metabolic diseases.

Termination of hot-flush period over menopause transition

If gonadal steroid concentrations remain undetectable after ovary failure and kisspeptin production by KNDy neurons is consequently exacerbated (Fig. 2B), why do hot flushes taper off sometime in postmenopause? This is important in the light of possible obscure chronic inflammatory diseases that are associated with the flushing time. Hot flushes typically last for 0.5–5.0 years after natural menopause, but they may persist for as long as 15 years in a small percentage of postmenopausal women, whilst lasting longer and being more severe in surgically menopausal women (Kronenberg, 1990; Bachmann, 1999). Even so, hot flushes may reoccur if oestrogen is administered and then withdrawn (Jensen and Christiansen, 1983; Kronenberg, 2010), which means that KNDy neurons should still be functioning and responsive to oestrogen (Rossmanith *et al.*, 1991; Hall *et al.*, 2000; Gill *et al.*, 2002a, 2002b; Hall, 2007).

Desensitization of the set of receptors involved in KNDy-GnRH neuron function appears to be part of the answer to this question. Kisspeptin receptors undergo desensitization after prolonged periods of exposure to high-kisspeptin concentrations (Ramaswamy *et al.*, 2007; Jayasena *et al.*, 2009, 2013; Pampillo *et al.*, 2009; Roa *et al.*, 2009; Scott *et al.*, 2013; Min *et al.*, 2014; Clarke *et al.*, 2015). The same happens in respect to NKB receptors (Schmidlin *et al.*, 2002; Ramaswamy *et al.*, 2007; García-Galiano *et al.*, 2012; Navarro, 2013). Long-term disruption of Dyn/κ-OR signalling compromises the rise in LH after ovariectomy (Navarro *et al.*, 2009), whilst in PCOS, a syndrome associated with elevated KNDy neuron function and hyperactivity of hypothalamic-pituitary axis (Lehman *et al.*, 2010; Witchel and Tena-Sempere, 2013), desensitization of GnRH secretion is also noticed (Waldstreicher *et al.*, 1988). Therefore, it may be that, after sometime under high NKB/kisspeptin levels, desensitization becomes permanent. Concomitantly, thermoneutral zone tends to acclimatize to the previous ambient temperature, thereby terminating the 'hot-flush period', as illustrated in Fig. 3C. Nonetheless, if this is the case, any protective effect of the kisspeptin-dependent NO-centred HSR should fade at the same pace. Also, kisspeptin-modulated regulation of leptin, growth hormone and prolactin secretion may be compromised. Thus, when hot flushes stop happening, women may be more predisposed to chronic degenerative diseases of inflammatory nature (Fig. 3C).

KNDy Neuron Circuitry as a Backup System for Triggering HSR in the Absence of Oestrogen

If oestrogen directly induces moderate NO-dependent vasodilation (avoiding overheating) and platelet anti-aggregation (low-CVD risk) during the fertile period, NO supply is given by different and indirect

