Peptides 88 (2017) 32–36

Contents lists available at ScienceDirect

Peptides

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

Research Paper

Phoenixin is negatively associated with anxiety in obese men

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ARTICLE INFO

Article history:
Received 3 September 2016
Received in revised form 3 December 2016
Accepted 14 December 2016
Available online 15 December 2016

Keywords:
Emotion
Gut-brain axis
Psychobiology
Psychoneuroendocrine
Psychosomatic

ABSTRACT

Phoenixin was recently identified in the rat hypothalamus and initially implicated in reproductive functions. A subsequent study described an anxiolytic effect of the peptide. The aim of the study was to investigate a possible association of circulating phoenixin with anxiety in humans. We therefore enrolled 68 inpatients with a broad spectrum of psychometrically measured anxiety (GAD-7). We investigated men since a menstrual cycle dependency of phoenixin has been assumed. Obese subjects were enrolled since they often report psychological comorbidities. In addition, we also assessed depressiveness (PHQ-9) and perceived stress (PSQ-20). Plasma phoenixin levels were measured using a commercial ELISA. First, we validated the ELISA kit performing a spike-and-recovery experiment showing a variance of $6.7 \pm 8.8\%$ compared to the expected concentrations over the whole range of concentrations assessed, while a lower variation of $1.6 \pm 0.8\%$ was observed in the linear range of the assay (0.07–2.1 ng/ml). We detected phoenixin in the circulation of obese men at levels of 0.68 ± 0.50 ng/ml. These levels showed a negative association with anxiety scores ($r = -0.259$, $p = 0.043$), while no additional associations with other psychometric parameters were observed. In summary, phoenixin is present in the human circulation and negatively associated with anxiety in obese men, a population often to report comorbid anxiety.

1. Introduction

Phoenixin is a recently discovered 20-amino acid peptide described to have regulatory properties in the reproductive system [22] likely *via* the G-protein coupled receptor, the GPR173, as recently suggested [20]. Also a fragment of the peptide, phoenixin-14, retained its biological activity [22]. This modulating effect was exerted *via* increasing the gonadotropin-releasing hormone (GnRH) receptor expression in the pituitary *in vitro* [22]. Pointing towards a physiological role of endogenous phoenixin, the blockade of the peptide using small interfering RNA injected intracerebroventricularly delayed the onset of estrus in female rats associated with a reduction of GnRH receptor expression [22]. Further suggesting an important role of phoenixin, the peptide sequence of phoenixin is highly conserved across non-mammalian (fish, amphibians, chicken) and mammalian species (rodents, human) [22].

The initial landmark study identified phoenixin in several rat peripheral (*e.g.* heart, thymus, esophagus, stomach and spleen) and central tissues with highest peptide expression levels in the hypothalamus [22]. Immunostaining indicated the expression of phoenixin in the magnocellular and parvocellular parts of the paraventricular nucleus, supraoptic nucleus, arcuate nucleus, dorsal hypothalamus, zona incerta, ventromedial hypothalamus, lateral hypothalamus and the perifornical area [22]. Additional central expression was shown in the substantia nigra, Edinger-Westphal nucleus, nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve [22] pointing towards additional functions besides the initially proposed role in the reproductive system. A subsequent study detected even higher peptide levels in the spinal cord compared to the hypothalamus and described a predominant localization in sensory neurons of the dorsal root, nodose and trigeminal ganglia [11] giving rise to a role in pain processing. This hypothesis is supported by the observation of a reduced number of writhes following intraperitoneal injection of acetic acid in mice receiving a central injection of phoenixin [11].

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http://dx.doi.org/10.1016/j.peptides.2016.12.011
0196-9781/

Following these studies, a recent report further described the phenotype of phoenixin neurons indicating a large proportion (70–86%) of phoenixin neurons co-localizing with NUCB2/nesfatin-1 in rat hypothalamic nuclei (arcuate nucleus, paraventricular nucleus, ventromedial hypothalamus and lateral hypothalamus) [15]. NUCB2/nesfatin-1 was discovered a decade ago and first localized in the rat hypothalamus [14]. It is to note that early on an anxiogenic effect of NUCB2/nesfatin-1 has been described in male rats following intracerebroventricular injection of the peptide [12]. Interestingly, also in humans an association of NUCB2/nesfatin-1 and anxiety has been reported with a positive correlation in women [6] and a negative association in men [4,5] indicating a sex-specific regulation of the peptide. In contrast to NUCB2/nesfatin-1, phoenixin was recently shown to exert potent anxiolytic effects in male mice [7]. However, whether phoenixin is also involved in the mediation, modulation or perception of anxiety in humans is unknown so far.

Therefore, the aim of the present study was to investigate whether phoenixin is associated with anxiety in humans. Based on the involvement of the peptide in reproductive functions [22] a menstrual cycle dependency can be assumed (or at least cannot be excluded at this point). Therefore, only men were investigated in the present study. Moreover, since obese subjects often report comorbid anxiety [2] and depression [21] we investigated obese men displaying a broad range of psychopathology in the present study. We first validated the commercial phoenixin ELISA kit and then investigated the potential association between circulating phoenixin and psychometrically measured anxiety levels. Subsequently, we further characterized our study population with regards to depressiveness and perceived stress.

