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Effects of Orexin-A and Orexin-B on Renal Sodium Handling

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Abstract: Orexin-A and orexin-B are peptides produced specifically by neurones located in the lateral hypothalamus, a region involved in the central regulation of feeding behaviour. Recent studies documented the presence of orexins and orexin receptors in peripheral tissues e.g., in the kidney. Therefore we examined the influence of exogenous orexin-A and orexin-B on renal function. The natriuresis, diuresis and creatinine clearance were analysed after intraperitoneally administration of orexins. The study investigated the possible mechanism through which orexin affects renal sodium sparing. For this reason, we measured the Na^+, K^+ -ATPase activity in renal cortex and medulla after locally infusion of orexin-A through the catheter inserted into the abdominal aorta close to the renal arteries. The study was performed on male Wistar rats. Orexin-A and orexin-B were given i.p. in two doses: 2 nmol rat^{-1} or 10 nmol rat^{-1} . The conscious animals were placed in metabolic cages and the urinary collection was made after 2 h of orexin-A or orexin-B administration. The intra-arterial infusion of orexin-A (66.7 or $333.3 \text{ pmol min}^{-1}$) was performed 30 min on rats under anaesthesia. The urine to estimate diuresis and natriuresis was collected directly after the surgery. The Na^+, K^+ -ATPase activity was assayed in an isolated microsomal fraction of the renal cortex and medulla. The i.p. administration of orexin-A caused the decrease in natriuresis and fractional sodium extraction without change in glomerular filtration rate (GRF) estimated by calculating creatinine clearance. Neither low or high dose of orexin-B had effect on renal function. The Na^+, K^+ -ATPase activity was increased in renal cortex and medulla after infusion of orexin-A ($333.3 \text{ pmol min}^{-1}$) by 32.4 and 9.11%, respectively. These data indicate that orexin-A given i.p. can affect the renal sodium retention, probably by activating tubular sodium reabsorption mediated via an increase of Na^+, K^+ -ATPase activity.

Key words: orexin, natriuresis, creatinine clearance, Na^+, K^+ -ATPase

INTRODUCTION

Orexins are neuropeptides widely present in the brain structures, primarily in the lateral hypothalamus. These peptides are involved in regulating various brain functions such as feeding and sleeping (Sakurai *et al.*, 1998; Chemelli *et al.*, 1999). Two orexins were identified; orexin-A (a 33 amino acid peptide) and orexin-B (a 28 amino acid peptide), both of which are derived from the same precursor prepro-orexin by proteolytic processing (Sakurai *et al.*, 1998). Two different orexin receptors have been described, the orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), which belong to the G - protein coupled receptor family (Sakurai *et al.*, 1998; Katugampola and Davenport, 2003). Orexin-A has equal affinity to OX1R and OX2R whilst orexin-B has approximately 10-fold greater affinity to OX2R than to OX1R (Sakurai *et al.*, 1998).

Recent studies have documented that the orexin receptors and orexin immunoreactive cells are also distributed outside the brain. mRNA expression for prepro-orexin and orexin receptors are detected in various of peripheral organs such as adrenal gland, pancreas, stomach, heart and kidney (Voisin *et al.*, 2003;

Nakabayashi *et al.*, 2003). Moreover, in human and rat orexin-A was detected in plasma (Arihara *et al.*, 2001; Dalal *et al.*, 2001; Jöhren *et al.*, 2001).

Since cells outside the central nervous system express orexin receptors and orexin is detected in the plasma, these findings support the existence of a peripheral orexin system.

The orexin receptors have been shown in the kidney (Voisin *et al.*, 2003). However, to date there have been no reports indicating whether the orexin affects the regulation of renal function. The present study, therefore, was aimed at examining the effects of orexin-A and orexin-B on the sodium handling. For this reason the natriuresis and creatinine clearance were measured after i.p. administration of orexin-A and orexin-B. To explain the possible mechanisms mediating orexin effects in the kidney the activity of Na^+, K^+ -ATPase was assayed.

MATERIALS AND METHODS

Male Wistar rats with body weight 300-350 g were used for the study. The animals were maintained on a 12 h light-dark cycle in a temperature controlled

environment ($20\pm 2^{\circ}\text{C}$) with food and tap water available *ad libitum* for 2 weeks before being assigned to experimental protocols. All procedures were in accordance with the guidelines of the University Animal Research Committee.

