Journal of Science and Medicine in Sport 23 (2020) 1021–1027

Contents lists available at ScienceDirect

Journal of Science and Medicine in Sport

journal homepage: www.elsevier.com/locate/jsams

Original research

Variations in the *TRPV1* gene are associated to exertional heat stroke

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ARTICLE INFO

Article history:
Received 22 August 2019
Received in revised form 20 April 2020
Accepted 23 April 2020
Available online 18 May 2020

Keywords:
Heat exhaustion
Malignant hyperthermia
Whole exome sequencing
Thermogenesis
In vitro contracture test

ABSTRACT

Objectives: Exertional Heat Stroke (EHS) is one of the top three causes of sudden death in athletes. Extrinsic and intrinsic risk factors have been identified but the genetic causes still remain unclear. Our aim was to identify genes responsible for EHS, which is a necessary step to identify patients at risk and prevent crises.
Design: Genetic and functional laboratory studies
Methods: Whole Exome Sequencing (WES) was performed to search for candidate genes in a cohort of 15 soldiers who had a documented EHS episode. *In silico* and *in vitro* functional studies were performed to evaluate the effect of mutations identified in the candidate gene *TRPV1*.
Results: WES led to the identification of two missense variations in the *TRPV1* gene. These variations were very rare or unreported in control databases and located in critical domains of the protein. *In vitro* functional studies revealed that both variations induce a strong modification of the channel response to one of its natural agonist, the capsaicin.
Conclusions: We evidenced mutations altering channel properties of the *TRPV1* gene and demonstrated that *TRPV1*, which is involved in thermoregulation and nociception, is a new candidate gene for EHS. Our data provide the bases to explore genetic causes and molecular mechanisms governing the pathophysiology of EHS.

Practical implications

- *TRPV1* is a new candidate gene in Exertional Heat Stroke.
- The identification of molecular bases of EHS will help to propose efficient genetic screening to individuals at risk of EHS.
- Because of the identification of variations in *TRPV1* and *RYR1* genes both in Exertional Heat Stroke and Malignant Hyperthermia, the cross-risk between the two diseases should still be considered.

1. Introduction

Exertional Heat Stroke (EHS) is a life-threatening disease that occurs in young individuals who engage in prolonged and strenuous activity.[1] First cases have been reported in army soldiers[2] but many cases are now described in civilian population. Indeed, the popularity and increased competitiveness in summer road races and pre-season American football are likely associated with the number of EHS in athletics in recent years. The incidence of EHS is around 3 cases/10,000 person-years[3] but it can reach up to 2/1000

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https://doi.org/10.1016/j.jsams.2020.04.018
1440-2440/

athlete-exposures[4]; it was responsible for 30 deaths over the last 10 years among American football players.[5]

EHS is defined by a severe hyperthermia, with a body temperature above 40 °C, associated with a sudden neurological dysfunction, observed in healthy individuals exposed to intensive physical exercise.[6] Ultimately it results in a multiple organ failure leading to a high mortality rate that can be drastically reduced with early and quick cooling of the patient. The pathophysiology of EHS is based on a dysfunction of thermoregulation that leads to an uncontrolled increase of internal heat production exceeding compensation mechanisms. The core body temperature rises continuously and the patient evolves into an uncompensable heat stress state.[7] A hypermetabolic state, especially in the skeletal muscle, and the development of a systemic inflammatory syndrome are also part of the pathophysiology.[6] Extrinsic risk factors such as hot and humid environment have been identified, as well as intrinsic factors such as dehydration or sleep deprivation. Some studies have suggested that the impact of these risk factors could be modulated by training or acclimatization, which should help the patient to adapt to exercise and environment conditions.[8] However, several evidence support the existence of inherited genetic mutations that predispose some patients to EHS,[9–11] despite these adaptation mechanisms.

