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ORIGINAL ARTICLE

Heat stress alters metabolic pathways and nitric oxide signaling in keratinocytes under hyperglycemia

Syeda Tayyiba Rahat[1], Ilkka Miinalainen[2], Anni I. Nieminen[3], Seppo Vainio[1,4,5,6,7], Nsrein Ali[1,7]*

[1]Faculty of Biochemistry and Molecular Medicine, Laboratory of Developmental Biology, University of Oulu, Oulu, Finland, [2]Biocenter Oulu, University of Oulu, Oulu, Finland, [3]Stem Cell and Metabolism Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [4]Flagship GeneCellNano, University of Oulu, Oulu, Finland, [5]Kvantum Institute, University of Oulu, Oulu, Finland, [6]Infotech Oulu, University of Oulu, Oulu, Finland, and [7]NETSKINMODELS COST Action CA21108, University of Oulu, Oulu, Finland

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***Correspondence**
Nsrein Ali
Tel.: +358 (0) 50 34 94 212
E-mail address:
nsrein.ali@oulu.fi

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ABSTRACT

Background: Diabetic patients are particularly vulnerable to heat exposure due to impaired thermoregulation and reduced sweating ability. The impact of heat on skin cell function, particularly keratinocytes, is poorly understood. Recent studies highlight the critical role of nitric oxide (NO) in thermoregulation and heat stress responses, but its specific involvement in keratinocyte responses and metabolic profiles remains unexplored.

Objective: This proof-of-concept study investigates the metabolic profiles of HaCat keratinocytes under normal and high-glucose conditions during varied heat exposures.

Methods: We conducted experiments using a metabolomics approach, NO levels assessments, western blot analysis, and evaluations of mitochondrial morphology.

Results: Our findings indicate that acute heat exposure over 90 minutes significantly alters metabolic pathways, particularly amino acid metabolism (including arginine, valine, leucine, and serine), the pyrimidine metabolite uracil, and glycolysis, notably lactate production. Arginine metabolism was uniquely affected by high glucose combined with heat, aligning with previous clinical observations.Furthermore, we discovered that changes in NO production correlated with heat exposure duration, and that NO levels in extracellular vesicles (EVs) from HaCat cells were inversely related to intracellular NO levels. Additionally, we observed alterations in HSP-70 protein expression and mitochondrial morphology, supporting cellular adaptation to thermal stress.

Conclusion: This study is the first to demonstrate heat-induced metabolic changes in keratinocytes involving arginine and NO, highlighting their potential as clinical biomarkers for thermal stress adaptation, with implications for both healthy individuals and diabetic patients.

INTRODUCTION

Diabetes is a metabolic disorder characterized by elevated blood glucose levels, insulin resistance, and damage to multiple organs[1,2]. Emerging evidence suggests that diabetic individuals are more vulnerable to heat exposure than non-diabetics, with studies reporting higher mortality rates in this population during heat events[3,4].

The skin, through its sweat glands, plays a crucial role in thermoregulation by facilitating heat dissipation via sweat production[5,6]. In individuals with diabetes, this thermoregulatory function is impaired due to several complications. These include endothelial and non-endothelial signaling dysfunction linked to cardiovascular issues; metabolic abnormalities such as chronic hyperglycemia and irregular insulin distribution, altered autonomic nervous function, and reduced skin blood flow. Collectively, these factors contribute to a diminished ability to sweat[7,8].

Interestingly, clinical studies have reported therapeutic benefits of heat treatments in diabetes, including improved blood glucose control and reduced insulin resistance[9,10]. One

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hypothesis is that heat exposure influences both the rate and composition of sweat. Furthermore, the interaction between nitric oxide (NO) and heat shock protein (HSP) pathways has been identified as a potential molecular mechanism promoting sweat production under heat stress[11–13].

NO, a free radical generated by the oxidation of L-arginine[14–16] has gained increasing attention for its diverse biological roles, particularly in the skin[17–19]. It plays a crucial role in regulating oxidative stress and mitochondrial activity due to its biphasic nature. At low concentrations, NO exerts protective effects on mitochondria by promoting mitochondrial biogenesis, enhancing efficiency, and modestly regulating reactive oxygen species (ROS). However, at elevated concentrations, NO can lead to oxidative stress by inhibiting the electron transport chain, increasing ROS and reactive nitrogen species. This can trigger mitochondrial fragmentation and dysfunction, resulting in cell injury and death[20–22]. Additionally, NO is vital for thermoregulation, contributing to both central and peripheral mechanisms of body temperature control. It is involved in the body's responses to heat stress, fever, and immune-related temperature changes, with concentrations of NO rising during heat exposure. This increase is believed to influence neuronal activation in the hypothalamus, further affecting the body's thermal response[16,23–24].

HSPs, particularly HSP-70, are critical molecular chaperones involved in protein homeostasis and cytoprotection. Their expression is dynamically induced under stress conditions, including heat[14,25–26]. Altered HSP levels have been associated with diabetic complications[11,27]. HSP-70, specifically, has been linked to keratinocyte responses to heat, promoting anti-inflammatory mechanisms and protection against photodamage[28].

In our recent work, we identified metabolites in sweat extracellular vesicles (EVs) using a novel extraction and collection technique. These metabolites were influenced by heat and correlated with glucose levels in healthy individuals, suggesting a role for sweat in systemic metabolic signaling[29].

In the present study, we aim to establish a causal relationship between heat exposure and skin responses in the context of diabetes. Using an *in vitro* model of human immortalized keratinocytes (HaCaT), cultured under normal (5 mM) and high (25 mM) glucose conditions to simulate healthy and diabetic environments, respectively, cells were exposed to heat for varying durations to investigate metabolic profiles and further elucidate the underlying molecular pathways involved.

Our findings demonstrate that heat exposure induces distinct metabolic alterations in keratinocytes, particularly within the arginine-NO axis. Notably, in high-glucose conditions, arginine metabolism and downstream NO production were significantly modulated. Interestingly, the NO levels in EVs displayed an inverse pattern compared to intracellular levels. Repeated heat exposure further amplified NO production, which was associated with upregulated HSP-70 expression and enhanced mitochondrial integrity.

This study provides novel insights into the role of skin in thermoregulation, particularly under diabetic conditions. It identifies arginine and NO as potential clinical biomarkers for monitoring skin response to heat stress and suggests that targeting their pathway through HSPs may offer therapeutic strategies for improving heat management in patients with diabetes.

