ORIGINAL ARTICLE

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In vivo investigations of neurotensin receptors in adipocytes, hepatocytes and enterocytes of rat

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Abstract

Introduction: Atherosclerotic vascular disease is currently the biggest threat and the highest cause of death worldwide, approaching almost 60%.The development of atherosclerosis is affected by ecological factors associated with industry and pollution of the environment. Neurotensin (NT) is a peptide acting via 3 kinds of neurotensin receptors (NTR) localized in target tissues. In several studies, the presence of its receptors has been shown in chicken liver, and the influence of NT on the metabolism of this organ was confirmed (glycogenolysis stimulation through sympathetic nervous system, entero-hepatic circulation of bile acids, metabolism of lipoproteins).

Materials and methods: Healthy male WISTAR rats weighing 300±30 grams, were used for the experiments. The animals were divided into 4 groups: 1) control group, to which 0.9% NaCl was administrated (i.p.); 2) the second group was given levocabastine 1mg/kg i.p.; 3) the third group received SR 48692 0.4mg/; 4) the fourth group was given NT analog [D-Trp 11]-neurotensin 15nM/kg. Plasmatic membranes of liver, small intestine and adipose tissue were prepared according to the method of Havrankova. Analysis of results obtained in the investigation of NT receptors was performed using the Scatchard method from LIGAND-Pc, v. 3.1 software.

Results: The investigation of antigenity of I^{125}NT showed proper antigen-antibody reaction. No binding of the I^{125}NT with plasmatic membranes of adipocytes or enterocytes was observed. Unspecific binding of I^{125}NT with 10 µmol/L of free NT was observed in the plasmatic membranes of hepatocytes.

Conclusion: The presence of NT receptors only in the membranes of hepatocytes may suggest their role in the regulation of lipid metabolism via receptor – ligand way.

Key words

neurotensin receptors, liver, adipose tissue, small intestine

INTRODUCTION

Atherosclerotic vascular disease is currently the biggest threat and the highest cause of death worldwide, approaching almost 60%.The development of atherosclerosis is affected by ecological factors associated with industry and pollution of the environment. Uncontrolled development of industry, including energy, communications and the service sector, as well as the wide use of pesticides in agriculture, is causing toxic pollution of the air, water, soil and food. Chemical toxic substances enter the environment as gaseous or liquid substances. Pollution of the environment is regarded as the cause of a number of diseases, including atherosclerosis, causing a deterioration of the health status of the population by as much as 5-10%.

The disturbance in the area of the social environment known as the psychosocial stress could be distinguished as an independent risk factor for many diseases, including the diseases of the circulatory system. There are a number of well known endogenic factors regulating lipid metabolism which can affect the development of atherosclerosis. In our previous studies we were able to show that NT administered intra-peritoneally also influenced lipid metabolism in rats [1,2].

Neurotensin (NT) is a 13 amino acids containing the peptide-playing a role of neuromodulator in the central nervous system. This substance also shows paracrine and endocrine activities in the whole organism [3]. On the cellular level, NT stimulates production of cyclic guanosine-3,5-monophosphate (cGMP) [4], phosphatidylinositol (IP) [5] and cyclic adenosine-3,5-monophosphate (cAMP) [6]. To date, 3 kinds of receptors for NT have been identified: NTR1, NTR2 and NTR3. All 3 kinds of receptors bind NT via C-terminal hexapeptide of known sequence [7]. NTR1 belongs to the G family of receptors and shows high affinity to NT. A specific blocker of NTR1 is a non-peptide – SR 48692. Several known biologic effects of NT are blocked by SR 48692. In the future, perhaps this agent could be used in the therapy of schizophrenia or Parkinson's disease, combined with the activity of NTR1 [8-10] or malignant disorders [11, 12]. The second type of NT receptor (NTR2) is a low affinity receptor, and this specifically blocking substance is an antagonist of

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the H1 histamine receptor – levocabastin. The presence of NTR3 receptor has been shown not only on neurons of the central nervous system, but also on cells of prostate cancer, skeletal muscles, cardiac muscle and adipocytes, and its functions are being studied at present [13]. It has been shown that NT was also present in the alimentary tract of birds and mammals [3,14-16]. A high concentration of NT has also been described in the portal vein of rats and chickens, and chicken liver is an organ rich in high affinity receptors for NT [17].