EHS shares many clinical and biological similarities with Malignant Hyperthermia (MH),[12] a life-threatening genetic disease triggered by volatile halogenated agents. MH is characterized by a hypermetabolic state in skeletal muscle with hyperthermia and can lead to multiple organ failure.[13] MH can be diagnosed by a positive *In Vitro* Contracture Test (IVCT), which quantifies the excessive contraction of a skeletal muscle biopsy in response to triggering agents, or by genetic testing. Mutations are detected in the *RYR1* gene in 60% of MH cases,[14] and rarely in the *CACNA1S* and *STAC3* genes.[15,16] All these genes encode proteins that are involved in calcium homeostasis in skeletal muscle. Interestingly, half of EHS patients displays a positive IVCT which evidences a calcium release disorder in these patients.[3] Contrary to MH, the genetic bases of EHS remain poorly documented. Mutations in *RYR1* have been associated with EHS with a much lower prevalence than in MH, estimated at 10%,[9] which suggests the involvement of other genes in the disease.

We report here the genetic study of a cohort of soldiers presenting with an EHS episode in whom mutations in *RYR1* had been previously excluded. Whole Exome Sequencing analysis and downstream functional studies led to the identification of *TRPV1* as a new gene associated with EHS. *TRPV1* encodes a cationic channel implicated in thermoregulation and has been recently associated with MH phenotypes, arguing in favor of an overlap between the two diseases.[17]

2. Methods

Samples were obtained from EHS soldiers of the French national cohort recruited between January 2004 and December 2006 by the Military Hospital Lavéran reference center for EHS. EHS occurred during or immediately after a military exercise consisting in a timed race (8-km running with boots and combat jacket) or a commando race (8-km running with complete battle dress, including an 11-kg combat rucksack and weapon). These exercises were part of soldiers' sessional exams. Patients were included on the basis of a characterized EHS crisis with 1) body temperature $\geq$ 39 °C recorded at the time of the crisis either rectally or, when not possible in the field, by ear (less accurate method), and 2) neurological dysfunction such as mental confusion or loss of consciousness.[18] The temperature threshold used to defined a crisis was 39 °C in this study in order to increase diagnostic sensitivity, because of the inevitable delay between the crisis and the temperature measurement in the field and to anticipate care of patients with neurological dysfunction with hyperthermia.[2] All selected patients displayed a positive *In Vitro* Contracture Test according to the European Malignant Hyperthermia Group (EMHG) guidelines. IVCT was considered positive when contraction was equal to or above 0,2 g at 2% halothane concentration or 2 mM caffeine.

Genetic analyses were performed after written informed consents were obtained. Whole Exome Sequencing (WES) was performed (n = 15) using Illumina HiSeq© 2000/2500 technology (Illumina, USA). The Ingenuity© software (Qiagen, Germany) was used to filter the variations. Based on the estimated prevalence[3] of EHS, variants with an allele frequency of at least 0.1% in common controls databases (ExAC and gnomAD) were excluded and only missense, frameshift (indels) or splice site variations were analyzed. Because our cohort consisted of sporadic cases, WES data analysis was performed with either autosomal recessive or dominant mode of inheritance hypotheses. Genes with 1-at least two patients harboring a variant matching with previous criteria and 2- a tissue expression in the skeletal muscle, were further analyzed. Variants were confirmed by Sanger sequencing. Segregation studies were not performed because relatives clinical evaluation and paraclinical assessment could not be performed and DNAs were not available. Additional EHS probands (n = 45) were analyzed by Sanger sequencing for the *TRPV1* (NM_018727.5) candidate gene.

In vitro functional studies of *TRPV1* variations identified by WES were performed with a plasmid containing the human *TRPV1* sequence in fusion with the mCherry tag that has already been described.[17] The p.Gly684Val and p.Arg772Cys mutations were introduced by site-directed mutagenesis, following the instruction of the QuickChange XL Site-Directed Mutagenesis kit (Agilent, USA). Plasmids encoding the Wild-Type or a mutant TRPV1 were transfected in HEK-293 cells according to the DharmaFECT Duo Transfection Reagent protocol (Dharmacon, USA). Cells were cultured in standard conditions and experiments were performed after 48 h of *TRPV1* expression.

