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Ultrastructural localisation of substance P and choline acetyltransferase in endothelial cells of rat coronary artery and release of substance P and acetylcholine during hypoxia

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Summary. Substance P and choline acetyltransferase have been localised in a small proportion of endothelial cells of rat coronary arteries using electron microscopic immunocytochemistry. During a hypoxic period of 1 min, coronary vasodilatation was produced in the Langendorff heart preparation and increased levels of substance P and acetylcholine were released into the perfusate. The possibility that these substances are released from endothelial cells during hypoxia and contribute to the hyperaemic response is discussed.
Key words. Heart; hypoxia; endothelial cells; substance P; acetylcholine; ultrastructure.

Introduction

It has been proposed that acetylcholine (ACh), substance P and many other neurohormonal substances act via receptors on endothelial cells causing release of endothelium-derived relaxing factor (EDRF) which acts on the underlying smooth muscle to cause vasodilatation[1–3]. Evidence has now been presented to suggest that EDRF

is nitric oxide[4], although it seems likely that there may be more than one EDRF[5].

The presence of endothelial cells is a prerequisite for vasodilatation of dog coronary arteries due to hypoxia[6,7]. In perfused heart preparations ACh relaxes precontracted rabbit coronary arteries only in the presence of an intact endothelium[8]. Vasodilatation of coronary arteries by substance P[9] is also endothelial-dependent[10]. Choline acetyltransferase (ChAT) has been localised in vascular endothelial cells in rat brain[11]. More recently serotonin and substance P have been localised in endothelial cells of rat femoral and mesenteric arteries[12], and serotonin has been localised in rat coronary artery endothelial cells[13].

In this study we have examined electron microscopically the immunocytochemical distribution of ChAT and substance P in rat coronary arteries and sought to establish a pathophysiological role for these substances by measuring their release from the perfused Langendorff rat heart preparation during hypoxia.

Methods

Localisation of substance P and ChAT in endothelial cells

Eight adult Wistar rats were anaesthetised with ether and perfused via the left ventricle with 37 °C phosphate-buffered saline (PBS) followed by a 4 °C fixative containing 3% paraformaldehyde, 0.1% glutaraldehyde and 150 ml saturated picric acid in 1000 ml of 0.1 M phosphate buffer (pH 7.3). Segments of coronary vessels were removed and placed in glutaraldehyde-free fixative overnight at 4 °C. Sections (40 µm) were cut on a Vibratome and were rinsed for 24 h in several changes of PBS. Sections were incubated with either antiserum to substance P or rat monoclonal antibody to porcine ChAT tested for specificity as previously described[14,15]. Antibody against substance P was generated in the rabbit against synthetic substance P conjugated to bovine serum albumin with glutaraldehyde, No. 16076 (CRB U.K.). Immunostaining was performed according to the peroxidase-antiperoxidase (PAP) technique of Sternberger et al.[16]. The peroxidase activity was visualised with DAB (3,3′-diaminobenzidine tetrahydrochloride) and H_2O_2. After peroxidase reaction, the Vibratome sections were postfixed in osmium and embedded in Epon. The primary antiserum was diluted 1:500 or 1:1000. Incubation was performed at 4 °C for 24 or 48 h. Normal serum and PAP were used in dilutions 1:50. Control experiments were carried out with antiserum preincubated for 1 h at 37 °C and for an additional 24 h at 4 °C with either substance P (Bachem Inc. R., 2910; 0.5 mg/ml) or purified ChAT (from bovine brain; 10 units per mg protein; Sigma, U.K.) or omitting the antibody. No reaction product was observed in control sections.

