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IMMUNOHISTOCHEMICAL EVIDENCE FOR THE PRESENCE OF A VASOACTIVE INTESTINAL PEPTIDE, NEUROPEPTIDE Y AND SUBSTANCE P IN RAT ADRENAL MEDULLA AFTER HEAT STRESS

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Abstract - Immunohistochemistry revealed the presence of VIP-, NPY- and SP-immunoreactivity in the rat adrenal medulla. VIP- and NPY-immunoreactivity was detected in chromaffin and ganglion cells and in nerve fibers, but SP-immunoreactivity was found only in chromaffin cells. After acute heat stress, VIP- and NPY- immunoreactivities in cells and nerve fibers were reduced, probably as a result of the release of these peptides with catecholamines. The absence of SP-immunoreactive ganglion cells in the adrenal medulla suggests that the SP-immunoreactive nerve fibers are extrinsic in origin.

Key words: Rat, adrenal medulla, heat stress, vasoactive intestinal peptide, neuropeptide Y and substance P

INTRODUCTION

The adrenal gland is the primary peripheral endocrine gland in the sympathetic stress response. The adrenal medulla is derived from sympathetic ganglion cells from the neural crest during development. After neural crest cell migration, the chromaffin cells are engulfed by the cortical cells and subsequently differentiate into neuroendocrine secretory cells (Nussey and Whitehead, 2001). The chromaffin cells are responsible for the secretion of catecholamines noradrenaline (NA) and adrenaline (A) as well as a host of neuroactive peptides.

Catecholamine biosynthesis and secretion from the chromaffin cells is controlled by sympathetic nerve activity (Souvatzoglou, 2005). The adrenal medulla is innervated by preganglionic sympathetic fibers of the splanchnic nerve (Aunis, 1998). These sympathetic fibers enter the medulla and disperse to innervate adrenal chromaffin cells (Coupland, 1962; Carmichael, 1986; Tomlinson and Coupland, 1990; Souvatzoglou 2005). Upon presympathetic stimulation, chromaffin cells release their contents into the circulation. Stress also affects the synthesis and release of several neuropeptides co-stored with the catecholamines in chromaffin cells.

Vasoactive Intestinal Peptide (VIP) is a 28 amino acid peptide. VIP-immunoreactivity has been reported to occur in nerve fibers in the adrenal superficial cortex and medulla, and in some medullary cells (Hökfelt et al., 1981; Holzwarth, 1984). Immunohistochemical studies have demonstrated a small number of VIP-immunoreactive cells in the adrenal medulla where neuropeptide colocalizes with adrenaline (Hökfelt et al., 1981; Holzwarth, 1984; Kondo et al., 1986). Several lines of evidence suggest that the adrenocortical VIP-ergic nerves originate from VIP-containing medullary cells as well as from the

splanchnic nerve (Linnoila et al., 1980; Kondo, 1985; Maubert et al., 1990).). A large body of evidence suggests that VIP plays a role in the control of the hypothalamo-pituitary-adrenal (HPA) axis, almost exclusively acting in a paracrine manner since its blood concentration is very low (Nussdorfer and Malendwicz, 1998).

Neuropeptide Y (NPY) is a 36 amino acid peptide. NPY-immunoreactivity has been demonstrated in approximately 50% of the total chromaffin cells of the rat adrenal medulla (Kuramoto et al., 1986). This peptide is colocalized with catecholamines in noradrenaline- and adrenaline-producing adrenomedullary cells (Schalling et al., 1988). Stressors have been found to increase plasma NPY levels in humans and laboratory animals (Zukowska-Grojec et al., 1988). Besides its presence in nerve endings, NPY is produced by the chromaffin cells of the adrenal medulla in different species, including human (Cavadas et al., 2001).

Substance P (SP), a 11 amino acid peptide, belongs to a family of tachykinins. Immunohistochemical study demonstrated a relatively small number of cells with SP-like immunoreactivity and it is suggested that SP co-exists with catecholamines in a population of chromaffin cells of the rat adrenal medulla. Very few SP-immunoreactive nerve fibers with varicosities were found in the adrenal medulla of rats. They extended between small clusters of chromaffin cells and had their dot-like terminals around and within the cell clusters (Kuramoto et al., 1985). SP-immunoreactive varicose nerve fibers, which were found in the medulla, are in contact with a cluster of the NA cells showing catecholamine fluorescence, which suggests that SP from medullary nerve fibers may regulate the secretory activity of the NA cells (Murabayashi et al., 2007).

Taking all this into consideration, as well as the fact that there are no reports of semiquantitative analysis of neuropeptides in the adrenal medulla after acute heat exposure, we decided to determine the effect of high ambient temperature on VIP, NPY and SP in rat adrenal medulla.