To evaluate the calcium release in the cytoplasm after TRPV1 activation, cells were loaded with Fluo-4 AM dye for 30 min at 37 °C and next maintained in a calcium-free medium containing 1 mM EGTA, what allowed to assess exclusively calcium release from intracellular stores. The stimulation with the agonist (capsaicin 20 μM, Merck, Germany) was performed at room temperature and the emission of fluorescence of the Fluo-4 AM dye was followed by microscopy on a DMI6000 Leica FRAP (Leica Microsystems) with a 10x objective. Images were taken every second during 80 s and stimulation was performed at 10 s. Data were analyzed with the ImageJ software to select regions of interest and acquire the fluorescence intensity that was normalized to baseline fluorescence (F0). An average of 20 cells was analyzed for each condition and experiments were repeated 3 times.

To evaluate the resting calcium in the cytosol, cells were loaded with Fura-2 (2.5 μM) for 30 min at 37 °C in Tyrode buffer. After loading, cells were washed and maintained in Ca^{2+}-free Tyrode buffer. Fura-2 measurements were performed at room temperature using a wide field Leica DMI6000B microscope equipped with a 40x oil-objective and an Orca-Flash4.0 digital camera (HAMAMATSU). Using a Lambda DG-4+ filter (Sutter instruments), Fura-2 AM was excited at 340 and 380 nm and their respective emitted fluorescence lights were measured at wavelength 510 nm. Images were taken with 5 s interval. Fluorescence ratios were analyzed with MetaFluor 6.3 software (Universal imaging).

Data were analyzed with GraphPad Prism 6.0 (USA) using a one-way ANOVA after checking for Gaussian distribution. Normally-distributed data were analyzed through parametric ANOVA followed by Tukey's post-hoc tests while non-normally-distributed data were analyzed through non-parametric Kruskal-

Wallis ANOVA followed by Dunn's post-hoc tests. Differences were considered significant when $p < 0.05$.

3. Results

Among the 15 patients studied by Whole Exome Sequencing (WES), two possibly damaging heterozygous variations were identified in the *TRPV1* gene in two patients: the c.2051 G > T; p.Gly684Val and the c.2314C > T; p.Arg772Cys variations (Table 1, Fig. 1). Sequencing of the entire *TRPV1* coding sequence in 45 other patients did not reveal additional potentially deleterious variations.

Patients harboring a variation in *TRPV1* were young men, with body temperature recorded at the time of the crisis above 40 °C. They both presented neurological signs resulting in a loss of consciousness. Both patients experienced rhabdomyolysis and patient 2 developed hepatorenal failure. Interestingly, patient 2 also harbored elevated CPK at rest (three times the normal level). According to ICVT result, patient 1 was diagnosed as MHS (susceptible to Malignant Hyperthermia) and patient 2 was MHSh (susceptible to Malignant Hyperthermia only to halothane stimulation). The patients did not report any others symptoms. These clinical features were similar to the other patients of the cohort analyzed by WES (Table 1, supplemental table A).

The c.2051 G > T; p.Gly684Val variation identified in patient 1 was very rare with a Minor Allele Frequency (MAF) of 0.000004063 in the ExAC database and the c.2314C > T; p.Arg772Cys variation identified in patient 2 was not reported in genomic databases. Both variations were also predicted damaging by four different software (CADD,[19] SIFT,[20] Polyphen2,[21] Mutation Taster[22]) and modified highly conserved amino acids in functional domains of the protein (Table 1, Fig. 1).

Mapping of the Glycine 684 onto the crystal structure of TRPV1 (PDB number: 5IRZ, residues 335 to 751[26]) showed that this residue is localized in the critical pore domain of the TRPV1 channel. The *in silico* structure prediction showed that replacement of the Glycine 684 by a Valine induces a major steric hindrance in the pore of the channel (supplemental figure A).

The p.Arg772Cys variation affects a residue located in a regulatory domain of the C-terminal region of TRPV1, whose structure is so far unavailable.

