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Altered *in vitro* uptake of norepinephrine by cardiovascular tissues of young spontaneously hypertensive rats*

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A number of different observations indicate that the sympathetic nervous system is involved in the development of spontaneous hypertension in the Okamoto strain [1–5], and the young spontaneously hypertensive rat (SHR) is thought to be a suitable model for the study of sympathetic activity at a prehypertensive stage.

Prior to 3 weeks of age, systemic arterial pressure of SHR is reported to be not much different from that of control rats [6]. Since the rapid rise of blood pressure is detected normally at 4 weeks [3, 6–8], the period of initiation should be considered to precede 4 weeks of age [6].

Plasma norepinephrine (NE) levels are higher in SHR at 3–4 weeks of age than in age-matched WKY controls [2, 4, 7, 9], but no significant difference is present between the two strains by 12 weeks of age [2]. Dopamine β-hydroxylase (DBH) is secreted by the process of exocytosis from sympathetic nerve endings with NE [10]. Serum DBH activity in SHR after 6 weeks of age and also at 16 weeks is not significantly different from that of WKY [11, 12]; however, at 3 weeks of age, both mesenteric vessel and serum DBH activities of SHR are about two times higher than those of WKY [7, 12, 13].

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Cardiac NE turnover, which may reflect sympathetic activity, is increased in SHR from 30 to 60 days after birth [14]. The turnover of NE in the kidney and skeletal muscles of SHR, compared to WKY, is also significantly higher at 5 weeks of age but it is no longer elevated at 9–14 weeks when hypertension is stabilized [5, 14]. No such changes are seen in these organs of WKY or Wistar rats when turnover of NE is compared at 5–9 weeks [5].

We have previously reported neuronal incorporation of [^{3}H]NE by isolated storage vesicular fractions of mesenteric arteries [15] and portal-mesenteric (P-M) veins and atria [16] of adult SHR and age-matched WKY controls. [^{3}H]-NE, measured in storage vesicular fraction, was found to be significantly greater in the mesenteric arteries but those of atria and P-M veins were reduced significantly [16]. In the present study, we investigated [^{3}H]NE uptake by mesenteric artery and atria of SHR and their age-matched WKY at 3 and 4 weeks of age. Our previous reports of adult rats excluded cytoplasm from the storage vesicle pellet and, therefore, dealt with [^{3}H]NE uptake and retention by storage vesicles and not total [^{3}H]NE uptake into nerve terminals. The present study, on the other hand, represents the total [^{3}H]NE uptake by the synaptosomal fraction including uptake of NE both from synaptic cleft to neuronal cytoplasm and from cytoplasm to the interior of storage

vesicles of mesenteric arteries and atria. We have observed a trend from 3 to 4 weeks of age for a progressive increase of NE uptake by mesenteric arteries from SHR starting with suppressed uptake at 3 weeks and increased uptake at 4 weeks as compared to WKY. Atria did not show this trend; rather, considerably larger amounts of [^{3}H]NE were incorporated into the synaptosomal fraction of SHR atria than those of the age-matched WKY rats at both ages.

Methods

Male SHR of the Okamoto strain [17] and age-matched WKY rats, 3–4 weeks of age, were used (Charles River Breeding Laboratories, Wilmington, MA.

Preparation and incubation of tissues. Rats were decapitated, and tissues from one SHR and one WKY were used for each experiment. The superior mesenteric artery with most of its branches and mesentery and atria (both left and right atria were used) were preincubated for 15 min at 37° in buffer, containing in g/l: NaCl, 8.06; KCl, 0.35; $CaCl_2 \cdot 2H_2O$, 0.30; $MgSO_4 \cdot 7H_2O$, 0.294; KH_2PO_4, 0.162; and dextrose, 2.0, and 1 mM pargyline. This medium was adjusted to pH 7.4 before each experiment [18]. The samples were then incubated for 60 min in the same medium in the presence of [^{3}H]-*l*-NE (Amersham Corp., Arlington Heights, IL). We used 1 nmole of [^{3}H]-*l*-NE (sp. act. 30 Ci/mmole) in 2 ml of buffer.

After incubation, the tissues were blotted, washed five times in ice-cold incubation medium less [^{3}H]-*l*-NE, and weighed. The mesenteric arteries were resuspended in cold 0.32 M sucrose solution containing 10^{-3} M EDTA, 0.1 M potassium phosphate, pH 7.4, and placed in a glass homogenizer with a loose-fitting pestle; the pestle was gently rotated by hand to remove fat [15]. The defated mesenteric arteries and atria were then minced and homogenized in approximately 10 vol. of ice-cold sucrose medium. A cooled Teflon-in-glass homogenizer with a clearance of 0.004 to 0.006 inch was used; 30 passes of the pestle were made at 10/min. The pestle rotated at approximately 1500 rpm for mesenteric artery and 1000 rpm for atria.

