Forensic Science International 232 (2013) 16–24

Contents lists available at SciVerse ScienceDirect

Forensic Science International

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

Heat stress and sudden infant death syndrome—Stress gene expression after exposure to moderate heat stress

Marianne Cathrine Rohde [a,*], Thomas Juhl Corydon [b,1], Jakob Hansen [a], Christina Bak Pedersen [c], Stinne P. Schmidt [c], Niels Gregersen [c], Jytte Banner [a]

[a] Institute of Forensic Medicine, Department of Forensic Pathology, Aarhus University, Denmark
[b] Department of Biomedicine, Aarhus University, Denmark
[c] Research Unit for Molecular Medicine, Aarhus University, Denmark

ARTICLE INFO

Article history:
Received 8 October 2012
Received in revised form 21 April 2013
Accepted 3 June 2013
Available online 27 July 2013

Keywords:
Heat stress
Fibroblast culture
Infant death
SIDS
QRT-PCR

ABSTRACT

The aim of the present study was to investigate stress gene expression in cultured primary fibroblasts established from Achilles tendons collected during autopsies from sudden infant death syndrome (SIDS) cases, and age-matched controls (infants dying in a traumatic event). Expression of 4 stress responsive genes, *HSPA1B*, *HSPD1*, *HMOX1*, and *SOD2*, was studied by quantitative reverse transcriptase PCR analysis of RNA purified from cells cultured under standard or various thermal stress conditions. The expression of all 4 genes was highly influenced by thermal stress in both SIDS and control cells. High interpersonal variance found in the SIDS group indicated that they represented a more heterogeneous group than controls. The SIDS group responded to thermal stress with a higher expression of the *HSPA1B* and *HSPD1* genes compared to the control group, whereas no significant difference was observed in the expression of *SOD2* and *HMOX1* between the two groups. The differences were related to the heat shock treatment as none of the genes were expressed significantly different in SIDS at base levels at 37 °C. *SOD2* and *HMOX1* were up regulated in both groups, for *SOD2* though the expression was lower in SIDS at all time points measured, and may be less related to heat stress. Being found dead in the prone position (a known risk factor for SIDS) was related to a lower *HSPA1B* up-regulation in SIDS compared to SIDS found on their side or back.

The study demonstrates the potential usefulness of gene expression studies using cultured fibroblasts established from deceased individuals as a tool for molecular and pathological investigations in forensic and biomedical sciences.

1. Introduction

Sudden infant death syndrome (SIDS) has been investigated vigorously in numerous studies. Epidemiological and pathological studies have shown that prone sleeping is the single most important risk factor in SIDS [1], and modifiable by simple intervention [2–4]. Thermal stress has been linked to the prone position [5,6]. The front surface of a body allows greater heat loss than the back, so if placed prone, heat loss may be compromised. Additional covering by bedding and/or heavily wrapping can leave the head and face the only area of excessive heat loss [6–8].

In the later years research has increasingly focused on the genetic background of SIDS. SIDS is most likely a combination of both extrinsic and intrinsic factors, gene factors and external or environmental factors, and several gene defects may be responsible for cases previously classified as SIDS [9–11].

Thermal stress causes damage to several constituents including lipids, DNA and proteins, and is associated with decreased cell viability and death [12–15]. The cellular levels of heat shock proteins increase during thermal stress through transcriptional activation of heat shock protein encoding genes, a cell protective mechanism called the heat shock response [16].

The HSP70 family is comprised of several members localized in different cellular compartments [17–21]. They serve a major role in stabilization of nascent polypeptides as well as in refolding of denatured proteins into native conformations and in preventing aggregation of mis-folded proteins [16,22,23].

HSP60 is a mitochondrial chaperone essential for the folding and assembly of newly imported proteins in the mitochondria and

* Corresponding author. Tel.: +45 87168319.
E-mail addresses: mcr@forensic.au.dk (M.C. Rohde), tjc@hum-gen.au.dk (T.J. Corydon), Jah@forensic.au.dk (J. Hansen), cbak@ki.au.dk (C.B. Pedersen), stinne.schmidt@gmail.com (S.P. Schmidt), nig@ki.au.dk (N. Gregersen), Jb@forensic.au.dk (J. Banner).
[1] Tel.: +45 87167775x2.

