Original Article

Stress Perception and (GT)n Repeat Polymorphism in Haem Oxygenase 1 Promoter Are Both Risk Factors in Development of Eating Disorders

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Abstract. Haem oxygenase 1 (HO-1) plays a pivotal role in metabolic stress protecting cells in dependence on reactive oxygen species. This study investigated a potential gene environment interaction between the (GT)n repeat HO1 polymorphism and the stress perception in patients with eating disorder and in controls. Stress perception and (GT)n polymorphism were measured in 127 patients with eating disorders and in 78 healthy controls using Stress and Coping Inventory and genotyping. Based on the inventory, overall, specific and weighted stress scores were defined. Clinical stress score was generated according to the patient’s history and interviews. According to our hypothesis, 1) all stress scores describing subjective stress perception were significantly higher in patients compared to controls (P ≤ 0.001; P ≤ 0.002; P ≤ 0.001), 2) the L/L genotype of GT promoter repeats (L > 25 GT repeats, S < 25 GT repeats) in the patients was associated with higher overall (P ≤ 0.001), specific (P ≤ 0.010) and weighted stress score (P ≤ 0.005) compared to the L/S variant, and 3) Pearson’s correlation of clinical versus objective stress scores showed not very tight relationship (0.198; 0.287; 0.224, respectively). We assume potential risk of the L allele of HO1 promoter polymorphism for the stress response and contribution of the subjective stress perception together with the L/L genotype to the development of eating disorder. Decreased HO1 expression in the presence of L/L genotype plus more intensive stress perception in the patients can lead to secondary stress, with increasing severity of the symptoms and aggravation of the disease.

Introduction

Restricted and insufficient food intake results in malnutrition with the lack of vital nutrients including vitamins and minerals (ascorbic acid, retinol, α-tocopherol, selenium, zinc, etc.). Altered balance between oxidants and antioxidants results in oxidative stress, which has been described in anorexia nervosa, obesity, or type 2 diabetes (Chen et al., 2002; Agnello et al., 2012; Faienza et al., 2012). Disturbed feeding behaviour has serious consequences on the cell and complex metabolism affecting energy metabolism, thermogenesis or hormone secretion. Nutritive factors can also influence gene expression. For example, an animal model of anorexia exhibits disturbed oxidative phosphorylation via down-regulating assembly factor of complex 1 Ndulafl (Lindfors et al., 2011). Another very important up- and down-regulated gene that responds sensitively to metabolic stress is haem oxygenase 1 (HO1, Abraham and Kappas, 2008).

Eating disorders (ED) including anorexia nervosa (AN), bulimia nervosa (BN) and unspecified eating disorder are multifactorial diseases with a clear genetic
component. As in other complex disorders the pathophysiology remains unknown. EDs are very heterogeneous diseases and the endophenotype approach with detailed focus on the clinical subgroups and their characteristics provides more opportunities for ED therapy. We used this approach to investigate the influence of stress perception and (GT)n repeat polymorphism in haem oxygenase 1 in ED patients.

**HO-1**

Haem oxygenase 1 (HO-1 or HMOX-1), the key enzyme of haem degradation, catalyses the oxidative cleavage of haem into biliverdin, Fe^{2+} and CO. Subsequent rapid conversion of biliverdin to bilirubin by biliverdin reductase makes HO-1 the only rate-limiting enzyme of bilirubin formation (Tenhunen et al., 1968). Besides the role of the enzyme in catalysing haem degradation, the indirect effect of its reaction products (CO, biliverdin, bilirubin) are of great importance in maintaining cell homeostasis and regulating cell metabolism (Shibahara et al., 1987). Expressed in many tissues and up-regulated by multiple stimuli, HO-1 is involved in numerous anti-oxidant, anti-proliferative and anti-inflammatory processes with the same aim: to protect the cell against oxidative stress (Tenhunen et al., 1969; Poss and Tonegawa, 1997; Exner et al., 2004, 2006; Abraham and Kappas, 2008; Grochot-Przeczek et al., 2012).

