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β-ENDORPHIN INVOLVEMENT IN THE REGULATORY RESPONSE TO BODY SODIUM OVERLOAD

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Abstract—The present study was performed to examine the role of the endogenous β-endorphinergic system on blood pressure regulation, sympathetic and brain activity during body sodium overload.

β-Endorphin knockout ($\beta end^{-/-}$), heterozygous ($\beta end^{+/-}$) and wild-type ($\beta end^{+/+}$) mice were submitted for two weeks to either a normal- or a high-sodium diet (NSD and HSD, respectively), and systolic blood pressure (SBP), urinary catecholamines (as an index of sympathetic nervous system activity), and the brain pattern of Fos-like immunoreactivity (as a marker of neuronal activation) were evaluated in each group.

HSD caused a significant increase in SBP in $\beta end^{-/-}$ mutant mice compared with $\beta end^{+/+}$ mice kept in the same experimental conditions ($P<0.01$), but no statistical differences were observed between $\beta end^{+/-}$ and $\beta end^{+/+}$ on a HSD. Moreover, when animals from the three genetic lines were fed with a NSD no changes in SBP were evidenced.

With regard to brain activity, $\beta end^{-/-}$ mice maintained on a HSD showed a significant increase in Fos-like immunoreactive neurons in the median preoptic nucleus ($P<0.01$) compared with $\beta end^{+/-}$ and $\beta end^{+/+}$ animals.

Additionally, $\beta end^{-/-}$ mice had higher levels of urinary epinephrine excretion ($P<0.05$) on a HSD in comparison to $\beta end^{+/+}$ and $\beta end^{+/-}$ animals in the same experimental conditions. No differences, however, were registered in norepinephrine and dopamine urinary excretion in animals from the three genetic lines after two weeks on either a HSD or a NSD.

In summary, our results indicate that the β-endorphinergic system may play a part in the compensatory response to sodium overload, since the absence of β-endorphin causes an increase in systolic blood pressure, and increases median preoptic nucleus neural activity and urinary epinephrine excretion.

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Abbreviations: ArcLP, lateral posterior arcuate nucleus; BP, blood pressure; DA, dopamine; Dahl S, Dahl sensitive rat (s); $\beta end^{-/-}$, β-endorphin knockout; $\beta end^{+/-}$, β-endorphin heterozygous; $\beta end^{+/+}$, β-endorphin wild-type; EPI, epinephrine; Fos-LI, Fos-like-immunoreactivity; HPLC, high-pressure liquid chromatography; HSD, high-sodium diet; MnPO, median preoptic nucleus; MSH, melanocyte-stimulating hormone; NE, norepinephrine; NSD, normal-sodium diet; NTS, nucleus of the solitary tract; PB, phosphate buffer; PCR, polymerase chain reaction; POMC, proopiomelanocortin; RVLM, rostral ventrolateral medulla; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat(s).

Key words: β-endorphin knockout mice, systolic blood pressure, catecholamines, sympathetic activity, neuronal activity, median preoptic nucleus.

In normal conditions of cardiovascular regulation, increased dietary salt triggers multifunctional mechanisms (both central and peripheral) in order to maintain electrolyte and cardiovascular homeostasis (Leenen et al., 2002). One of these mechanisms may involve an increase in proopiomelanocortin (POMC) levels in the pituitary gland and in the enzymes involved in the processing of POMC, the proconvertases PC1 and PC2 (Mosqueda-Garcia and Kunos, 1987; Chandranohan et al., 2001; Humphreys, 2004). These enzymes are responsible for the increase in α–melanocyte-stimulating hormone (MSH), γ-MSH and β-endorphin among others (Zhou et al., 1993).

Knockout mice for PC2 show an increase in blood pressure (BP) when placed on a high sodium diet (HSD). Thus, these data indicate that the normal activity of PC2, and in consequence the normal levels of POMC-derived peptides, may participate in the maintenance of normal BP levels during sodium overload (Ni et al., 2003).

Increased dietary salt increases BP in many hypertensive patients and in animal models of experimental hypertension, producing salt-sensitive hypertension (SSH) (Brooks et al., 2005).

A series of studies have revealed that Dahl sensitive rats (Dahl S) and spontaneously hypertensive rats (SHR) which develop an increase in BP when fed with a HSD (Dahl et al., 1968; Huang and Leenen, 1996), show lower levels of pituitary POMC mRNA compared with control animals (Felder and Garland, 1989; Hao and Rabkin, 1996).

Clinical studies also show that patients with essential hypertension have lower levels of β-endorphin and, after treatment with clonidine (antihypertensive drug), plasma levels of β-endorphin increase significantly and negatively correlate with the decrease of BP (Zheng et al., 1995). The antihypertensive action of clonidine, either in hypertensive animal models or in patients with essential hypertension, can be reversed by naloxone (Farsang et al., 1984; van Giersbergen et al., 1989; Zheng et al., 1995).

Taking into account that opioids modulate sympathetic nervous activity (Kimura et al., 1988; Kienbaum et al., 1998; Kitamura et al., 2002) and, as mentioned earlier, lower levels of β-endorphins have been reported in experimental models of hypertension as well as in patients with essential hypertension, the aim of this study was to test the hypothesis that β-endorphin deficiency is involved in the

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increase of BP and of sympathetic activity during sodium overload.

