DEVELOPMENT OF NEUROPEPTIDE Y-LIKE IMMUNOREACTIVITY IN THE RAT HEART

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The time of appearance and distribution of neuropeptide Y (NPY) in the developing and adult rat heart has been studied by immunohistochemical method using the Avidin biotin technique. At 18 days of gestation and postnatal day 1, no immunoreactivity was detected. Immunoreactivity was first observed on postnatal day 5 around the large blood vessels, developing coronary arteries and very sparsely along the blood vessels in the musculature. At later stages up to adulthood the intensity of immunostaining and density of fibres along the intramyocardial blood vessels increased. After postnatal day 10 the NPY immunoreactive fibres were also seen amongst the muscle fibres. Immunopositive NPY neurons were seen in the ganglionic clusters close to the great vessels at postnatal day 20 and were visualised in between the ventricular muscle bundles near the atrioventricular region only in the adult heart. The ventricular musculature was always more richly innervated than the atria by NPY immunopositive nerve fibres. Thus the present study demonstrates that NPY-like immunoreactivity makes its appearance postnatally, first around the blood vessels and later on the muscle fibres too.

The mammalian heart is classically known to be innervated by the postganglionic fibres of sympathetic adrenergic and parasympathetic cholinergic nerves. The past decade has shown the presence of regulatory peptides such as substance P, vasoactive intestinal polypeptide, neurotensin, neuropeptide Y and enkephalin in the autonomic nerves innervating the heart. Of these, neuropeptide Y (NPY), a 36-amino-acid peptide, is shown to be abundantly present in the cardiovascular system of mammals (7, 11, 19). NPY is known to have potent vasoconstrictor effects (17) and play a regulatory role in coronary blood flow and contractility of heart musculature (1, 2, 9, 10). There is some indication of a possible relationship of NPY with the development of high blood pressure in stroke prone spontaneously hypertensive rats (13) and rat models of renovascular hypertension (3) because of the high density of NPY nerves found in the peripheral blood vessels. In human infants, pulmonary hypertension has been shown to be associated with a premature innervation of arteries of the respiratory system by NPY (4). This also makes it important to know the

developmental profile of NPY innervation in cardiovascular system. The present study is aimed at providing baseline data on the ontogeny and distribution of NPY-like immunoreactivity in rat hearts with a view to aid the understanding of its role in development of normal cardiac physiology, coronary vasospasm and perhaps in congestive heart failure in which the NPY levels, in particular, are reported to be elevated (15).

MATERIAL AND METHODS

Three albino rats (Wistar strain) of either sex in each of the age groups of 18 days gestation (E 18), postnatal day (DPN) 1, 5, 10, 20 and adults were used for the study. The hearts from rats of ages E 18 and DPN 1 were immersion fixed after killing the pups with overdose of ether anaesthesia. The rats of DPN 5, 10, 20 and adult age periods were perfused intracardially with a mixture of 4% paraformaldehyde and 1% glutaraldehyde after anaesthetizing with ether. The hearts were removed and fixed overnight in the same fixative. The tissues after washing and equilibration in 30% sucrose in 0.1 M phosphate buffer were cut in the cryostat at -20 degree C. Sections of 40 μ m thickness were taken in glass vials and free floating sec-

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tions were processed for immunostaining. The sections were treated with 1 : 9 solution of 3% hydrogen peroxide in methanol for 10 min to suppress the endogenous peroxidase and then washed with 0.1 M phosphate buffer saline containing 0.1% triton X. After buffer washes the sections were treated with normal goat serum at 1 : 10 dilution for six hours or overnight. The sections were incubated at 4 degree C for 48–72 hr with 1 : 100 to 1 : 500 working dilution of polyclonal NPY antibody (gift from Drs. A. Balasubramaniam and S. Sheriff, USA), characterised earlier for specificity and used to determine postprandial changes in plasma NPY levels in awake dogs (6). The reaction was visualised by avidin-biotin-peroxidase DAB technique. In some sections staining was

enhanced by using 2.5% nickel II sulphate in 0.2 M acetate and 0.1 M imidazole buffer alongwith DAB. The control sections were processed either without incubation in NPY antiserum or in antiserum preadsorbed with synthetic NPY (5) at concentrations of 10 μ g/ml of antiserum.

RESULTS

At embryonic day 18 (E 18) and postnatal day 1 (DPN 1) no immunoreactivity was observed. On DPN 5, immunoreactive nerve fibres were found around the large blood vessels, developing coronary arteries and very sparsely along the blood vessels in the ventricular musculature (Figs. 1a, b). At later age

FIG. 1a. Photomicrograph of the ventricular musculature of DPN 5 rat showing the black stained NPY immunoreactive fibres in the wall of a medium sized blood vessel (bv).

FIG. 1b. Photomicrograph of tangentially cut ventricular musculature of DPN 5 rat showing some varicosities of stained nerve fibres on the small blood vessels indicated by arrowheads. Inset shows the same at low magnification.

FIG. 2a. Photomicrograph of ventricular musculature of DPN 10 rat. Note the increase in NPY stained nerve fibres around the blood vessels (bv) and few fibres indicated by arrowhead on the ventricular musculature.

