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Sympathectomy alters acetylcholinesterase expression in adult rat heart

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Abstract

Study objective **– The aim of the study was to determine the significance of adrenergic nerve associated acetylcholinesterase for the pool of total acetylcholinesterase molecules.**

Design **– Acetylcholinesterase was analysed after destruction of adrenergic nerves by 6-hydroxydopamine or bilateral stellate sympathectomy. Effectiveness of treatment was verified by determining noradrenaline concentrations in right ventricle. Acetylcholinesterase activity was assayed in homogenates of atria and portions of left ventricular free wall.**

Subjects **– Adult male Sprague-Dawley rats were used, weight 225-260 g, n = 5 per experimental group.**

Main results **– Sympathectomy caused a small decrease in acetylcholinesterase activity, due to a decrease in the activity of the tetrameric globular form of the enzyme. Choline acetylcholinesterase activity was not altered by sympathectomy, which is an indication that cholinergic nerves were not affected.**

Conclusions **– The contribution of adrenergic neurones to the cardiac pool of acetylcholinesterase is measurable and consists primarily of the tetrameric globular form of the enzyme.**

Acetylcholinesterase, which hydrolyses the transmitter acetylcholine used at both the pre- and postganglionic synapses of the parasympathetic system, both present in heart, is a secreted glycoprotein that exists as multiple molecular forms.[1] Two classes of molecular forms exist in heart: asymmetrical forms, which consists of catalytic subunits covalently bound to a collagen-helical peptide, and globular forms which lack collagen tails .[2 3] The importance of the multiple molecular forms of this enzyme is that they allow it to have more than one subcellular location.[1] In heart, acetylcholinesterase is thought to have both neuronal and muscle cell locations.[4–6] In rodent and rabbit heart, it has been shown to be located along the sarcolemma of the muscle cells.[4 5] These same studies showed that acetylcholinesterase is associated with adrenergic nerves as well as with cholinergic nerves. To determine the extent of its association with the adrenergic nerves of rodent heart, acetylcholinesterase expression was investigated in adult rats after both chemical and bilateral stellate sympathectomy. Both of these methods have been shown to destroy the adrenergic postganglionic fibres of heart.[7–10] Experiments were also performed to determine which molecular forms are associated with adrenergic nerves.

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Methods

6-HYDROXYDOPAMINE TREATMENT AND TISSUE PREPARATION

Adult male Sprague-Dawley rats (225-260 g) were injected intraperitoneally with 100 $mg \cdot kg^{-1}$ 6-hydroxydopamine-HBr (in 0.9% NaCl and 0.1% ascorbic acid) on the first day and with 200 $mg \cdot kg^{-1}$ of this neurotoxin one week later. Control animals received vehicle on the same schedule. Animals were given water and regular lab chow ad libitum, and were housed under a 12-12 h light-dark cycle. The principles governing the care and treatment of animals as laid down by the National Institutes of Health were followed at all times during the study.

Two weeks after the initial injection each animal was anaesthetised by an intraperitoneal injection of sodium pentobarbitone (36 $mg \cdot kg^{-1}$). The thoracic activity was opened and the heart was perfused with 50-100 ml ice cold heparinised saline (0.9% NaCl and 10 $IU \cdot ml^{-1}$ sodium heparin) through the left ventricle to remove blood. This treatment does not reduce the amount of asymmetrical acetylcholinesterase present in the heart (unpublished observation). The heart was subsequently removed, rinsed with ice cold heparinised saline, and dissected into four chambers. Tissue samples were stored at –70°C until use. On the day of assay, tissue samples were weighed, minced and homogenised in glass-glass conical homogenisers at a dilution of 1:10 (w/v) in 5 $mmol \cdot litre^{-1}$ potassium phosphate, pH 7.4, containing 0.1 $mmol \cdot litre^{-1}$ EDTA.

ACETYLCHOLINESTERASE HOMOGENATE ASSAYS

Aliquots of homogenates were diluted 1:1 (v/v) with 50 mmol·litre^{-1} potassium phosphate buffer, pH 7.4, with 2% Triton X-100, NaCl 2 mol·litre^{-1} and EDTA 10 mmol·litre^{-1}. Triplicate samples of the diluted homogenates were preincubated for 15 min at 37°C in the acetylcholinesterase assay buffer consisting of potassium phosphate 50 mmol·litre^{-1}, pH 7.4, and ISO-OMPA (tetraisopropylpyrophosphoramide) at a final concentration of 0.1 mmol·litre^{-1}. The enzyme reaction (400 μl total reaction volume) was initiated by the addition of [^{3}H]-acetylcholine iodide (10-100 mCi·mmol^{-1}) at a final concentration of 0.1 mmol ·litre^{-1}. Assays were performed at 37°C and stopped after 20 min by the addition of 400 μl of 50 mmol·litre^{-1} glycine containing 1 mmol·litre^{-1} NaCl at pH 1.25. The [^{3}H]-acetate product was quantified by liquid scintillation spectrometry.[11] Activity is expressed as pmol [^{3}H]-acetate formed per min. Protein levels were determined by the method of Markwell *et al.*[12]