The development of a β-endorphin-deficient mouse line (Rubinstein et al., 1996) provides a useful tool for investigating the role of this opioid peptide in the regulatory response to sodium overload. In the present study, we have used mice with gene dosage–dependent variations in brain β-endorphin, expressing either 100%, 50%, or 0% of normal β-endorphin content (Rubinstein et al., 1996), to evaluate whether varying levels of endogenous β-endorphin may alter BP, sodium balance, sympathetic nervous system activity (measured as changes in urine catecholamine levels), and the brain Fos-like immunoreactive pattern (as a marker of neuronal activation), after both normal and HSD.

EXPERIMENTAL PROCEDURES

Animals (β-endorphin mutant mice)

β-Endorphin wild-type ($\beta end^{+/+}$), heterozygous ($\beta end^{+/-}$) and knockout ($\beta end^{-/-}$) mice, aged from 6 to 9 weeks old, were used in the present experiments. The generation of β-endorphin mutant mice has been described previously in Rubinstein et al. (1996). In brief, a point mutation was introduced by site-directed mutagenesis into exon 3 of the POMC gene to generate a premature translational stop codon. The resultant truncated prohormone lacks the carboxyl-terminal 31 amino-acids composing β-endorphin, but is expressed at normal levels and correctly processed to adrenocorticotropic hormone, melanocyte-stimulating hormones and gamma-lipotropic hormone. The mutant allele was originally introduced into 129S2/SvPas-derived D3 embryonic stem cells and subsequently backcrossed for five successive generations to C57BL/6N mice (Simonsen, Gilroy, CA, USA) and then an additional two or four to C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA).

As previously shown by Rubinstein et al. (1996), $\beta end^{+/-}$ and $\beta end^{-/-}$ mice express 50% and 0% respectively of the normal β-endorphin content reported in $\beta end^{+/+}$ mice in both hypothalamus and pituitary gland.

The $\beta end^{+/+}$, $\beta end^{+/-}$ and $\beta end^{-/-}$ mice used in the present experiments were derived at the N9 generation on a C57BL/6 background from heterozygote mating pairs. Mice were born and reared in the breeding colony at the Instituto Ferreyra (Córdoba, Argentina), and were housed in groups of five to eight per cage in a temperature-controlled environment, maintained on a 12-h light/dark cycle, and fed and watered *ad libitum*.

All experimental protocols were approved by the appropriate animal care and use committees at our institute, following the guidelines in the public Health Service Guide for the care and use of Laboratory Animals, to minimize both the suffering and the number of animals used.

Animal genotyping

Genotyping was performed on genomic DNA samples obtained from mouse tails by polymerase chain reaction (PCR) (Franchini et al., 2003). For DNA extraction, mice were anesthetized with 2,2,2-tri-bromoethanol (Aldrich, Milwaukee, WI, USA) 300 mg/kg i.p. Then, tail samples were obtained and incubated with 0.5 ml digestion buffer (50 mM Tris–HCl, pH 8.0; 100 mM EDTA; 0.5% sodium dodecyl sulfate; 0.5 mg/ml proteinase K, at 55 °C overnight (12–14 h). DNA was purified using standard procedures.

In the PCR protocol we used a combination of three oligonucleotide primers, 889: 5′-ACCTCCGAGAAGAGCCAG-3′ (POMC exon 3), 187: 5′-ACATGTTCATCTCTATACATAC-3′ (3′-flanking sequence in POMC gene), 186: 5′-GAGGATTGGGAAGACAATAGCA-3′ (PGK-neo cassette, specific for the targeted allele Cibersyn, Aston, PA, USA). The set of oligonucleotides 889–187 amplified a 1.4 kb product corresponding to the wildtype allele and the pair of oligonucleotides 186–187 amplified a 1.2 kb product corresponding to the mutated allele.

PCR conditions were hot start 94 °C for 3 min followed by 35 cycles at 94 °C for 0:45 min, 60 °C for 0:45 min, 72 °C for 1:20 min and an additional elongation cycle at 72 °C for 20 min. Amplified products were electrophoresed on 1.0% agarose gels and identified by their size.

BP monitoring

Non-invasive pulse tail-cuff system. Systolic blood pressure (SBP) was measured in conscious mice using a pulse-based tail-cuff instrument (Kent, Litchfield, CT, USA; RTBP2005 Single Animal System). SBP values were derived from an average of 10 measurements per animal at each time point. Seven preliminary training-day sessions were performed during the week before starting the experiment. The investigator who measured the tail-cuff SBP was blinded for experimental groups.

Despite the fact that the non-invasive pulse-based tail-cuff system for measuring SBP in mice has been previously validated by Whitesall et al. (2004), we have also validated this method in our laboratory, comparing measurements using a direct intra-arterial method with simultaneous indirect pulse tail-cuff measurements in the same unanesthetized mice.

Intra-arterial BP measurements. Mice were anesthetized with a mixture of ketamine (162 mg/kg i.p., Holliday-Scott, Buenos Aires, Argentina) and xylazine (1 mg/kg i.p., König, Argentina). A middle incision was made in the neck. Taking care to avoid the vagus nerve and carotid sinus, the left carotid artery was isolated below the level of the bifurcation and was tied off distally with 5.0 silk suture, and a clamp was applied proximally. Approximately 1.5 mm of polyethylene tubing PE-10 (0.025 inch OD, 0.011 inch ID, Clay Adams, Parsippany, NJ, USA) welded to PE-50 (0.039 inch OD, 0.023 inch ID, Clay Adams), was inserted into the vessel so that the tip was approximately at the junction of the aorta and the carotid. The catheter was then firmly sutured in place. The cannula was tunneled s.c. to the area between the shoulder blades and connected to a stainless steel “elbow” made of 23-gauge hypodermic tubing. One end of the elbow protruded through the skin. The cannula was filled with sterile saline, and the external end of the elbow closed with a plastic cap. After a 48-h minimum recovery period, polyethylene tubing filled with sterile saline containing heparin (50 U/mL) was connected to the external end of the elbow. SBP was measured by connecting the arterial cannula to a strain gauge transducer (BLPR, WPI) connected to a Grass Polygraph (Quincy, MA, USA; 79 D model).