The intraperitoneally administration of orexins: The study was performed on conscious rats, placed individually in metabolic cages. Rats were classified into five groups, each containing 8 rats. Orexin-A and orexin-B (Bachem, CH) were given intraperitoneally (i.p.) in two doses (2.0 or $10.0\text{ nmol rat}^{-1}$) dissolved in 0.5 mL 0.9% NaCl. Control group was given saline without orexin.

Two hours before (adaptation period) and after orexins or saline administration (experimental period) the urine collection was made. To avoid urine contamination, food was not available during urine collection. Urine was centrifuged to remove particulate matter, frozen and stored at -70°C until analysis.

The animals were anaesthetised with pentobarbital (50 mg kg^{-1} i.p.) at 2 h after orexins/saline treatment and Systolic Blood Pressure (SBP) and heart rate were measured using the tail-cuff method. Then, the blood for biochemical analysis was taken from abdominal aorta into tubes containing EDTA. Plasma was frozen and stored at -70°C .

Biochemical studies: Creatinine in plasma and urine was assayed colorimetrically using Sigma Diagnostics kit (Sigma-Aldrich, St. Louis, MO, USA). Sodium concentrations in plasma and urine were measured by flame photometry. Glomerular filtration rate (GFR) was estimated by calculating creatinine clearance. Fractional excretion of Na^{+} was counted as the ratio between urinary excretion and the amount filtered ($\text{GFR}\times\text{plasma concentration}$).

Infusion of orexin-A into abdominal aorta: The rats were anaesthetised with pentobarbital (50 mg kg^{-1} i.p.) and the thin catheter was placed through the femoral artery into the abdominal aorta close to the renal arteries. After the surgery, the infusion of physiological saline was started at the rate of $66\text{ }\mu\text{L min}^{-1}$ for 30 min (stabilisation period). Then, the solution of orexin-A in 0.9% saline was infused ($66\text{ }\mu\text{L min}^{-1}$, though 30 min) at the total dose 2 nmol rat^{-1} and 10 nmol rat^{-1} (66.7 and $333.3\text{ pmol min}^{-1}$). The control animals received 0.9% NaCl during the whole experiment (60 min). Immediately after orexin-A infusion, 5 mL of physiological saline was infused through a catheter to remove erythrocytes from the kidney. The kidneys were removed and placed on the ice.

During the experiment the urine was taken directly from urinary bladder: 1) before saline infusion, 2) after saline infusion (adaptation period), 3) at the end of

orexin-A infusion (experimental period). The urine was centrifuged and stored in -70°C .

The microsomal fraction of the renal cortex and medulla was isolated by method of Jorgensen (Jorgensen, 1974) with slight modification. The medulla and cortex were separated by dissection and homogenised using glass homogenizer in sucrose-histidine solution (10 mL g^{-1} tissue) containing 0.25 M sucrose and 0.03 M histidine (pH 7.2). The homogenate was centrifuged at $8000\times g$ for 20 min at 4°C . The sediment was resuspended in 6 mL of sucrose-histidine solution and centrifuged again. The combined supernatants from two centrifugations were centrifuged at $50000\times g$ for 30 min at 4°C . The pellet (microsomal fraction) was resuspended in 4 mL of sucrose-histidine solution and stored in -70°C .

Na^{+} , K^{+} -ATPase assay: ATP-ase activity was assayed by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of the microsomal fraction at 37°C . The assay medium (1 mL) contained: 100 mM NaCl, 20 mM KCl, 4 mM MgCl_2 , 40 mM Tris-HCl (pH 7.1) and $50\text{ }\mu\text{g}$ of microsomal protein. Preincubation was carried out for 10 min and then, 3 m MATP was added. After 15 min of incubation time, the enzymatic reaction was terminated by adding 0.35 mL of ice-cold 1 N HClO_4 . Then inorganic phosphate was assayed by the spectrophotometric method of Hurst (Hurst, 1964). According to this method, 0.2 mL of the incubation medium was diluted with water to 3 and 0.6 mL of sodium molybdate reagent (0.31 M Na_2MoO_4 in 3.0 N H_2SO_4) was added to form yellow phosphomolybdate complex which was subsequently reduced to molybdenum blue by adding 0.6 mL of stannous chloride-hydrazine sulphate reagent containing SnCl_2 ($1.3\text{ }\mu\text{M}$), hydrazine sulphate (0.023 M) and H_2SO_4 (1.0 N). Absorbance was read after 20 min at 700 nm . Na^{+} , K^{+} -ATPase activity (ouabain-sensitive fraction) was calculated as difference between total ATPase (assayed in the absence of ouabain) and ouabain-resistant fraction, assayed in the presence of 1 mM ouabain. ATPase activity was expressed in $\mu\text{mol Pi}$ liberated from ATP by 1 mg of microsomal protein during 1 h ($\mu\text{mol Pi/mg protein/h}$). Protein was assayed by the method of Lowery *et al* (1951) using bovine serum albumin as a standard.