CHOLINE ACETYLTRANSFERASE HOMOGENATE ASSAY

Choline acetyltransferase activity was determined by a modification of the method of Roskoski *et al.*[13] Aliquots of the homogenates were diluted 1:20 (w/v) in potassium phosphate buffer 5 mmol·litre^{-1}, pH 7.4, containing EDTA 0.1 mmol·litre^{-1}. Aliquots (20 μl) of the diluted homogenates were assayed in duplicate at 37°C. The assay was initiated by the addition of 20 μl of an assay mixture containing [^{14}C]-acetyl coenzyme A for a final total concentration of 0.4 mmol·litre^{-1}, choline chloride 2 mmol·litre^{-1}, physostigmine 0.5 mmol·litre^{-1}, potassium phosphate 50 mmol·litre^{-1}, pH 7.4, EDTA 0.1 mmol·litre^{-1}, potassium chloride 100 mmol·litre^{-1} and 0.1% Triton X-100. After 15 min, reactions were terminated by the addition of 20 μl of a solution consisting of acetylcholine 200 mmol·litre^{-1}, acetylcarnitine 100 mmol·litre^{-1} and 0.1 N formic acid. To separate the [^{14}C]-acetylchloline product from the radioactive substrate and other radioactive products, aliquots of the terminated reaction mixture were subjected to low voltage paper electrophoresis. Levels of [^{14}C]-acetylcholine were determined by liquid scintillation spectrometry.

NORADRENALINE ASSAY

Noradrenaline concentrations were determined in samples of the right ventricular free wall by the radioenzymatic method of Henry *et al.*[14] Hearts were removed as described above. Portions of the right ventricular free wall were homogenised 1:10 (w/v) in 0.1 N perchloric acid. Homogenates were centrifuged at 10000 *g* for 10 min at 4°C. Aliquots of 50 μl were assayed in duplicate at 37°C in 15 ml glass conical tubes. The assay was initiated by the addition of 50 μl of an assay mixture consisting of Tris-HCl 1 mol·litre^{-1}, pH 8.6, 5% EDTA, dithiothreitol 0.1 mmol·litre^{-1}, [^{3}]-S-adenosylmethionine 2.5 μCurie, and phenylethanolamine-N-methyltransferase 0.22 IU. After 30 min, the reaction was stopped by the addition of 2 ml of 0.5 mol·litre^{-1} sodium buffer, pH 10, containing 5% EDTA, dithiothreitol 0.1 mmol·litre^{-1} and alumina 150 mg. The alumina was washed and then the [^{3}H] adrenaline was eluted in 1 ml of 0.1 N perchloric acid, after the addition of 50 μl of 0.2 N acetic acid with 25 μg adrenaline and 100 μg S-adenosylmethionine chloride. Subsequently, 100 μl of a saturated solution of phosphotungstic acid was added. Tubes were centrifuged for 5 min at a low speed and 1 ml aliquots of the supernatant were transferred to another tube, which contained 1 ml potassium phosphate 1 mol·litre^{-1}, pH 7.15, and 10 ml of 1% v/v diethylhexylphosphoric acid-toluene. Tubes were vortexed and centrifuged at a low speed and 9 ml of the organic phase was counted in 400 μl of Liquifluor. Standard curves were linear in the range of 0.1-10 ng noradrenaline.

BILATERAL STELLATE SYMPATHECTOMY

Four male rats subjected to bilateral stellate sympathectomy and four male rats with sham surgeries were obtained from Zivic-Miller, inc. All rats were of the Sprague-Dawley strain and were used for experimentation 12 d after surgery. Aliquots of the right ventricle of each animal were subjected to the noradrenaline assay as described above, while left ventricle and atrial homogenates were centrifuged at 20000 *g* for 30 min to obtain supernatants for assay of acetylcholinesterase activity and molecular forms.

