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

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Abstract - Immunohistochemistry revealed the presence of Vasoactive Intestinal Peptide (VIP), Neuropeptide Y (NPY), and the absence of Substance P (SP) immunoreactivity in the rat adrenal cortex. VIP- and NPY-immunoreactivity were detected in nerve fibers around the small blood vessels projecting into the capsule and cortical zones surrounding blood vessels and cortical cells. After acute heat stress, VIP- and NPY-immunoreactivities in the nerve fibers were reduced, probably as a result of the release of these peptides.

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

INTRODUCTION

The adrenal gland consists of a centrally positioned medulla, surrounded by cortex, together enclosed within connective tissue capsule (Whitworth et al., 2003; Hammer et al., 2005). The cortex is further roughly subdivided into the outer zona glomerulosa (ZG), medial zona fasciculata (ZF) and inner zona reticularis (ZR).

There is still some confusion as to the distribution of the different types of axon terminals found in the adrenal gland. Evidence of peptide-containing terminals was found for: VIP (Oomori et al., 1994), NPY (Varndell et al., 1984), substance P (Pfister and Gorne, 1983; Gorne et al., 1984; Livett et al., 1990). With regard to the classical autonomic terminals, cholinergic terminals are found throughout the cortex and medulla and are still regarded as the main type of innervation.

Vasoactive Intestinal Peptide (VIP) is a 28-amino-acid peptide. An abundant network of nerve fibers with VIP-immunoreactivity is observed in the adrenal medulla and in the capsule and adjacent to ZG. Several lines of evidence suggest that adrenocortical VIP-ergic nerves originate from VIP-containing medullary cells as well as from the splanchnic nerve (Linnoila et al., 1980; Holzwarth, 1984; Maubert et al., 1990). Some 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 Maledonowicz, 1998).

Neuropeptide Y (NPY) is a 36-amino-acid peptide. In the adrenal gland NPY is present both in a population of chromaffin cells and in nerves. NPY-immunopositive fibers are found around the capsular or subcapsular blood vessels and in the ZG, where they can form plexuses (Pelto-Huikko, 1989; Maubert et al., 1990). Single NPY-immunoreactive fibers were sparsely distributed in the deeper regions of the cortex. At an ultrastructural level, the NPY-immunoreactive nerve fibers contained abundant small clear vesicles mixed with a few small and large granular vesicles. The immunoreactive material appeared on the granular cores as well as in the axoplasm. The NPY fibers were closely apposed to smooth muscle cells and pericytes of small blood vessels in the cortex (Kuramoto et al., 1986).

Substance P (SP), an 11-amino-acid peptide, belongs to a family of tachykinins. Immunohistochemistry revealed a small number of SP-immunoreactive cells and fibers in the adrenal medulla. These fibers were observed only in the medullary part of the gland (Linnoila et al., 1980), but a recent study revealed SP immunoreactivity in the adrenal capsule and cortex, in some nerve fibers around blood vessels and in thick nerve bundles passing through the cortex directly into the medulla (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 cortex after acute heat exposure, we decided to determine the effect of high ambient temperature on VIP, NPY and SP in rat adrenal cortex.

MATERIALS AND METHODS

In this study male rats of Wistar strain (*Rattus norvegicus* Berkenhout 1769), weighing 320 ± 30g, were acclimated to 22±1°C and kept under a 12 light:dark cycle. Animals were provided with commercial rat food and tap water *ad libitum*. The rats were divided into two groups, each consisting of ten rats. The animals in 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, Belgrade), immediately before sacrifice.

After measuring of body mass and temperature, the animals were sacrificed 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. Adrenal glands were fixed in 4% formalin solution and embedded in paraplast, according to standard procedure, after which they were serially cut into 5μm thick sections on a 'Reichert' rotation microtome.

The avidin-biotin immunocytochemical method was used for the detection and localization of neuropeptides in the adrenal cortex. 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 one hour, after which sections were incubated with avidin-biotin complex. Sections were rinsed in phosphate buffered saline (PBS) after every 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 in accordance with the rules of animal care proposed by the Serbian Laboratory Animal Science Association (SLASA), which is a member of the Federation of European Laboratory Animal Science Association (FELASA).