MATERIALS AND METHOD

Cell culture and glucose treatment

HaCat cell lines were maintained in normal glucose (5 mM D-Glucose) and high-glucose (25 mM D-Glucose) DMEM (Thermo Fisher) to mimic normoglycemic and diabetic conditions, respectively. DMEM media was supplemented with 1% Penicillin/Streptomycin (Sigma), 10% FBS (Gibco) and maintained at 37°C in a humidified incubator under 5% CO_2. The media was replaced every 48 h after the seeding of the cells (seeding density 2.2×10^6 cells). When needed the cells were maintained in different passages after detachment with Trypsin–EDTA (Gibco Life Technology).

A cell viability assay was performed following heat exposure treatment using trypan blue dye (Cat# T10282 Invitrogen™, CA, USA). Cell counting was conducted using the TC20™ automated cell counter (Bio-Rad Laboratories, CA, USA), which provides an accurate count of both viable and nonviable cells. A representative figure relevant to this assay is presented in Figure S1B, showing the results 48 h after heat exposure.

Heat exposure

Acute and chronic heat exposures

On the day of treatment, HaCat cell cultures were rinsed with growth media to eliminate dead cells. Following this, fresh growth media were added to all control and treatment plates. Control cell plates were incubated for 90 min and 48 h at 37°C in a humidified incubator with 5% CO_2, while treatment plates were incubated at 40°C under the same conditions. To ensure precise control of temperature and humidity during heat exposure, a temperature data logger with an integrated USB interface (LOG32TH, Carl Roth) was employed.

After heat exposure, cells were rinsed with 1× PBS and subsequently collected for protein, metabolomics, and electron microscopy analyses, in which each was conducted according to its respective protocols. This procedure was repeated twice using two independent cell passages, with three biological replicates conducted for each passage.

Repetitive heat exposure

On the day of the treatment, HaCat cells maintained in 5 and 25 mM glucose levels were subjected to repeated heat exposure. In this experimental setup, the cells were exposed to 40°C in a humidified incubator under 5% CO_2 for 90 min every 24 h, repeated three times. Following each heat exposure, the cells were returned to an incubator at 37°C under the same incubation conditions. Cells for analysis were collected immediately

after the third heat exposure and used for further analyses as described in the section above.

Isolation of extracellular vesicles from keratinocytes

Following 48 h of heat exposure, EVs were isolated using our previously published protocol with some modifications[29,30]. Briefly, the culture medium was collected and filtered through 40 µm Sterile Cell Strainers (CLS431750, Corning™) to remove dead cells. The supernatant was cleared from cellular components by centrifugation twice at 2,500 × *g* for 15 min at 4°C. The filtrate was then refiltered through 0.8 µm vacuum filter units (Thermo Fisher Scientific, Waltham, MA, USA). The filtrate was centrifuged in a Sorvall AH-629 rotor at 100,000× *g* for 12 h, in a Sorvall Ultracentrifuge Machine WX ultra 90 (VWR, Thermo Electron Corporation, Radnor, PA, USA). The supernatant was collected; the pellets were washed using 1× PBS followed by centrifugation at 100,000× *g* for 2 h and then were resuspended in 1× PBS. The characterization of EVs was performed using nano tracking analysis and electron microscopy (negative staining), as described in our previous studies[29,30] and detailed in the Data S1, as previously described.

Electron microscopy–negative staining of HaCat cells

HaCat cells were fixed in a 1% glutaraldehyde and 4% formaldehyde mixture in 0.1 M phosphate buffer (pH 7.2) for 10 min. Cells were detached and fixation continued for 1 h and stored at +4°C in the fixative. After fixation, cells were centrifuged to form a pellet, immersed in 2% agarose in distilled water, and postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries, Vermont, USA). Thin sections (70 nm) were cut with a Leica Ultracut UCT ultramicrotome, stained using uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit transmission electron microscope (FEI Europe, Eindhoven, The Netherlands) operated at 100 kV. Images were captured with a Quemesa bottom-mounted CCD camera (Olympus Soft Imaging Solutions GMBH, Münster Germany) using Radius software (EMSIS GmbH, Münster, Germany).

The number of mitochondria was counted in ten cells from both normal and high-glucose conditions. Mitochondria were classified into two groups: (1) "normal mitochondria," which can exhibit various shapes (elongated or round) with organized cristae and an electron-dense matrix, and (2) "abnormal mitochondria," which are swollen, enlarged, and round, characterized by disarrangement of their cristae and a partially or completely electron-lucent matrix, as previously reported in our publication[31].

Nitrite concentration determination

HaCat cells were exposed to heat as explained earlier in the experimental design. Cell lysate was prepared using RIPA buffer (Cell Signaling Technology). Nitrite concentration was determined using a Griess reagent kit (G7921, Thermo Scientific). The standard nitrite concentration solutions were prepared up to 100 mg. Cell lysate samples and standards were combined with Griess reagent following the protocol provided with the kit, and incubated for 30 min. After incubation, the absorbance at 544 nm was measured with a spectroscope (Victor V3, Perkin Elmers, Massachusetts, USA). The standard curve was plotted using nitrite standards and their respective absorbance at 544 nm. Nitrite concentration in cell lysate was calculated by the corresponding standard curve equation.

Statistical analysis

All analyses were performed with GraphPad Prism software, version (9.3.1), which was used for the statistical analyses. The two-tailed Student *t*-test was employed and *P-values less than 0.05 were considered significant.

RESULTS

Thermal stress induces metabolic remodeling in skin keratinocytes

Recently, we demonstrated that metabolite levels in sweat-derived EVs are modulated by heat exposure, highlighting their potential as noninvasive indicators of metabolic changes in both healthy and diabetic individuals[29,30]. A key limitation of our clinical study, however, was the lack of access to baseline metabolite profiles under conditions of high glucose alone or heat exposure alone under normal glucose conditions. To overcome this, and building on evidence that eccrine sweat glands play a central role in maintaining skin homeostasis[6,32–33], and given their ectodermal origin with keratinocytes, we sought to further explore these metabolic responses in a controlled *in vitro* setting.

We used HaCaT keratinocyte cells as a model system to investigate the individual and combined effects of heat and glucose stress on cellular metabolism. To mimic normoglycemic and diabetic environments, cells were cultured under normal (5 mM) and high (25 mM) glucose conditions, as previously reported[34–38]. Afterward, the cells were exposed to heat stress at 40°C for 90 min. We assessed the impact of temperature changes on cell numbers and viability using trypan blue staining to distinguish living from dead cells. Our results indicated no significant difference in cell survival between cells cultured in normal- or high-glucose conditions when exposed to heat compared with those maintained at room temperature of 37°C (Figure S1A,B). These findings were further supported by ultrastructural electron microscopy analyses presented in a later section of this article.