ANALYSIS OF ACETYLCHOLINESTERASE MOLECULAR FORMS

For molecular form analysis, tissue samples were homogenised 1:15 (w/v) in Tris-HCl 50 mmol·litre^{-1}, pH 7.3, 1% Triton X-100, NaCl 1 mol·litre^{-1} and EDTA 5 mmol·litre^{-1}. Homogenates were centrifuged at 20000 *g* for 30 min at 4°C, after which 400 μl aliquots of supernatants were layered on linear 5-20% sucrose gradients containing the homogenate buffer. Velocity sedimentation was performed at 4°C and 230000 *g* maximum in an SW40 Ti rotor to an $\omega^2 t$ value of 1.09×10^{12} rad^2·s^{-1} or approximately 22 h. Forty 300 μl fractions were collected from the top of each gradient using an ISCO-185 fractionator. Acetylcholinesterase sedimentation coefficients were estimated by comparison with those of bovine serum albumin (4.41S) and catalase (11.3S), added to the gradient prior to centrifugation. The relative proportions of the acetylcholinesterase molecular forms, expressed as percentages, were calculated by comparing the enzymatic activity under each peak with that under the entire sedimentation profile.[6]

MATERIALS

Biochemicals were obtained as follows: [^{3}H]-acetylcholine iodide, [^{14}C]-acetyl coenzyme A and [^{3}H]-S-adenosyl methionine from NEN Research Pro-

ducts (Dupont), ultra pure sucrose from Schwartz-Mann, Co, and all other biochemicals from Sigma Chemical Company. Liquifluor came from NEN Research Products (Dupont). Other chemicals were obtained from Fisher Scientific Co and Aldrich Chemical Co.

STATISTICAL ANALYSIS

Data are expressed as mean (SEM). Statistical analysis was performed by the Student's *t* test.

Results

The effectiveness of the 6-hydroxydopamine treatment was determined by assaying for noradrenaline concentrations in samples of the right ventricular free wall. The mean noradrenaline concentration in the right ventricular samples of animals injected with control vehicle was 1.44(SEM 0.23) $\mu g \cdot g^{-1}$ wet weight. Treatment with 6-hydroxydopamine resulted in a reduction of noradrenaline levels to below 5% of controls in the right ventricle on both day 9 and day 14.

Acetylcholinesterase activity was examined as a function of 6-hydroxydopamine treatment and the data are presented in table I. In animals that were killed on the ninth day after the start of treatment, acetylcholinesterase activity was lower than normal by 23-24%, but there was no difference in activity between groups on the 14th day after the start of treatment. The results were the same when the data were expressed per unit protein (data not shown).

To determine if the decrease in acetylcholinesterase was the result of changes in the cardiac cholinergic nerves, the activity of the cholinergic nerve marker choline acetyltransferase was examined in the atria and left ventricle. The activity of this enzyme was not altered by 6-hydroxydopamine treatment (table II).

Since the results of 6-hydroxydopamine treatment were non-conclusive regarding the presence of acetylcholinesterase in the adrenergic nerves, animals with bilateral stellate sympathectomies of 12 d duration were examined for acetylcholinesterase activity and compared to animals subjected to sham surgery. The level of noradrenaline in the right ventricle was reduced to between 10 and 15% of control by this surgical procedure. This treatment also reduced acetylcholinesterase activity. For example, in the left ventricle the activity was 53(2) $pmol \cdot min^{-1} \cdot mg^{-1}$ for the control group, while this value was 46(1) for the sympathectomised group ($p<0.01$, $n = 5$). In the atria, values were 106(4) for the control group and 92(4) for the treatment group ($p<0.01$, $n = 5$).

The effect of bilateral sympathectomy on the molecular forms of acetylcholinesterase was also examined. Supernatants obtained from the left ventricular free wall and atria were subjected to density gradient sedimentation analysis. Activities of molecular forms of acetylcholinesterase were calculated as outlined in Methods and the results are shown in table II. In both heart regions, G_1 (the monomeric globular form), G_4 (the tetrameric globular form), and A_{12} (the

Table I Effect of 6-hydroxydopamine (6-OHDA) pretreatment on acetylcholinesterase (AChE) and choline acetyltransferase (CHaT) activity in adult rat heart. Data are means (SEM)

			AChE ($pmol \cdot min^{-1} \cdot mg^{-1}$)	CHaT ($nmol \cdot min^{-1} \cdot g^{-1}$)
Atria	d9	Control	116(7)	4.66(0.23)
		6-OHDA	89(9)*	5.11(0.40)
	d14	Control	116(5)	4.63(0.22)
		6-OHDA	120(5)	4.76(0.48)
Left ventricle	d9	Control	34(3)	1.2(0.10)
		6-OHDA	24(2)*	1.3(0.11)
	d14	Control	34(3)	1.33(0.07)
		6-OHDA	34(2)	1.46(0.12)

Enzymatic activity is expressed per unit wet weight and represents activity in homogenates of rat heart; d9 and d14 refer to day 9 and day 14 after the start of treatment.
*$p<0.01$ between control and treatment groups, $n=5$.