The highly conserved HO1 gene is strongly expressed especially in the liver and spleen. Constitutive expression of HO-1 provides a fast and effective tool in regulating signal cascades via HO-1 reaction products; the signalling molecule, CO, is involved in anti-inflammatory pathways via modulation of MAPK, JNK, transcription factor AP-1 or nuclear factor NF-κB (Otterbein et al., 2000; Megias et al., 2007, 2009). Besides the stimulated expression of HO1 in response to oxidative stress, transcription of the gene is modulated via genetic variants. So far, three functional polymorphisms in the HO1 gene have been identified: (GT)n variable tandem repeat polymorphism and two single-nucleotide polymorphisms, G-1135A and T-413A, with the main interest focused on GT repeats (Hirai et al., 2003).

**(GT)n repeat polymorphism**

The variable tandem repeat polymorphism in the promoter of the HO1 gene is based on different numbers of consecutive GT nucleotides. It is located approximately 250 bp upstream from the transcription start site. The size of the GT repeats varies from 12 to 40. *In vitro* studies demonstrate reduced transcriptional activity of the HO1 gene in cell lines with a larger size of (GT)n repeats (L allele) compared to shorter variants (S allele). This leads to an impaired induction of HO-1 exposed to reactive oxygen species and a decreased response to oxidative stress (Yamada et al., 2000; Chen et al., 2002; Taha et al., 2010). Several classifications according to the allelic size are used, with the most frequent alleles of 23 and 30 GT repeats (Yamada et al., 2000; Baan et al., 2004; Schillinger et al., 2004).

**Stress perception and its inheritance**

Recent research has supported the role of lifetime severe stress and trauma in the development, maintenance and relapses of eating disorders (Johnson et al., 2002). The stress contributes not only to the development of ED, mainly their specific symptoms (bulimic symptoms and drive for thinness), but also to subsequent stress generation, negative life stressors and depressive symptoms (Alda et al., 1989; Bodell et al., 2012). Negative stressors, anxiety or depression have been shown to have high heritability. Therefore, a gene-environment (G x E) approach is now being applied in many studies (Wade et al., 2009). In addition to the shared environmental influence of behavioural patterns there are many inherited traits resulting from the altered function of the genes of interest (e.g., serotoninergic, dopaminergic, opioidergic) (Trace et al., 2013). AN or BN are accompanied by distorted body self-perception, anxiety, fear of gaining weight combined with restricting or sometimes lifetime adverse events. Taken together with the lack of nutrients and metabolic consequences of the stress itself, secondary stress is generated. Genetic factors may play both, protective and non-protective roles in the stress response, depending on the presence of functional polymorphisms or otherwise influenced gene expression or epigenetic modifications resulting from gene-environment interactions (Nagata et al., 2000; Abraham et al., 2007; Sachs-Ericsson et al., 2012).

**Material and Methods**

**Ethics and Samples**

A total of 127 patients and 78 control samples were analysed. All patients and controls were Caucasian females from the Czech Republic. The study was approved by the Ethics Committee of the First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague. All probands agreed with their participation in the study and signed the informed consent. The control group was recruited from healthy female students and young healthy woman working in hospital matched by age, sex and socio-economic status. All patients and controls answered the inventories and gave their DNA samples anonymously.

**Measures**

The Stress and Coping Inventory (SCI) was designed for health promotion, as an educational instrument and a research tool, and its reliability and validation were carried out by Holmes and Rahe (Holmes and Rahe, 1967; Rahe et al., 2000). SCI Questionnaires for Youth and Adults with 49 questions were answered by patients during the first two weeks of their hospitalization. The same questionnaires were answered by healthy controls. SCI data were evaluated via the models of stress scores. Objective Stress Score (OSS) was calculated as a cumulative sum of positive answers (adverse event present =
1, absent = 0) to all SCI items (with the maximal value of 49 points). Specific Stress Score (SSS) was counted as a sum of selected items known as the risk factors for ED specific items; event present = 1, absent = 0. These items (1, 2, 4, 5, 8, 12, 13, 15, 18, 21, 25, 26, 27, 29, 34, 39, 44, 45, 49) are known to play important roles in the onset or relapse of ED (Pavlova, 2010). Finally, Weighted Stress Score (WSS) was evaluated according to Rahe, giving each item different value (e.g. personal injury or illness 53 points, change in social activities 18 points, etc.) (Holmes and Rahe, 1967; Rahe et al., 2000).