Subsequently, metabolite profiling was conducted using a targeted metabolomics approach as described by Rahat *et al.*[29] (Figure 1a). Our analysis revealed that heat exposure significantly decreased six metabolites under high-glucose conditions compared to normal glucose. Notably, arginine and valine (amino acids), along with uracil (a pyrimidine metabolite), were markedly downregulated (Figure 1b–d). Conversely, levels of leucine and serine (amino acids), as well as lactate (a glycolysis product), were significantly elevated (Figure 1e–g). Interestingly,

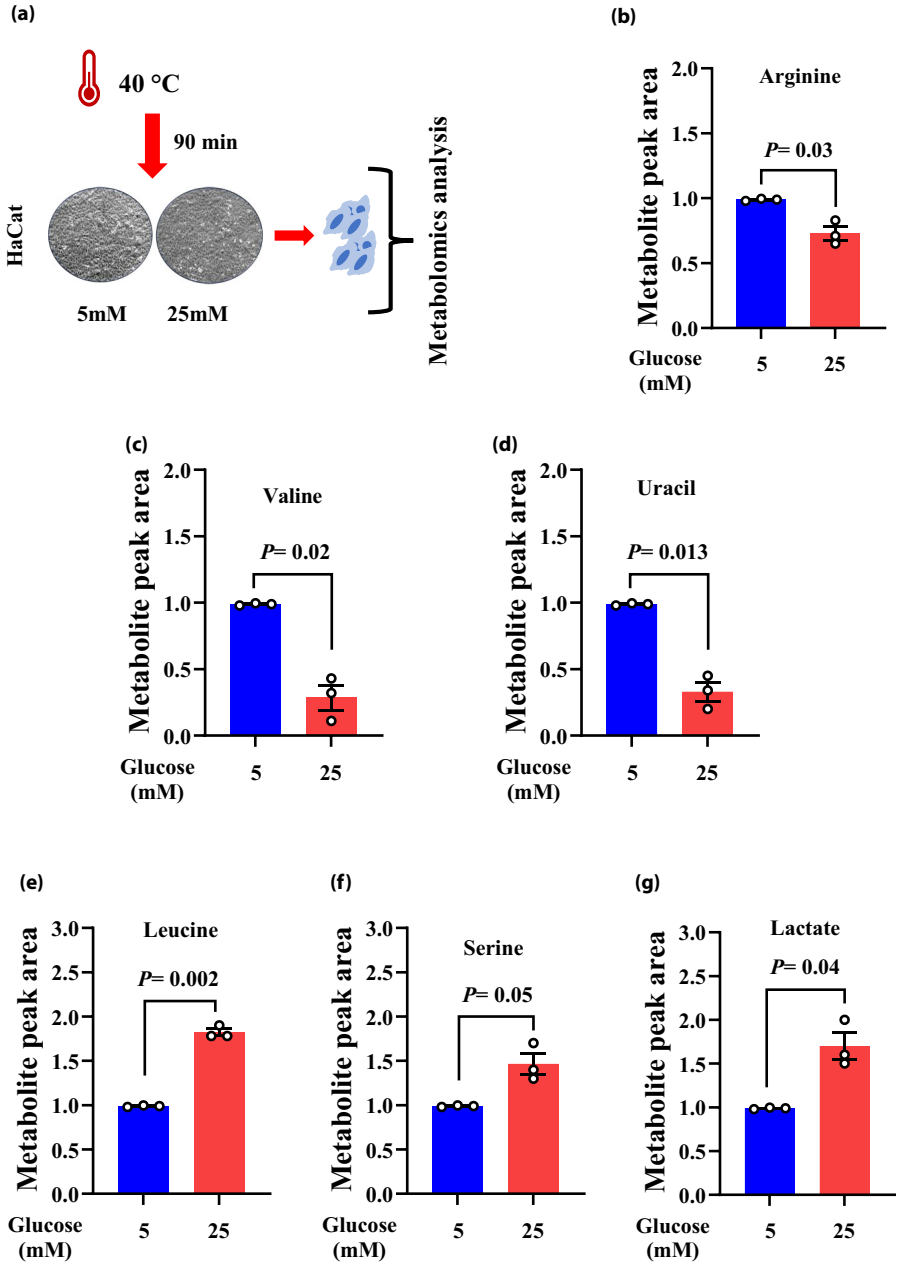

Figure 1 | Metabolic composition of keratinocytes upon acute heat exposure. (a) Schematic overview of the experimental design. (b–g) Relative levels of arginine (b), valine (c), uracil (d), leucine (e), serine (f), and lactate (g) in keratinocytes maintained under high- versus normal glucose conditions following 90 min of heat exposure. Data represent fold changes in metabolite peak areas ± SEM, normalization to the cell number and expressed relative to non-heated control cells. Two-tailed Student's *t*-test was used. *$P < 0.05$.

these intracellular shifts contrast with our previous observations in sweat EVs from individuals with type 2 diabetes[29].

To determine whether the observed metabolic changes were specifically induced by the combined effect of high glucose and heat, we examined cells exposed to acute heat under normal glucose (5 mM) conditions. In this setting, none of the previously identified metabolites, including arginine, uracil, leucine, serine, or lactate, were significantly altered, although arginine displayed an opposite trend compared to the combined condition (Figure S2A–F). Similarly, exposing cells to high glucose at 37°C did not significantly affect the levels of arginine, leucine, serine, or lactate. However, the latter three metabolites exhibited trends consistent with those observed under the combined stress condition, albeit to a lesser extent (Figure S2G,J–L).

Notably, valine and uracil were significantly decreased in high-glucose compared to normal glucose conditions (Figure S2H,I).

Collectively, these findings suggest that arginine is uniquely responsive to the combined effects of heat and high-glucose exposure, highlighting its potential as a specific marker of this dual stress interaction in clinical settings. This also confirms our previous clinical observation reported in Rahat *et al.*[29].