Table II Effect of bilateral sympathectomy on the activities of molecular forms of acetylcholinesterase (AChE) in rat heart. Data are means (SEM)

		G_1	G_4	A_{12}
Left ventricle	Control	22(0.29)	26(1.4)	4.7(0.23)
	Sympathectomised	21(1)	20(0.75)*	4.0(0.34)
Atria	Control	43(2)	51(2)	8.1(0.35)
	Sympathectomised	38(2)	45(1)*	7.1(0.83)

Enzymatic activity was calculated as described in Methods and is expressed per unit wet weight. G_1 is the monomeric globular AChE form that sediments at 4S; G_4 is a tetrameric form of AChE that sediments at 10S; and A_{12} is the asymmetrical form of AChE that consists of three tetramers covalently bound to a collagen like tail and that sediments at 16S in our gradients.
*$p<0.01$ *v* control.

asymmetrical form with 12 catalytic subunits), were the predominate forms. G_2, the dimeric globular form, is present in small amounts and cannot be adequately separated from G_1 acetylcholinesterase in our gradient system. Thus when calculating G_1 activity, roughly 5-15% is due to contamination with G_2. In samples of the left ventricle free wall and atria, bilateral sympathectomy resulted in a decrease in G_4 acetylcholinesterase activity, but no significant changes in the other molecular forms.

Discussion

The results of the present investigation show that sympathectomy either by chemical or surgical means, resulting in the destruction of cardiac nerves,[7–9] causes the loss of acetylcholinesterase activity. These results suggest that adrenergic nerves contribute measurable amounts of acetylcholinesterase activity to the cardiac pool of this enzyme. Interestingly, not all treatment paradigms resulted in the same findings. For example, on the ninth day after the start of chemical sympathectomy, a decrease in acetylcholinesterase activity was seen, but by the 14th day the decrease was not apparent. This discrepancy may indicate that a compensatory increase in acetylcholinesterase can occur after sympathectomy and stresses the need to do more than one time point in these types of studies. A discrepancy in the amount of acetylcholinesterase loss was also seen between the chemical and surgical sympathectomy paradigms. This difference is most likely because the extent of degeneration, indicated by variations in noradrenaline levels, was different between the two groups. After bilateral stellate sympathectomy, noradrenaline levels were reduced by 85-90% and acetylcholinesterase levels were reduced by 15%. 6-Hydroxydopamine treatment resulted in a 95% or better reduction in noradrenaline levels on treatment day 9, concurrent with a 24-30% reduction in acetylcholinesterase levels. Thus the amount of acetylcholinesterase loss seen in our studies correlates with the amount of noradrenaline level reduction. Radioligand studies, after chemical sympathectomy, have shown a 30% reduction in [^{3}H]-quinuclidinyl benzilate binding sites[15] suggesting that 30% of the muscarinic receptors may be associated with cardiac adrenergic nerves. This reduction is similar to our finding that up to 30% of acetylcholinesterase is associated with cardiac adrenergic nerves.

An association of acetylcholinesterase with cardiac adrenergic nerves in rat, suggested by the present work, confirms the results from ultrastructural studies in rabbit and mouse[4 5] and indicates that this association may occur in many species including man. The presence of acetylcholinesterase in these nerves may be important for the termination of the action of acetylcholine at cardiac adrenergic nerves. Stimulation of muscarinic receptors in these nerves results in decreased release of noradrenaline upon sympathetic stimulation[16–20] and is one of the bases for the parasympathetic modulation of sympathetic effects.[16 21] Additionally, acetylcholinesterase in adrenergic nerves may relate to the putative non-cholinergic functions of this enzyme, such as processing neuropeptides,[22] or the inactivation of peptides.[23] In this regard, adrenergic nerves have been shown in heart to contain neuropeptides such as neuropeptide-Y.[7 8]

In the present investigation, only the globular forms of acetylcholinesterase exhibit decreased activity after sympathectomy. It would appear that G_4 acetylcholinesterase is associated with the adrenergic nerve fibres. G_4 acetylcholinesterase is the predominant form in nervous tissue and is thought to be the physiological functional form.[24] The monomeric form, G_1, is predominantly intracellular in many tissues[25 26] and may represent a precursor pool.[27 28] The amount of this precursor form in adrenergic nerves is probably relatively small compared to the amount in other cellular pools and therefore no measurable decrease was seen after sympathectomy. A_{12} acetylcholinesterase is thought to bind to the external lamina in muscle.[1]

In conclusion, our findings show that there is a small but measurable pool of the globular forms of acetylcholinesterase that are associated with the adrenergic nerve fibres in both ventricles and atria. The association of acetylcholinesterase with adrenergic nerves as well as with cholinergic nerves must be kept in mind when identifying cholinergic nerves by methods using acetylcholinesterase staining.

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