To further explore the impact of both variations, calcium release of mutated TRPV1 after stimulation with the specific agonist capsaicin was performed. The TRPV1-Arg772Cys mutant induced a modification in the kinetics of calcium release resulting in an increase in the total calcium released as compared to TRPV1-WT (Fig. 2.1 and 2.2). The TRPV1-Gly684Val mutant did not respond to capsaicin stimulation. Ectopic expression of TRPV1 in HEK-293 cells induced an elevation of the resting cytosolic calcium concentrations even in absence of TRPV1 stimulation (Fig. 2.3) due to a calcium leak from intracellular stores. The expression of the TRPV1-Arg772Cys induced a similar elevation of basal cytosolic calcium concentration (Fig. 2.3). On the contrary, the expression of the TRPV1-Gly684Val mutant did not change the resting concentration of calcium (Fig. 2.3), confirming this mutant channel is not functional. The expression and localization of recombinant TRPV1 in HEK-293 cells were not altered by variations according to Western-Blot and immunofluorescence results (supplemental figure B), and we conclude that both genetic variations alter *in vitro* the global channel function of TRPV1, at rest or after specific agonist stimulation.

4. Discussion

Our work led to the identification of variations in the *TRPV1* gene in association with Exertional Heat Stroke phenotype in two patients. The *TRPV1* gene encodes a non-selective cation channel with a broad spectrum of tissue expression. In the skeletal muscle, TRPV1 is localized in the sarcoplasmic reticulum membrane and is especially involved in calcium transport,[24] which is consistent with the hypothesis of a calcium release disorder in patients who display a positive *In Vitro* Contracture Tests. TRPV1 is a nociceptor that can be activated by pungent ingredients like capsaicin, the active molecule of chili pepper, or heat and acidic environment,[25,26] which are produced during exercise and muscle contraction. TRPV1 is also stimulated by halogenated anesthetics,[27] and interestingly a recent study reported mutations in the *TRPV1* gene in patients with Malignant Hyperthermia.[17]

The two variants that we report present the standard criteria of pathogenicity. They are unreported or very rare (MAF < 0.00.1%) in control databases while we chose to keep variants with a MAF < 0.1%, which is compatible with a rare and triggered disease such as EHS. They are also predicted as pathogenic *in silico* and located in critical functional domains of the protein. The p.Gly684Val mutation is located in the S6 transmembrane domain (residues 559–687 according to Uniprot website: https://www.uniprot.org/uniprot/Q8NER,) which is part of the highly conserved pore domain of TRPV1 and is crucial for the channel function. The region including the pore domain is implicated in selectivity, opening and closure of the channel but is also implicated in conformational change in capsaicin-induced activation and heat-induced desensitization.[28] The steric hindrance resulting from the replacement of the small amino acid by a larger one in the pore is likely to impact the channel function as shown in X-ray structure (supplemental figure A). The absence of response observed after capsaicin stimulation, as well as its lack of effect on calcium levels at rest, strongly suggest that the p.Gly684Val mutation blocks the channel function of TRPV1. The p.Arg772Cys mutation is located in the C-terminal region of TRPV1,[23] which is responsible for the interaction with calcium binding proteins, such as calmodulin and the S100A1 protein. Previous work demonstrated that the Arg772 residue is involved in binding of the S100A1 molecule that plays a role in the regulation of TRPV1, particularly in capsaicin-induced desensitization.[29,30] Together with our results showing modified kinetics of calcium release in ectopic expression, this strongly suggests that the p.Arg772Cys mutation alters the regulation of the channel function of TRPV1. These data suggest that both variations induce a modification of the function of TRPV1 and classify these variations as likely pathogenic according to the recommendations of the American College of Medical Genetics and Genomics (ACMG).[31]