Differential centrifugation and cell fractionation. Tissue homogenates were subjected to successive centrifugation, which led to separation of three pellets, P_1, P_2, and P_3, and the corresponding supernatant fractions, S_1, S_2, and S_3, by a procedure identical to that used for the mesenteric arteries in Part 1 of our report [15]. The initial centrifugation at 600 *g* and the second step at 9000 *g* for 10 min each were performed in an SS-34 rotor in a Sorvall RC-2 centrifuge. The last step to obtain the P_3 and S_3 was performed in a Beckman SW60 Ti swing-out rotor at 105,000 *g* for 120 min on a Beckman L5-65 ultracentrifuge.

Discontinuous sucrose density gradients were prepared in 4.8-ml Beckman nitrocellulose tubes by layering eight successive concentrations of sucrose which ranged from 0.4 M at the top of the gradient to 1.4 M at the bottom. The P_3 pellet was resuspended in 0.5 ml of glass-distilled water and transferred to the top of the discontinuous sucrose gradient and centrifuged in a Beckman SW60 Ti swing-out rotor at 53,000 *g* for 90 min.

Ten-drop (125 μl) fractions were collected from the bottom of the gradient through a small hole. The radioactivity associated with the [^{3}H]NE in each fraction was counted in a Beckman model LS-335 liquid scintillation counter.

Protein assay. The protein content of samples was determined by the method of Lowry *et al.* [19] using bovine serum albumin as the standard.

Data analysis. Age- and sex-matched SHR littermate and WKY littermate control rats were always killed simultaneously, and tissues from each strain were fractionated, incubated, and assayed identically and in parallel, thereby obtaining paired data from each study [20]. The NE incorporation data were analyzed by the paired *t*-test for paired observations [21].

Results

Figure 1 shows that peak radioactivity of the synaptosomal fraction was associated with a discrete region banded around the 0.4 to 0.5 M sucrose interface in both SHR and WKY samples, which indicates that [^{3}H]NE was incorporated into NE storage vesicles [15]. An additional radioactivity peak was also associated with a heavier band at the 0.7 to 0.8 M sucrose interface in SHR preparation indicating presence of a possible aggregate of storage vesicles [15].

The amounts of incorporated NE calculated from the radioactivity per mg protein of synaptosomal fractions (P_3) from both mesenteric arteries and atria are shown in Table 1. The mean value of different paired experiments conducted on different days is shown together with their standard errors. In four of six paired studies, incorporation of [^{3}H]NE by the mesenteric arteries of 3-week-old SHR was less than that of controls, and the mean value was 10.3% less than that in WKY tissues ($P > 0.05$, NS). In contrast, incorporation of [^{3}H]NE by the mesenteric arteries of 1 week older SHR was higher than that of controls in six of seven paired experiments, where the mean difference was 46.7% higher in SHR in comparison to that of WKY samples, the differences being statistically significant ($P < 0.05$).

The NE incorporation data obtained with the synaptosomal fractions from atria of 3- and 4-week-old SHR and WKY rats are also shown in Table 1. In both age groups, incorporation of [^{3}H]NE by the atria of SHR was higher than that of controls in all but one experiment. The mean difference between paired atria samples of 3-week-old SHR