MATERIALS AND METHODS

In this study male Wistar rats (*Rattus norvegicus* Berkenhaut 1769), weighing 320 ± 30g, were acclimated to 22±1°C and kept under a 12 h light/dark cycle. The animals were fed with commercial rat food and drank tap water *ad lib*. The rats were divided into two groups, each consisting of ten rats. The animals from the first group were intact controls. The rats from the second group were exposed to an ambient temperature of 38°C for 60 min in a hot chamber (Sutjeska, Beograd, YUG), immediately before sacrifice.

After measuring the body mass and temperature, the animals were killed by decapitation using a guillotine (Harward-Apparatus, Holliston, MA, USA). The left adrenal gland from each animal was removed, freed of fat on ice and weighed. The adrenal glands were fixed in 4% formalin solution and embedded in paraffin according to the standard procedure, after which they were serially cut into 5 μm thick sections on a 'Reichert' rotation microtome.

The avidin-biotin immunocytochemical method was used for the detection and localization of neuropeptides in the adrenal medulla. After deparaffinization, antigen retrieval was performed in sodium citrate for 21 min in a microwave and endogenous peroxidase was suppressed in 3% H_2O_2. The primary antibody was incubated (1:500 VIP; 1:800 NPY; 1: 250 SP; Abcam, Cambridge, UK) over night (4°C); the secondary antibody (1:200; Santa Cruz biotechnology, Inc., Heidelberg, Germany) was incubated for 1 h, after which the sections were incubated with avidin-biotin complex. The sections were rinsed in phosphate buffered saline (PBS) after each step. Visualization was done by using 3,3'-diaminobenzidine (DAB). Mayer hematoxylin was used for counterstaining. After dehydration, the sections were mounted in DPX.

The experiments were performed according to the rules of animal care proposed by Serbian Labora-

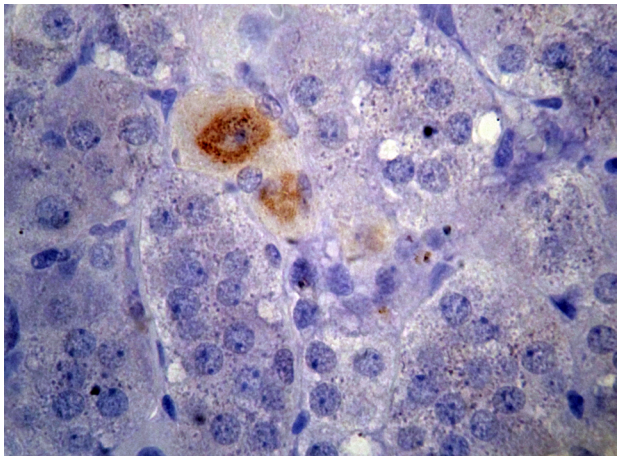

Fig. 1. VIP-immunoreactive chromaffin cells in control rats. Avidin-biotin method.

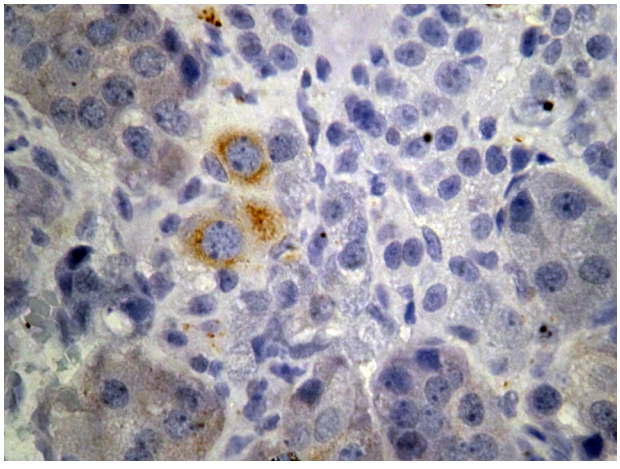

Fig. 2. VIP-immunoreactivity in ganglion cells in control rats. Avidin-biotin method.