Urine collection

Urine samples were collected from $\beta end^{-/-}$, $\beta end^{+/+}$ and $\beta end^{+/-}$ mice housed separately in metabolic cages during a 24-h period in a tube with a preservative solution containing 25 mg metabisulfite, 25 mg EDTA, 80 ml of a solution of 2 g/l of boric acid and 20 ml of HCl acid (6 N). After centrifuging the urine samples they were stored at −70 °C until processing.

Measurement of urinary catecholamines and creatinines

Urinary epinephrine (EPI), norepinephrine (NE) and dopamine (DA) levels were determined by high-pressure liquid chromatography (HPLC) analysis with an electrochemical detector. Urine samples were first extracted with alumina (Merck, Al_2O_3 II–III according to Brockman). The analysis of the catecholamines was

performed in duplicate, by reverse phase HPLC using a Zorbax Eclipse XDB-C8 rapid resolution column (4.6×150 mm, 3.5 μm, Agilent, USA), with electrochemical detector (HP 1049). The mobile phase was monochloroacetic acid (4.7 g), NaOH (1.8 g), Na_2EDTA (0.51 g), sodium octane sulfonate (0.15 g), citric acid monohydrated (10.5 g), made up to 1 l with a water/ACN mixture (96:4) at a flow rate of 0.8 ml/min. The pH of the solution was adjusted to 3.0 with monochloroacetic acid or NaOH. The column temperature was held at 25 °C and the potential used for detecting all the compounds was 0.8 V. Samples of each animal were analyzed in the same assay to eliminate interassay variability. For each sample, the ratios of peak-height to internal standard were calculated and introduced into a calibration curve equation obtained by linear regression analysis. Spiked biological samples gave a linear response in the range of interest for each compound. The intra-assay coefficient of variation of the method was 5.5%.

Urinary creatinines were determined by use of a commercially available colorimetric kit (Jaffé Method) from Roche Diagnostics Corporation, Indianapolis, IN, USA.

Immunohistochemistry

Mice of the three genotypes corresponding to normal and HSD groups were anesthetized with 2,2,2-tri-bromoethanol (Aldrich; 300 mg/kg i.p.) and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2. The brains were then removed, fixed overnight in the perfusion solution, and stored at 4 °C in PB containing 30% sucrose. Coronal sections of 50-μm were cut using a freezing microtome. Fos-like immunoreactivity (Fos-LI) procedures have been previously described (Franchini and Vivas, 1999). The Fos antibody used in this study was raised in rabbits against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, batch no. 60950101; Oncogene Science, Manhasset, NY, USA) and diluted 1:20,000 in PB containing 2% normal horse serum and 0.3% Triton X-100. As we cannot exclude cross-reactivity of this c-Fos antibody with other proteins of the Fos family (such as Fos-B, Fra-1 and Fra-2) we consider the term Fos-LI to be more appropriate for the interpretation of Fos protein immunostaining.

The brain nuclei evidencing Fos-LI were identified and delimited according to the mouse brain atlas of Paxinos and Franklin (2001), and the counting was done in four animals of each condition and repeated at least twice on each section analyzed, to ensure that the numbers of profiles obtained were similar. The investigator who conducted the counting of Fos-LI-positive cells was blinded for the experimental groups.

Statistical analysis

The data, expressed as mean±S.E., were analyzed using one-, two- and three-way ANOVA with repeated measures as appropriate, with the LSD and Tukey post hoc tests when significant differences were recognized. A P value of less than 0.05 was used to indicate the presence of a significant difference.

SBP data were analyzed by using three-way ANOVA with repeated measurements: BP levels (within factors), genotype and diet (between factors). Tukey tests were used as follow-ups to identify significant differences between groups.

Fos-LI data, catecholamines and balance study data were analyzed with two-way ANOVA, using genotype and diet as between factors, followed by the LSD post-test when significant differences were recognized. Simultaneous SBP correlation (tail-cuff and intra-arterial) were analyzed by Pearson's r analysis.

Experimental protocol

Training period. Experiments were performed on male mice of the three genotypes ($\beta end^{-/-}$, $\beta end^{+/+}$ and $\beta end^{+/-}$), aged 6–9 weeks old. To acclimatize the animals to single housing they were placed individually in cages for a week with free access to normal rodent chow and distilled water. Following another week of BP measurement training, mice were housed in metabolic cages and for the next seven days were given a liquid normal sodium diet (NSD) (0.08% NaCl, Ensure, Abbott, Holand) instead of normal rodent chow. It is important to note that a liquid diet was used instead of normal rodent chow in order to be able to collect enough urinary volume to measure urinary catecholamines from individual mice.

Experimental period. The day before starting the experimental period, SBP was measured in mice from the three genetic lines and 24-h urine samples were obtained in order to measure catecholamine excretion in animals from each genotype (Baseline period). The mice from the three genotypes ($\beta end^{+/+}$, $\beta end^{-/-}$ and $\beta end^{+/-}$) were then immediately randomized to either continue on the NSD or be subjected to an otherwise nutritionally identical HSD (4% NaCl, Ensure, Abbott) for the following two weeks (Experimental period: days 1–14).