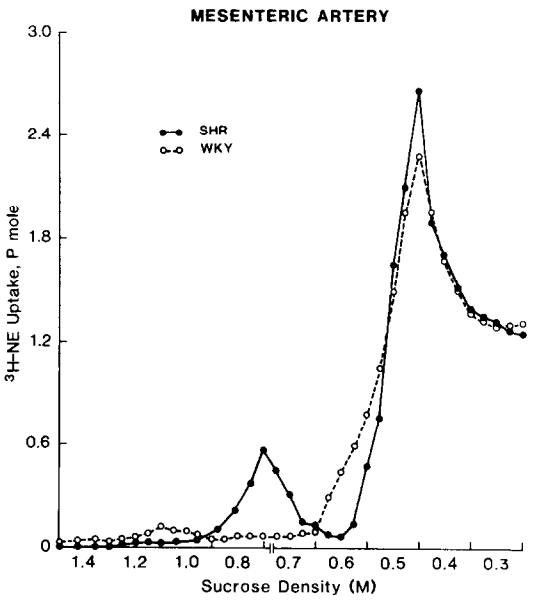

Fig. 1. Distribution of [^{3}H]NE in sucrose density gradient subfractions of synaptosomal fraction (P_3). Mesenteric arteries of 4-week-old SHR and WKY rats were used. The P_3 pellet was hypo-osmotically ruptured by resuspending in 0.5 ml of glass-distilled water and was transferred to the top of the discontinuous sucrose gradient and centrifuged in a Beckman SW60 Ti rotor at 53,000 *g* for 90 min. Ten-drop fractions were manually collected from the bottom of the gradient through a small hole.

Table 1. Incorporation of [^{3}H]NE into synaptosomal fraction (P_3)*

Tissue	Age (weeks)	No. of studies	[^{3}H]NE uptake (pmoles × 10^{-1}/mg protein P_3) SHR	WKY	Difference (% of WKY)
Mesenteric Arteries	3	6	8.26 ± 1.71	9.21 ± 1.91	−10.3
			NS†		
	4	7	6.88 ± 1.31	4.69 ± 0.83	+46.7
			$P < 0.05$		
Atria	3	8	39.51 ± 2.47	34.66 ± 1.63	+14.0
			$P < 0.05$		
	4	7	46.93 ± 3.07	38.03 ± 2.95	+21.7
			$P < 0.05$		

* Each paired incubation was done in triplicate. Values are means ± S.E.M.
† Not significant.

rats was 14.0% higher than that of WKY controls and the difference in 4-week-old rats was even greater (21.7%) than that of 3-week-old animals. The differences between paired atria samples of both age groups were statistically significant ($P < 0.05$).

Discussion

The amount of [^{3}H]NE taken up into the synaptosomal fractions of mesenteric artery and its branches was substantially higher (46.7% higher, $P < 0.05$) in a 4-week-old SHR as compared to an age-matched control WKY, whereas at 3 weeks of age no difference in uptake was observed. Within each strain, NE uptake declined between 3 and 4 weeks as shown in Table 1. However, the decline was substantially greater (49%) in WKY than in SHR (17%). This resulted in the relatively greater uptake of NE by synaptosomal fractions of SHR than by those of age-matched WKY ($P < 0.05$). This age-related decline in NE uptake supports earlier reports by Nagatsu *et al.* [13] and Nagaoka and Lovenberg [7] of a decline in other indices of sympathetic activity in these rats between 3 weeks and later age.

The relatively higher uptake of NE by 4-week-old SHR fits with our previous report of enhanced incorporation of [^{3}H]NE into storage vesicles of mesenteric arteries of adult SHR [15]. Interestingly, the latter data were based on uptake into storage vesicle fraction only, whereas in the present study results were based on uptake by the synaptosomal fraction, both uptake of NE from synaptic cleft to neuronal cytoplasm and from cytoplasm to the interior of storage vesicles of mesenteric arteries.

Our data showing reduced uptake followed by increased uptake in mesenteric arteries at 3 and 4 weeks, respectively, are compatible with other studies of young SHR in that levels of plasma NE were higher in 3- but not 6-week-old SHR than age-matched WKY [7, 9, 13]. A significant fraction of circulating NE is considered to be derived from blood vessels [22], so that a reduced NE uptake into synaptosomal fractions of 3-week-old SHR vessels could contribute to higher levels of serum NE, while a progressively higher uptake of NE by mesenteric arteries of older rats tends to lower NE in serum.

The present study shows that the amount of [^{3}H]NE incorporated into the synaptosomal fraction of atria from 3- to 4-week-old SHR was considerably larger than those of the age-matched WKY rats. The average amount of [^{3}H]-NE uptake in 3-week-old SHR was about 14% higher than that of WKY and maintained a 21.7% higher uptake at 4 weeks of age. An enhanced incorporation of [^{3}H]NE by the atria of young SHR is in contrast to that by the adult samples in which the NE incorporation was less (average 77.7%) than that of controls as we reported previously [16]. Our data on young SHR appear to be compatible with increased cardiac NE turnover in the pre-and early hypertensive stages of SHR [14]. Yamori [14] observed that cardiac NE turnover was increased in SHR in the pre- and early hypertensive stages but it was no longer elevated at 100 days after birth when hypertension was stabilized. Our results confirm this, in that what we are observing is a progressively increased NE uptake activity by SHR at the 3–4 week stage, compatible with observations of increased cardiac NE turnover by SHR at this stage. At the adult stage, the increased NE uptake activity of SHR may still be present. But results as expressed by pmoles NE/mg protein are affected by the adaptive hypertrophy that occurs in adult SHR as suggested by their high atrial pressures [23, 24].