and MnPO nuclei, which are highly responsive to cold and the febrile effects of PG E_2 (PGE_2), are strongly affected by body temperature changes but also receive information from different parts of the hypothalamus involved in pituitary function and reproduction as well. KNDy neurons, which are located in human infundibular nucleus (the equivalent of arcuate nucleus in other mammals) directly project to MPO and MnPO neurons, so that, signals from KNDy nuclei influence temperature control and the limits of thermoneutral zone, i.e. the range of ambient temperature in which neither heat-sparing mechanisms nor heat-defence ones are activated. In parallel, KNDy cells project to GnRH secreting neurons in order to regulate gonadotropin release by the hypophysis. Accordingly, kisspeptin has a pulsatile pattern of liberation that dictates GnRH production being susceptible to oestrogen feedback (both positive and negatively). Kisspeptin induces the activation of nNOS thus favouring the production of the neuroprotective gas NO, which is an inducer of HSR and HSP70 expression. KNDy neuron activity determines also the function of neuropeptide Y (NPY)-secreting neurons, which induces NPY-elicited NO release and HSP70 expression. (**A**) In premenopausal women, at the same time that oestrogen stimulates peripheral NO release, which blocks platelet aggregation and avoids hypertension thus conferring cardiovascular protection, it represses the function of KNDy nuclei maintaining thermoregulation within a neutral range with just a moderate tone of sympathetic activity. (**B**) During the climacteric, as the ovary function gradually decreases leading to diminished oestrogen levels in the circulation, KNDy neuron function commences to be enhanced at the same pace. Notably, in the absence of oestrogen, the expressions of NKB, NKB receptors (NK_3R), and of kisspeptin are remarkably enhanced, particularly due to the fact that oestrogen-dependent dynorphin expression is dramatically reduced. This leads to augmented flux of information towards GABAergic neurons of MnPO and the consequent overactivation of sympathetic heat-defence mechanisms, which include rise in the tone of the sympathetic vasodilator system as well as cholinergic sweating systems, which are mediated by NO, acetylcholine (Ach) and vasoactive intestinal polypeptide (VIP) co-transmission. Together, such 'protecting' responses produce hot flushes. As a corollary, if, on the one hand, minimal rises in ambient temperature may trigger the flushes, because the thermoneutral zone is found to be narrowed due to dysregulation of MnPO and circumjacent nuclei, on the other hand, enhanced NO production (both central and peripherally) can support HSP70 expression and cytoprotective HSR, provided that flushes are in the scene. (**C**) In late menopause, the long-term stimulation of NK_3R is likely to determine its desensitization, which is followed by suppression of kisspeptin secretion by KNDy neurons. These long-term events also desensitize GPR54 kisspeptin receptors and tend to normalize GnRH, thus suppressing LH plasma levels. Since, at the same time, KNDy neurotransmission is ineffective (desensitized), the thermoneutral zone tends to acclimatize to the previous ambient temperature, terminating the 'hot-flush period'. Consequently, HSP70-based cytoprotection and thermotolerance is abolished, making symptomatic women now prone to poor glycaemic control, neurodegenerative diseases, endothelial dysfunction and augmented risk of CVD. CVD, cardiovascular disease; NKB, neurokinin B; PVN, paraventricular nucleus; DMN, dorsomedial nucleus; OVLT, organum vasculosum of the lamina terminalis. Thickness of arrows indicate the degree of intensity of both inhibitory and stimulatory effects depicted. POA, optic area; MPO, medial preoptic; MnPO, median preoptic; nNOS, neuronal nitric oxide synthase.

mechanisms during the climacteric. Accordingly, as oestrogen levels decrease in perimenopause, its suppressive effects over KNDy neurons tend to vanish accordingly. However, the consequent rise in KNDy neuron activity leads to NKB- and kisspeptin-dependent stimulation of NO and HSP70 production, both directly and indirectly via neuropeptide Y (NPY)-secreting neurons. Therefore, oestrogen-dependent NO (and HSP70) production can be replaced with KNDy-elicited NO secretion in menopause to drive HSR, thereby preserving cytoprotection. This occurs, however, at the expense of a boost in the flux through KNDy-heat defensive circuitry that leads to flushes after minimal upwardly changes in ambient temperature due to the narrowing of thermoneutral zone.

KNDy neurons and the NO-HSP70 axis

Oestrogen induces NO release in the median eminence in seconds, via KNDy membrane surface G_q protein-coupled mER receptors and this mechanism is responsible for neuroprotection, GnRH release and regulation of body temperature and energy homoeostasis (Roepke *et al.*, 2010; Kelly and Rønnekleiv, 2015). This occurs because KNDy-derived kisspeptin signals on to NO-secreting neurons of POA, through GPR54 receptors, to activate neuronal nitric oxide synthase (nNOS) through its phosphorylation at Ser1412 via the Akt pathway, leading to central and peripheral NO-based vasodilation (Figtree *et al.*, 2003; Micevych and Dominguez, 2009).

Additionally, stimulation of KNDy mER receptors reduces the postsynaptic inhibitory GABAergic tone (Roepke *et al.*, 2010), whilst NO release is also required for the kisspeptin-dependent positive feedback of oestrogen, during preovulatory activation of GnRH neurons *in vivo* (Fig. 4) (Hanchate *et al.*, 2012). Consequently, in menopause, NO-kisspeptin mutual positive feedback stagnates, as if it were in late-follicular phase/ovulation ad infinitum, so that kisspeptin alone assumes the NO-based GnRH secretion and LH surge. However, as kisspeptin concomitantly regulates temperature set point, any tiny elevation of ambient temperature may activate heat-defence mechanisms during the climacteric, because the KNDy system is now much more sensitive to the input from peripheral warm sensors due to the narrowing of thermoneutral zone (Fig. 3B). Conversely, both oestrogen-dependent and independent kisspeptin-mediated NO secretion (and the protective HSR) is preserved, albeit this may represent the appearance of VMS if thermoneutral zone is outdone (Fig. 4). Leptin, which acts upstream of KNDy neurons (Skorupskaite *et al.*, 2014), coordinates GnRH secretion by nNOS-dependent NO secretion in POA (Bellefontaine *et al.*, 2014), being strongly associated with incidence and duration of hot flushes (Alexander *et al.*, 2010).