Next, the Clinical Stress Score (CSS) was established as the objective stress measure which resulted from complex clinical evaluation by an independent psychiatrist, trained in ED, trauma research and treatment. CSS was based on the observations on the ward, clinical interviews during the hospitalization, and evaluation of the present and past case history; all information available was considered (from the patient’s outpatient and inpatient documentation). The life adverse events were rated absent = 0, present = 1, severe and/or repeated = trauma 2.

### Clinical assessment

The study cohort consisted of female patients with primary DSM-IV diagnoses of AN or BN, successively hospitalized at the Specialized Unit for Eating Disorders in General University Hospital in Prague in the years 2006–2013. Patients with ED and comorbid primary diagnosis of alcohol abuse or personality disorders were excluded from the study.

Eating pathology was assessed with the 36-item Eating Disorder Examination Questionnaire (EDE-Q), semi-structured interview that monitors the severity of specific eating-related pathology over 28 days before assessment. It contains a Global Scale, which is the average of the four subscales: Shape Concern, Weight Concern, Eating Concern and Restraint. This measure has been demonstrated to have acceptable reliability and validity (Rizvi, et al., 2000; Berg, et al., 2012; Kelly et al., 2012). The diagnoses were confirmed by two experienced psychiatrists specialized in ED.

Average age was 25 ± 7.1 years in patients with ED and 26 ± 5.1 years in controls. Average body mass index (BMI in kg/m²) was 15.4 ± 2.7 in AN patients; 21.7 ± 3.1 in BN patients and 19.9 ± 3.9 in controls. Clinical data from the patients with ED were collected during their six- to eight-week hospitalization at the Specialized Unit for Eating Disorders, with a comprehensive specialized ED programme (re-alimentation, regime, psychotherapy and rehabilitation).

### Genotyping and statistical analyses

Genetic analyses were performed after the DNA extraction using a standard desalting method. HO1 promoter polymorphisms of GT repeats were genotyped according to Král et al. (2011). For genotype distribution in the HO1 gene, we classified the cases according to long (L ≥ 25 GT repeats) and short alleles (S < 25 GT repeats). Statistical analyses were performed with SPSS software version 17.0 (SPSS Inc., Chicago, Ill.). To estimate the differences of subjective stress perception among the groups – patients compared to controls – t-test was used. The same t-test was used for the comparison of stress scores in L/L and L/S genotypes. Correlation of the objective and subjective stress indexes was evaluated with Pearson’s correlation.

### Results

#### Stress indexes

Subjective perception of stressful events in patients and controls was described using cumulative stress scores OSS (Overall), SSS (Specific) and WSS (Weighted). Subjective evaluation of the presence of stressful life events in patients according to SCI was significantly higher compared to controls (P ≤ 0.001 for OSS; P ≤ 0.002 for SSS and P ≤ 0.001 for WSS), see Table 1. The average of Weighted Stress Scores for subjective stress perception was 471 points in patients compared to 359 points in the controls (see Fig. 1 for details).

#### G x E interaction of (GT)n repeats and stress perception

Association of the HO1 promoter polymorphism and subjective stress perception was tested in 127 patients and 78 controls (t-test). The distribution of HO1 genotypes in the patients and controls did not deviate from Hardy-Weinberg equilibrium (Wellek et al., 2010). Also, the frequency of the minor allele S in both groups of patients and controls was higher than 0.05 cut-off for rare alleles. The genotype frequencies found in our cohort are consistent with the population data from other Caucasians (Denschlag et al., 2004; Rueda et al., 2007; Weis et al. 2012; Gregorek et al., 2013). To analyse the subjective stress perception and its score in particular genotypes, S/S genotype carriers were excluded involving 11 patients and eight controls. Even when the rare incidence of S/S genotype was in accordance with the epidemiological data, the group size was not sufficient to be included into further statistical analyses.

Significant differences among patients with the genotype L/L compared to L/S were found in the Overall Stress Score (P ≤ 0.001), Specific Stress Score (P ≤ 0.01)

| Table 1. Subjective stress perception differences between ED patients and controls |
|--------------------------------------|-----|-----|-------|-----|
|                                      | N   | Mean | t     | P value |
| Overall Stress Score                 | controls patients 78 | 10.03 | -3.726 | 0.001 |
| Special Stress Score                 | controls patients 78 | 3.45  | -3.07  | 0.002 |
| Weighted Stress Score                | controls patients 78 | 359   | -3.318 | 0.001 |

Degrees of freedom are the same for all scores, df = 203
Subjective versus objective stress perception

Subjective stress perception expressed by OSS, SSS, and WSS was compared with the Clinical Stress Score. This analysis was performed only in the patients (N = 127). Pearson’s correlation of subjective (OSS, SSS, WSS) and objective stress (CSS) did not reveal a tight relationship between the subjective and objective stress scores (.198* for OSS, .287* for SSS and .224* for WSS; *P value for all cases ≤ 0.025), see Table 4. This represents the validity of all three indexes on one side and their different focus on the other.