0379-0738/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.forsciint.2013.06.003

is thus an essential part of the mitochondrial stress response. Several studies have shown that HSP60, like other heat shock proteins, is necessary for cellular survival under toxic or stressful circumstances [24,25].

Heat shock factor 1 (HSF-1) is the major regulator of heat shock protein transcription. In the absence of cellular stress, HSF-1 is inhibited by association with heat shock proteins [26]. When the level of mis-folded proteins increase, heat shock proteins dissociates from HSF-1, followed by up regulation of heat shock proteins [27,28].

Oxidative stress in cells refers to a state where the production of reactive oxygen species (ROS) exceeds the buffer and detoxification capacity of the cell, resulting in accumulation of ROS that are reactive and damaging to cellular constituents such as protein, DNA and lipids [29]. Enzymes react and detoxify ROS and constitute an essential cellular protection mechanism. Failure of this system has been related to several different cellular dysfunctions involved in aging, cancer and several other diseases [24,30–32]. Heme oxygenase 1 (HO-1), and MnSOD (SOD2) are part of the antioxidant defence system and catalyze essential detoxification reactions after various stressors including heat [33–41]. The superoxide dismutase activity reduces the damaging reactions of superoxide, thus protecting the cell from superoxide toxicity and is essential for cell survival [29].

Cellular stress response is interesting in relation to SIDS studies, since common risk factors for SIDS are infection, hyperthermia and hypoxia. Gozal suggested that failure to express heat shock proteins may lead to reduced tolerance, and enhance inappropriate physiologic responses and vulnerability which ultimately may lead to infant death [42]. However, only two studies have followed up on this [43,44] and focused on polymorphisms in genes rather than functionality. Rahim et al. found polymorphisms in the Hsp60 encoding gene in SIDS compared to controls, but no differences in the genes encoding Hsp70 and Hsp90 [44]. Bross et al. examined polymorphisms in the genes encoding the chaperonin complex Hsp60/Hsp10 and found no higher associations with SIDS, than the other cases examined [43].

In the present study we investigated the hypothesis that SIDS may be associated with decreased ability to respond to an external stressor with an appropriate protective stress response. We have addressed this by assessing the expression of selected genes involved in the cellular heat shock and antioxidant responses in cultured Achilles tendon fibroblasts obtained at post mortem autopsies from SIDS cases and controls. The cultured cells were subjected to heat stress and the expression profiles of the genes *HSPA1B* (*Hsp70*) *HSPD1* (*Hsp60*), *HMOX1* (*HO-1*) and *SOD2* (*MnSOD*) were assessed at mRNA level by reverse transcriptase quantitative PCR (RT-qPCR) analysis.

2. Materials and methods

SIDS was defined according to the Nordic classification [45], as the sudden and unexpected death of an infant 1 week to 12 months old, where the cause of death is unknown after thorough post mortem examination. Full autopsy, history, circumstances and supplemented examinations as microbiological testing were included. Achilles tendon biopsies had been sampled and cultured from SIDS victims and age-matched controls; cases of unnatural death with a known cause of death aged 0–1 year, examined at the Institute of Forensic Medicine, Aarhus, Denmark, from 1992 to 2005. Only 11 controls were available due to age mismatch, as Achilles tendon biopsy was only performed for research purposes in this group.

Documents from the Forensic Department, including police reports, were reviewed for information on child position, bedcover, head position at the time of death, possible symptoms of minor nonlethal disease in the days prior to death, besides information on the circumstances of death, time of death, post mortem time interval from death to sampling of the Achilles tendon, pathological findings at autopsy and supplemented microscopic examinations, microbiological results and chemical analyses when examined. The history of the child was often missing detailed information on sleeping position, symptoms in the last few days, family relations and other social issues.