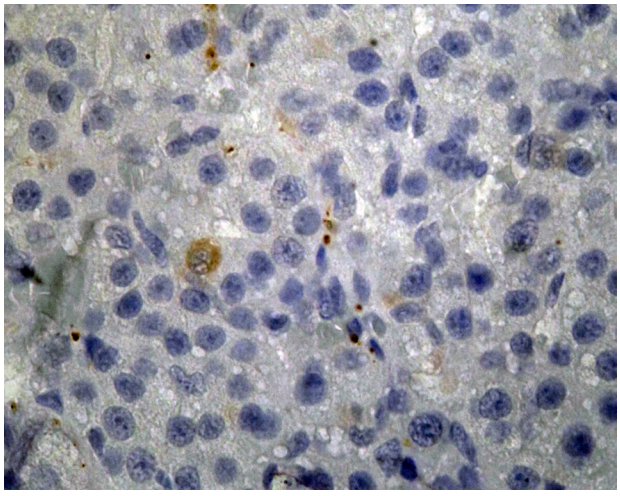

Fig. 3. VIP-immunoreactive chromaffin cells in heat stressed rats. Avidin-biotin method.

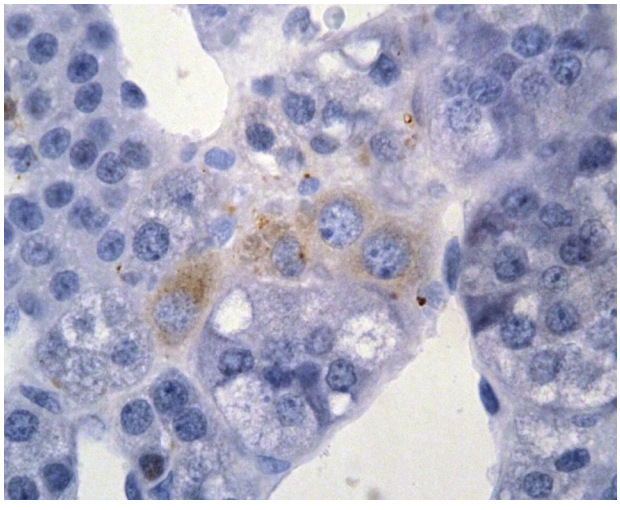

Fig. 4. VIP- immunoreactivity in ganglion cells in stressed rats. Avidin-biotin method.

tory Animal Science Association (SLASA), which is a member of the Federation of European Laboratory Animal Science Association (FELASA).

RESULTS

Immunocytochemistry revealed the presence of VIP-immunoreactivity in the medulocytes, ganglion cells and nerve fibers in the adrenal medulla. These VIP-immunoreactive cells were found as single cells, or in small groups of several cells, which contained numerous granules in their cytoplasm (Fig. 1). In the ganglion nerve *cells,* the immunoreactive material was localized diffusely throughout the pericarion and axoplasm (Fig. 2). VIP-immunoreactivity was also detected in nerve fibers in the adrenal medulla. These fibers were seen among non-immunoreactive medullary cells and juxtamedullary cortical cells. After the heat stress, only a few chromaffin cells with VIP-immunoreactivity were observed, but VIP-immunoreaction in the medulocytes and ganglion cells was weaker than in the control (Fig 3 and Fig. 4). Treatment with heat changed the nerve fibers so that they were much thicker.

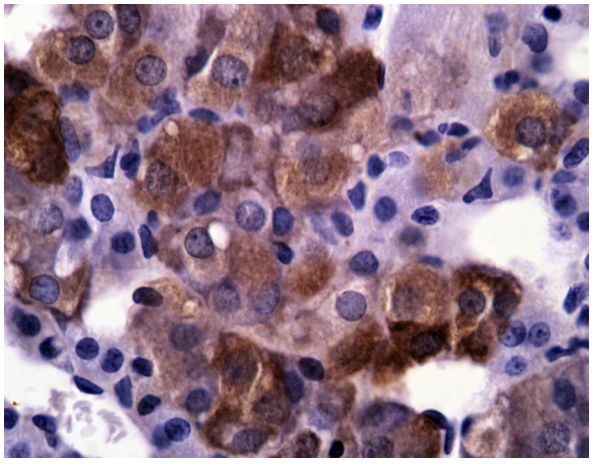

Fig. 5. NPY-immunoreactive medulocytes in control rats. Avidin-biotin method.

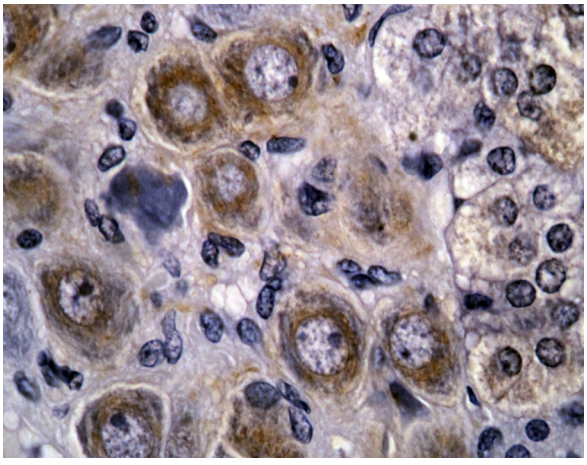

Fig. 6. Ganglion cells with NPY-immunoreactivity in control rats. Avidin-biotin method.