Thermal regulation of nitric oxide in skin keratinocytes

Building on our observation that arginine levels significantly decreased following heat exposure under high-glucose conditions, we sought to investigate the underlying molecular mechanism. Arginine serves as a precursor for nitric oxide (NO), which is synthesized via its oxidation by nitric oxide synthase (NOS)[13–16]. Given the reduction in arginine upon acute heat exposure, we hypothesized a concomitant decrease in NO production. To test this, NO levels were quantified using the Griess assay as previously described[39] (Figure 2a). Following 90 min of heat exposure, NO concentrations were substantially lower in keratinocytes cultured under high-glucose conditions compared to their normal glucose counterparts (Figure 2b), suggesting a link between decreased arginine availability and reduced NO production in heat-stressed hyperglycemic keratinocytes.

Previous studies have suggested that glucose concentration may influence NO production[35,40–41]. To investigate this in our model and determine whether the observed NO reduction was specific to the combined effects of heat and glucose, HaCat cells were incubated at 37°C in media containing 5, 12, or 25 mM glucose, and NO levels were measured (Figure S3A). While a moderate, nonsignificant increase in NO production was observed at higher glucose concentrations, the differences were not statistically significant (Figure S3B). These results suggest that NO production is not solely driven by glucose concentration but rather by a synergistic interaction between hyperglycemia and heat stress.

Building on the growing evidence that the molecular composition of EVs does not necessarily mirror the molecular profile of their parent cells or tissues[42,43], and our current findings showing that keratinocytes' metabolic profiles under heat exposure contrast with those observed in sweat EVs collected from healthy and type 2 diabetic patients in clinical settings[29], we aimed to assess NO levels in keratinocytes' EVs following heat exposure and compare their patterns with those observed in keratinocytes themselves. Due to insufficient EV yield after 90 min in our experimental setup, which differs from clinical conditions, we extended the culture duration to 48 h based on evidence indicating this timeframe optimally enriches EVs[31,44–46]. Keratinocytes were cultured under normal- and high-glucose conditions with heat treatment. Following exposure, both cells and culture media were collected: cells were used for intracellular NO analysis, while the media were processed for EV isolation and subsequent NO quantification (Figure 2c). A significant reduction in NO production was observed in high-glucose cells compared to normal glucose controls (Figure 2d), confirming the sustained effect of thermal stress on NO synthesis. In contrast, EVs isolated from culture supernatants after 48 h, characterized using nano tracking analysis (NTA) and electron microscopy (Figure S4A,B), revealed that high-glucose conditions induced a greater release of EVs containing significantly elevated NO levels (Figure 2e). These results support the role of EVs as a compensatory mechanism regulating cellular stress responses in hyperglycemic keratinocytes.

Altogether, our data reveal NO as a potential biomarker in keratinocytes and their EVs for monitoring the cellular effects of heat exposure in diabetic conditions, with contrasting levels observed in the two matrices.

Thermal stress alerts HSP-70 signaling and mitochondrial morphology in human keratinocytes

Heat Shock Protein 70 (HSP-70) plays a protective role in response to heat stress by functioning as a molecular chaperone to maintain cellular integrity[26,47]. Recent studies have also proposed that HSP-70 may modulate the NO pathway[14]. To investigate the impact of acute and chronic heat exposure on HSP-70 expression, we conducted western blot analysis (Figure 3a).

Following 90 min of heat exposure, western blot images and subsequent band intensity quantification revealed a significant decrease in HSP-70 levels in high glucose-maintained cells compared to those in normal glucose conditions (Figures 3b,c and S5). Similarly, after 48 h of heat exposure, HSP-70 expression was substantially downregulated, indicating a progressive decline with increasing heat (Figure 3d,e and S6). To confirm that high glucose alone affects HSP-70 expression, we examined its levels under basal conditions (37°C). The results showed no significant difference, suggesting that glucose concentration alone does not modulate HSP-70 expression (Figure S7A). These findings align with our ongoing study, which indicates that high glucose induces metabolic reprogramming in keratinocytes through specific molecular modulators, as identified using a 2D gel-based proteomics approach (Ali *et al., In Preparation*). Collectively, the data suggest that the observed reductions in HSP-70 expression under heat stress are temporally correlated with changes in arginine and NO levels in both acute and chronic heat exposure scenarios.

Considering the established role of HSP-70 in maintaining mitochondrial homeostasis by supporting protein folding and preserving aggregation[25,26,48–49], we investigated whether the heat-induced decline in HSP-70 expression might trigger mitochondrial phenotype alterations under high-glucose conditions. Electron microscopy images revealed that the majority of mitochondria in cells maintained in 25 mM glucose appeared structurally normal after acute heat exposure, exhibiting various shapes (elongated or round), well-organized cristae, and an electron-dense matrix. In contrast, the majority of mitochondria

