Up-regulation of NPY gene expression in hypothalamus of rats with experimental chronic renal failure

Elzbieta Sucajtys-Szulc a, Joanna Karbowska b, Zdzislaw Kochan b, Wojciech Wołyniec a, Michal Chmielewski a, Boleslaw Rutkowski a, Julian Swierczynski b,⁎

a Department of Nephrology, Transplantology and Internal Medicine Medical University of Gdansk, 80-211 Gdansk, Poland
b Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland

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Abstract

Anorexia is possibly one of the most important causes of malnutrition in uremic patients. The cause of this abnormality is still unknown. Considering that: (a) NPY is one of the most important stimulants of food intake; (b) eating is a central nervous system regulated process and (c) NPY is expressed in hypothalamus, we hypothesized that the decrease of NPY gene expression in the hypothalamus could be an important factor contributing to anorexia associated with uremic state. In contrast to the prediction, the results presented in this paper indicate that the NPY gene expression in the hypothalamus of chronic renal failure (CRF) rats was significantly higher than in the hypothalamus of control (pair-fed) rats. Moreover, we found that serum NPY concentration in CRF rats was higher than in control (pair-fed) animals. The increase of plasma NPY concentration in CRF rats may be due to the greater synthesis of the neuropeptide in liver, since higher level of NPY mRNA was found in liver of CRF rats. The results obtained revealed that experimental chronic renal failure is associated with the increase of NPY gene expression in hypothalamus and liver of rats.

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Keywords: NPY; Hypothalamus; Liver; Chronic renal failure

1. Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide from pancreatic polypeptide family, is one of the most abundantly expressed peptides in the nervous system [1]. Due to five different NPY receptors (named as Y1 to Y5), expressed in many tissues, this neuropeptide seems to be involved in the regulation of variety of biological functions [1,2]. Moreover, it has been suggested that NPY is associated with some diseases, including obesity, hypertension, dementia and epilepsy [1]. One major function of NPY is a regulation of appetite and body weight homeostasis [1,2]. These actions of NPY appear to be mediated at hypothalamic sites by Y1 and Y5 NPY receptors [2]. NPY appears to stimulate not only food intake per se, but specifically the preference for carbohydrates [2], which in turn increase NPY mRNA level in arcuate nuclei (ARC), a prime controlling center of food intake [2].

Poor nutritional status has been demonstrated in some uremic patients [3,4], which has been related to high morbidity and mortality both in hemodialysis [5] and peritoneal dialysis patients [6]. Moreover, it has been reported that food consumption declines as renal function is deteriorating [7]. Several factors can contribute to a poor nutritional status [8]. Anorexia is possibly one of the most important causes of malnutrition in the uremic state [9]. Although the cause of uremic state associated anorexia is unknown, several factors can contribute to this pathological state [10]. Considering that NPY is one of the most important stimulants of food intake, one can suppose that this hormone is contributing to malnutrition observed in uremic patients [2,11]. Aguilera et al. [12] found normal plasma NPY concentration in 66%, lower in 22% and higher in 12% of peritoneal dialysis patients. Moreover, they found that peritoneal dialysis patients with anorexia display lower NPY concentration than those without anorexia [12].
Other authors reported that chronic renal failure is associated usually with an elevated plasma NPY concentration [13–20]. These discrepancy could be associated to different stage of renal failure and/or to the criteria used to define anorexia [21]. Recently, it has been concluded that anorexia in CRF patients was not due to deficient plasma NPY concentration [22]. However, regarding that (a) eating is a central nervous system-regulated process, and (b) NPY gene is expressed in the hypothalamus [1], it cannot be excluded that uremia related anorexia may result from the diminished hypothalamic NPY gene expression. The main hypothesis of this study is that the inhibition of NPY gene expression in hypothalamus of CRF rats may occur and that this phenomenon could play an important role in the pathogenesis of uremia-associated anorexia. To check this hypothesis in the present study we examine the NPY mRNA level in the hypothalamus of CRF failure rats and compare to the NPY mRNA level in the hypothalamus of sham-operated pair-fed rats. Additionally, to answer the question whether changes in NPY gene expression are limited to the hypothalamus or occur in other tissues of CRF rats, we analyzed NPY mRNA level in liver of CRF and the pair-fed animals.