Volume of water and liquid diet intake, body weight and urine volume were recorded after the first and second week of the experimental period (7th and 14th day respectively). The sodium concentration of fluid intake and urine output was determined by flame photometry in order to calculate sodium balance.

Baseline measurements of SBP were registered as mentioned before, during the baseline period, that is to say the day before starting the experimental period, and after 2 weeks of either HSD or NSD (end-point measurement).

Urinary EPI, NE and DA concentrations of 24 h urine samples were obtained both during the baseline and at the end point (the day before starting the experimental period and two weeks after the beginning of the experimental period) as indicators of systemic sympathetic activity.

Two days after the end-point BP and catecholamine measurements (16th day of the experimental period), mice from the three genotypes in the NSD and HSD groups were perfused and their brains subjected to Fos-LI procedure.

RESULTS

Balance study

Table 1 shows the results of the balance study during the experimental period after one and two weeks of either HSD or NSD in mice of the three genetic lines.

ANOVA showed a main effect of genotype and diet on body weight, after one and two weeks of the experimental period ($P<0.01$ and $P<0.05$ respectively). Thus, $\beta end^{-/-}$ mice showed a greater body weight than $\beta end^{+/-}$ and $\beta end^{+/+}$ animals and $\beta end^{+/-}$ mice were significantly heavier than $\beta end^{+/+}$ animals.

The analysis showed no effect on water intake due to genotype, but showed a significant effect of diet after one and two weeks of NSD or HSD (both $P<0.001$). As expected, animals that were kept during the experimental period on a NSD consumed less water than mice of the HSD group.

In relation to liquid diet intake, the analysis revealed a main effect of diet ($P<0.001$) and a non-effect of genotype or of the interaction (diet×genotype) after one week of treatment, however, after the 2nd week of the experimental period no effect was observed. It is important to note that after the first week, animals that were kept under a HSD

Table 1. Balance study during the experimental period after 1 (A) and 2 weeks (B) of HSD or NSD in βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ mice

Variable	Effect	NSD			HSD		
		βend$^{+/+}$	βend$^{-/-}$	βend$^{+/-}$	βend$^{+/+}$	βend$^{-/-}$	βend$^{+/-}$
Body weight (g)							
A	*g*, *d*	33.9±0.9	39.8±1.3	35.7±0.8	32.5±0.6	35.1±0.7	34.4±1.2
B	*g*, *d*	35.2±0.9	40.9±1.3	38.2±1.1	34.5±0.7	37.7±1.4	35.9±0.8
Water intake (ml/10 g bw/24 h)							
A	*d*	0.5±0.2	0.1±0.1	0.3±0.1	8.2±0.6	7.4±0.4	7.0±0.6
B	*d*	0.1±0.0	1.2±0.6	0.3±0.3	6.8±0.5	6.8±0.9	6.9±0.6
Liquid diet intake (g/10 g bw/24 h)							
A	*d*	7.1±0.6	6.0±0.3	7.4±0.4	5.4±0.2	5.3±0.3	5.6±1.1
B		5.4±0.2	5.4±0.4	5.1±0.3	5.1±0.2	5.0±0.5	5.4±0.4
Urine volume (ml/10 g bw/24 h)							
A	*d*	4.30±0.54	3.23±0.20	4.47±0.43	9.78±0.61	8.81±0.68	9.27±0.58
B	*d*	2.92±0.21	3.79±0.47	2.50±0.21	8.93±0.43	8.75±0.53	9.33±0.69
Na balance (mEq/10 g bw/24 h)							
A	*d*	0.16±0.01	0.14±0.01	0.18±0.02	0.18±0.21	0.67±0.34	0.64±0.17
B	*d*	0.17±0.01	0.14±0.02	0.18±0.03	0.46±0.11	0.47±0.11	0.70±0.08

Values are mean±S.E., n=8–15/group. *d*, main effect of diet; *g*, main effect of genotype.

consumed smaller amounts of food (independently of the genotype) compared with those that were maintained on a NSD; but after the 2nd week of treatment no differences in diet intake were recorded.

As regards sodium balance and urinary volume excretion, the ANOVA showed a significant main effect only of diet after both, the 1st week ($P<0.001$ and $P<0.01$ respectively) and 2nd week (both $P<0.001$) of the experimental period but showed no effects of genotype or of the interaction (genotype×diet).

Effect of high-sodium intake on SBP

The statistical analysis of SBP revealed a triple main effect of the interaction (genotype, diet, level of SBP) ($P<0.05$) and the Tukey post hoc analysis showed that βend$^{-/-}$ animals placed on a HSD showed a significant increase in SBP compared with βend$^{+/+}$ mice kept under the same experimental conditions; however, no statistical differences were observed between βend$^{+/-}$ and βend$^{+/+}$ mice on HSD (see Fig. 1). Post hoc analysis, comparing the SBP of animals of each genotype on a HSD or NSD, showed an increase in SBP in both βend$^{-/-}$ and βend$^{+/-}$ mice on a HSD when compared with animals from the same genetic lines on a NSD. However, no statistical differences in SBP were observed in βend$^{+/+}$ mice on either HSD or NSD (Fig. 1).