Although the pathophysiology of EHS is still largely unclear, results obtained in the recent years on TRPV1 function could provide new research avenues. First, the fact that EHS patients show abnormal response to the MH *in vitro* contracture test may rely on calcium channel properties of TRPV1 in muscle. Pioneer work demonstrated that heat stress activates TRPV1 and induces calcium accumulation in myocytes during muscle contraction.[32] Recent studies suggested that TRPV1 may crosstalk with RyR1, amplifying calcium release in skeletal muscle.[24] Therefore, an abnormal function of TRPV1 could modify calcium release from RyR1, explaining the massive cytosolic calcium release that occurs during an EHS or MH crisis due to TRPV1 mutations. This functional relationship with RyR1 and the demonstrated sensitivity of TRPV1 to halothane[17] may explain the fact that EHS patients with TRPV1 mutations have a positive IVCT. However, this hypothesis may only partially explain our results since the TRPV1 mutant p.Gly684Val does not release calcium after capsaicin stimulation. Further experiments with other stimuli such as halogenated anesthetics, and muscle specific expression system, would help to clarify the patient positive *in vitro* contracture test. However, *RYR1* independent mechanisms

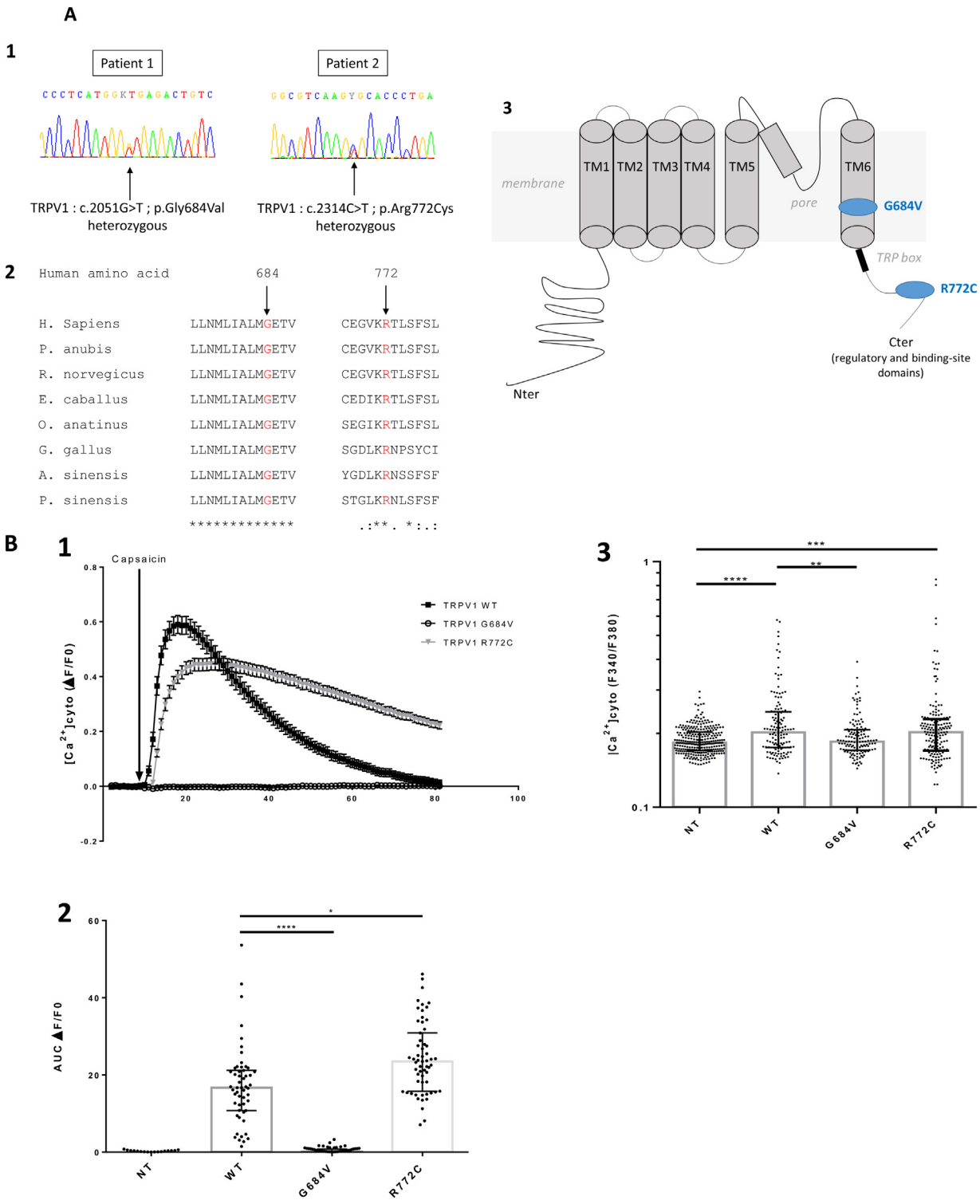

Fig. 1. (1) TRPV1 sequences with heterozygous c.2051 G > T; p.Gly684Val (patient 1) and c.2314C > T; p.Arg772Cys variations (patient 2). (2) Multiple sequence alignment of TRPV1 protein regions surrounding the p.Gly684Val and p.Arg772Cys residues (red) in various species. An asterisk (*) indicates a position which has a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties. A period (.) indicates conservation between groups of weakly similar properties. (3) Locations of c.2051 G > T; p.Gly684Val and c.2314C > T; p.Arg772Cys variations on a TRPV1 schematic representation. The p.Gly684Val variation is located in the conserved transmembrane segment 6 of the pore domain and the p.Arg772Cys variation is located in the C-terminal part of the protein that includes regulatory domain and interacts with ligands and proteins such as PIP2, calmodulin or protein S100[35] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Clinical and genetic features of the patients with variations in the *TRPV1* gene.