Statistical analysis: Data are presented as the mean $\pm$ SEM. The results obtained in control and orexins treated groups were compared by ANOVA followed by Student's t-test for unrelated variables. Values were considered statistically significant when $p<0.05$.

RESULTS

The systolic arterial pressure was significantly reduced after orexin-A (10 nmol rat^{-1} i.p.) by 26.15%

Table 1: The cardiovascular effects of orexin-A and orexin-B after i.p. administration

Parameters	NaCl	OXA (2 nmol)	OXA (10 nmol)	OXB (2 nmol)	OXB (10 nmol)
Blood pressure (mm Hg)	121.8±39.2	92±27.5	90±20.6*	92.4±24.1	89.4±25.9
Heart rate (beats/min)	371.7±27.9	383±40.1	413±37.5	388.9±38.5	369±50.8

Values represent mean ± SEM. *p<0.05 compared to saline administration by ANOVA and Student's t-test

Table 2: The effects of orexin-A and orexin-B on fractional extraction of sodium (FENa⁺) and urine output after i.p. administration

Parameters	NaCl	OXA (2 nmol)	OXA (10 nmol)	OXB (2 nmol)	OXB (10 nmol)
FENa ⁺ (%)	1.069±0.261	0.520±0.142	0.495±0.073*	1.102±0.321	0.948±0.299
Diuresis (mL h ⁻¹)	0.884±0.332	0.650±0.143	0.541±0.163	0.703±0.179	0.414±0.169

Values represent mean ± SEM. *p<0.05 compared to saline administration by ANOVA and Student's t-test

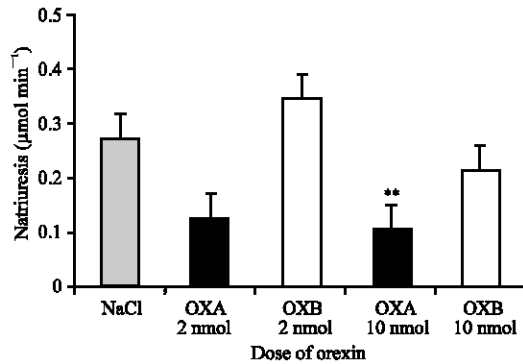


Fig. 1: The effect of orexin-A and orexin-B on urinary sodium extraction. Orexin-A and orexin-B were administered i.p. at two doses (10 or 2 nmol per rat). Control group received 0.9% NaCl. Results are expressed as the means±SEM. **p<0.01 compared with saline administration by ANOVA and Student's t-test

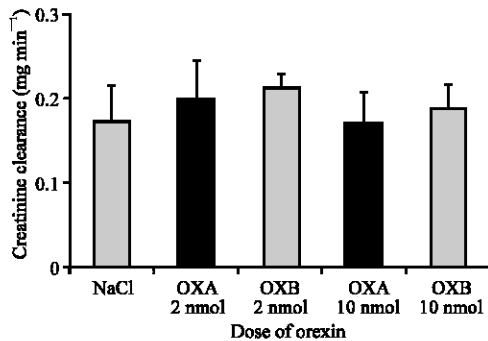


Fig. 2: The effect of orexin-A and orexin-B on creatinine clearance. Orexin-A and orexin-B were administered i.p. at two doses (10 or 2 nmol per rat). Control group received 0.9% NaCl. Results are expressed as the means±SEM. The statistical analysis was performed by ANOVA followed by Student's t-test. There were no significant differences compared with control

(p<0.05). Following the injection of orexin-B the arterial blood pressure tended to be lower, although not significantly. The heart rate did not change significantly after orexin-A and orexin-B administration (Table 1).