In relation to the average change in SBP (end-point SBP−baseline SBP), a significant double interaction was observed between genotype and diet ($P<0.01$). On a HSD, the average increase in SBP of mice lacking β-endorphin was 17±3 mm Hg, while the change of SBP in βend$^{+/-}$ was 7±3 mm Hg and 1±3 mm Hg for βend$^{+/+}$ mice. However, when animals from the three genetic lines were maintained with a NSD, no statistical changes in SBP were reported (change in SBP for: βend$^{+/+}$=−4±2; βend$^{-/-}$=−4±1 and βend$^{+/-}$=3±2).

Correlation of intra-arterial and pulse tail-cuff SBP measurements

In a separate group of mice (n=32) we compared indirect (tail-cuff) and direct (intra-arterial) SBP. The correlation (r=.71, $P<0.0001$) of the results, shown in Fig. 2, confirms the accuracy of BP measurement by both methods.

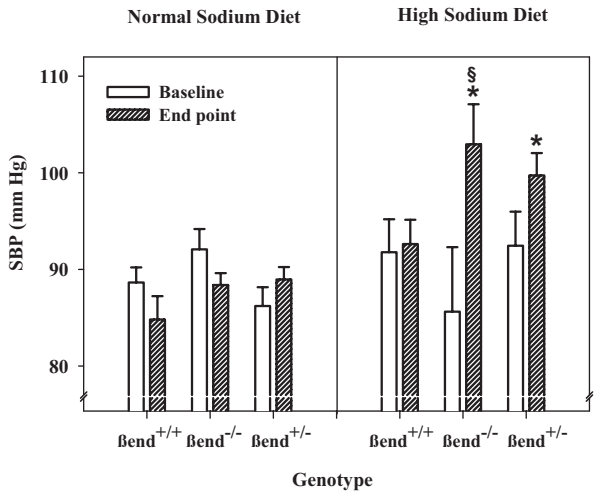

Fig. 1. Effect of dietary salt on SBP in conscious βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ mice. Open bars denote baseline SBP measurements; striped bars, the end point SBP measurements. Left panel shows the SBP measurements, baseline and end-point measurements respectively, of animals of the three genetic lines subjected to NSD during the whole experimental period (2 weeks). Right panel shows baseline and end-point SBP measurements of mice of the three genotypes maintained during the experimental protocol on a HSD. The statistical analysis of SBP revealed a triple main effect of the interaction (genotype, diet, level of SBP) {$F(2,45)=6.28$, $P<0.05$}. § $P<0.01$ βend$^{-/-}$ versus βend$^{+/+}$ both under HSD. * $P<0.05$ βend$^{-/-}$ and βend$^{+/-}$ under a HSD compared with the values of SBP in their counterparts under a NSD. Values are means±S.E. Number of animals in each group is given in parentheses: βend$^{+/+}$ (n=9), βend$^{-/-}$ (n=8), βend$^{+/-}$ (n=10).

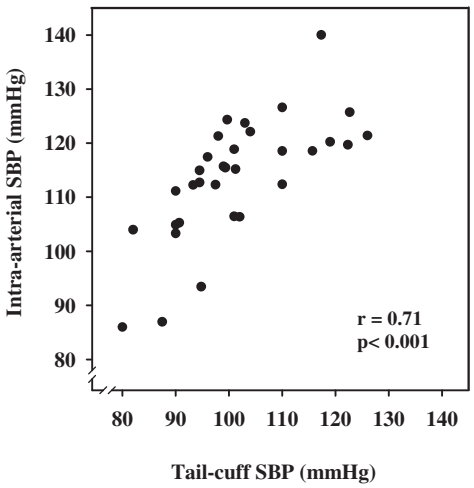

Fig. 2. Scatterplot shows the relationship of SBPs determined in conscious mice by both intra-arterial and tail-cuff system (n=32).

Effect of HSD on Fos-LI

As shown by Fig. 3, the analysis of the number of Fos-LI neurons in the median preoptic nucleus (MnPO) showed a significant double interaction between genotype and diet ($P<0.01$). The LSD post hoc test indicated that βend$^{-/-}$ mice fed during 16 days with a HSD showed a significant increase in the number of Fos-LI neurons in the MnPO compared with βend$^{+/+}$ and βend$^{+/-}$ mice kept on a HSD or a NSD (Fig. 3A).

A similar data profile was observed in the lateral posterior arcuate nucleus (ArcLP), although no statistical differences were observed in the double interaction (genotype×diet). The statistical analysis revealed, however, a main effect of genotype and diet in the ArcLP (both $P<0.05$). The post hoc test indicated that βend$^{-/-}$ mice had a higher number of Fos-LI neurons in this subdivision compared with βend$^{+/-}$ and βend$^{+/+}$ mice. On the other hand, when mice were kept on a HSD (whatever the genetic line) an increase in the number of Fos-LI neurons was evident (Fig B).

As expected, independently of the genetic line, an increase in the sodium content of diet was accompanied by a rise in Fos-LI expression in several forebrain and brain stem nuclei (Table 2). Among these, in the vascular organ of the lamina terminalis (VOLT), the supraoptic nucleus (SO), posterior paraventricular nucleus (PVN-p), median posterior arcuate nucleus (ArcMP) and the nucleus of the solitary tract (NTS), the ANOVA exhibited a significant main effect of diet ($P<0.05$). A genotype main effect was observed in the dorsal and ventral subdivisions of the dorsal raphe nucleus (DRD and DRV) and in the lateral parabrachial nucleus (LPB) ($P<0.01$). The LSD post hoc analysis showed an increased number of Fos-LI neurons within both nuclei in mice lacking β-endorphin versus wild-type and heterozygous mice.