Patients	Patient 1	Patient 2
Gender	Male	Male
Age	31	25
Personal and family history	NA	No
Exertional Heat Stroke crisis		
Trigger	timed race	commando race
Corporal temperature during crisis	41 °C	40 °C
Date of crisis	December (French Guyana)	February
Neurological signs	Spatial and temporal disorientation, loss of consciousness	Loss of consciousness
Hepatorenal failure	No	Yes
Rhabdomyolysis	Yes	Yes
CPK at crisis	232 UI/L	NA
CPK at rest (N: 55–170 UI/l)	76 UI/L	427 UI/l
In Vitro Contracture Test		
Status	MHS	MHSh
halothane (2%)	positive (0,9 g and 0,2 g)	positive (0,3 g and 0,2 g)
caffeine (2 mM)	positive (0,4 g and 0,3 g)	negative (0,1 g)
Genetic features		
Gene	*TRPV1*	*TRPV1*
cDNA change	c.2051 G > T	c.2314C > T
Amino acid change	p.Gly684Val	p.Arg772Cys
Status	heterozygous	heterozygous
RS number	rs759094783	rs1217651219
Prevalence in databases		
ExAC frequency	0.000004063	absent
GnomAD frequency	absent	absent
Prediction tools		
CADD phred score	25,9	34
SIFT	Damaging	Damaging
PolyPhen	Damaging	Damaging
MutationTaster	Damaging	Damaging
Orthologue conservation	Conserved across sarcopterygians species	Conserved across tetrapods species
Protein localization	Pore domain	S100A1 binding domain

MHS: Malignant Hyperthermia Susceptible with a positive *In Vitro* Contracture Test to halothane and caffeine. MHSh: Malignant Hyperthermia Susceptible with a positive *In Vitro* Contracture Test to halothane only. NA: not available.

could also explain the pathophysiology of EHS due to *TRPV1* mutations.

The link between the mutations identified in *TRPV1* and EHS could be based on a dysfunction of the thermosensitive role (heat detection) or thermoregulatory role (the control of core body temperature) of TRPV1 during exercise. *In vivo*, the thermosensitive role of TRPV1 has been largely described with TRPV1-/- mice models presenting reduced sensitivity to noxious heat.[33,34] The effect of TRPV1 deficiency on thermoregulation in knock-out mice is more debated, but several studies have demonstrated abnormal thermogenesis in response to the agonist capsaicin[34,35] and distinct thermoregulatory phenotypes in terms of behavior and metabolism in mice lacking *TRPV1*.[36]