2. Materials and methods

2.1. Animals

Male Wistar rats (10 week old, weight approximately 250 g at the beginning of the study) were used in all experiments. Animals were kept in individual cages with free access to water, and controlled lighting schedule (illuminated from 7 am to 7 pm). Experimental chronic renal failure (CRF) was induced by two-stage (5/6) subtotal nephrectomy as described previously [23]. Average daily food intake was measured by the difference in weight between the amount of food provided and the amount remaining over a 1 day period. Pair-fed (sham-operated) rats received the amount of food (commercial diet containing 23% of protein, composition of which has been described previously [24]) corresponding to what had been consumed by the matched chronic renal failure (CRF) animals. The non-fasted overnight rats were killed (between 8 am to 10 am) 6 weeks after induction of renal failure. Blood samples were collected from abdominal aorta and determination of blood pH, pCO2, urea, creatinine, albumin, transferrin, triacylglycerols and cholesterol (total, HDL-cholesterol, LDL-cholesterol) was performed. Hypothalamic portion of the brain and liver specimens were dissected and rapidly frozen in liquid nitrogen. The tissues were stored at −80 °C until analysis. All animal procedures were conducted in agreement with our institutional guidelines for the care and use of laboratory animals.

2.2. RNA isolation

Total cellular RNA was extracted from frozen hypothalamus and liver by the guanidinium isothiocyanate-phenol/chloroform method [25] and finally dissolved in dimethyl pyrocarbonate-treated water. The RNA concentration was determined from the absorbance at 260 nm and all samples had the 260/280 nm absorbance ratios about 2.0.

2.3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First strand cDNA was synthesised from 1 μg of total RNA (RevertAid™ First Strand cDNA Synthesis Kit—Fermentas). Prior to amplification of cDNA, each RNA sample was treated with RNase-free DNase I (Fermentas) at 37 °C for 30 min. The cDNA was used as a template in Multiplex PCR reaction with the housekeeping β-actin gene, as an internal control. The cDNA samples are amplified for 30 cycles (94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s) at a final volume of 20 μl containing 1× PCR buffer, 3.5 mM MgCl2, 0.5 mM dNTP Mix, 0.5 mM of sense and antisense primers and 0.5 U Taq DNA Polymerase (Fermentas). Specific sense and antisense primers used for the preparations of respective cDNA was: NPY 5′-TAAGTAAACAAAGAATGGG-3′ and 5′- AGTATGAGTAAGTGTG-3′ [26]; β-actin 5′-GAAATCCTGCGTGCAATTAGA-3′ and 5′-GCTAGAAACATTGGCGTTGIA-3′ [27]. The RT-PCR products were analysed by 1.5% agarose gel electrophoresis. Band intensities were compared by imaging of ethidium bromide staining and quantified using Sigma Scan software program (Jandel Scientific Co.). Results are expressed in arbitrary units.

2.4. Determination of mRNA levels by real-time RT-PCR

Hypothalamus NPY mRNA expression was quantified by real-time RT-PCR using iCycler iQ Real Time Detection System (Bio-Rad, Hercules, USA). Primers were designed with Sequence Analysis software package (Informagen, Newington, USA) from gene sequences obtained from Ensembl Genome Browser (www.ensembl.org). The following oligonucleotide primer pairs were used: TCA TACCA CAGACAGAGATATG (sense) and CCATCAC CACATCGGA AGG (antisense) for NPY; CTCTACAG ACCATTGCACC (sense) and AGGTCTCAGAGTCTCC (antisense) for β-actin. Real-time PCR amplifications were performed in a 25 μl volume using iQ SYBR Green Supermix (Bio-Rad), as previously described [28]. Each reaction contained 2 μl of cDNA and 0.3 μM of each primer. Samples were incubated for an initial denaturation and polymerase activation at 95 °C for 5 min, followed by 40 PCR cycles each consisting of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 40 s. Controls without RT and with no template cDNA were performed with each assay and all samples were run in triplicate. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-Actin mRNA was quantified and results were normalized to these values. Relative quantities of transcript were calculated using the 2−ΔΔCt formula [29]. The results are expressed in arbitrary units, with one unit being the mean mRNA level determined in the control group. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles and subjecting the amplification products to agarose gel electrophoresis.

2.5. Plasma NPY concentration

Plasma NPY concentration was estimated by radioimmunoassay method using kit from Peninsula Laboratories, USA after extraction of this hormone by Sep-Pac C18 Cartridges (Waters Associates, Milford Massachusetts, USA).

2.6. Statistical analysis

The statistical significance of differences in hypothalamic and liver NPY mRNA level, serum NPY concentration and serum creatinine, urea and serum lipids concentrations were analyzed using ANOVA followed by Student’s t test using the systat software (Systat). The data are expressed as mean±SD. Significance was defined by p<0.05.