No main effects were detected in the number of Fos-LI neurons in other nuclei analyzed, such as the rostral paraventricular nucleus, central amygdaloid nucleus, ventrolateral subdivision of the DRN or locus coeruleus (data not shown).

Effect of HSD on urinary catecholamine excretion

During the baseline period, no differences were observed in urinary EPI, NE, DA and creatinine levels in βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ mice (Table 3).

However, after the 2 week experimental period (end point measurement) the statistical analysis of 24-h urinary EPI excretion showed a double interaction between genotype and diet ($P<0.05$); and the LSD post hoc analysis showed that βend$^{-/-}$ mice on a HSD had an increased level of urinary EPI compared with βend$^{+/+}$ and βend$^{+/-}$ mice maintained either on a HSD or a NSD (Fig. 4A). On the other hand, no significant differences were registered in the end-point measurements in the urinary NE and DA excretions in βend$^{-/-}$, βend$^{+/-}$ and βend$^{+/+}$ mice on either a HSD or a NSD (Fig. 4B and C, respectively).

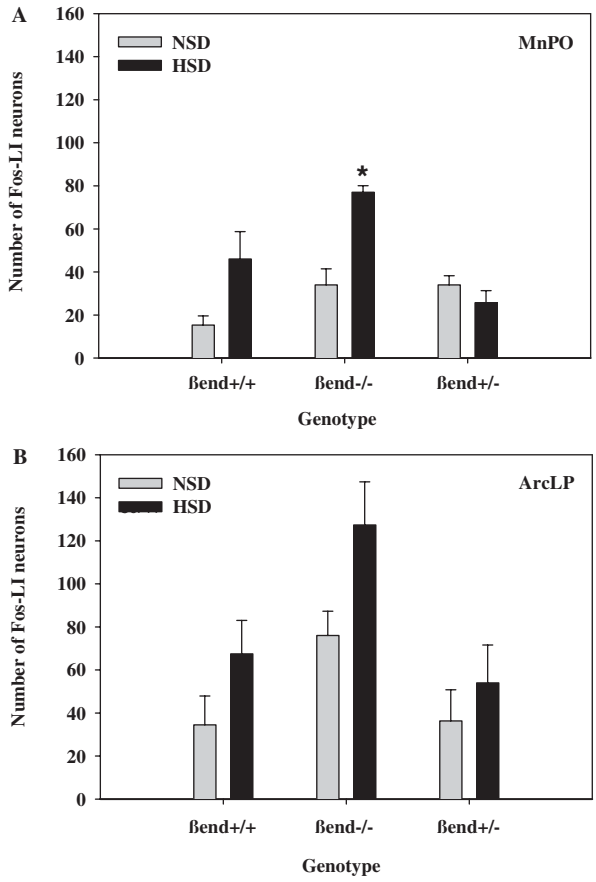

Fig. 3. Effect of dietary salt on brain activity in conscious βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ mice. Bar graphs indicate the number of Fos-LI neurons in the MnPO, median preoptic nucleus (A; 0.50 mm from the bregma) and ArcLP, arcuate nucleus, lateroposterior section (B; −2.54 mm from the bregma) of mice of the three genetic lines after being kept during 16 consecutive days on either a HSD or a NSD. Gray bars: NSD group, black bars: HSD group. The analysis of the number of Fos-LI neurons in the MnPO showed a significant double interaction between genotype and diet {$F(2,13)=7.05$, ($P<0.01$)}. * $P<0.05$. Data are presented as means±S.E., n=4 mice/group.

Table 2. Number of Fos-LI neurons in βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ mice maintained on NSD or HSD during 16 consecutive days

Brain nucleus	Effect	NSD			HSD		
		βend$^{+/+}$	βend$^{-/-}$	βend$^{+/-}$	βend$^{+/+}$	βend$^{-/-}$	βend$^{+/-}$
VOLT	*d*	1±1	21±13	15±7	37±19	48±19	27±24
SO	*d*	1±1	4±2	0±0	43±27	91±28	28±13
PVN-p	*d*	33±5	47±9	37±11	46±14	81±9	61±22
ArcMP	*d*	16±5	27±4	16±6	38±3	41±12	54±18
NTS	*d*	44±17	84±12	65±32	110±41	165±28	80±14
DRD-DRV	*g*	30±11	130±67	41±16	61±3	97±23	70±27
LPB	*g*	148±26	279±34	141±62	217±32	273±55	131±22

Values are mean±S.E., n=4/group. ArcMP, median posterior arcuate nucleus (distance from the bregma [db]: −2.54 mm); *d*, main effect of diet; DR, dorsal raphe nucleus (db: −4.6 mm); DRD, dorsal part of the dorsal raphe nucleus; DRV, ventral part of the dorsal raphe nucleus; *g*, main effect of genotype; LPB, lateral parabrachial nucleus (db: −5.2 mm); NTS, nucleus of the solitari tract (db: −7.48 mm); PVN-p, posterior paraventricular hypothalamic nucleus (db: −1.06 mm); SO, supraoptic nucleus (db: −0.82 mm); VOLT, vascular organ of the lamina terminalis (db: 0.50 mm).

The analysis showed a main effect of diet on urinary creatinine levels ($P<0.05$) and the LSD post hoc test revealed that regardless of the genotype, mice on a HSD showed increased urinary creatinine levels compared with animals on a NSD.