RESULTS

In the control rats, a plexus of VIP-immunoreactive nerve fibers with varicosities was seen to encircle small blood vessels penetrating the capsule and running in the subcapsular region of the adrenal cortex (Fig. 1). Some of the varicose fibers were also found in the fasciculata and reticularis zones (Fig. 2). Treatment with heat changed the nerve fibers, so that they

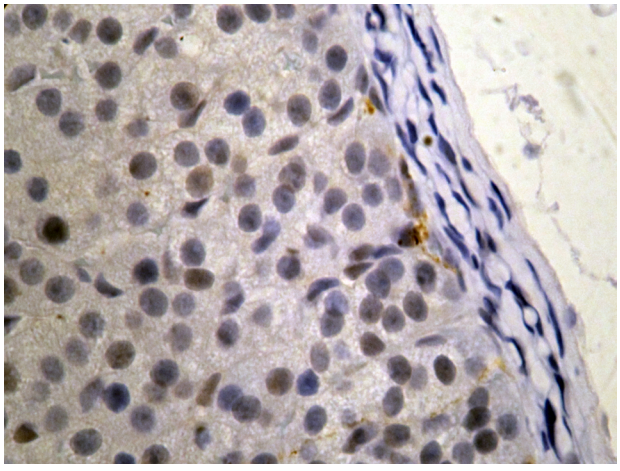

Fig. 1. VIP-immunoreactivity in nerve fibers of capsule in control rats. Avidin-biotin method. Scale bar 20 μm.

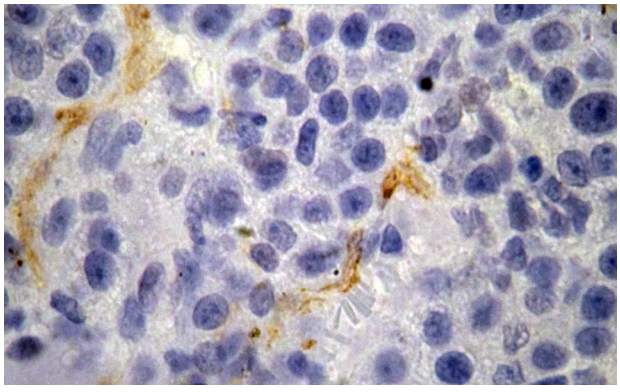

Fig. 2. VIP-immunoreactivity in nerve fibers of cortex in control rats. Avidin-biotin method. Scale bar 20 μm.

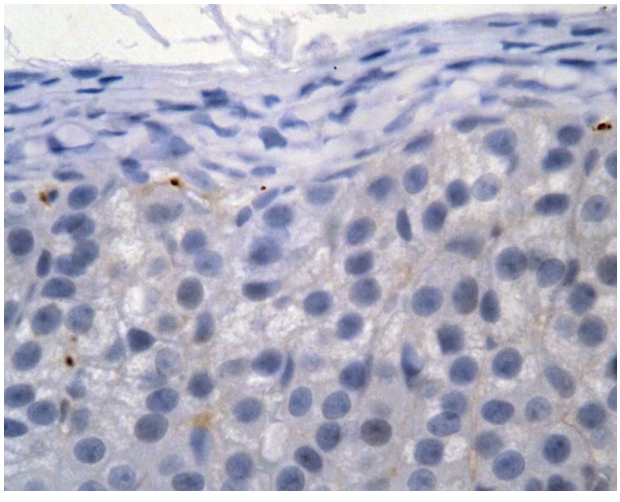

Fig. 3. VIP-immunoreactivity in nerve fibers of capsule in heat stressed rats. Avidin-biotin method. Scale bar 20 μm.

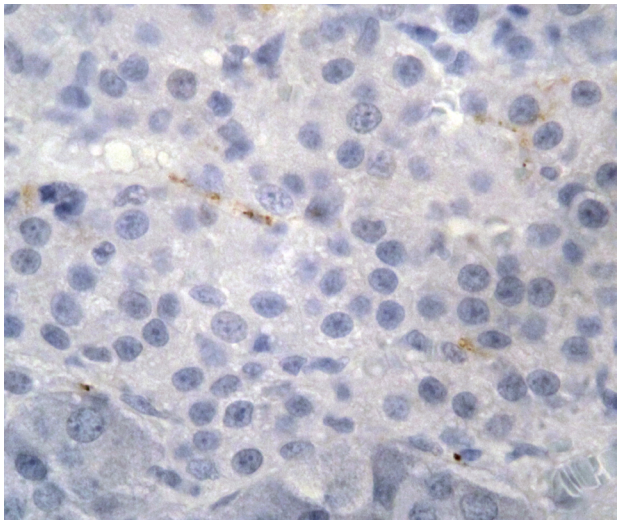

Fig. 4. VIP-immunoreactivity in nerve fibers of cortex in stressed rats. Avidin-biotin method. Scale bar 20 μm.