3. Results

Table 1 displays some parameters characteristic for CRF found in nephrectomized rats when compared to control (pair-fed, sham-operated) animals. Serum concentrations of both urea and creatinine increased approximately 3- and 4-fold respectively in CRF rats as compared to pair-fed animals. Simultaneously, the induction of CRF resulted in 3-fold increase in serum triacylglycerol concentration as compared to pair-fed rats. Moreover, we observed approximately 2- to 3-fold increase of serum total cholesterol concentration, HDL-cholesterol concentration and LDL-cholesterol concentration. At the end of experiment, body weights were lower in CRF rats as compared to the pair-fed animals. This was due to smaller body weight gain, since at the start point, all tested animals had

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (pair-fed)</th>
<th>CRF (nephrectomized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>200±5 g</td>
<td>150±5 g</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.4±0.2</td>
<td>7.2±0.2</td>
</tr>
<tr>
<td>pCO2</td>
<td>35±2</td>
<td>38±2</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>5±1</td>
<td>20±3</td>
</tr>
<tr>
<td>Serum urea (mg/dL)</td>
<td>10±2</td>
<td>30±5</td>
</tr>
<tr>
<td>Serum albumin (mg/dL)</td>
<td>5±1</td>
<td>8±2</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dL)</td>
<td>150±20</td>
<td>200±30</td>
</tr>
<tr>
<td>Serum HDL-cholesterol (mg/dL)</td>
<td>40±5</td>
<td>60±7</td>
</tr>
<tr>
<td>Serum LDL-cholesterol (mg/dL)</td>
<td>100±15</td>
<td>150±20</td>
</tr>
<tr>
<td>Serum triacylglycerol (mg/dL)</td>
<td>100±15</td>
<td>200±30</td>
</tr>
</tbody>
</table>

Other authors reported that chronic renal failure is associated usually with an elevated plasma NPY concentration [13–20]. These discrepancy could be associated to different stage of renal failure and/or to the criteria used to define anorexia [21]. Recently, it has been concluded that anorexia in CRF patients was not due to deficient plasma NPY concentration [22]. However, regarding that (a) eating is a central nervous system-regulated process, and (b) NPY gene is expressed in the hypothalamus [1], it cannot be excluded that uremia related anorexia may result from the diminished hypothalamic NPY gene expression. The main hypothesis of this study is that the inhibition of NPY gene expression in hypothalamus of CRF rats may occur and that this phenomenon could play an important role in the pathogenesis of uremia-associated anorexia. To check this hypothesis in the present study we examine the NPY mRNA level in the hypothalamus of CRF failure rats and compare to the NPY mRNA level in the hypothalamus of sham-operated pair-fed rats. Additionally, to answer the question whether changes in NPY gene expression are limited to the hypothalamus or occur in other tissues of CRF rats, we analyzed NPY mRNA level in liver of CRF and the pair-fed animals.
similar body weight. The average daily food intake was 25±2 g in both groups. Considering that body weight was significantly lower in CRF rats than in pair-fed animals (365±40 g versus 426±23 g; \( p < 0.01 \)), one can conclude that other factors than the amount of food consumption contribute to lower body weight gain in CRF animals. Blood bicarbonate concentration and pH was not different in CRF rats and control (pair-fed) animals (Table 1). The above data indicate that CRF rats were uremic but not acidotic when sacrificed. Serum concentrations of some nutritional markers such as albumin and transferrin were slightly lower in CRF rats as compared to control (pair-fed) animals (Table 1). Serum glucose concentration was not different in CRF rats and control (pair-fed) animals (Table 1). Most of the abnormalities found in our experimental model of CRF resemble those observed in chronic renal failure patients.

The results presented in Fig. 1 indicate that serum NPY concentration was markedly elevated in CRF rats. In this respect the results obtained with experimental model of CRF resemble those observed in patients with chronic renal failure [13–20].

Fig. 2A (top panel) shows a representative experiment of NPY mRNA abundance in the hypothalamus of the pair-fed and CRF rats. NPY mRNA level was significantly higher in the hypothalamus of CRF rats as compared to NPY mRNA level in the hypothalamus of pair-fed animals. \( \beta\)-Actin mRNA (used as an internal control) was expressed at relatively high, essentially similar level in the hypothalamus of pair-fed and CRF rats (Fig. 2A). Fig. 2B (bottom panel) provides a semiquantitative analysis of the data determined from densitometric scan (obtained from 10 animals used in experiments for each treatment). Although the results displayed in Fig. 2B represent relative estimates, they show that hypothalamic NPY mRNA level was higher in CRF rats than in pair fed animals (Fig. 2B). To confirm the results presented above, we used real-time RT-PCR which is considered as more quantitative (and sensitive) method to quantify the steady state NPY mRNA level in the hypothalamus of pair-fed and CRF rats. Application of this method provides essentially similar results as conventional RT-PCR (not shown).