In summary, NE uptake by mesenteric artery and atria of SHR at 3 and 4 weeks of age was compared with that of age-matched WKY. The arteries and atria were incubated in the presence of [^{3}H]NE; subcellular fractions were isolated by means of differential and sucrose density gradient centrifugation. The amount of [^{3}H]NE taken up into the synaptosomal fractions of mesenteric artery in 3-week-old male SHR was considerably lower than that of the age-matched WKY, but it was substantially higher in 4-week-old SHR as compared to control WKY in the paired experiments. Such a trend for a relative increase of NE uptake fits with our previous report of enhanced incorporation of [^{3}H]NE into storage vesicles of mesenteric arteries from adult SHR. It is also compatible with other studies of young SHR which found that plasma levels of NE were higher in SHR as compared to age-matched WKY at 3–4 weeks but not at 6 weeks. Atria did not show this trend. Rather, considerably larger amounts of [^{3}H]NE were incorporated into the synaptosomal fractions of SHR atria than those of the age-matched WKY rats at both ages.

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Absence of a requirement for long-range DNA torsional strain in the production of protein-associated DNA strand breaks in isolated mammalian cell nuclei by the DNA intercalating agent 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA)

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DNA intercalating agents, such as the clinically active antineoplastic drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA), produce in mammalian cells [1] or in isolated nuclei [2] DNA single- and double-strand breaks that appear to be associated with covalently linked proteins [3]. These protein-associated strand breaks were hypothesized to represent topoisomerase–DNA complexes which might be induced by the torsional strain generated in the DNA helix by the reduced helical twist which accompanies intercalation [3]. The topoisomerase–DNA complexes were proposed to act as swivels that would relieve this strain [4].

In the present study, the possible involvement of torsional strain was tested by examining the effects of X-ray-induced DNA breaks. X-ray-induced strand breaks can relieve torsional strain over DNA lengths of at least 10^{10} daltons, as indicated by the effects of low doses of X-ray on the sedimentation velocity of mammalian cell nucleoids [5]. If strand breaks can relieve torsional strain over long distances in nuclear chromatin and if this strain is required for the formation of protein-associated strand breaks, then it should be possible, by X-irradiation of cells or isolated nuclei prior to treatment with intercalator, to prevent the formation of the protein-associated strand breaks.

In practice, this experiment cannot readily be performed using intact cells, because cells would repair the X-ray-induced strand breaks during the subsequent treatment with intercalator. The experiment, however, can be conducted using isolated nuclei, because isolated nuclei do not repair X-ray-induced strand breaks, but do form protein-associated strand breaks in response to intercalators [2, 6]. Using isolated nuclei, we have studied the effect of X-rays on the production by m-AMSA of DNA single- and double-strand breaks and DNA–protein crosslinks. We conclude that long-range torsional strain does not play a role in the formation of protein-associated strand breaks by m-AMSA.

Materials and methods

Cells and radioactive labeling. L1210 mouse leukemia cells were grown in suspension cultures in RPMI 1630 medium plus 15% (v/v) fetal calf serum as described previously [1]. Cellular DNA was radioactively labeled in exponentially growing cells by incubation for 20 hr at 37° with [2-^{14}C]thymidine (0.02 μCi/ml) or with [*methyl*-^{3}H] thymidine (0.1 μCi/ml; 10^{-6} M unlabeled thymidine added) (New England Nuclear Corp., Boston, MA).

Isolation of L1210 cell nuclei. L1210 cells were centrifuged and resuspended in nucleus buffer [150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA), 0.1 mM dithiothreitol, pH 6.4] at 0° as described previously [2, 6]. Cells were centrifuged and resuspended in nucleus buffer plus 0.27% Triton X-100, incubated for 10 min at 0°, and pelleted by centrifugation (1200 rpm for 5 min). The nuclei were then resuspended in nucleus buffer.

Drug treatment and irradiation of cells and nuclei. m-AMSA (NSC 249992), obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, was dissolved at 10 mM in dimethyl sulfoxide and stored frozen at −20°. m-AMSA treatments were for 30 min at 37° and were stopped by a 20-fold dilution of treated nuclei in drug-free nucleus buffer at 0°. Control nuclei were treated with dimethyl sulfoxide at the same final concentration as used in the m-AMSA treatments.