Table 2. Subjective stress perception in L/L and L/S genotypes in the patients

<table>
<thead>
<tr>
<th>N</th>
<th>Mean</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Stress Score</td>
<td>L/S</td>
<td>57</td>
<td>11.18</td>
</tr>
<tr>
<td>Special Stress Score</td>
<td>L/S</td>
<td>57</td>
<td>3.86</td>
</tr>
<tr>
<td>Weighted Stress Score</td>
<td>L/S</td>
<td>57</td>
<td>398</td>
</tr>
<tr>
<td>Overall Stress Score</td>
<td>L/L</td>
<td>59</td>
<td>15.66</td>
</tr>
<tr>
<td>Special Stress Score</td>
<td>L/L</td>
<td>59</td>
<td>5.37</td>
</tr>
<tr>
<td>Weighted Stress Score</td>
<td>L/L</td>
<td>59</td>
<td>527</td>
</tr>
</tbody>
</table>

Table 3. Subjective stress perception in L/L and L/S genotypes in the mixed cohort

<table>
<thead>
<tr>
<th>N</th>
<th>Mean</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Stress Score</td>
<td>L/S</td>
<td>87</td>
<td>10.37</td>
</tr>
<tr>
<td>Special Stress Score</td>
<td>L/S</td>
<td>87</td>
<td>3.53</td>
</tr>
<tr>
<td>Weighted Stress Score</td>
<td>L/S</td>
<td>87</td>
<td>364</td>
</tr>
<tr>
<td>Overall Stress Score</td>
<td>L/L</td>
<td>99</td>
<td>13.62</td>
</tr>
<tr>
<td>Special Stress Score</td>
<td>L/L</td>
<td>99</td>
<td>4.73</td>
</tr>
<tr>
<td>Weighted Stress Score</td>
<td>L/L</td>
<td>99</td>
<td>470</td>
</tr>
</tbody>
</table>

Table 4. Objective versus subjective stress perception

<table>
<thead>
<tr>
<th>OSS</th>
<th>SSS</th>
<th>WSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSS Pearson’s Corr.</td>
<td>0.198</td>
<td>0.287</td>
</tr>
<tr>
<td>P value</td>
<td>0.025</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Stress scores: CSS – Clinical, OSS – Overall, SSS – ED Specialized, WSS – Weighted

Discussion

Development of ED can be interpreted as a maladaptive defence reaction against a perceived threat or trauma (relational, physical, sexual stress or trauma). In ED, physical and psychological stress is closely related (in malnutrition, cognition or concentration). Stress perception or sensitivity to stress, the very important factors in ED pathology, are influenced just by the coincidence of physical and psychical factors, often interacting in the gene environment interactions.

In our study we focused on stress perception described via SCI (Rahe et al., 2000). Several stress scores have been developed including Overall, Specific, Weighted (subjective stress perception) and Clinical Stress Score (objective stress perception). All scores describing the subjective stress perception regardless of their different methodology showed significantly in-

Fig. 1. Values of objective WSS according to HO1 genotypes in the patients and controls are shown on the horizontal axis. Legend of the point distribution on the axis: rhombus – controls, square – mixed cohort of patients and controls, circle – patients; blue – L/S genotype, green – L/L genotype, white – all genotypes mixed.