In our previous studies, we were able to show that NT administered intra-peritoneally influenced lipid metabolism in rats [1,2].

MATERIALS AND METHODS

Healthy male Wistar rats weighing 300±30 grams, were used for the experiments. The research schedule was accepted by the local Ethical Committee. For the investigation, the animals were divided into 4 groups, each consisting of 3 animals. NT receptors research was conducted on 4 groups of rats:

1) control group to which 0.9% NaCl was administrated (i.p.);
2) the second group was given levocabastine 1mg/kg i.p. (courtesy of Jansen-Cilag USA);
3) the third group received SR 48692 0.4mg/kg (kindly provided by Sanofi Recherche, France);
4) the fourth group was given NT analog [D-Trp 11]-neurotensin 15nM/kg (Sigma- Aldrich, USA).

The animals were fed with LSM type standard food and given water *ad libitum*. Rats were kept in an air-conditioned room, at a temperature of 22±2°C, with the day-night cycle of 12/12 hours. All rats were next anesthetized with Thiopental (Biochemie GmbH, Austria), 25-35 mg/kg of body mass, and the time from intra-peritoneally administration to complete anesthesia was 5±2 minutes. After anesthesia and section, tissue samples were immediately frozen in liquid nitrogen and kept at -80°C until investigation.

Isolation of plasmatic membranes fraction. Plasmatic membranes of liver, small intestine and adipose tissue were prepared according to the method of Havrankova [18]. The tissue was homogenized in the proportion of 1 gram per 20 ml of 0.001M $NaHCO_3$. Homogenates were centrifuged at 600g for 30 minutes. Supernatants were centrifuged at 20,000g for 30 minutes. The pellets were then washed twice with 0.001M $NaHCO_3$. After measurement of membrane protein, the samples were centrifuged again at 20,000g for 30 minutes. The pellets containing plasmatic membranes were suspended in a constant volume of incubation buffer: 0.04M Tris-HCl, pH 7,4, containing 0.1% of bovine serum albumin (BSA). All procedures of membrane preparation were conducted at +4°C. The chemicals used were provided by SIGMA Chemicals, USA.

Iodination of Neurotensin. Labeling was conducted in the Isotope Laboratory of the Endocrinology Clinic, University of Medical Sciences in Poznań, Poland. For labeling, 15 µg of NT were used. The loss of I^{125}NT was estimated for 3 µg and was caused by absorbance on the walls of the test tube, Sephadex column and micropipets. From the total amount, 12 µg of I^{125}NT were obtained, at the activity of 900 µCi. Thus, 1 µg of I^{125}NT had activity of 75 µCi. One nanogram of I^{125}NT gave 50,000 impulses per minute in the gamma counter.

The separation of I^{125}NT was conducted on a Sephadex column (Pharmacia, Sweden).

To confirm the biological activity of the labeled I^{125}NT, the reaction with antibody against NT was conducted. The reaction was detected with immunoblotting. As antigen, the labeled I^{125}NT was used, and polyclonal antibodies against NT, Neurotensin – C-19 (Santa Cruz Biotechnology Inc., USA). Antigen was run in the poliacrylamide gel and identified in Western blotting.

Statistical analysis of results. For each group of animals, the mean ± SD was calculated. Analysis was performed with Statistica 6.0 software. Analysis of results obtained in the investigation of NT receptors was performed using the Scatchard method from LIGAND-Pc, v. 3.1 software [19]. The figures were prepared in Statistica 6.0.