Preparation of rat hearts

Adult male Wistar rats (200–425 g) were injected with heparin (2500 units i.p.) before being killed by a blow to the head and exsanguination. The heart was removed and cannulated via the aorta for constant flow perfusion at a starting perfusion pressure of 50–60 mm Hg. This procedure has been described in detail previously[17]. The heart was paced at 4 Hz with electrical pulses of 5-ms duration and supramaximal voltage. The hearts were perfused for 20 min before a 200 µl bolus of 10^{-4} M ergotamine was given. This raised the perfusion pressure by an average of 50 mm Hg. Hypoxia was induced by switching from Krebs Henseleit solution equilibrated with 95% O_2, 5% CO_2 to one equilibrated with 95% N_2, 5% CO_2 via a second perfusion system with a rate-matched pump. After 1 min the hypoxia perfusion was ended by switching back to the original oxygenated solution. Control experiments were carried out using exactly the same procedure, except that normoxic Krebs solution replaced the hypoxic Krebs solution in the second perfusion system. Throughout the experiments, perfusion pressure and left ventricular pressure were monitored. Fractions (12-s duration) of cardiac effluent were collected in ice-cold polypropylene tubes and kept on ice until biochemical assay.

Substance P determinations

Ten fractions were collected before the hypoxic period, five during hypoxia and ten during recovery. Immediately after collection, aprotinin (final concentration 100 Kallikrein inhibitory units/ml) was added to an aliquot of each fraction in polypropylene tubes. Quantitation of substance P levels was by an inhibition enzyme-linked immunosorbent assay (ELISA) as previously described[18] (antibody to substance P raised in rabbits from CRB U.K.) with the following modifications: the standards were prepared in the perfusion buffer (Krebs Henseleit) and the antiserum raised in rabbits to substance P was diluted in 0.2% gelatin, 0.1% Tween 20, 0.04% sodium azide in PBS. Results were calculated as pmol substance P released per fraction per g wet weight heart.

ACh determinations

Fractions of perfusate were collected before, during and after the hypoxic period for ACh assay. ACh content in the perfusate was assayed by a chemiluminescent assay described by Israel and Lesbats[19]. The assay was modified so that the ACh was all converted to its breakdown products and the levels of choline determined. This precluded the necessity of incorporating an esterase inhibitor to prevent loss of ACh through degradation. Prior to the assay, the enzyme stock solution consisting of 4 ml of 0.2 M sodium phosphate buffer (pH 8.6), containing 100 µl of choline oxidase (250 U/ml), 50 µl of horseradish peroxidase (2 mg/ml), 1.04 ml of acetylcholinesterase (70 U/ml) and 100 µl of luminol (1 mM) was left to equilibrate for 1 h in the dark at room temperature. Automatic injection of 200 µl of the enzyme stock to 50 µl of perfusates and standard solutions produced chemilu-

minescence proportional to the amount of ACh and choline present, which was measured by a Packard Picolite Luminometer (United Technologies Packard, Berks, U.K.). The detection limit for ACh was 0.8 pmol/50 µl. Results were calculated as total amount of choline per fraction per g wet weight heart, and expressed as mean ± SEM.

Statistical comparison

Statistical comparison of biochemical data from both substance P and ACh determinations was carried out using a one-tailed Student's t-test and a two-tailed paired t-test.

Results

Localisation of substance P and ChAT in endothelial cells

Electron microscopic immunocytochemical examination of ultrathin sections of coronary arteries from all 8 animals showed immunoreactivity of a small proportion (approximately 5–10%) of endothelial cells for substance P (fig. 1) and ChAT (fig. 2A, B). The immunoreactive cells could be clearly seen usually sandwiched between unlabelled endothelial cells. Various organelles were detectable in the labelled cytoplasm including nuclei and Golgi apparatus (figs 1, 2A). Many caveolae or vesicles were observed, particularly associated with the abluminal surface of the cells (figs 1, 2A, B).