FIG. 2b. Ventricular musculature of DPN 10 rat showing NPY immunopositive nerves on muscle fibres. Scale bar = 20 μ m.

periods the intensity of staining and density of fibres along the large vessels and intramyocardial blood vessels increased (Figs. 2a, 3b, 4a). At DPN 10, immunoreactivity was mostly present close to the serosal surface of the ventricular musculature with few NPY immunostained fibres being seen on the ventricular and atrial musculature (Figs. 2a, b). After DPN 10, the NPY immunoreactive fibres were increasingly found on and between the muscle fibres of both the ventricles and the atria (Figs. 3c, d); the ventricles being always more richly innervated than the atria. The fibres were arranged parallel to and between the muscle bundles, singly or in fascicles forming a latticework. The single fibres showed varicosities. The

immunopositive NPY neurons were seen amidst the ganglionic clusters near the large vessels at DPN 20 (Fig. 3a) and were visualised between the ventricular muscle fibres near the atrioventricular junctional zone only in the adult heart (Figs. 4e, f). In the adult animals the walls of the atria and the ventricles displayed dense innervation of blood vessels and musculature by NPY immunopositive fibres.

DISCUSSION

The study demonstrates a postnatal appearance of neuropeptide Y (NPY) immunoreactive nerve fibres in the rat heart musculature with intrinsic NPY

FIG. 3a. A ganglionic cluster close to the large blood vessels of DPN 20 rat showing some NPY immunoreactive ganglion cells (arrowheads) and nerve fibres (arrow) amidst the non immunoreactive ones (open arrows). nfb : nerve fibres.

FIG. 3b. Photomicrograph of ventricular musculature of DPN 20 rat showing further increase of NPY immunoreactivity on the intramyocardial blood vessels (bv).

FIG. 3c. Ventricular musculature of DPN 20 rat with striations showing NPY immunostained nerve fibres on the muscle (nickel intensification).

FIG. 3d. Transverse section through atrial wall of DPN 20 rat showing the NPY nerve fibres nearer the serosal surface. Scale bar = 20 μ m.

FIG. 4a. Photomicrograph of adult rat heart ventricular musculature. Note the increased density of NPY nerve fibres around the vascular wall and on the ventricular musculature.

FIG. 4b. Control section from adult rat ventricle using preadsorbed antiserum shows absence of NPY immunostained nerve fibres.

FIG. 4c. Tangential section through the atrial wall of adult rat showing the NPY immunostained varicose fibres.

FIG. 4d. Photomicrograph of the adult rat ventricular musculature. Note the NPY immunostained varicose terminals (arrowhead) on the muscle fibres.

FIG. 4e. Tangential section through adult rat ventricular muscle close to the atrioventricular junctional region showing increased density of NPY nerve fibres on the muscle as well as scattered NPY immunostained (arrowhead) and non immunostained neurons (arrow).

FIG. 4f. A small cluster of NPY neurons and nerve fibres in the ventricular musculature in the adult rat heart (nickel intensification). Scale bar = 20 μ m.

neurons being visualised only in the adult. The heart is known to be richly innervated by NPY. The NPY immunoreactive nerves in the adult rat are present around the nodal tissues, surround the coronary arteries and arterioles and are found in close association with the cardiac muscle (11). In the present study, development of NPY immunoreactivity in the rat heart is seen to appear in a distinctive manner. The nerves surrounding and innervating the arteries display NPY immunoreactivity earlier than those in the neighbourhood of the cardiac musculature. The neuronal cell bodies intrinsic to the heart demonstrate the NPY immunopositivity still later. In the human fetal heart, NPY nerves were not detected until 11–12 weeks of gestation when sparse nerve fibres were found in the atrial epicardium and in association with the developing coronary arteries. Subsequently there was increase in the number of immunostained nerves with innervation of atrial myocardium, ventricular epicardial arteries and ventricular muscle. By 21–24 weeks the overall distribution was similar to that found at birth (19).

The cardiac NPY nerves are shown to be largely of postganglionic sympathetic origin as demonstrated by disappearance of these nerves consequent to stellate ganglionectomy and chemical sympathectomy after use of 6-hydroxydopamine (7, 14, 18). Co-existence of NPY with tyrosine hydroxylase like immunoreactivity (adrenergic) also has been demonstrated in the adult guinea pig (18). NPY fibres also come from the intrinsic neurons (12) which are demonstrated in the adult rat heart in the present study as also by Gu *et al.* (11); although they are found to be apparently lacking in the human heart (19).

It is interesting to note that while immature looking adrenergic neurons are seen to be present at birth in the rat myocardium (16), NPY neurons were seen only in the adult in our study. Similarly adrenergic nerves and receptors (8) are found to be present and functional in the prenatal life, while we have observed that NPY fibres are demonstrable only postnatally as also the NPY receptors in the rat myocardium (Sheriff, personal communication).

Thus there seems to be a relatively later appearance and perhaps expression of NPY as compared to adrenergic innervation in the cardiac musculature. Alterations from this normal pattern of development of NPY and its role in disease conditions of the heart would be of significant importance to analyse.

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