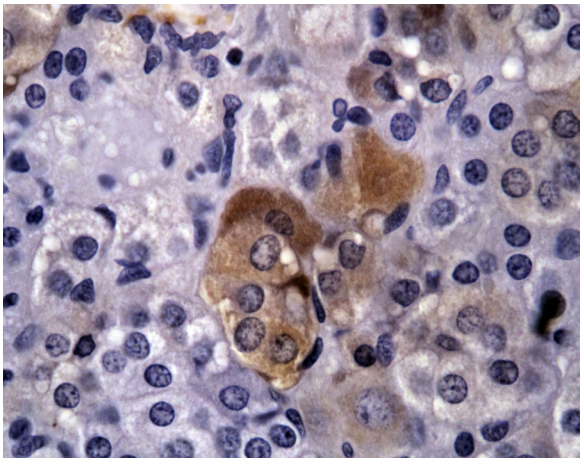

Fig. 7. NPY-immunoreactive chromaffin cells in stressed rats. Avidin-biotin method.

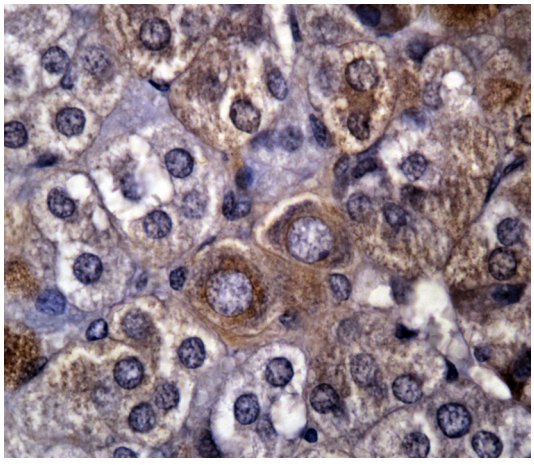

Fig. 8. NPY-immunoreactivity in ganglion cells in heat stressed rats. Avidin-biotin method.

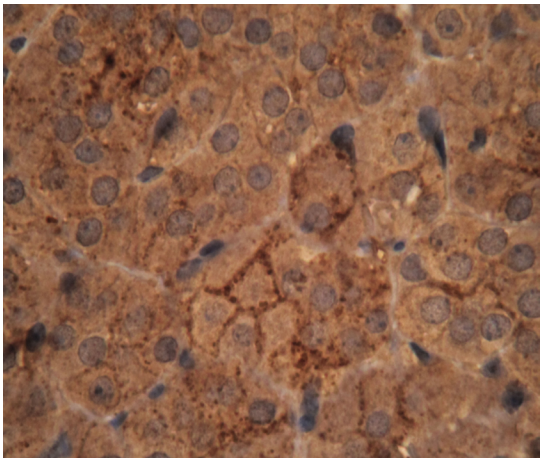

Fig. 9. SP-immunoreactive chromaffin cells in control rats. Avidin-biotin method.

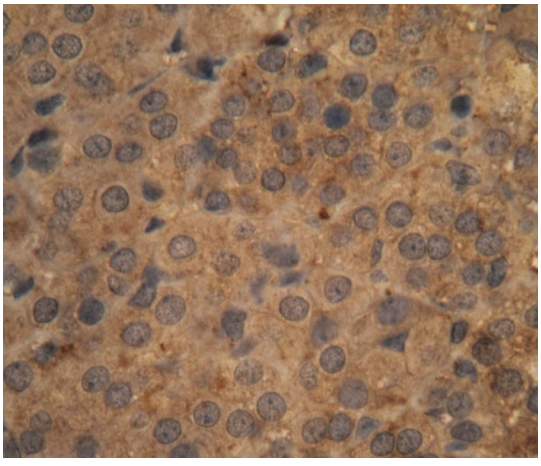

Fig. 10. Chromaffin cells with SP-immunoreactivity in stressed rats. Avidin-biotin method.

NPY-immunoreactivity was observed in medulocytes and in ganglion cells. The immunoreactive material was localized diffusely in the cytoplasm of chromaffin cells (Fig. 5). These cells were dispersed all over the medulla. The immunoreactive material in the ganglion *cells* was localized diffusely throughout the axoplasm (Fig. 6). NPY-immunoreactivity was also detected in nerve fibers. After the heat stress, NPY-immunoreactivity was weaker in the cells as well as in the ganglia, in comparison to those in the control (Fig. 7 and Fig. 8). Like the VIP nerve fibers, NPY nerve fibers were much thicker after heat stress.