Recently, the molecular role of TRPV1 in thermosensation was dissected. In most mammals, the heat-induced activation of TRPV1 is rapidly followed by a desensitization of the channel, in order to protect cells against damages if noxious heat persists.[28] This desensitization is regulated by the heat-induced interaction between the N and C-terminal regions of TRPV1 which is coupled to the outer pore domain, inducing a conformational change of the channel and closing the pore.[28] Point mutations in key residues in these regions can abolish this interaction, and the desensitization phenomenon. The p.Arg772Cys mutation we described here is located in the C-terminal region of TRPV1 delineated by the authors of this work and it could alter the physiological interaction between C- and N-terminal parts of TRPV1, thus impacting the desensitization mechanism. This hypothesis fits with the delay observed in the kinetics of calcium release in our calcium imaging experiments with capsaicin stimulation. Inefficient heat-induced inactivation of the TRPV1 channel during a strenuous exercise due to a mutation such as p.Arg722Cys could therefore participate in the pathophysiology of EHS.

The role of TRPV1 in the control of body temperature is less understood. It was evidenced during pharmacological screening to search for new analgesics targeting TRPV1.[25,34] Clinical trials established that some TRPV1 antagonists tested as analgesics can cause thermogenesis dysfunction (either hyperthermia or hypothermia) in patients, depending on their ability to selectively inhibit capsaicin-, heat- or proton-induced TRPV1 activity. These results suggested that TRPV1 could participate in the regulation of the systemic thermogenesis pathways and reflected the complexity of the thermoregulation function of TRPV1.[36,37] Interestingly, compounds able to block TRPV1 channel function after any type of stimulation (capsaicin, heat of acidic pH) trigger hyperthermia. Along with this result, one can hypothesize that a complete block of TRPV1 function by a loss of function mutation such as the p.Gly684Val may also result in an abnormal regulation of thermogenesis during strenuous muscle activity.

5. Conclusions

In conclusion, our study involves *TRPV1* mutations as a new cause of Exertional Heat Stroke. The *TRPV1* gene, as well as *RYR1*, may both be linked to Exertional Heat Stroke and Malignant Hyperthermia, which supports the hypothesis of a continuum between the two diseases.[38] We identified two missense mutations in EHS patients that presented positive IVCT and demonstrated that these mutations impact capsaicin–induced calcium release. However, precise mechanisms by which TRPV1 participates in MH and EHS pathophysiology and alters calcium homoeostasis in muscle inducing altered response in IVCT need further explorations. It is possible that each of the mutations of *TRPV1* we describe here impairs one of the functions TRPV1 is involved in, thermosensitivity or whole body thermoregulation.