RESULTS

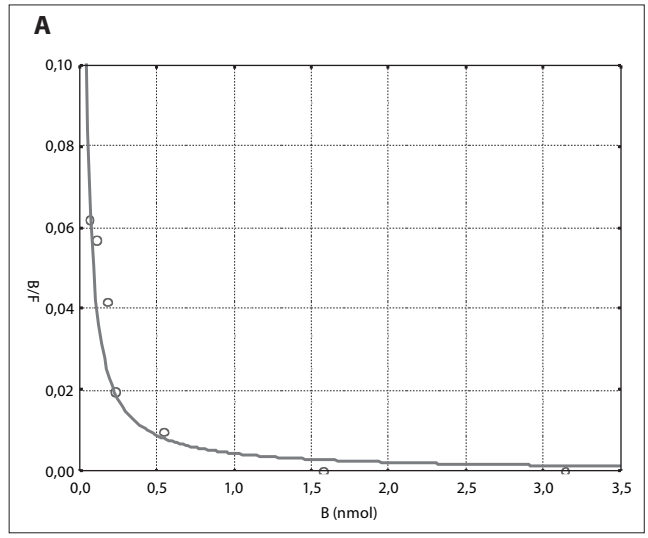
Investigation of antigenity of I^{125}NT showed proper antigen-antibody reaction.

No binding of the I^{125}NT with plasmatic membranes of adipocytes or enterocytes was observed. Unspecific binding of I^{125}NT with 10 µmol/L of free NT is shown in Table. 1.

Table 1. Values of binding sites parameters for NT in plasmatic membranes of hepatocytes from Scatchard curve in control rats and animals that obtained NT analog (Trp-NT), NTR1 and NTR2 blocking agent, (levocabastine, SR 48692) 1 h before taking tissue samples. Values are the means ± S.E. from 3 independent experiments performed in triplicate

Group of animals	Kd_1 [pM]	R_1 [pmol/mg of protein]	Kd_2 [pM]	R_2 [pmol/mg of protein]	Bsp (% of control group)
0.9% NaCl	37±2	1.6±0.1	391±25	33±3	(8.27±0.4) 100 %
SR 48692	35±3	1.5±0.1	370±22	30±2	(8.11±0.4) 98%
Trp-NT	18±1	1.1±0.1	176±12	27±2	(5.77±0.3) 70%
Levocabastine	0	0	0	0	0

K_d – equilibrium dissociation constant.
R – total concentration of specific receptor sites.
Bsp – maximal binding capacity (% of control group).



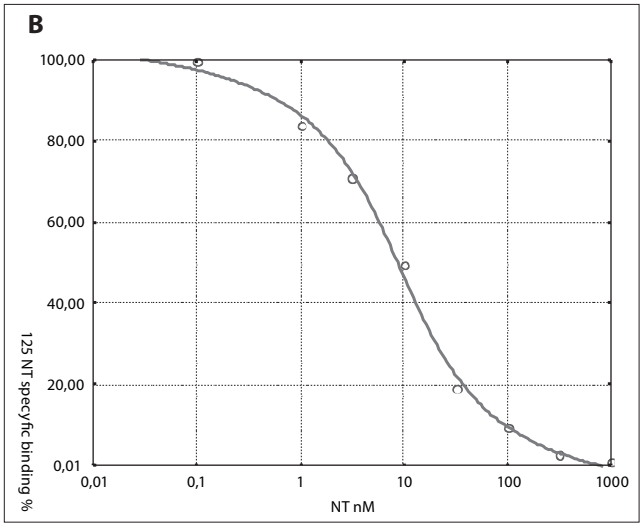
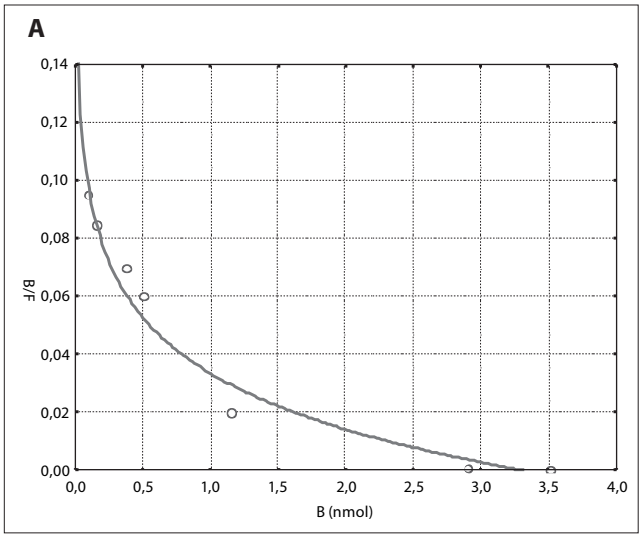