In NPY-secreting neurons of POA and paraventricular nucleus (PVN) of hypothalamus, kisspeptin directly regulates the synthesis and secretion of NPY (Kim *et al.*, 2010), which enhances GnRH-binding sites in anterior pituitary that potentiates gonadotropin release (Parker *et al.*, 1991). Conversely, NPY neurons are activated by stressful signals of reduced energy availability increasing NPY release to stimulate feeding behaviour while suppressing GnRH release (Acosta-Martinez *et al.*, 2007), whilst mER receptor stimulation rapidly enhances GABAergic signalling in NPY neurons (Smith *et al.*, 2013). Therefore, NPY neurotransmission is expected to be enhanced in menopause because oestrogen naturally inhibits NPY secretion via mER (Dhillon and Belsham, 2011) and, because of this,

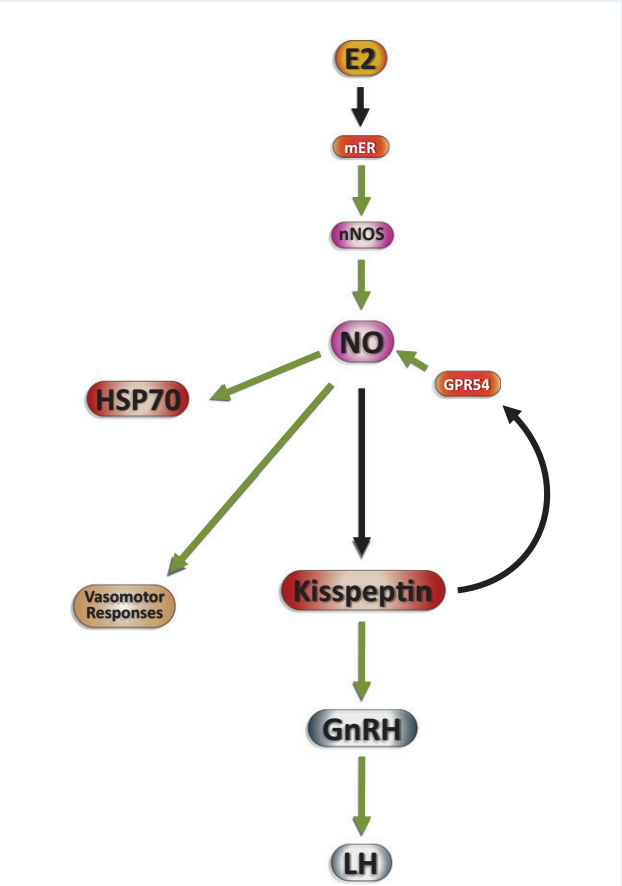

Figure 4 Oestrogen-NO-kisspeptin axis. Nonclassical membrane surface oestrogen (E2) receptors (mER) signal towards the activation of nNOS that produces large amounts of the gas NO, which, in turn, enhances the expression of HSP70 as well as vasomotor responses. In parallel, NO stimulates kisspeptin production leading to GnRH secretion and LH surge. However, kisspeptin positively feeds back to NO-producing neurons, via GPR54 (also known as KISS1 receptor), increasing NO production whenever oestrogen is bound to mER.

plasma NPY levels increase during hot flushes (Wyon *et al.*, 2000). However, NPY is a direct activator of neuronal HSP70 production and secretion *in vitro* (Asea *et al.*, 2013). Whence, there is an additional dismemberment of the oestrogen-kisspeptin-NO axis because, under heat-stress conditions and during long-term oestrogen ablation, kisspeptins may increase HSP70 expression either directly (via NO generation) or indirectly (via NPY), which may explain why kisspeptin possesses neuroprotective action against Alzheimer's β-amyloid toxicity (Chilumuri and Milton, 2013). Consequently, as NPY pathways may be enhanced in the POA of menopausal women, it is also expected that NO-based HSR should be elevated, albeit accompanied of hot flushes.