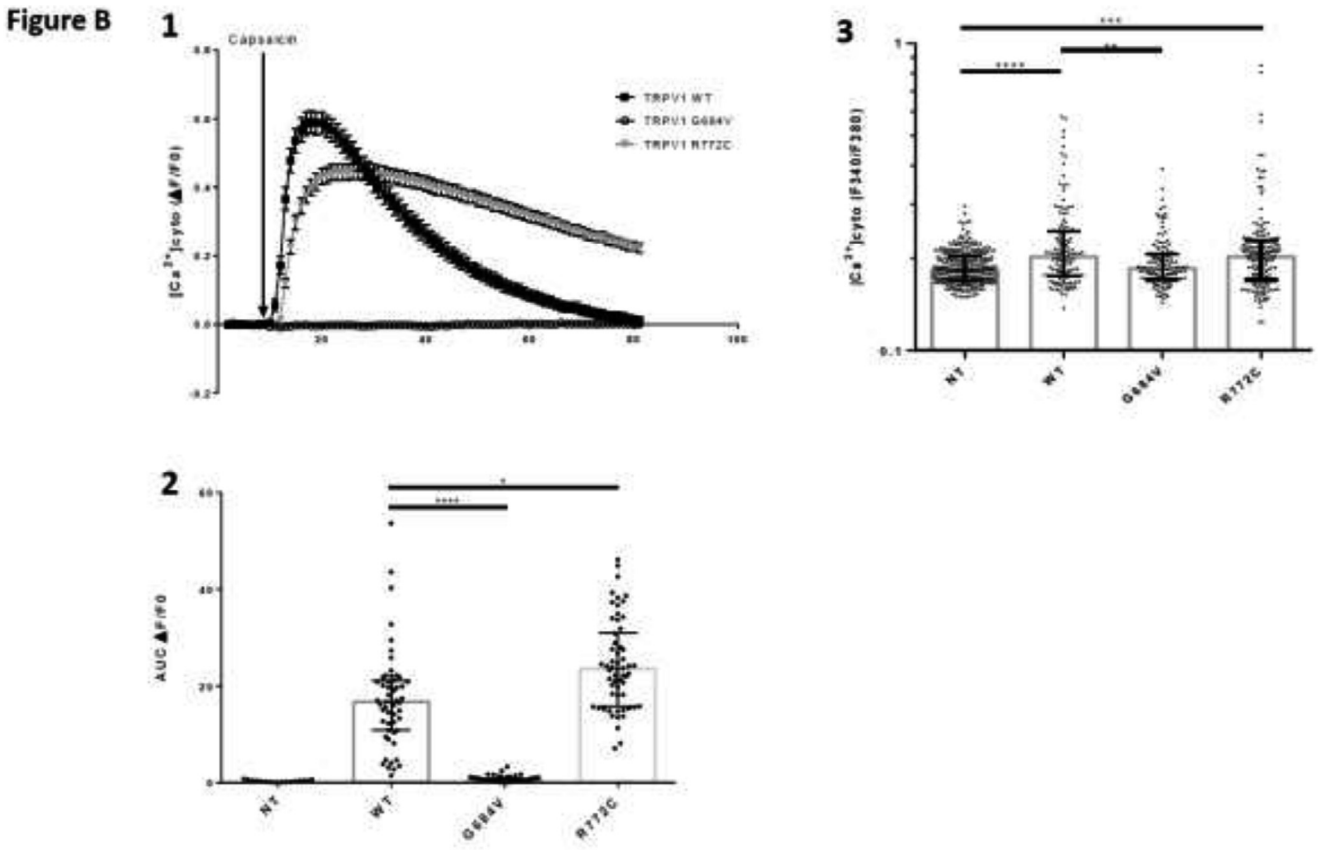

Fig. 2. Calcium imaging studies in HEK-293 cells after TRPV1 mutants expression. (1) Representative time course of cytosolic calcium concentrations using the fluorescence ratio of the Fluo4 indicator dye (ΔF/F0, arbitrary units) after injection of 20 μM capsaicin ; minimum of 40 cells for each condition (mean +/− SEM). (2) Corresponding Aeras Under the Curve (AUC) (median +/− interquatile ranges). (3) Measurement of cytosolic calcium concentration at rest using the fluorescence ratio of the Fura2-AM indicator dye (F340/F380, arbitrary units) (median +/− IQR). Non transfected cells (NT) were used as control. Non parametric ANOVA followed by post-hoc test were performed: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Nevertheless, mutations found so far in *RYR1* and *TRPV1* show a low prevalence. It can be first explained by the fact that EHS is a multifactorial disorder, and not solely caused by genetic predispositions. It can also reflect that mutations in other genes may be involved in EHS.

Funding

Whole Exome Sequencing was funded by the Fondation Maladies Rares (grant N° FONDATION_WES-20150602) and functionnal characterisation was supported by grants from INSERM and Association Française contre les Myopathies (AFM-Téléthon).

Acknowledgements

We thank the APHM CRB-TBM *Centre de Ressources Biologiques – Tumorothèque et Banque de Muscles* (authorization number: AC-2018-31053; CRB BB-0033-00097) for providing samples and Dr Catherine FOUTRIER-MORELLO for providing the results of the *In Vitro* Contracture Tests. We thank the Fondation Maladies Rares, France which supported the Whole Exome Sequencing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsams.2020.04.018.

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