In the adrenal medulla, there were many SP-immunoreactive chromaffin cells. These cells were polygonal in shape and contained numerous granules in their cytoplasm, which were located near the cell membranes (Fig. 9). No SP-immunoreactivity was seen in the large ganglion cells. After the heat stress, SP-immunoreactivity was weaker in the chromaffin cells, in comparison to those in the control (Fig. 10). We did not find SP varicose nerve fibers in the adrenal medulla.

DISCUSSION

Immunoreactivity to neuropeptide Y and vasoactive intestinal peptide has been demonstrated in intra-adrenal ganglion cell bodies and nerve fibres of the rat (Pelto-Huikko, 1989; Oomori et al., 1994).

Our study showed that VIP-immunoreactivity in two cell types, medulocytes and ganglion cells, is in accordance with the results of Kondo et al. (1986). These VIP-like immunoreactive chromaffin cells were polygonal in shape and they appeared solitarily or as a group of several cells. The large ganglion immunoreactive cells had a round nucleus that was poor in chromatin. Weaker VIP-immunoreactions after heat stress might be explained by the fact that this peptide is co-released with adrenaline because the blood concentration of this catecholamine was higher after stress (data not shown). Studies on the dog adrenal gland indicate that VIP is released along with catecholamines from the gland in response to direct splanchnic nerve stimulation *in vivo* (Gaspo et al., 1995). In the conscious calf, VIP is released from the gland in response to stimulation of the peripheral end of the splanchnic nerve (Bloom et al., 1988). VIP is involved in the maintenance of the normal growth and steroidogenic capacity of the rat adrenal cortex. However, indirect evidence suggests that this peptide might play a relevant role under paraphysiological conditions, e.g. in the mediation of HPA-axis responses to cold and inflammatory stresses (Nussdorfer and Malendowicz, 1998). Nowak et al. (1994) have shown that endogenous VIP does not regulate HPA-axis function under basal conditions, but it plays a pivotal role in the mechanisms involved in the activation of the HPA axis induced by cold exposure. Afterwards the heat stress nerve fibers were much thicker, indicating their increased secretory activity (Tanelian and Markin, 1997).

NPY-like immunoreactivity was detected in medulocytes and ganglion cells. A previous finding has reported that the adrenaline cells can be immunostained for NPY (Lundberg et al., 1986), but recent studies have shown that NPY is a sympathetic neurotransmitter which is co-stored and co-released with noradrenaline and adrenaline (Ruohonen et al., 2009). Weaker NPY-immunoreactions after heat stress could be explained by the fact that this peptide is co-released with adrenaline and noradrenaline, because the blood concentrations of these catecholamines were significantly higher after stress (data not shown). Findings indicate that NPY is co-released with catecholamines under a variety of stimuli, including splanchnic nerve and cholinergic- and nicotinic-receptor activation (Spinazzi et al., 2005). Immobilization, cold stress and shaker stress all caused increases in rat adrenal NPY mRNA (Hiremagalur et al., 1994; Levenson et al., 1998). After heat stress, nerve fibers were much thicker, indicating their increased secretory activity (Tanelian and Markin, 1997).

The existence of SP-immunoreactive chromaffin cells in the adrenal medulla has been reported in various mammals (Kuramoto et al., 1985; Heym

et al., 1995). The double immunostaining revealed that SP-immunoreactive chromaffin cells were present in the adrenal medulla of the rat and demonstrated that they were either phenylethanolamine N-methyltransferase (PNMT) immunoreactive or PNMT-immunonegative, suggesting that SP may be synthesized in both A and NA cells (Murabayashi et al., 2007). Because the blood concentrations of these catecholamines were significantly higher after stress (data not shown), this could explain the weaker SP-immunoreactivity after heat stress since this peptide is co-released with adrenaline and noradrenaline. SP and other peptides may be synthesized in both types of chromaffin cells and released from the granules by exocytosis under appropriate stimuli (Kuramoto et al., 1985; Murabayashi et al., 2007).

Since no ganglion cells with SP immunoreactivity were found in the rat adrenal gland (Kuramoto et al., 1985), the SP-immunoreactive nerve fibers are regarded as extrinsic in origin. Previous studies using retrograde tracing methods revealed that nerve fibers innervating the adrenal medulla arose from the sensory neurons of the dorsal root ganglia and nodose ganglia (Mohamed et al., 1988; Heym et al., 1994; Dun et al., 1996).

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