HSR as a negative feedback for NKB and kisspeptin during hot flushes and its consequences in menopause

Although, NK_3R is the main NKB receptor involved in the oestrogen-GnRH axis in POA, NK_1R is also expressed in the same nuclei

(Rupniak, 2002; Pennefather *et al.*, 2004; Navarro *et al.*, 2015) signalling downstream through NF-κB activation (Steinhoff *et al.*, 2014). Furthermore, NK_3R seems also to be involved in NF-κB activation at least peripherally (Pinto *et al.*, 2002; Veron *et al.*, 2004). In rodent arcuate nucleus, NKB actions via NK_3R are also partially dependent on kisspeptin-producing neurons (Navarro *et al.*, 2015). However, kisspeptin is a well-known tumour suppressor that acts via GPR54 to block NF-κB activation in different cell types (Cho *et al.*, 2009; Ji *et al.*, 2014). Concomitantly, kisspeptin coordinates NO synthesis through the activation of nNOS in rodent POA (Hanchate *et al.*, 2012) thus enhancing the synthesis of NO, which, *per se*, is an NF-κB blocker (Matthews *et al.*, 1996). Therefore, KNDy-dependent NO production works to circumvent excessive NF-κB activation and disease, through different mechanisms that enhance HSR (Calabrese *et al.*, 2007; Pósa *et al.*, 2015; Shen *et al.*, 2017). Thus, in the climacteric, enhanced KNDy neuron function may enhance NF-κB activity through NKB (augmented predisposition to inflammatory diseases) but, on the other hand, increased NKB-dependent secretion of kisspeptins blocks NF-κB directly via NO and, indirectly, via NO- and NPY-induced expression of HSP70 (Fig. 5).

In other words, as paradoxical as may appear (because the prevalence and severity of hot flushes are associated with chronic inflammatory diseases), the above observations support the concept that there is some degree of cytoprotection (e.g. against CVD, neurodegeneration, neuromuscular and metabolic abnormalities; Fig. 3B) whenever kisspeptin is counteracting NKB effects during the flushes (Fig. 5). Hot-flush-associated adrenergic stimulation secondary to long-term oestrogen deprivation and narrowing of the thermoneutral zone (Freedman, 2014; Krause and Nakajima, 2015) should also confer cytoprotection due to the stimulatory nature of α-adrenergic signalling over HSR and HSP70 production. Therefore, the KNDy system works as a backup of HSR in the absence of gonadal hormones whenever hot flushes take place. As a corollary, the opposite should be valid: when hot flushes cease sometime after menopause, it is expected that the protective KNDy neuron-dependent NO-elicited HSR should decrease in parallel (Fig. 3C). Consequently, heat treatment may be able to restore HSR in oestrogen-deficient states by interrupting the vicious cycle that decrease HSF1 availability due to cellular senescence. In fact, exercise, which is one of the most powerful inducers of HSR (Krause *et al.*, 2015b) has, in parallel, provided promising results as a non-pharmacological approach for hot flushes (Krause and Nakajima, 2015). Heat treatment is paradoxically envisaged to re-equilibrate KNDy autoregulation, by increasing the production of NO, an NF-κB blocker (Fig. 5), thus alleviating hot flushes.