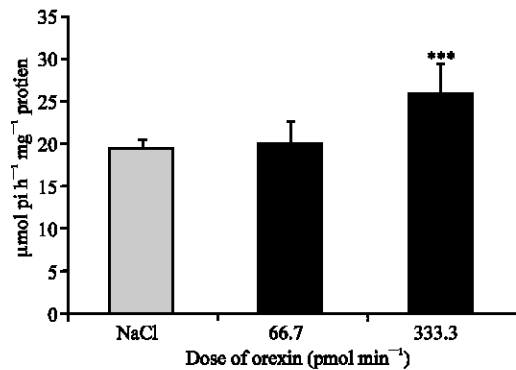


Fig. 3: The effect of orexin-A on Na⁺,K⁺-ATPase activity in renal cortex. Orexin-A was infused for 30 min at the dose 66.7 pmol min and 333.3 pmol min. Orexin-A infusion was preceded by 30 min period of 0.9% NaCl infusion. Enzyme activity is expressed in μmol of inorganic phosphate liberated by the enzyme contained in 1mg of microsomal protein during 1 hour. The values represent means±SEM. ***p<0.001 compared with saline administration by ANOVA and Student's t-test

After the administration of the high dose of orexin-A (10 nmol rat⁻¹ i.p.) natriuresis was markedly decreased (by 61.3%, p<0.001) in comparison with the control response to saline. Both doses of orexin-B did not change significantly the sodium output (Fig. 1). In animals receiving orexin A (10 nmol rat⁻¹ i.p.) the fractional extraction of sodium was statistically significantly lowered (by 77.1%, p<0.05). Orexin-B at the high dose decrease the fractional sodium extraction but the difference was not statistically significant (Table 2).

In rats receiving orexin-A or orexin-B the urine output, compared with control, tended to be reduced but these differences were not significant (Table 2). There were no statistical differences in the creatinine clearance after orexin-A and orexin-B administration (Fig. 2).

The Na⁺, K⁺-ATPase activity was increased in renal cortex and medulla after infusion of high dose of orexin-A (10 nmol; 333.3 pmol min⁻¹) by 32.4%, p<0.001 and 9.11%, p<0.05, respectively (Fig.3 and 4). At the low dose,

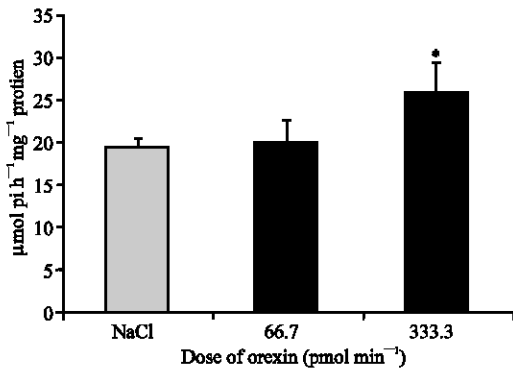


Fig. 4: The effect of orexin-A on Na⁺, K⁺-ATPase activity in renal medulla. Orexin-A was infused for 30 min at the dose 66.7 and 333.3 pmol min⁻¹. Orexin-A infusion was preceded by 30 min period of 0.9% NaCl infusion. Control animals received 0.9% NaCl for 60 min. Enzyme activity is expressed in µmol of inorganic phosphate liberated by the enzyme contained in 1mg of microsomal protein during 1 h. The values represent means±SEM. *p<0.05 compared with saline administration by ANOVA and Student's t-test

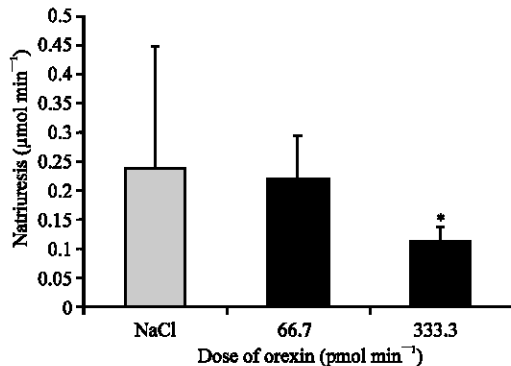


Fig. 5: The effect of orexin-A infusion on urinary sodium extraction. Orexin-A was infused for 30 min at the dose 66.7 and 333.3 pmol min⁻¹. Orexin-A infusion was preceded by 30 min period of 0.9% NaCl infusion. Control animals received 0.9% NaCl for 60 min. Results are expressed as the means±SEM. *p<0.05 compared with saline administration by ANOVA and Student's t-test

orexin-A did not stimulate Na⁺, K⁺-ATPase in cortex as well as in renal medulla. The orexin-A infusion (333.3 pmol L⁻¹) decreased the natriuresis by 46.1%, p<0.05 (Fig. 5). Neither high or low dose of orexin-A had significant effect on urine extraction (Fig. 6).