To answer the questions: (a) whether the stimulatory effect of CRF on NPY gene expression is limited to the hypothalamus or is observed in other tissues? and (b) what is the cause of the increase in serum NPY concentration? We analyzed NPY mRNA level in liver of CRF and the pair-fed rats. The liver was chosen because this organ makes a major contribution to circulating NPY concentration [30]. Fig. 3A (top panel) shows a representative experiment of NPY mRNA abundance in the liver of pair-fed and CRF rats. NPY mRNA level was significantly higher in the liver of CRF rats than in pair-fed animals. \( \beta\)-Actin mRNA abundance was essentially similar in the liver of pair-fed and CRF rats (Fig. 3A). Fig. 3B (bottom panel) provides a semiquantitative analysis of the data determined from densitometric scan (obtained from 10 animals used in experiments for each treatment). The results displayed

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chronic renal failure rats ((n=10))</th>
<th>Pair-fed rats ((n=10))</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dl±SD)</td>
<td>24±0.9</td>
<td>0.6±0.1</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>Urea (mg/dl±SD)</td>
<td>295.9±71.1</td>
<td>840.4±4.5</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>pH</td>
<td>7.40±0.08</td>
<td>7.39±0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>Bicarbonate (mmol/l±SD)</td>
<td>27.2±2.7</td>
<td>26.4±1.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glucose (mg/dl±SD)</td>
<td>151.4±16.8</td>
<td>163.8±14.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Albumin (g/l±SD)</td>
<td>33±4</td>
<td>38±2</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>Transferrin (g/l±SD)</td>
<td>0.8±0.2</td>
<td>1.1±0.1</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dl±SD)</td>
<td>229.6±97.1</td>
<td>74.2±26.9</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl±SD)</td>
<td>176.3±20.5</td>
<td>69.8±20.5</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl±SD)</td>
<td>79.6±22.5</td>
<td>35.3±11.2</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl±SD)</td>
<td>50.7±14.2</td>
<td>19.8±5.5</td>
<td>( p &lt; 0.01 )</td>
</tr>
</tbody>
</table>

Fig. 1. Serum neuropeptide Y (NPY) concentration in chronic renal failure rats (CRF) and pair-fed rats. Data are presented as mean±SD \((n=10)\) *\( p < 0.05 \).
as described in Materials and methods. The cDNA amplified from diminished anorexia may result from hypothalamic dysfunction, especially appetite. We hypothesized that chronic renal failure associated anorexia is caused by defective hypothalamic regulation of uremic toxins [21,31]. Since hypothalamus plays essential role including interactions among cytokines, neuropeptides and pathogenesis of anorexia in uremic patients is multifactorial, arbitrary units (*p<0.01).

in Fig. 3 show that in liver NPY mRNA level was higher in CRF rats than in pair fed animals.

4. Discussion

Anorexia is likely an important factor contributing to malnutrition observed in chronic renal failure [9]. The pathogenesis of anorexia in uremic patients is multifactorial, including interactions among cytokines, neuropeptides and uremic toxins [21,31]. Since hypothalamus plays essential role in the regulation of food intake, it is likely that CRF associated anorexia is caused by defective hypothalamic regulation of appetite. We hypothesized that chronic renal failure associated anorexia may result from hypothalamic dysfunction, especially from diminished NPY gene expression in hypothalamus, despite of normal [12], lower [12] or elevated plasma concentration of this neuropeptide [12–20] in uremic patients. (The apparent discordance between plasma NPY concentration in CRF patients reported previously could be associated to different stage of renal failure and/or the criteria used to define anorexia). In contrast to the prediction, the results presented in this paper indicate for the first time that the NPY gene expression in the hypothalamus of CRF rats is significantly higher than in the hypothalamus of the pair-fed rats. Higher NPY mRNA level in the hypothalamus of CRF rats is unlikely to be due to assay problem, since it was confirmed by two methods (RT-PCR and real time PCR). Moreover, we found that serum NPY concentration in CRF rats was much higher than in the pair-fed animals. The increase in serum NPY concentration in CRF rats may be a result of the increased production and/or decreased renal excretion and/or degradation. Since higher NPY mRNA level was found in liver of CRF rats than in pair-fed animals (Fig. 3), it is very likely that higher synthesis in liver (and possibly in other tissues) is contributing to elevated serum NPY concentration. Several investigators reported that circulating NPY concentration was significantly increased in CRF patients [13–20]. Therefore, the effect of chronic renal failure on serum NPY concentration in CRF patients and CRF rats is essentially similar. Higher serum NPY concentration, high plasma urea, creatine and some lipids concentration (Table 1), as well as less food intake by uremic rats suggest that the experimental model of CRF mimics human disease. Thus, one can suppose that in hypothalamus (and possibly in other tissues) of CRF patients, similarly as in hypothalamus and in liver of CRF rats, higher NPY gene expression is taking place. Assuming that higher NPY mRNA level is strictly associated with the higher peptide level in the hypothalamus, one can suppose that anorexia associated with CRF is not due to insufficient NPY production in hypothalamus. Measuring serum NPY concentration, Lim group [22] suggested recently that in CRF patients, anorexia was not due to deficient plasma NPY. Thus our findings are consistent with, and extend, the studies of Lim group [22], who did not examine NPY gene expression in hypothalamus.