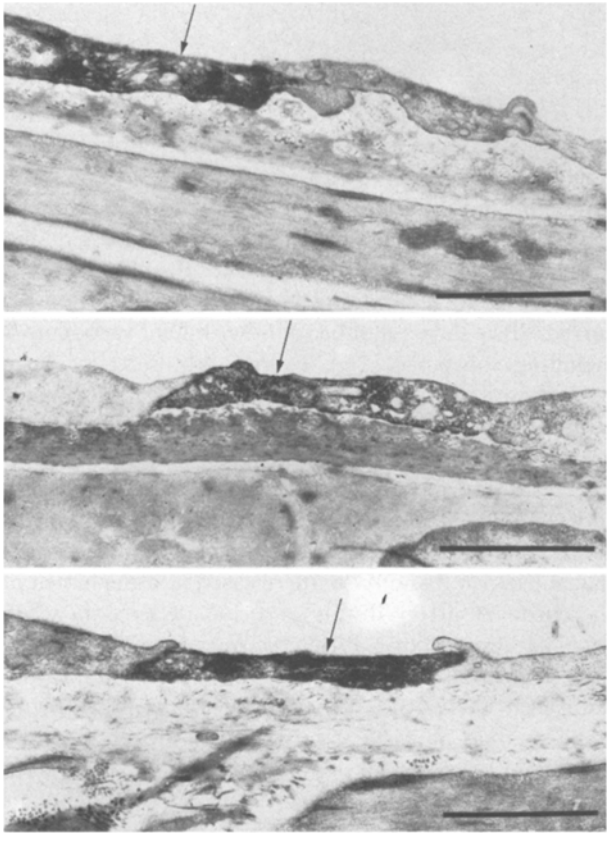

Figure 1. Ultrastructural localisation of substance P-like immunoreactivity in endothelial cells of rat coronary arteries (arrows). Note that in all 3 examples the adjacent cells are completely unlabelled. Calibration bar = 1 µm.

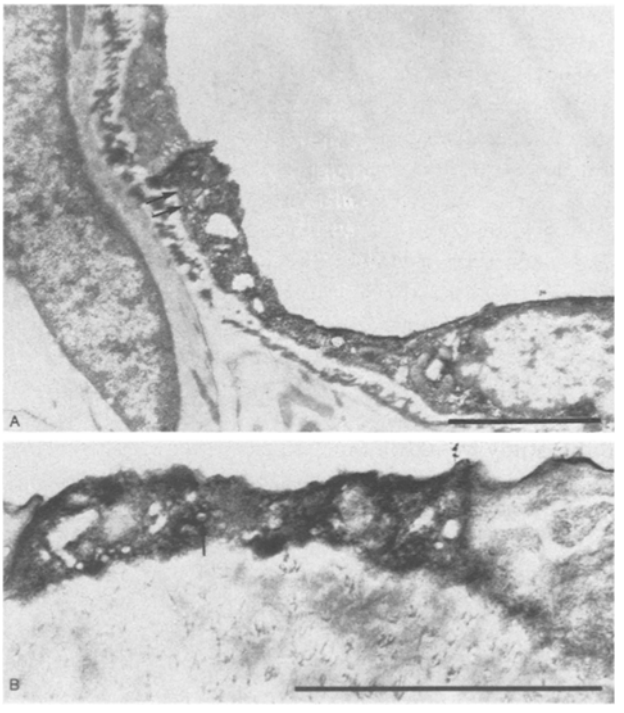

Figure 2. Ultrastructural localization of ChAT immunoreactivity in endothelial cells. *A* Cross-section through part of a small coronary artery. Note that the endothelial cell labelled with antibody to ChAT contains many caveolae (vesicles) on the abluminal surface (arrows). *B* Higher magnification of a ChAT-labelled endothelial cell. Arrows point to vesicles in the cytoplasm. Calibration bar = 1 µm in A and B.

Effect of hypoxia on the Langendorff heart preparation

Switching to hypoxic Krebs solution rapidly produced coronary vasodilatation as indicated by a decrease in perfusion pressure (fig. 3A) which was followed by a recovery phase on returning to oxygenated Krebs solution. In the control experiment using normoxic Krebs solution there was no vasodilatation (fig. 3B), thus eliminating any technical procedure as the causative factor for the response. Heart weights ranged from 0.74 to 1.56 g.