Data were analyzed in Stata 10.1, and epidata was used for administrating the data collected and data handling. We accepted results of the tests to be statistically significant if we found p-values <0.05. We used non-parametric statistical analyses, as the data were not following a Gaussian distribution. Mann–Whitney (Ranksum) was used instead of the t-test to test for differences in two independent measurements, according to groups. Wilcoxon Signed-Rank was used to test paired observations and Spearman's rank correlation coefficient was used instead of a regression to look for dependence between estimates.

2.1. Cell culturing and harvesting

Achilles tendons had all been removed during post mortem examinations prior to the study and stored at −135 °C [46]. Following thawing, cells were incubated in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were cultivated to confluence in 150 cm^2 culture flasks, representing approximately 1×10^6 cells. There were six culture flasks per case and control included in the study (total of 246 culture flasks). The minimum growth time was 4 weeks and maximum 10 weeks. During the growth period cells were sub-cultivated when they reached confluence to avoid growth inhibition, and cells were in passage 4–6, when used in the heat exposure study. For sub-culturing, cells were detached in trypsin–EDTA solution 0.5% (v/v) (Gibco), phosphate-buffered saline (PBS, pH 7.4), centrifuged for 3 min at 1500 rpm and seeded in culture flasks. Cells were harvested by scraping, centrifuged for 3 min at 1500 rpm and washed twice in PBS. Following removal of PBS the fibroblast pellets were stored at −80 °C.

2.2. Heat stress exposure

The growth medium was changed on the day before the stress treatment. The stress experiment was carried out by incubating the cells in a humidified heat incubator at 40 °C with 5% CO_2. At the starting point ($t = 0$) one set of flasks were harvested as controls. The rest of the cells remained heat exposed and were removed at selected time points (3; 6; 12; 18 and 24 h after incubation). Cells were harvested immediately after removal from heat stress and cell pellets were stored at −80 °C for later RNA-isolation.

2.3. RNA isolation and cDNA synthesis

Total cellular RNA was isolated using TRIzol® Reagent (Invitrogen, Denmark). The RNA quality and purity was evaluated by analyzing 500 ng total RNA on a 1.5% agarose gel, as well as measuring the absorbance of a diluted RNA sample A260:A280 ratio on a spectrophotometer. RNA was frozen immediately and stored at −80 °C. First strand cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kits from BIORAD with oligo(dT) and random primers in a final volume of 20 μl, according to the manufacturer's instruction. The cDNA was stored at −20 °C.

2.4. Quantitative real-time PCR (Q-PCR)

Q-PCR analyses were carried out in 96-wells plates with 2 μl cDNA (diluted 1:20 or 1:10) as template using the TaqMan® Universal PCR Master Mix, containing AmpliTaq Gold® DNA polymerase, dNTPs, and optimized buffer components (Applied Biosystems), 900 nmol/l of each primer, and 250 mmol/l probe in a total reaction volume of 25 μl. PCR was performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) with the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. All samples were measured in triplicates, and means and standard deviations were estimated. Quantitative PCR was performed using primers and hydrolysis probes (TaqMan®, Applied Biosystems) commercially designed to quantify *HSPA1B* (Hsp70; Hs00271244_s1, Applied Biosystems), *SOD2* (MnSOD; Hs00167309_m1, Applied Biosystems), *ACTB* (human *β-actin:* (4310881E)a, no information, Applied Biosystems), and self-engineered designs for *HSPD1* (HSPD1) [47] and *HMOX1* (HO-1) [48]. RNA expression was performed using a standard curve method [49], with pooled cDNA from fibroblasts included in the study as reference (1:1, 1:10, 1:100, and 1:1000) used for constructing the dilution curves. The amounts of *HSPA1B*, *HSPD1*, *MnSOD* and *HMNOX1* RNA were normalized to the endogenous/housekeeping gene *ACTB* (human β-actin), which has shown to be a suitable reference gene for thermal stress experiments in fibroblasts [48].

3. Results

The four target genes are presented relative to the reference gene *ACTB* (human β-actin) before heat stress ($t = 0$), and at five successive measurements after heat stress exposure (3, 6, 12, 18 and 24 h). The individual responses from all cases and controls are displayed in Fig. 1 and Appendix.