DISCUSSION

The major new finding of the present study is that peripheral administration of orexin-A decreased

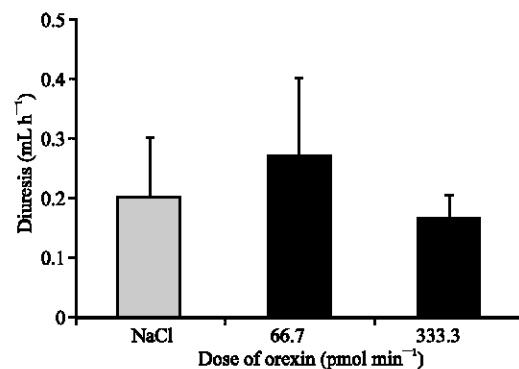


Fig. 6: The effect of orexin-A infusion on urine output. Orexin-A was infused for 30 min at the dose 66.7 and 333.3 pmol min⁻¹. Orexin-A infusion was preceded by 30 min period of 0.9% NaCl infusion. Control animals received 0.9% NaCl for 60 min. Results are expressed as the means±SEM. The statistical analysis was performed by ANOVA followed by Student's t-test. There were no significant differences in the urine output compared with saline infusion

natriuresis and FENa⁺ without affecting creatinine clearance indicating that orexin-A stimulates tubular Na⁺ reabsorption. The increase of Na⁺, K⁺-ATPase activity after locally infusion of orexin-A suggests that this enzyme is (at least in part) the mediator of orexin action on sodium sparing.

At the present time the mechanisms mediating orexin effects on sodium balance are not characterised. Although, there are many possible links between the orexin system and renal function.

First, since the mRNA for orexin receptors (OX1R) and the mRNA expression for prepro-orexin were detected in the kidney (Jöhren *et al.*, 2001; Nakabayashi *et al.*, 2003; Voisin *et al.*, 2003) it is likely that orexin directly acts on renal function. Our results indicate that orexin-A given into abdominal aorta close to the renal arteries is able to increase Na⁺, K⁺-ATPase activity in renal cortex and medulla. Na⁺, K⁺-ATPase plays the pivotal role for sodium reabsorption in all tubular segments. The activity of this enzyme is regulated by phosphorylation and dephosphorylation which can be mediated by adenylate cyclase/protein kinase A (PKA) or phospholipase C/protein kinase C (PKC)-dependent cascade (Feraille and Doucet, 2001; Beltowski *et al.*, 2002). Many hormones and peptides stimulating G-protein-coupled receptors, such as catecholamines, angiotensin, are able to stimulate these signalling pathways and modulate sodium absorption (Holmqvist *et al.*, 2005; Yang *et al.*, 2003; Lund *et al.*, 2000). As OX1R is linked to G-protein and the biological actions of orexins in many tissue can be mediated by the cAMP/PKA or PLC/PKC

pathways (Mazzocchi *et al.*, 2001; Nanmoku *et al.*, 2000; Randeva *et al.*, 2001; Malendowicz *et al.*, 1999), it might be possible, that orexin like other hormones activating G-protein coupled receptors can be involved in regulation of sodium pump activity.

Second, the observed reduction of systolic blood pressure after orexin-A administration (Table 1) could be responsible for impaired pressor natriuresis. In our study high dose of orexin A administered i.p. caused slight, but statistically significant decrease in systolic blood pressure without change in heart rate. This result is in agreement with the recent studies show rather elevation of blood pressure than the reduction. This difference in results may be explained by differences in experimental design. In the earlier study orexin was usually administered centrally (intracerebroventricular or intracisternal). Moreover, the doses of orexins used in these studies were lower. Available data show that the intracerebroventricular or intracisternal administration of orexin A or B increases mean arterial pressure (MAP), heart rate (HR), renal sympathetic nerve activity (RNSA) and elevates plasma level of norepinephrine and epinephrine in conscious rat (Shirasaka *et al.*, 1999; Matsumura *et al.*, 2001) as well as in anaesthetised rat (Chen *et al.*, 2000).