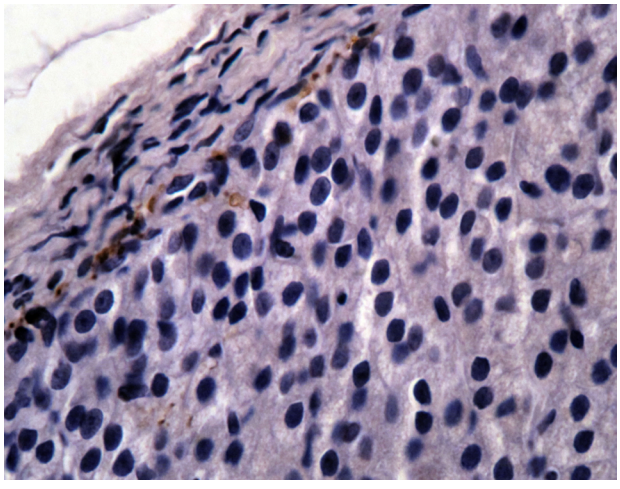

Fig. 5. NPY-immunoreactivity in nerve fibers of capsule in control rats. Avidin-biotin method. Scale bar 20 μm

were much thinner, both in the capsule (Fig. 3) and in the cortex (Fig. 4).

NPY-immunoreactivity was detected in the nerve fibers. Nerve fibers with varicosities were observed around the small blood vessels, which penetrated the capsule and coursed in the subcapsular regions of the adrenal gland (Fig. 5). These nerve fibers extended into the cortical zones where they surrounded blood vessels and cortical cells. Single varicose nerve fibers were sparsely distributed in the fasciculata and

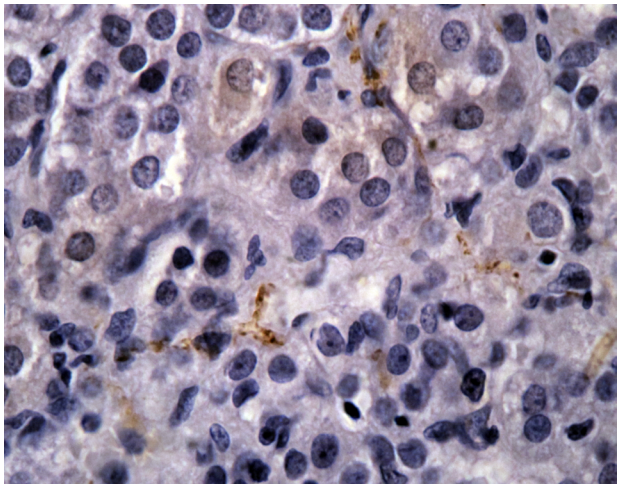

Fig. 6. NPY-immunoreactivity in nerve fibers of cortex in control rats. Avidin-biotin method. Scale bar 20 μm

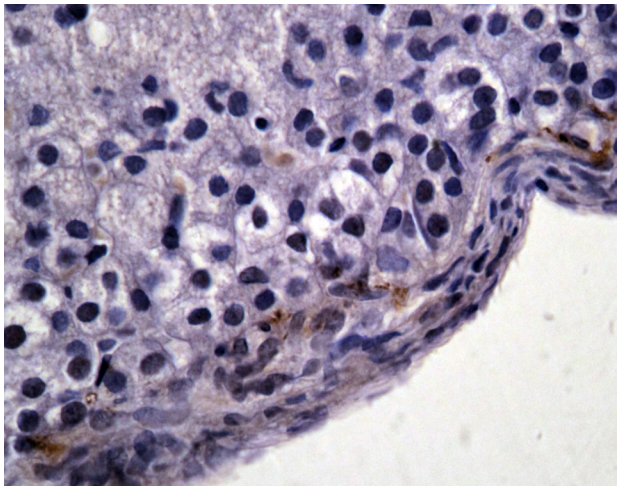

Fig. 7. NPY-immunoreactivity in nerve fibers of capsule in heat stressed rats. Avidin-biotin method. Scale bar 20 μm.

reticularis zones of the cortex (Fig. 6). Like the VIP nerve fibers, the NPY nerve fibers were much thinner in the capsule (Fig. 7) and in the cortex (Fig. 8) after heat stress.