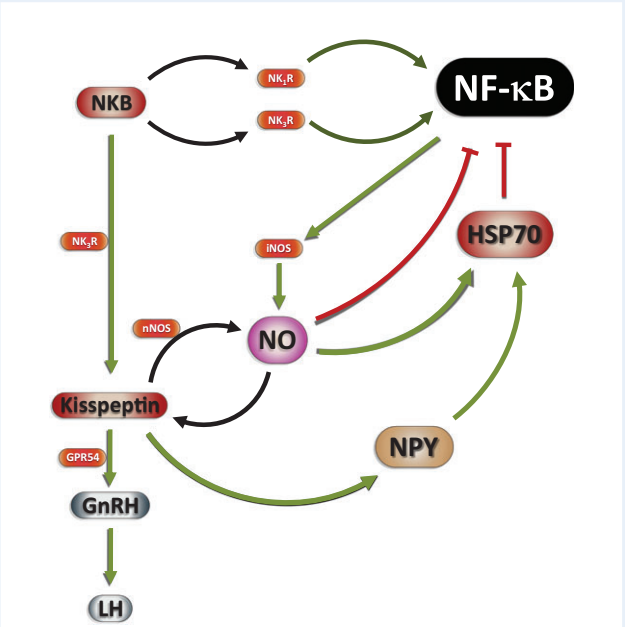

Figure 5 Kisspeptin-NKB feedback route. NKB possesses two antagonistic responses in relation to nuclear factor κB (NF-κB) in the hypothalamus. After binding to either Type-1 or Type-3 NKB receptors (NK_1R and NK_3R, respectively), NKB evokes a series of NF-κB-mediated responses, which include pro-inflammatory pathways and the expression of inducible nitric oxide synthase (iNOS). On the other hand, NKB activates kisspeptin secretion, leading to both GnRH-mediated LH surges and NO production via nNOS. NO positively feeds back to kisspeptin neurons, amplifying NO production. As a result, excess NO counteracts NF-κB downstream effects both directly (blocking NF-κB activation and transcribing activity) and indirectly, as NO is a powerful inducer of HSP70 expression, which in turn, impairs NF-κB signals and pro-inflammatory effects. Kisspeptin also increases the activity of neuropeptide Y (NPY) neurons that produce a NPY-elicited HSP70 expression and cytoprotection.

Predicted Interventions in Low-Grade Chronic Inflammatory Diseases Associated with Deranged HSR in Symptomatic Flushing Women

Depressed HSR secondary to decreasing oestrogen concentrations may be causative for several chronic degenerative diseases of inflammatory nature that are observable in menopausal women. Therefore, we discuss in this section possible strategies to improve such unhealthy states via HSR during menopausal transition considering the incidence of hot flushes.

Heat treatment

It is a natural conjecture that heat treatment, the most powerful inducer of HSR, could physiologically re-adequate homoeostasis in this group of women, including (and, perhaps, especially) those symptomatic for hot flushes, as the course of VMS may be associated with deranged HSR. However, this has never been assessed in flushing women, which is understandable because elevation of ambient temperature is a known risk factor to prompt hot flushes, so that heat treatment for this group of women may have been thought inconceivable.

Warm baths with therapeutic purposes were well appreciated by ancient Greek citizens, and were very popular amongst Roman citizens >2500 years ago. The first notice of possible metabolic effects of heat treatment in modern times came, however, with the observation that Finnish saunas results in continuing hypoglycaemia in insulin-

treated diabetic patients (Koivisto, 1980a, 1980b). While this was thought to be related to cutaneous vasodilation, Prof. Philip L. Hooper and his colleagues theorized that partial immersion in a hot tub could mimic the beneficial effects of exercise (Hooper, 1999), as exercise increases body temperature whilst improving glycaemic control. In fact, in a small sample of T2DM patients, 3 weeks of 30 min hot tub sessions (6 days per week) produced a significant reduction in fasting glycaemia (~13%), which was accompanied by decreased % HBA_{1c} (~1 unit) and mean body weight (~1.5%).

It is now well accepted that heat therapy (for 15 min, at least three times a week; sauna: 80–100°C; hot tub: 40°C), by enhancing HSR, is a promising, safe and inexpensive tool for treating diabetes, obesities and related metabolic diseases (Hooper *et al.*, 2014; Krause *et al.*, 2015c). Heat treatment is also successfully employed to treat fibromyalgia (Matsushita *et al.*, 2008), which is frequently encountered in menopausal women and associated with fluctuations in oestrogen levels (Freeman *et al.*, 2007; Blümel *et al.*, 2012; Bernik *et al.*, 2013). This is noteworthy because a series of pain disturbances, including fibromyalgia and other CNS-centred diseases of low HSR, tend to develop soon after menopause (Kahn, 2006; Kuchinad *et al.*, 2007; Hassett *et al.*, 2015; Lukkahatai *et al.*, 2015). Hot tub treatment may improve life quality and hemodynamic function in chronic heart failure patients, including postmenopausal women (Michalsen *et al.*, 2003). Remarkably, by using a mouse model of atherosclerosis, we have heat-shocked the animals (41.5°C for 15 min) once a week, over 8 weeks, observing a spectacular reduction on the animal death rate, an impressive remission of vascular disease, enhanced blood flow and reversion of the depressed HSR (Bruxel *et al.*, manuscript in preparation).