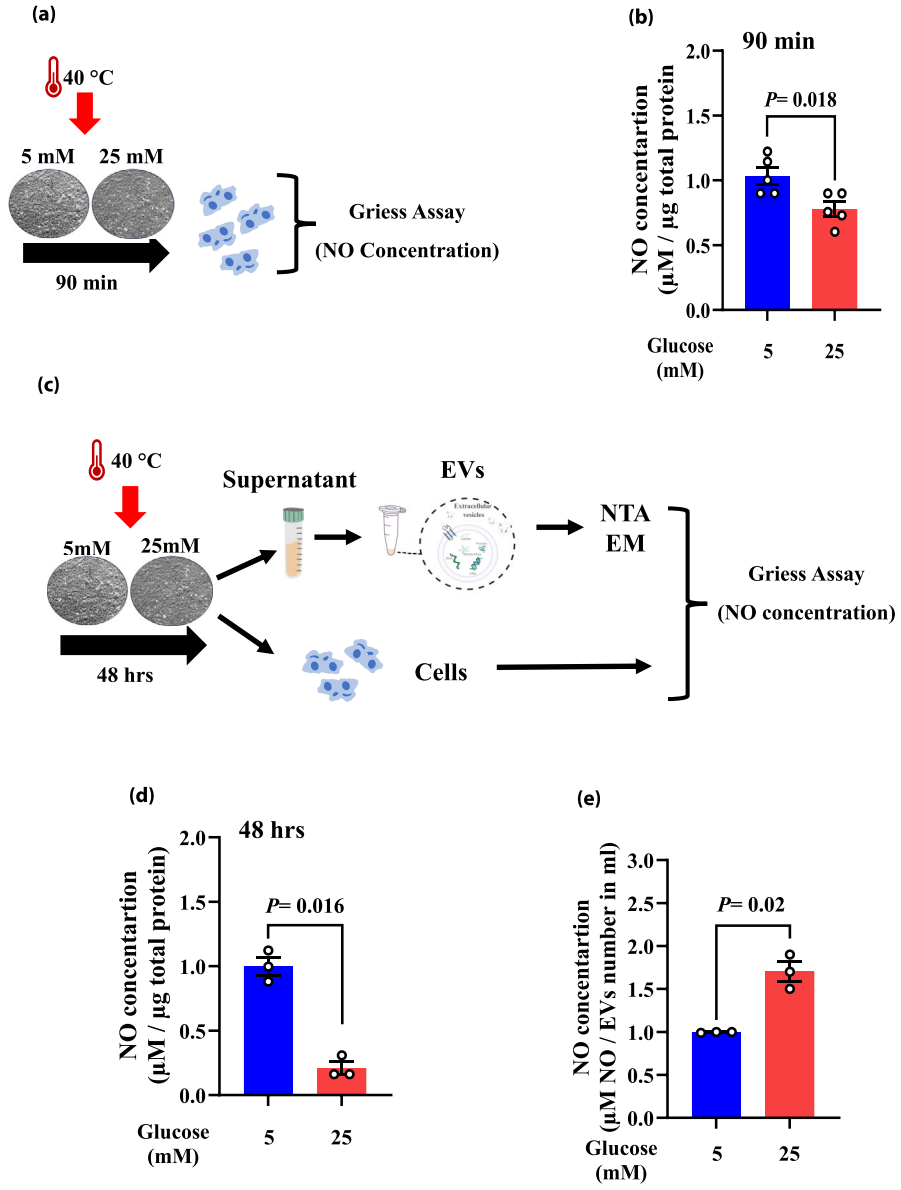

Figure 2 | Nitric oxide is a downstream target of both acute and chronic heat exposure in high glucose-maintained keratinocytes and their EVs. (a) Schematic representation of the experimental workflow for assessing NO levels under acute heat exposure. (b) NO concentration in HaCat cells maintained in normal- and high- glucose conditions after 90 min of acute heat exposure. (c) Schematic representation of the experimental workflow for assessing NO levels under chronic heat exposure. (d) NO concentration in HaCat cells after 48 h of chronic heat exposure under normal- and high- glucose conditions. (e) NO concentration in EVs isolated from HaCat cells maintained in normal- and high- glucose conditions after 48 h of heat exposure. Data are presented as mean ± SEM from five independent experiments in (b) and three in (d) and (e), normalized to either total cellular protein (µg) or EV particle concentration. Two-tailed Student's *t*-test was used. *$P < 0.05$.

in cells maintained in 5 mM glucose appeared abnormal, displaying swelling, enlargement, and rounded shapes, along with irregularly organized cristae and a partially or completely electron-lucent matrix (Figure 3f, dashed rectangle). These observations were supported by the low magnification of electron microscopy images showing the ultrastructural details of 15–20 cells and their total mitochondria and indicating a healthy cellular state (Figure S8A,B). These findings align with our viability data, showing that heat did not induce cytotoxicity, as presented in Figure S1A,B. Subsequently, the number of normal and abnormal mitochondria under both glucose conditions after 90 min of heat exposure was quantified using ten cells per condition, based on our previously published data[31]. The results demonstrated an increase in abnormal mitochondria in cells