The lowest stress score was observed in the “more protective” L/S genotype in the controls, followed by controls with the L/L genotype. A higher stress score was found in patients with the L/S genotype and the highest value in L/L patients. The Weighted Stress Score was measured according to Rahe (Holmes and Rahe, 1967; Rahe et al., 2000).
creased subjective stress perception in the patients compared to controls (see Table 1). This was consistent with our hypothesis assuming that ED patients are more sensitive to stress than healthy controls. Higher sensitivity to stress is accompanied with malnutrition, exhaustion, neuro-endocrinological changes or psychological factors such as high demands on one’s own body, restraining, perfectionism and other stress-related behavioural factors (Kyrou and Tsigos, 2007, 2009; Hague et al., 2013; Patterson and Abizaid, 2013). Malnutrition can have, and in ED patients often has, fatal consequences. We can also speculate that long-term stress may have impact on the brain function. Recently, reduction in the 5-HT1A receptor binding potential has been shown in long-term occupational stress (Blix et al., 2013). Stressful life events resilience is also associated with the Val66Met polymorphism in brain-derived neurotrophic factor (BDNF) or Val158Met in the catechol-O-methyltransferase gene. Val66Met in BDNF has also been studied in ED, but with inconsistent results (Trace et al., 2013).

Because of the importance of G x E interactions in ED pathogenesis, we investigated the HO1 promoter repeat polymorphism and its association with subjective stress perception in patients with ED an in controls. HO-1 is a crucial enzyme whose expression is strongly regulated in response to stress stimuli. Decreased expression of the HO1 gene with L allele compared to the S allele has been demonstrated in in vitro studies (Yamada et al., 2000; Chen et al., 2002; Taha et al., 2010). To our knowledge, no data investigating the HO1 gene polymorphism and stress response or eating disorders are available. In this study we hypothesize that the combined effect of HO1 (GT)n repeat polymorphism and subjective stress perception may play a significant role in the development of eating disorders or in their stress response. We assume that carrying the L/L genotype with the lowest level of HO-1 expression results in less efficient protection against metabolic stress. Several studies describe the HO-1 impact on certain metabolic disorders including obesity, type 2 diabetes, hepatic injury or hormonal regulation (Bakken et al., 1972; Abraham et al., 2007; Eipel et al., 2007; Abraham et al., 2008).

Our data suggest a significantly increased tendency of stress perception in subjects with the “non-protective” L/L genotype and its contribution to ED pathology (Table 2, Fig. 1). For genotype environment interactions of HO1 and subjective stress perception the evaluated weighted stress score according to Rahe was used as a representative (Fig. 1) (Holmes and Rahe, 1967; Rahe et al., 2000). Very similar results of overall and specific stress scores according to the HO1 genotype were obtained (Table 2).

The same association of subjective stress perception and HO1 (GT)n repeat polymorphism was documented in a mixed cohort of patients and controls with interesting results. Even in the mixed cohort, a significant association of the L/L genotype with higher subjective stress perception was observed. This confirms the solid association of the L/L genotype with subjective stress perception in all three scores (Table 3). The importance of stress perception and HO1 repeat polymorphism in ED pathology is summarized in Fig. 1. First, the L/L genotype is associated with higher stress perception in the patients and also in the mixed cohort. Second, the controls always show a lower stress score than ED patients. Taken together, the lowest stress score was observed in the suspected more protective L/S genotype in the controls (300 points), followed by 388 points in the controls with L/L genotype, next in the patients with L/S (398) and finally 527 points in L/L patients.

Another aim of the study was to compare the subjective and objective stress perception, which included very detailed clinical observations and careful history. Lifetime exposure to stressful events (here represented by objective stress perception or clinical stress score) and subjective stress perception, even when each describes a different perspective, are both important factors in ED pathology. Stress perception and response in ED are tightly related to hormonal balance, as it has been demonstrated in animal models (Heiman et al., 1997; Hague et al., 2013; Patterson et al., 2013). In addition, several studies report stressful life events heritability (Plomin et al., 1990; Kendler et al., 2007; Power et al., 2013). The correlation of objective and subjective stress scores confirmed the hypothesis that the patients with ED have altered perception of one’s own body or stress (Cash and Deagle, 1997; Skrzypek et al., 2001; Stice et al., 2002; Keizier et al., 2013; Madsen et al., 2013). For the subjective perception stress scores, the highest level of consistency with the clinical score was shown by ED Specific Stress Score (SSS = 0.287; WSS = 0.224; OSS = 0.198). The Specific Stress Score has been established by ED specialized psychiatrists based on the clinical relevance of the items from Rahe’s SCI.

In conclusion, the current study showed that the objective stress perception together with (GT)n promoter repeat polymorphism in the HO1 gene influence ED pathology or stress-related disorders. Considering the limited size of our cohort, further studies are needed to confirm the G x E interactions of stress perception and the HO1 gene.

References


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