The mechanism(s) by which CRF causes the increase of NPY gene expression in the hypothalamus and liver is uncertain and requires further investigation. Several potential mechanisms can be invoked to explain CRF-related increase of NPY mRNA level in the hypothalamus and liver. In rodents, food restriction for 2 weeks, or food deprivation for 4 days, does increase NPY gene expression in the central nervous system [32]. Since pair-fed rats did eat the same amount of food as CRF rats and displayed lower level of NPY mRNA than CRF animals, the amount of food intake cannot explain the differences in NPY gene expression in pair-fed and CRF rats. This work has some important limitation. We have not analyzed some factors frequently associated with uremia, including proinflammatory cytokine [33–35], sympathetic overactivity [36], hyperinsulinemia and hyperleptinemia (21) which may influence the NPY gene expression in hypothalamus and liver. Despite the limitations, this work clearly indicated that experimental CRF is associated with increased NPY gene expression in hypothalamus and liver. The possible mechanism(s) by which CRF may cause the increase of NPY gene expression in the hypothalamus and liver is discussed below.

Korner et al. [37], showed increased level of NPY gene expression in the presence of high insulin concentration in fed and fasted obese rats. However, insulin administration to central nervous system reduces NPY gene expression in the arcuate nucleus of food-deprived lean (Fa/Fa) Zucker rats [38]. There are also conflicting results on plasma insulin concentration in CRF rats. Some authors reported that uremic rats displayed hypoinsulinemia [39,40], while others indicate that uremic rats had increased plasma insulin concentration [41,42]. Thus, the
considerable variation in published reports on plasma insulin concentration in CRF rats and on the effect of insulin on NPY gene expression in hypothalamus does not allow to make a clear conclusion about the role of insulin in the regulation of NPY gene expression in the hypothalamus of CRF rats.

It has been shown that leptin, the adipocyte-derived hormone, which seems to play an important role in the regulation of appetite and energy balance, down-regulates the NPY gene expression in the hypothalamus [2]. Recent studies suggest that leptin inhibits NPY gene transcription in the hypothalamus through STAT3 and SOCS3 [43]. Thus, leptin in healthy subjects is engaged in negative feedback control of energy balance. Moreover, the ARC neurons expressing NPY are important target of leptin actions. One can suppose that leptin can be an important factor regulating NPY gene expression in the hypothalamus of CRF rats. It is believed that after secretion by adipocytes, leptin is transported to the ARC via the general circulation, acts on leptin receptors located in the ARC and in this way plays a critical role in inhibiting food intake.

Hormones other than insulin and leptin might be also involved in the regulation of hypothalamic NPY gene expression. For instance elevation of serum corticosterone in CRF [22] may affect transcriptional control of NPY gene expression [44].

Finally, the question arises why the increase of NPY gene expression in hypothalamus of CRF rats did not prevent the decrease in food intake of these rats. One possible explanation is that uremic toxins may somehow modify the sensitivity of NPY receptors, consequently modulating the effect of NPY on food consumption.

During preparation of this manuscript, an elegant paper appeared, which suggests that elevated plasma leptin concentration may be an important cause of CRF associated anorexia via signaling through the central melanocortin system [45]. Thus, leptin may play an important role in the pathogenesis of uremia associated anorexia by a mechanism which is independent of the NPY regulation of appetite. This conclusion corroborates with our results.

In conclusion, the data presented in this paper indicate for the first time that NPY mRNA abundance in the hypothalamus of CRF rats was significantly higher than in the hypothalamus of sham operated, pair-fed animals. Moreover, our data indicate that the effect of CRF on NPY mRNA level is not limited to the hypothalamus but was also observed in liver. The increase in NPY mRNA abundance in the liver may contribute to the increase in NPY concentration in serum of CRF animals.

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References