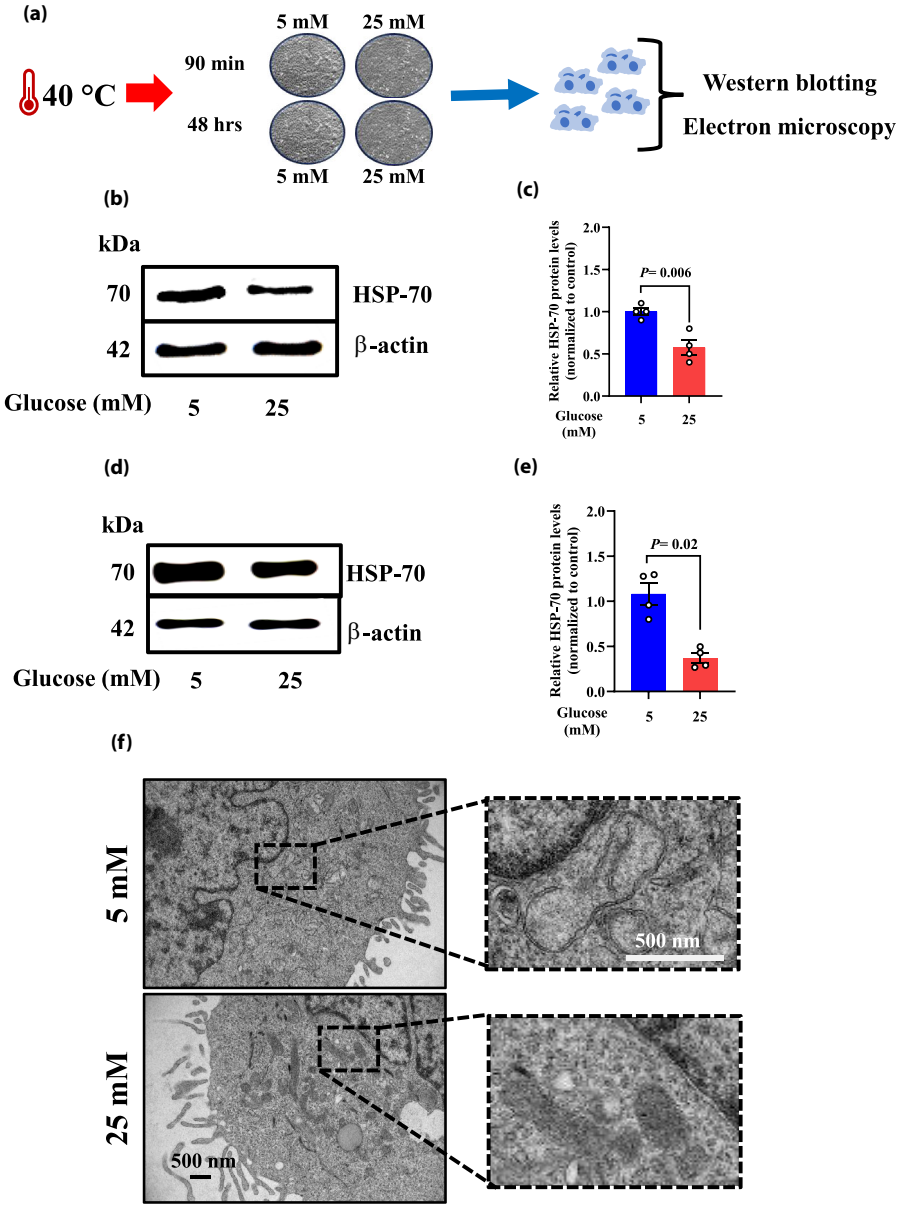

Figure 3 | HSP-70 protein levels are altered in response to acute and chronic heat exposure and are associated with changes in mitochondrial morphology. (a) Schematic representation of the experimental methodology. (b) Western blotting analysis of HSP-70 in HaCat cells after 90 min of acute heat exposure. (c) Quantification of HSP-70 protein expression levels following acute heat exposure during 90 min. (d) Western blotting analysis of HSP-70 expression in HaCat cells following 48 h of chronic heat exposure. (e) Quantification of HSP-70 protein expression levels after 48 h of heat exposure. Data are presented as averages, normalized to control ± SEM from three independent experiments. β-Actin was used as a loading control. Two-tailed Student's t-test was used. *$P < 0.05$. (f) Acute heat exposure induces abnormal mitochondrial phenotype in keratinocytes, characterized by swelling, enlarged round shapes, disorganized cristae, and a partially or completely electron-lucent matrix (black dotted rectangle). In contrast, mitochondria in cells maintained in 25 mM glucose retained a normal appearance, displaying various shapes (elongated or round), well-organized cristae, and an electron-dense matrix (black dotted rectangle). Scale bar = 500 nm.

under normal glucose conditions, whereas cells maintained under high-glucose conditions showed a significant decrease in abnormal mitochondria (Figure 4a). Building on our observations and the reported evidence linking HSP-70 to the maintenance of mitochondrial homeostasis[25,26,48–49], we propose that mitochondria in high-glucose conditions are more resilient than those in normal glucose conditions, reflecting a protective mechanism.

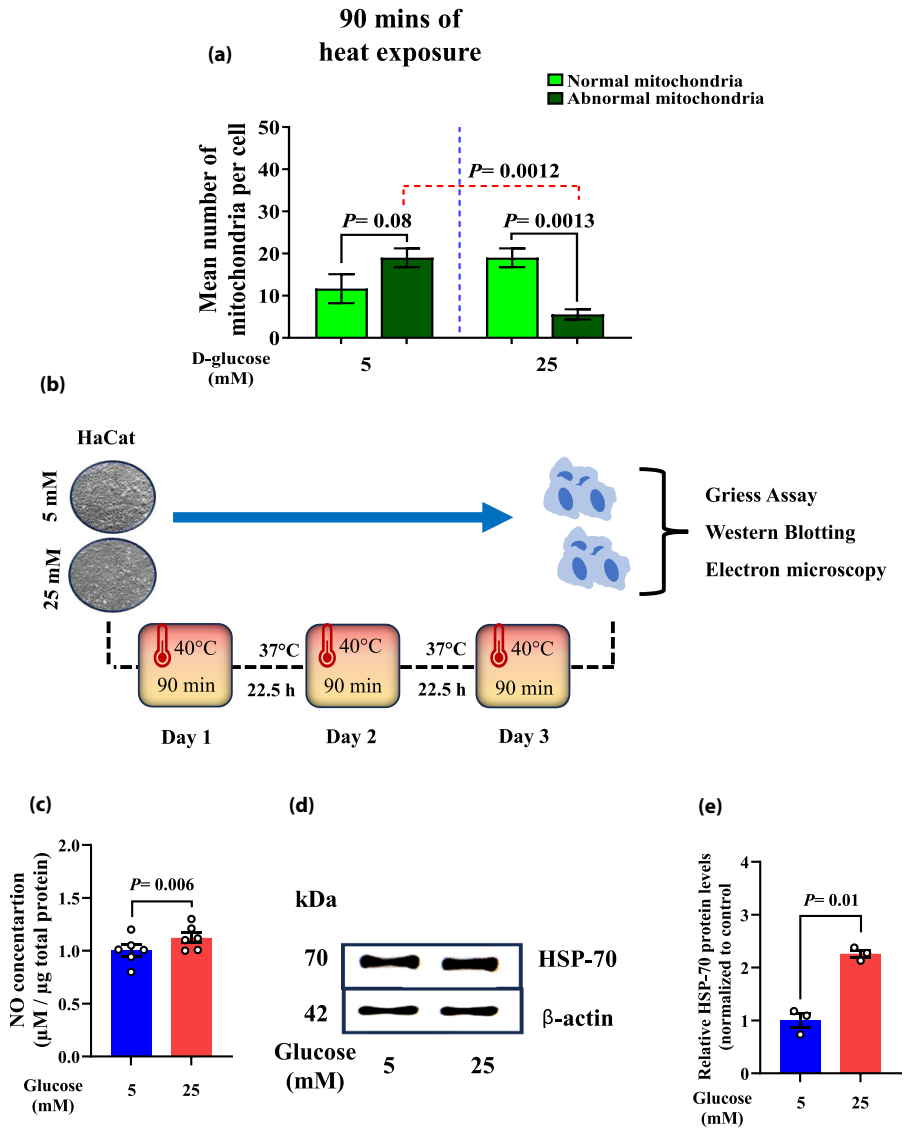

Figure 4 | Acute and repetitive heat exposure influence keratinocytes responses under high-glucose conditions. (a) Quantification of normal and abnormal mitochondria in ten cells from both normal- and high-glucose conditions after 90 min of heat exposure. Results are shown as the averages ± SEM using ten cells from each glucose condition. Two-tailed Student's *t*-test was used. **P* < 0.05. (b) Schematic representation of the experimental methodology. (c) Nitrate concentration in normal- and high glucose-maintained HaCat upon repetitive heat exposure. Data are presented as the average ± SEM from three independent experiments, normalized to protein concentration and control levels. (d) Western blotting analysis of HSP-70 expression in HaCat cells following three consecutive 90-min acute heat exposures. (e) Quantification of HSP-70 protein levels based on band intensity. Results are shown as the averages ± SEM from three independent experiments, normalized control levels. β-Actin was used as a loading control. Two-tailed Student's *t*-test was used. **P* < 0.05.

Supporting this hypothesis, further analysis of metabolites significantly influenced by high glucose revealed a marked decrease in hypoxanthine, an established marker of oxidative stress[44–46,50], alongside a trend toward increased levels of γ-Aminobutyric acid (GABA)-related metabolites, which are associated with antioxidant mechanisms[51,52], in high-glucose cells under basal conditions (37°C; Figure S7B,C). These observations align with our ongoing data indicating that high glucose induces metabolic reprogramming in keratinocytes through specific target modulators (Ali N. *et al.*, in preparation).