We did not find SP varicose nerve fibers in the adrenal cortex.

DISCUSSION

Immunohistochemical studies have demonstrated the existence of VIP-immunoreactive nerves in the

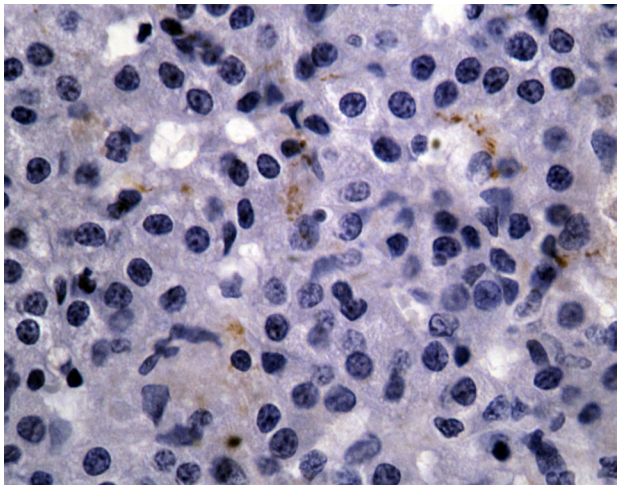

Fig. 8. NPY-immunoreactivity in nerve fibers of cortex in stressed rats. Avidin-biotin method. Scale bar 20 μm.

ZG and in the capsule (Hökfelt et al., 1981; Holzwarth, 1984). Studies have shown that binding VIP to its G protein receptors, VPAC1 and VPAC2-Rs-Rs, together with the adenylate cyclase and phospholipase C on the ZG cells, stimulates the secretion of aldosterone (Conconi et al., 2006). The effect of VIP on ZG cells was confirmed at the ultrastructural level by observation of a euchromatic nucleus, small lipid droplets and numerous mitochondria, which proved the intense synthetic activity of the cell (Rebuffat et al., 1994). There is also evidence that VIP may indirectly affect the synthesis of aldosterone, since it promotes the release of catecholamines, which may have a paracrine effect on the adrenal cortex cells (Nussdorfer and Malendowicz, 1998; Conconi et al., 2006). In this study, nerve thickness was reduced after heat stress, which could be explained by an increased secretory activity and the releasing of the peptides (Tanelian and Markin, 1997).

Immunohistochemical analyses detected NPY-immunoreactive fibers in the subcapsular regions of adrenal glands. These fibers are located around the capsular and subcapsular network of blood vessels, as well as in the ZG, where they form a plexus (Kondo, 1985; Pelt-Huikko, 1989), while in the ZF and in the ZR they pass without branching (Kuramoto et al., 1986). In rats, the NPY nerves are colocalized with

VIP nerves that innervate the ZG and blood vessels of adrenal cortex (Renshaw and Hinson, 2001). After the heat stress, the fibers were thinner, which can be interpreted by the secretion of regulatory peptides (Tanelian and Markin, 1997). Most *in vivo* and *in vitro* studies show that NPY may have an indirect stimulatory effect on aldosterone secretion, or through the release of catecholamines from nerve endings or from chromaffin cells that are located in the ZG (Renshaw and Hinson, 2001). In our study, the level of serum aldosterone was significantly increased in comparison to control animals (data not shown). NPY has no significant effect on corticosterone release from ZF cells (Renshaw and Hinson, 2001), but plays an important role in blood flow through the adrenal gland (Wahlestedt et al., 1990).

Immunohistochemical analysis did not detect the presence of SP-immunoreactive nerves in the capsule and in the adrenal cortex. So far, the SP nerves have been identified in humans (Linnoila et al., 1980) and rats (Kondo, 1985; Kuramoto et al., 1985), but in both species, innervation is scarce and limited to the adrenal medulla. Since no ganglion cells with SP immunoreactivity were found in the rat adrenal gland (Kuramoto et al., 1985), SP-immunoreactive nerve fibers are regarded as extrinsic in origin.

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