DISCUSSION

Our results indicate that the β-endorphinergic system is involved in the regulatory response to sodium overload, modulating BP, EPI levels and CNS activity. The fact that only a total deficit of β-endorphin, and not a partial one alters BP regulatory mechanism induced by sodium overload, suggests that β-endorphin is one among other factors working simultaneously to modulate target systems involved in the cardiovascular homeostasis. These results are consistent with previous evidence showing that under sodium overload lower levels of brain and peripheral POMC and β-endorphin have been found in hypertensive strains, Dahl S and SHR rats, as well as in a significant number of patients with essential hypertension (Felder and Garland, 1989; Zheng et al., 1995; Hao and Rabkin, 1996).

It is important to note that, as well as in our animal model of study, in these strains of salt-sensitive rats (Dahl S and SHR) a significant increase has been reported in neural activity within the MnPO when animals were placed on a HSD (Budzikowski and Leenen, 1997). Increased neural activity was observed within the MnPO of Dahl S rats fed with a HSD during the onset of hypertension (Budzikowski et al., 1998), and lesion of this nucleus during this period prevented or reversed the increase in BP (Johnson and Loewy, 1992).

Table 3. Baseline urinary catecholamine and creatinine levels in βend$^{+/+}$, βend$^{-/-}$ and βend$^{+/-}$ adult mice

Parameter	βend$^{+/+}$	βend$^{-/-}$	βend$^{+/-}$
EPI (nmol/mg Cr/24 h)	0.4±0.1	0.6±0.1	0.5±0.1
NE (nmol/mg Cr/24 h)	8.4±1.1	10.2±2.2	7.5±1.1
DA (nmol/mg Cr/24 h)	12.3±1.6	13.5±3.3	7.6±1.7
Cr (mg/24 h)	0.5±0.1	0.5±0.1	0.5±0.1

Values are mean±S.E., n=10/group. Cr, creatinine.

Although the sodium overload–induced increase in BP has often been attributed to renal pressor mechanisms associated with body sodium retention, in the present experiments no differences were observed in sodium balance between mice of the three genotypes on a HSD; suggesting that changes in BP may involve a different mechanism.

Over the past decade, there has been an increasing awareness that the CNS has a critical role in the development and maintenance of elevated arterial pressure. Dietary sodium overload normally triggers regulatory responses that involve the enhancement of central sympathoinhibitory mechanisms and the inhibition of the sympathoexcitatory ones, which results in a maintenance of normal sympathetic activity and BP. Reis and Talman (1984) suggested that hypertension may result from an imbalance in the interaction between the central neural networks that serve to excite sympathetic vasomotor neurons and those that inhibit them, with the imbalance favoring sympathetic discharge.

An important number of studies have reported that β-endorphinergic projections from the arcuate nucleus are present in nuclei involved in cardiovascular regulation. Anatomical studies demonstrated that the arcuate neurons send β-endorphin projections to the MnPO, PVN, NTS and rostral ventrolateral medulla (RVLM) among others (van der Kooy et al., 1984; Chu et al., 1991; Baker and Herkenham, 1995; Kawano and Masuko, 2000), where they may act to enhance the sympathoinhibitory or decrease the sympathoexcitatory mechanisms, leading to normalize sympathetic activity during a chronic high-sodium intake. In particular, within the NTS and the RVLM, β-endorphin has been reported to have a hypotensive effect (Mastrianni et al., 1989; Li et al., 1996; Ku and Chang, 2001).

In agreement with our findings, it has also been shown that during normal conditions of BP regulation, sodium overload produces a decrease in tyrosine hydroxylase and β-DA hydroxylase in the adrenal gland and an increase in β-endorphin and POMC mRNA levels in the pituitary gland (Saavedra et al., 1983; Mayan et al., 1996). However, in Dahl S and SHR rats, sodium overload induces an unexpected increase in sympathoexcitatory and an attenuated