Taken together, these findings reveal that heat exposure influences HSP-70 expression levels in a similar pattern to NO, suggesting a potential link between these two factors and the modulation of mitochondrial morphology in keratinocytes under heat stress.

Repetitive heat exposure enhances NO and HSP-70 levels and modulates mitochondrial phenotype in keratinocytes

Recent clinical studies suggest that the duration and frequency of heat exposure are critical factors for achieving therapeutic benefits, such as improved insulin sensitivity and glycemic control in diabetic patients[12–15]. Building on this, we investigated whether repeated heat exposure could enhance NO production and HSP-70 expression in high glucose-maintained keratinocytes. Cells underwent three rounds of 90 min heat exposure, as illustrated in Figure 4b. NO levels and HSP-70 protein expression were assessed using the Griess assay and western blotting, respectively.

Our findings demonstrate a significant increase in NO production in keratinocytes cultured under high-glucose conditions compared to those maintained in normal glucose environments (Figure 4c). Consistently, Western blot images revealed markedly higher expression levels of HSP-70 in high-glucose cells, which was further confirmed by densitometric quantification of band intensity (Figure 4d,e and S9).

Electron microscopy revealed altered ultrastructural mitochondrial morphology in both normal- and high-glucose-maintained cells following repeated heat exposure (Figure 5a). Disorganized cristae networks were observed, suggesting that repetitive heat exposure may enhance mitochondrial remodeling, similar to what we observed after acute heat exposure in cells maintained under normal glucose conditions (Figure 3f). Quantification of normal and abnormal mitochondria in ten cells from each condition (normal and high glucose) supports these histological observations, showing an increase in abnormal mitochondria compared to normal in both glucose conditions. This increase was significant in cells maintained in 25 mM glucose. Importantly, these structural changes may reflect an adaptive mechanism by which mitochondria engage in coping with repeated heat exposure, consistent with previous studies suggesting beneficial effects mediated through NO and HSP-70[11–13].

Collectively, these findings demonstrate, for the first time, the beneficial effects of repeated heat exposure on mitochondrial function in keratinocytes, mediated by increased NO production and HSP-70 expression.

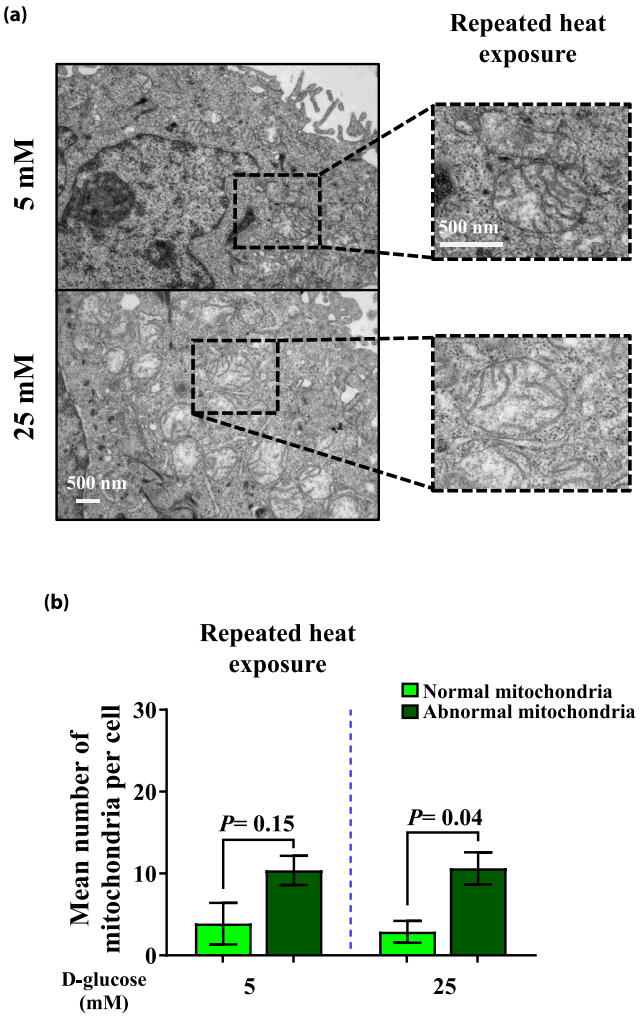

Figure 5 | Repeated heat exposure alters mitochondrial morphology in keratinocytes under high-glucose conditions. (a) Morphological examination of mitochondria in both keratinocytes incubated with normal and high glucose following repeated heat exposure. Scale bar = 500 nm. (b) Quantification of normal and abnormal mitochondria in ten cells from each condition (normal and high glucose). Results are shown as the averages ± SEM using ten cells from each glucose condition. Two-tailed Student's *t*-test was used. *$P < 0.05$.

DISCUSSION

In the present study, we focused specifically on keratinocytes, as a representative model for sweat gland cells to investigate their intrinsic response under diabetic-like conditions and in the absence of neuronal input. Using HaCat cells, our goal was to examine how different durations and frequences of heat exposure influence their metabolic patterns. We employed the same experimental design as in our recent clinical study[29]. Through targeted metabolomics, we were able to identify keratinocyte-autonomous thermoregulatory metabolic profile responses, distinguishing them from impairments typically attributed to neuropathic factors[7,8].

Sweat glands are extensively innervated, and proper neuronal signaling is essential for initiating sweat secretion[5,6,8,53]. In diabetes, this neuro–glandular interaction is disrupted, and numerous clinical reports have shown that neuropathic dysfunction is closely linked to impaired sweat gland function and, consequently, compromised thermoregulation[54–59]. Additionally, many clinical studies have demonstrated that heat may affect the production of sweat and its molecular composition in both healthy individuals and diabetic patients[56,60–64]. In agreement, our recently published article supports this statement, showing

that heat after exercise may influence the metabolic composition of sweat EVs[30].

In a previous clinical report, arginine was identified as the highest metabolite present in the sweat of healthy individuals following heat exposure. Additionally, the concentration of arginine in sweat was found to be independent of its variations in blood[65]. In line with this, our current study demonstrates that arginine is the unique metabolite associated with the dual effects of heat and high glucose, accompanied by a similar pattern of NO production. This finding aligns with previous clinical reports indicating that NO levels are altered during heat-induced exercise, which enhances sweat production[60,61,64,66–67]. One explanation for this phenomenon is linked to the activity of nitric oxide synthase (NOS), an enzyme expressed in sweat glands that produces NO through the oxidation of arginine. This process ultimately influences sweat production and composition[21,68–69], supporting a regulatory role for NO in thermal adaptation.