Besides inducing protective HSR *per se*, heat treatment imposes a hyperadrenergic state because, as heat-stress augments skin blood flow and sweat rate, the concomitant rise in cardiac output and vascular resistance of non-cutaneous beds (especially in skeletal muscle) warrant that blood pressure could be maintained within safe limits (Low *et al.*, 2011). Therefore, heat treatment chronically stimulates the adrenergic branch of HSR, which is associated with HSP70-based thermotolerance (King *et al.*, 2002).

Gut microbiota manipulation

HSR is also subject to the influence of gut microbiota. Faecal microbiota can produce NO from nitrate (Vermeiren *et al.*, 2009), thus boosting intestinal HSR. This is noteworthily because HSR at the intestinal wall level is critical, as the gut microbiota ecosystem influences low-grade inflammation and its associated chronic diseases (Cani *et al.*, 2007; Newsholme and Homem de Bittencourt, 2016), whilst obesity imposes modifications in gut microbial metabolism that are associated with pro-inflammatory SASP and tissue senescence (Yoshimoto *et al.*, 2013). This may be particularly important in menopausal transition since oestrogen and gut microbiota interact to regulate weight gain and lipid deposition.

Intestinal microbiota can metabolize oestrogen-like compounds that are consumed to biologically active forms (Chen and Madak-Erdogan, 2016) and may alleviates VMS promoting health benefits to menopausal women (Tousen *et al.*, 2011; Guadamuro *et al.*, 2015). Additionally, fermentation of carbohydrate prebiotics by gut microbiota modulates NOS/NO pathways and NO-producing bacteria while obviating systemic endothelial dysfunction (Roberfroid, 2005; Catry *et al.*, 2017). The opposite is true, as oestrogen modulates gut microbiota (Menon *et al.*, 2013) and epithelial barrier function (Looijer-van Langen *et al.*, 2011) thus combatting inflammatory bowel disease (Harnish *et al.*, 2004).

It is impressive that gut microbes do manipulate host-eating behaviour to increase their own fitness, sometimes at the expense of host fitness. It is believed that intestinal microbes may do this through two potential strategies: generating cravings for foods that they specialize on or foods that suppress their competitors, or inducing dysphoria until one eats foods that enhance their fitness (Alcock *et al.*, 2014). This may have crucial consequences for menopausal hot flushes because, as discussed above, flushing women tend to eat more sweets and carbohydrates predisposing them to metabolic risk. Whether gut microbiota does influence hot flushes is an unresolved point.

HSR inducers and co-inducers

Besides the physiological management of HSR by heat treatment (alone or in combination with physical exercise or prebiotics), pharmacological manipulation is an alternative. In this sense, hydroxylamine derivatives, such as bimoclomol and BGP-15, are promising resources for treating metabolic diseases of inflammatory nature (Henstridge *et al.*, 2014). Similar to that observed for glutamine, which after being metabolized through the HBP route prolongs the activation and transcribing activity of HSF1 (Leite *et al.*, 2016), bimoclomol and BGP-15 are HSR co-inducers, which means that the drugs do not initiate a bona fide HSR but rather require a prime (e.g. exercise, heat treatment and oxidative stress) to potentialise it. As expected, bimoclomol and BGP-15 induce tissue accumulation of HSP70 and HSR (Bíró *et al.*, 1997; Lubbers *et al.*, 2002; Polakowski *et al.*, 2002; Hargitai *et al.*, 2003; Sumegi *et al.*, 2017) in chronic inflammatory diseases, being clinically safe in human trials (Vígh *et al.*, 1997; Literáti-Nagy *et al.*, 2009, 2010, 2012, 2014; Crul *et al.*, 2013; Sapra *et al.*, 2014; Kennedy *et al.*, 2016).

Glutamine and its derivatives, which are also potentialisers of HSR (please, compare Fig. 1 in the present paper with Fig. 5 in Leite *et al.*, 2016), have been shown to improve metabolic status both *in vivo* and *in vitro* through the enhancement of HSR biochemical pathway (Newsholme *et al.*, 2003; Cruzat *et al.*, 2014a, 2014b, 2015; Keane *et al.*, 2015; Verdile *et al.*, 2015), alone or in combination with exercise (Petry *et al.*, 2014, 2015).