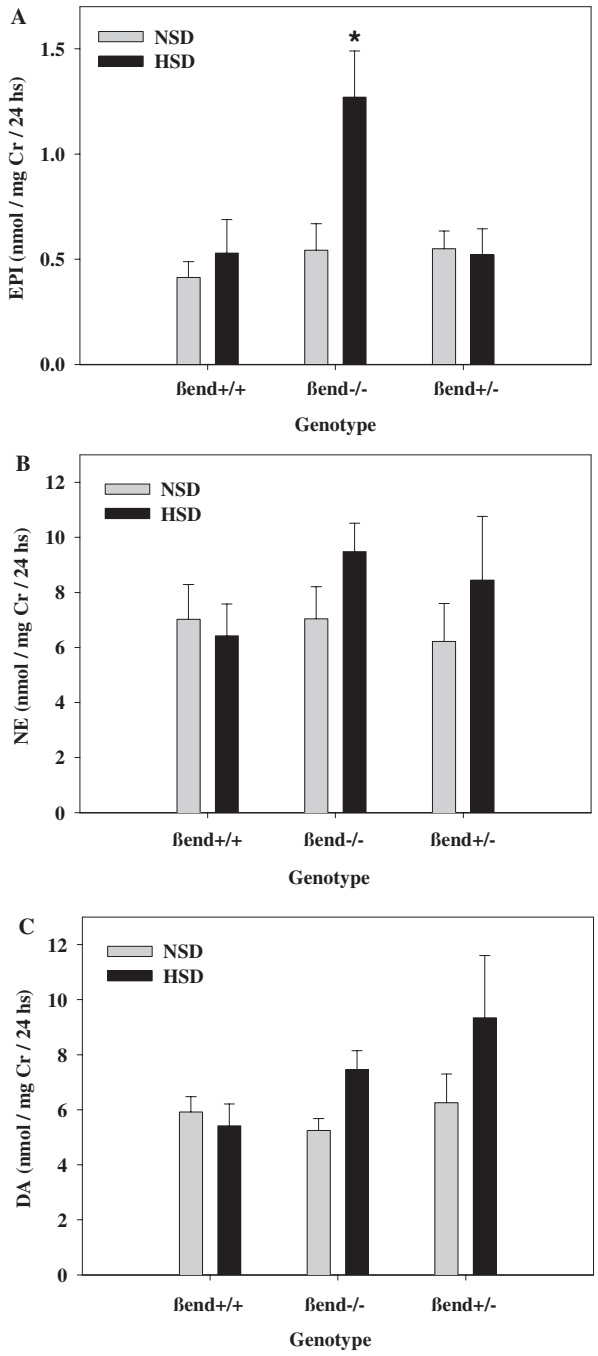

Fig. 4. Effect of dietary salt on urinary EPI, NE and DA excretion in conscious $\beta end^{+/+}$, $\beta end^{-/-}$ and $\beta end^{+/-}$ mice. Bar graphs show the urinary levels of EPI (A), NE (B) and DA (C) in mice after being kept during two weeks on either NSD or HSD. Gray bars: NSD group, black bars: HSD group. The statistical analysis of urinary EPI excretion showed a double interaction between genotype and diet {$F(2,45)=4.88$, $P<0.05$}; * $P<0.05$. Data are presented as means±S.E., $n=10$ mice/group.

sympathoinhibitory mechanism, which leads to an increase in BP (Huang and Leenen, 1994, 1996). In Dahl S rats, higher levels of EPI and NE have been reported in the adrenal medulla (Saavedra et al., 1983) and decreased POMC mRNA levels in the pituitary gland (Hao and Rabkin, 1996). On the other hand, several studies have indicated that patients with essential hypertension show an increased secretion of EPI from the adrenal gland (Jacobs et al., 1997) and, as mentioned earlier, lower levels of plasmatic β-endorphin (Zheng et al., 1995).

Likewise, $\beta end^{-/-}$ mice that were kept for two weeks on a HSD showed an increase in urinary EPI excretion compared with $\beta end^{+/+}$ and $\beta end^{+/-}$ mice even though the baseline levels of catecholamine excretions were similar in animals of the three genetic lines. Our preliminary results after one week of either NSD or HSD (data not shown) indicate that, independent of the genotype ($\beta end^{+/+}$ or $\beta end^{-/-}$), mice that were kept on a HSD showed an increase in urinary EPI levels, but as mentioned above, only $\beta end^{+/+}$ mice reached baseline EPI levels after 2 weeks of HSD. Conversely, β-endorphin mutant mice ($\beta end^{-/-}$) that showed an increase in SBP after 2 weeks of HSD, did not show the decrease in urinary EPI levels reported for β-endorphin control animals. Taking into account numerous studies indicating that sympathetic activity is decreased under conditions of chronic salt loading (Leenen et al., 2002; Brooks et al., 2005), our results show that this chronic sympathoinhibitory response to increased dietary salt is not observed in β-endorphin knockout mice, suggesting that a failure to suppress sympathetic activity in response to increased salt may be a pathogenic factor in this model.

β-Endorphin is synthesized and stored in corticotrophin cells of the pituitary gland (Li et al., 1976; Bloom et al., 1978) and is released in response to exogenous administration of EPI and clonidine (α2-adrenergic agonist), suggesting that the catecholaminergic system may be regulating the release of β-endorphin from the pituitary gland (Berkenbosch et al., 1981; Pettibone and Mueller, 1981). On the other hand, as has been shown by "*in vitro*" and "*in vivo*" experiments, the release of catecholamines from the adrenal medulla, in particular EPI release, can be modulated by the opioid system. *In vitro* studies indicate that catecholamine secretion from the adrenal medulla is decreased in the presence of mu-opioid agonists (Kitamura et al., 2002), and *in vivo* studies show how the administration of morphine in intact animals produces a decrease in EPI secretion from the adrenal medulla (Kimura et al., 1988).

Consistent with our results and the abovementioned studies, clinical evidence shows that the administration of naloxone in patients addicted to opioids induces a 30-fold increase in plasma EPI whereas NE only increases to a minor extent (threefold increase), accompanied by a simultaneous rise in heart rate and BP (Kienbaum et al., 1998). However, when these patients are pretreated with clonidine, an antihypertensive drug that induces hypotension, in part due to an increase in β-endorphin levels (Pettibone and Mueller, 1981), no changes in sympathetic activity or in BP are registered when naloxone is injected (Kienbaum et al., 2002).

Taking into account our data and that reported by other authors, we speculate that in normal conditions of regulatory response to sodium overload, the β-endorphinergic

system may be participating, centrally and/or peripherally, in the decrease of sympathetic activity to prevent an increase in BP. Conversely, the absence of this compensatory mechanism in β-endorphin knockout mice may lead to the increase in both SBP and sympathetic activity. However, further studies will be needed to be able to estimate whether the β-endorphinergic system is involved in the inhibition of sympathetic nervous activity during sodium overload. For example, it would be important to correlate the urinary EPI changes observed in mice lacking β-endorphin with plasma and adrenal medulla catecholamine levels.

CONCLUSION

In summary, the increases in SBP, EPI urinary output and in the activation of the MnPO during sodium overload of animals lacking β-endorphin, present new data on the contribution of the β-endorphinergic system to the compensatory mechanisms triggered in response to sodium overload.

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