Emerging clinical studies have demonstrated the therapeutic potential of heat exposure in improving insulin sensitivity, glycemic control, and inflammation in type 2 diabetes (T2D) patients, with frequent sessions being recommended[10,11,14,27,70]. In alignment with this, our findings show that repeated heat exposure restores NO levels and is accompanied by enhanced mitochondrial integrity in keratinocytes maintained under high-glucose conditions. However, the differing NO levels in sweat EVs from keratinocytes indicate that the EV molecular components may not mirror those of their parental cells or tissues, as previously reported[42,43]. Building on our results and the existing literature claiming heat as a potential therapeutic solution for diabetes management, our study proposes that arginine and NO may serve as potential clinical biomarkers, particularly in sweat EVs, offering a noninvasive approach for diabetes management and monitoring the physiological benefits of heat exposure.

While diabetes is associated with decreased levels of HSPs in insulin-sensitive tissues[71–74], repetitive heat exposure has been shown to induce HSP-70 expression in healthy keratinocytes[28]. Our study confirms this finding in keratinocytes maintained in high-glucose conditions. We observed that increased HSP-70 levels correlate with elevated NO production, suggesting a functional interplay between the HSP-70 and NO pathways as a molecular mechanism through which heat promotes cellular adaptation, as previously reported[11–14,27].

This interaction appears to support mitochondrial resilience, as evidenced by improved mitochondrial integrity in heat-exposed, high-glucose cells. Complementing these findings, we also detected shifts in redox-related metabolites such as hypoxanthine and GABA, molecules known to be involved in oxidative stress responses, indicating a broader rebalancing of the cellular redox state under thermal stress. These redox adaptations are particularly relevant in high-glucose environments, where excess reactive oxygen species (ROS) generation disrupts cellular homeostasis[75–77]. NO, acting as a redox-sensitive signaling molecule, is known to play a pivotal role in modulating oxidative stress and maintaining redox equilibrium[22]. Thus, the coordinated induction of HSP-70 and NO may represent a compensatory mechanism to counteract glucose-induced oxidative imbalance, with implications for diabetes management.

A more direct mechanism may link HSP-70 to mitochondrial protection. Previous studies have shown that repetitive heat exposure can protect keratinocytes from UVB-induced apoptosis by enhancing mitochondrial integrity through HSP-70 induction[78]. Similarly, in other cell types, HSP-70 overexpression has been found to inhibit cytochrome c release and prevent mitochondrial membrane permeabilization during stress, thereby reducing apoptosis[79,80].

These findings suggest that HSP-70 may support mitochondrial stability through both direct and indirect mechanisms, particularly by modulating nitric oxide (NO) signaling and preserving redox homeostasis. This aligns with the dual-pathway hypothesis outlined in our graphical abstract. This adapted mechanism is crucial for sustaining keratinocyte survival in the context of diabetes and the skin features associated with this metabolic disorder.

CONCLUSION

Our study identifies potential clinical biomarkers in keratinocytes in response to heat stress under diabetic conditions, which are dependent on the presence of neural connections, supporting our previous clinical observations. Our findings demonstrate an association between amino acid metabolism, specifically arginine and its downstream product nitric oxide (NO), as central players in the molecular mechanism by which keratinocytes respond to heat stress. The dynamic responses of NO to repeated heat exposure may also hold clinical value in managing diabetes. Elucidating the molecular mechanisms involving HSP-70 and mitochondrial activity supports an adaptive pathway utilized by keratinocytes to cope with heat stress conditions.

In conclusion, considering previous clinical observations that identify both arginine and NO as key players in beneficial heat therapies, our study provides proof-of-concept evidence for the use of these molecules as therapeutic markers and potential therapeutic routes involving HSP-70 and mitochondria.

Limitations and future directions

Despite these insights, several limitations should be acknowledged. First, although HaCaT cells are a widely accepted in vitro model for keratinocytes, they do not fully replicate the complexity of native sweat gland physiology. Second, our results are based on acute and repetitive heat exposure protocols under controlled conditions, which may not fully capture real-life thermal stress or long-term clinical outcomes, especially in diabetic individuals undergoing insulin therapy or other treatments. Third, while we identified changes in nitric oxide levels and associated metabolic shifts, the underlying signaling mechanisms, especially those involving calcium dynamics,

mitochondrial respiration, and membrane potential, remain to be elucidated. Additionally, the direct link between HSP-70 and mitochondrial function, as proposed in the graphical abstract, warrants further investigation.

Future studies involving primary human sweat gland cells, *in vivo* validation, and broader metabolic profiling are necessary to strengthen the translational potential of our findings. Additionally, exploring the interplay between Trisk95, calcium dynamics, and NO signaling may help uncover new regulatory mechanisms relevant to thermal adaptation and metabolic health.

AUTHOR CONTRIBUTION

NA and SJ-V created the idea. NA and ST-R designed the experiments. ST-R performed the experiments related to NO concentration and HSP-70 expression. A-IN performed the metabolomics analysis and provided the data. I.M. assessed mitochondrial morphology via electron microscopy. NA prepared the figures and wrote the paper with the help of ST-R. SV edited the paper. All authors reviewed and edited the paper.

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DISCLOSURE

The authors declare no conflict of interest.
Approval of the research protocol: N/A.
Informed consent: N/A.
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Animal study: N/A.

DATA AVAILABILITY STATEMENT

The data sets generated and/or analyzed during the current study are openly available from the University of Oulu data repository at https://oulurepo.oulu.fi/ or from the corresponding author on request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplementary Materials.

Figure S1. Morphological and viability assay of HaCat cells under heat exposure.

Figure S2. Metabolic profile of keratinocytes under acute heat exposure and high-glucose conditions.

Figure S3. Nitric oxide production remains unchanged across varying glucose concentrations.

Figure S4. Characterization of HaCat-derived EVs.

Figure S5. Uncropped western blot showing HSP70 expression in HaCat cells 90 min after heat exposure.

Figure S6. Uncropped western blot showing HSP70 expression in HaCat cells 48 h after heat exposure.

Figure S7. HSP-70 expression and metabolic profile under basal conditions (37°C) in cells maintained in normal- or high- glucose environments.

Figure S8. Acute heat exposure induces alterations in mitochondrial morphology under high-glucose conditions.

Figure S9. Uncropped western blot showing HSP70 expression in HaCat cells following repeated heat exposure.