Figure 1 A). Scatchard analysis **B)**. Inhibition of specific binding of 125-I-NT to cell membrane hepatocytes of rats given *in vivo* 0.9% NaCl 1 h before tissue sampling. Reported values are the means of 3 independent experiments performed in triplicate; variations did not exceed 5%



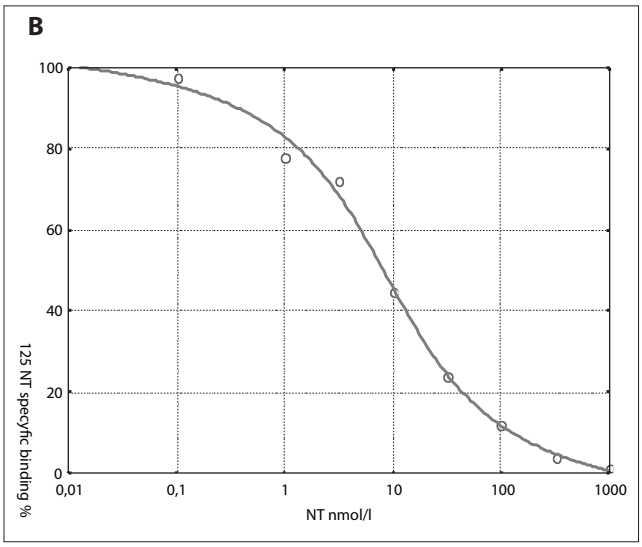
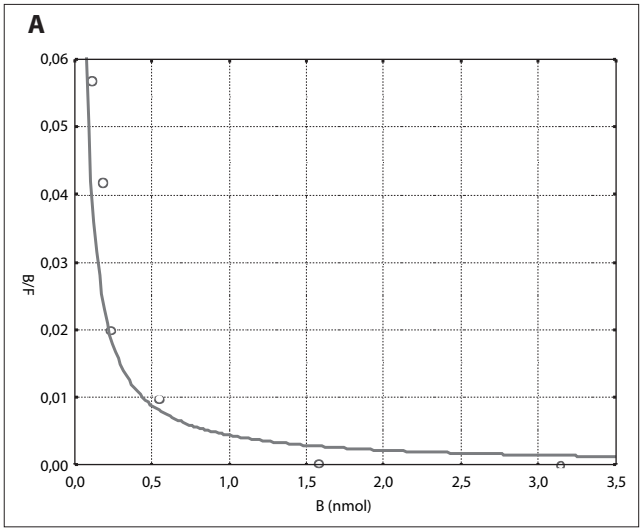


Figure 2 A). Scatchard analysis **B)**. Inhibition of specific binding of 125-I-NT to cell membrane hepatocytes of rats given *in vivo* SR 48692 1 h before tissue sampling at the dose of 0.4 mg/kg b.m. Reported values are the means of 3 independent experiments performed in triplicate; variations did not exceed 5%



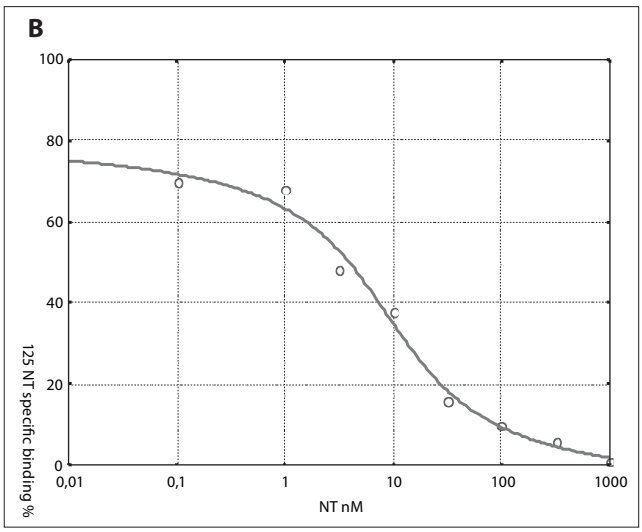


Figure 3 A). Scatchard analysis **B)**. Inhibition of specific binding of 125-I-NT to cell membrane hepatocytes of rats given in vivo Trp-NT one hour before sampling tissues in the dose of 15 nmol/kg b.m. Reported values are the means of three independent experiments performed in triplicate and variations did not exceed 5%