On the other hand, the microinjection of orexin-A into the nucleus of the solitary tract of the rat elicits a dose-dependent depressor and bradycardia response, mediated by activation of vagal cardiomotor neurones (de Oliveira *et al.*, 2003; Ciriello and de Oliveira, 2003). Thus, the effects following central injection of orexin seem to be site-specific and orexin can stimulate various structures acting antagonistically in CNS. Furthermore, it was demonstrated that plasma orexin-A (but not orexin-B) can rapidly cross the blood-brain barrier (Kastin and Akerstrom, 1999). Therefore, it is possible that orexin given peripherally could affect autonomic system via central mechanism. Nevertheless, recently it has been described that intravenous injection of both orexin-A and B was without significant effect on blood pressure and heart rate in anaesthetised (Chen *et al.*, 2000) and also in the conscious animals (Matsumura *et al.*, 2001). It seems that acute systemic administration of orexin induces plasma levels that are too low to influence the level of MAP and HR mediated by CNS. Also, it is likely, that orexins given peripherally can activate only some parts of autonomic nervous system. This speculation could be confirmed by the results described by Shirasaka *et al.* (1999) studying the cardiovascular and sympathetic responses produced by the central administration of orexins in conscious rats. They demonstrated that orexin-A at lower doses only selectively causes the activation of RNSA without the increase in plasma epinephrine and norepinephrine levels

(Shirasaka *et al.*, 1999). It should be noted that norepinephrine is one of the potent factors stimulating the renal tubular sodium reabsorption and Na^+ , K^+ -ATPase activity in the kidney.

It is believed that orexin besides autonomic system can also modulate function of endocrine system. Orexin is able to influence the release of hormones participating in electrolyte and water homeostasis such as catecholamines, steroid hormones, vasopressin and insulin. These hormones are well-known activators of Na^+ , K^+ -ATPase (Feraille and Doucet, 2001; Aperia *et al.*, 1994). For example, two orexin receptors OX1R and OX2R, as well as the prepro-orexin and its cleaved product orexin-A were detected in the rat adrenal gland (Lopez *et al.*, 1999) indicating that this gland is a target tissue for orexins (Malendowicz *et al.*, 1999; Malendowicz *et al.*, 2001; Mazzocchi *et al.*, 2001; Nanmoku *et al.*, 2000; Randeva *et al.*, 2001). Moreover, orexin A can increase the release from adrenal-cortex cells of both cortisol and aldosterone, the known antinatriuretic factors (Malendowicz *et al.*, 2001; Nanmoku *et al.*, 2002; Kumi *et al.*, 1999). Regarding the functional studies of OXRs in the adrenal medulla, there are conflicting reports showing the effects of orexins on catecholamine secretion. Some authors showed the stimulatory (Nanmoku *et al.*, 2002) other the inhibitory effect of orexins on catecholamine synthesis (Imashuku *et al.*, 1975) and secretion (Kawada *et al.*, 2003; Nanmoku *et al.*, 2000).

Finally, another interesting thing is the interaction between orexin and pancreas hormones. Insulin as well as glucagon are considered to be involved in sodium handling (Feraille and Doucet, 2001; Feraille *et al.*, 1999). The recent studies have shown that the endocrine cells in the pancreas, secreting glucagon or insulin, contain orexin A and express orexin receptors. Moreover, orexin-A can affect the release of these hormones (Kirchgessner and Liu, 1999; Ouedraogo *et al.*, 2003). It has been reported that plasma insulin in the rats (having free access to food) increased after subcutaneous injection of both orexin A and orexin B (Nowak *et al.*, 2000; Switonska *et al.*, 2002). Also *in vitro* experiments have shown that orexins stimulate insulin secretion (Nowak *et al.*, 2000). On the other hand, it has been shown that in rat during fasting condition after intravenous administration of orexin the plasma level of insulin either decreased (Ouedraogo *et al.*, 2003) or remained unaffected (Ehrstrom *et al.*, 2004).

To the best of our knowledge, this is the first study to demonstrate the influence of orexin-A and orexin-B on renal function. It seems that orexin effects on renal function may be direct, mediated by orexin per se through specific receptors or orexin may act indirectly by modulating other hormones secretion.

Sodium extraction is one of the important processes involved in the regulation of blood pressure. Nowadays it is known that some metabolic disorders such as obesity are associated with cardiovascular diseases e.g. hypertension. Since orexin and other peptides, participating in the appetite and feeding behaviour, can to interact with each other to regulate both appetite and energy balance as well as cardiovascular system, they may play a key role in the association between obesity and hypertension. Therefore, in order to prevent cardiovascular disease, it seems important to determine the mechanisms regulating feeding behaviour as well as water and electrolyte balance.

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