2. Materials and methods

2.1. Subjects

For this study, we enrolled male obese inpatients (from January 2011 to December 2014) hospitalized in the Department for Psychosomatic Medicine at Charité-Universitätsmedizin Berlin that received medical treatment for obesity and its somatic and mental comorbidities. The inclusion criteria were comprised of male sex, an age of ≥18 years and a body mass index (BMI) ≥ 0 kg/m^2. Patients with a current malignant disease, untreated psychotic disorders and preceding bariatric surgery were not enrolled in the study. In addition, patients with hypercortisolism or hypothyroidism were excluded from the study. The final number of patients analyzed in this study was n = 68.

All investigations in the present study were conducted according to the Declaration of Helsinki and all patients gave written informed consent. The study was approved by the institutional ethics committee of Charité-Universitätsmedizin Berlin (protocol number: EA1/114/10).

2.2. Laboratory analyses

Blood samples were obtained from patients within three days after hospital admission to prevent an influence of treatment on metabolic/hormonal and psychometric parameters. All blood samples were taken after an overnight fast between 07:00 and 08:00 in the morning. Patients were allowed to drink small amounts of water but were advised not to smoke or exercise in the morning (before blood withdrawal). After withdrawal from a forearm vein, venous blood was collected in pre-cooled standard EDTA tubes containing aprotinin (1.2 Trypsin Inhibitory Unit/1 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, USA) as peptidase inhibitor. The EDTA tubes were stored on ice directly after blood withdrawal and centrifuged for 10 min at 3000 rpm at 4 °C. Plasma was separated and stored at −80 °C until further processing.

Phoenixin levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA, linear range 0.07-2.1 ng/ml, catalog # EK-079-01, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) per the manufacturer's instructions. First, to validate the kit, a spike-and-recovery experiment was performed adding synthetic phoenixin-14 amide (079-01, Phoenix Pharmaceuticals) in increasing concentrations to pooled human plasma samples. Recovery was assessed and measured levels compared to the expected concentrations.

All plasma samples were processed in one batch; the intra-assay variability was 9%. The antibody used in this ELISA was generated in rabbit and recognizes both human phoenixin-14 as well as phoenixin-20 with 100% cross-reactivity (cross-reacting also with rat, mouse, bovine, porcine and canine phoenixin; manufacturer's information). No cross-reactivity was observed for adrenomedullin, alpha-atrial natriuretic polypeptide, angiotensin I and II, apelin-12, bradykinin, brain natriuretic peptide-32, gonadotropin releasing hormone, neuropeptide Y, orexin A, somatostatin and TLQP-21 (manufacturer's information). The concentrations of circulating phoenixin described in the present manuscript were well within the linear detection range of the kit (0.07–2.1 ng/ml) and the positive control using synthetic phoenixin-14 was detectable in the expected range of 0.2–0.5 ng/ml.

2.3. Anthropometric measurements

For calculation of the BMI (as kg/m^2), body weight and height of the participants were assessed at the same day of blood withdrawal between 07:00 and 08:00 in the morning in patients wearing light underwear.

2.4. Psychometric parameters

All psychometric parameters were obtained electronically. The patients were given electronic devices on the same day or the day before the blood withdrawal and were asked to fill in the following questionnaires within one day. Patients without psychometric data within six days after blood sampling were excluded.

For psychometric assessment two modules of the self-administered patient health questionnaire (PHQ) [17] were used in order to assess depression (PHQ-9) and general anxiety (GAD-7).

The PHQ-9 depression module is an established screening instrument for the diagnosis of major depression and evaluation of the severity of depressive symptoms. The PHQ-9 contains nine items representing the diagnostic criteria for DSM-IV depressive disorders. Each of them can be scored as "0" (not at all) to "3" (nearly every day) with a maximum of 27 points [17]. In a meta-analysis of 36 studies (21,292 patients) the PHQ-9 showed a specificity of 0.87 in detecting major depressive disorders [13]. Our patients were handed out the German version of the PHQ-9 [10]. For the current population Cronbach's alpha was calculated as 0.89.

For the examination of anxiety, the GAD-7 scale was used. GAD-7 is an efficient tool to identify generalized anxiety disorder, posttraumatic stress disorder, panic disorder as well as social anxiety disorder and to measure the severity of symptoms [18]. It consists of seven items with scores ranging from "0" (not at all) to "3" (nearly every day) and a maximum of 21 points [18]. In our study the German version was used [9]. In a recent meta-analysis of 12 studies (5223 patients) specificity was calculated with 0.84 and sensitivity with 0.83 at a cut-off point of eight [16]. In the present sample Cronbach's alpha was 0.90.

In order to assess stress the perceived stress questionnaire (PSQ) [8] was used in its revised German version with 20 items (PSQ-20) [1]. The PSQ-20 examines the level of subjectively perceived exac-