Unfortunately, however, there is yet no study specifically approaching the effects of bimoclomol, BGP-15 or glutamine in menopausal women or hot flushes.

Conclusion

If HSR may be compromised during the menopause transition and hot flushes might be a restorative reaction, it is imperative to clinically evaluate menopausal women for their HSR indices (H-index of eHSP70/iHSP70 and cellular responses to heat treatment *in vitro*) and thermotolerance (which is associated with intracellular accumulation of HSP70 and other chaperones). The H-index may be useful to

assess both longitudinal individual progression during clinical care (pharmacological and non-pharmacological interventions) and its status in comparison to flush features in a population. Notwithstanding, it is certain that some open questions must be addressed:

(1) Does the expected difference in the status of HSRs between symptomatic and asymptomatic women, with respect to hot-flush duration and severity, really exist?
(2) What is the status of HSRs in premenopausal and postmenopausal women? Do they indeed present differential responses? If so, can HRT change it?
(3) Since KNDy neuron activity and sympathetic tonus are altered during the flush episodes, are these changes associated with serum levels of eHSP70 in objectively controlled flushing patients?
(4) As both oestrogen and heat treatment block cellular senescence and SASP, can the antisenescent effects of oestrogen be at least partially restored by hot flushes in menopausal women? If so, can heat treatment mimic oestrogen-dependent antisenescent effects by enhancing the HSR?
(5) What are the principal effects of long-term heat treatment on cardiovascular, metabolic, neuroendocrine and neuromuscular systems during menopausal transition?
(6) Do calorie restriction-like associations between heat treatment, nutraceutical approaches (e.g. glutamine, resveratrol) and exercise improve flushes and their metabolically related changes? Do they interfere in gut microbiota?
(7) Is there a differential in thermotolerance between premenopausal and postmenopausal women, and between symptomatic and asymptomatic women with regard to hot flushes?
(8) Since chronic heat treatment may lead to adaptation of central and peripheral NO production, does NO metabolism take place in thermotolerance in heat-treated menopausal women thus contributing to improved HSR?
(9) As glucose ingestion reduces both VMS and serum eHSP70 in response to stressful challenges, is there a common underlying mechanism that could shed light on metabolic alterations observable in flushing women that could result in novel treatments for oestrogen-deficient states (including menopause transition and the premenstrual period)? If so, is there some involvement of gut microbiota?
(10) Because cellular deregulation that parallels KNDy dysfunction and VMS in oestrogen-deficient states are associated with Dyn/κ-OR signalling, is there additional sites where similar derangement could be happening thus contributing to pain-related diseases (e.g. fibromyalgia, migraine) that are commonly found in menopausal transition? If so, can HSR inducers (including heat treatment) alleviate these conditions?

> *'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)*

Acknowledgements

We are grateful to Dr Míriam Gil Sant'Helena (National Institute of Science and Technology of Hormones and Women's Health—INCT/CNPq/UFRGS) and Dr Mirna Stela Ludwig (Regional University of Northwestern Rio Grande do Sul State—UNIJUÍ) for critical and insightful discussions during the preparation of the present manuscript. Prof. Poli Mara Spritzer is also thanked for the availability of INCT/CNPq/UFRGS facilities for translational studies of the group. We would like to apologise for not mentioning important primary studies due to space constraints and to allow a better flow of information throughout the text. This paper is sincerely dedicated to all menopausal women, and their families, who know better than everyone else what menopausal transition is and what it means to spend at least a third of their lives in postmenopause. We hope we have been helpful.

Authors' roles

A.A.M. raised the matter and P.I.H.B. developed it. P.I.H.B. wrote the first draft of the paper and prepared the figures. A.A.M. and P.I.H.B. co-wrote the final version and approved the submitted and published versions.

Funding

The Brazilian National Council for Scientific and Technological Development (CNPq — Conselho Nacional de Desenvolvimento Científico e Tecnológico) (#551097/2007-8, 563870/2010-9, 402626/2012-5 and 402364/2012-0 to P.I.H.B.).

Conflicts of interest

The authors declare no conflict of interest and no competing interests such as consultancies, financial involvement, patent ownership, etc. in relation to the work described herein. CNPq (the funding organism) was not involved in the propositions presented in this manuscript.

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