DISCUSSION

The disorders of the lipid metabolism associated with endogenic or environmental factors are the best documented risk factors for atherosclerosis and it's complications. The most dangerous lifestyle disease which should be highlighted is obesity. One of the main causes of obesity is modern processed food, which is rich in lipids, sugar, salt, preservatives and emulgators (synthetic additives – E numbers). These substances could cause disturbances in the metabolic processes, leading to the diseases related to/dependent on the changes in the environment and lifestyle. Neurotensin is the endogenic substance examined by us affecting lipid metabolism, as well as being the key hormonal substance inhibiting cravings.

The most important cells involved in the metabolism and absorption of lipids and cholesterol are without doubt adipocytes, enterocytes and hepatocytes. Regarding the mass, the most important role is played by the liver and distal part of the jejunum. In the investigation conducted on the brain and liver cells of chicken, the presence of binding sites for $I^{125}NT$ was shown. Analysis of the Scatchard curve showed the presence of high (Kd of 25-80pM) and low (Kd 250-450 pM)

affinity receptors. The reduction of the binding capacity of 25-40% was described after incubation of the investigated tissue with pertussis toxin [17]. Radiolabeled NT was also used to detect the localization and characteristics of NT receptors in human sigmoid colon. Tissue samples were obtained from patients undergoing colectomy due to colorectal cancer. NT receptors were detected in the cell membrane of the circular layer of intestinal smooth muscle cells. I^{125}NT bound to high affinity sites (Kd 0.88 ± 0.09 nM and B_{max} =4.03 ± 0.66 fmole/mg of wet tissue). A specific binding site could be blocked by NT analog (NT 8-13), neuromedyne N and SR 48692 [20,21]. A similar investigation was conducted for NT receptors localized in cell membranes of intestine smooth muscle cells in chicken [22]. Research conducted on rats showed the presence of mRNA for NTR in such peripheral organs as the liver, kidneys, heart, brain and spleen [23].

Our results did not confirm the presence of I^{125}NT binding sites in the plasmatic membranes from adipocytes and enterocytes taken from the distal fragment of small intestine in rats. On the contrary, the presence of I^{125}NT binding sites was shown for hepatocytes. These binding sites hadthe following characteristics: Kd_1=37±2 pM, Kd_2 =391±25 pM, R_1 =1.6±0,1 [pmol/mg of protein], R_2 =33±3 [pmol/mg of protein] and Bsp =8.27±0,4

In vivo administration of levocobastine to the animals under study led to selective blocking of receptors NTR2. The consequence of this blocking was total inhibition of I^{125}NT binding sites (Table 1). This conclusion may be confirmed by the fact that in animals obtaining *in vivo* the NTR1 blocker SR 48692, a non-significant change was observed. The obtained results indicated the presence of NT receptors of the second type (NTR2) in the membranes of hepatocytes. In the membranes of hepatocytes from animals obtaining *in vivo* NT analog, Trp-NT, the reduction of the total binding capacity (Bsp) to 70%, and the change of characteristics of the remaining free receptors were noticed. These results suggest unspecific binding of Trp-NT, which may cause partial blocking of NT receptors in the cell membranes of hepatocytes.

It is known that NT influences stimulation of glycogenolysis in rat liver via the sympathetic nervous system [24, 25]. Although earlier studies suggested the influence of NT on the entero-hepatic circulation of bile acids and lipoproteins metabolism [26-28], a full explanation of this feature has not been described to date.

CONCLUSION

In the 3 investigated tissues (adipose tissue, small intestine and liver), low and high affinity binding sites of ^{125}I-NT were found only in the liver. In our study, we did not confirm the presence of I^{125}NT binding sites in cell membranes of adipocytes and enterocytes. The presence of NT receptors only in the membranes of hepatocytes may suggest their role in the regulation of lipid metabolism via receptor - ligand way.

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