Substance P release during hypoxia

Perfusates from 12 Langendorff hearts subjected to hypoxia were analysed for substance P content. There was a significant ($p < 0.025$) increase in release of substance P during the hypoxic period (0.43 ± 0.08 pmol/fraction/g heart) compared to the prehypoxic (basal level) and recovery periods (0.23 ± 0.05 and 0.22 ± 0.05 pmol/fraction/g heart, respectively). Paired comparisons of substance P levels between basal and hypoxic levels for each animal showed a significant increase above the basal level of 320 ± 202% ($n = 12$) ($p < 0.02$) during the hypoxic period which was not seen during the same peri-

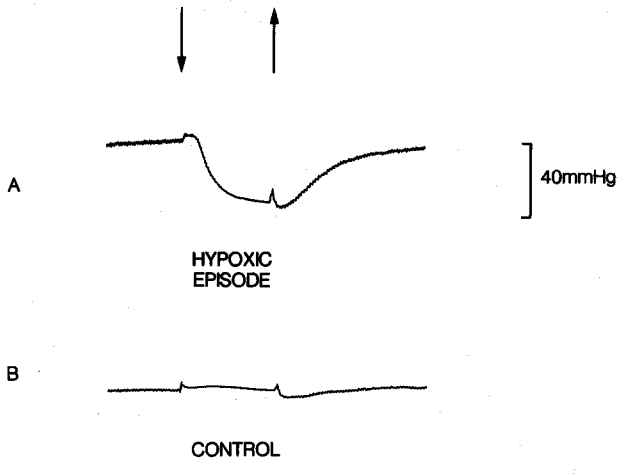

Figure 3. Trace of perfusion pressure through a heart preparation showing (*A*) vasodilatory response to hypoxia and (*B*) lack of response on switching to normoxic Krebs solution.

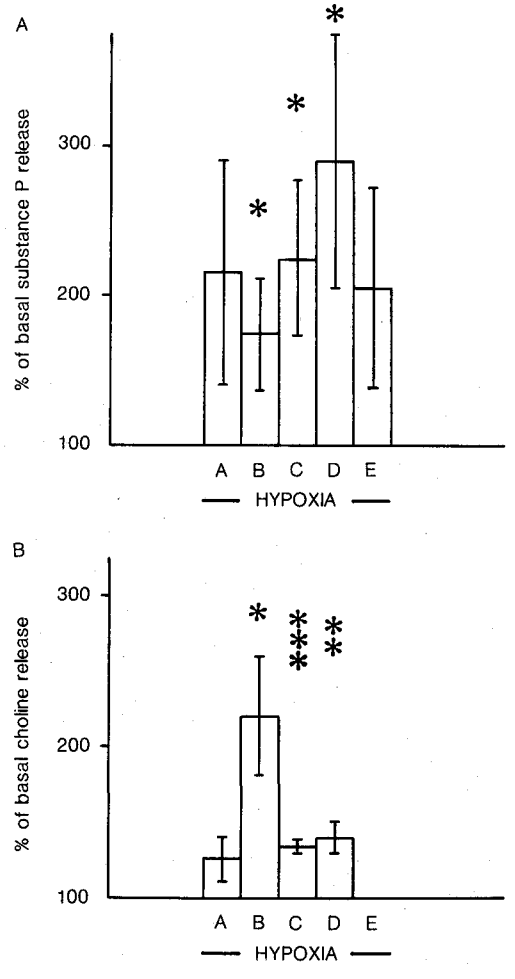

Figure 4. Release of (*A*) substance P (n = 12) and (*B*) ACh (n = 4) into the perfusion solution of rat Langendorff heart preparations during hypoxia, expressed as a percentage of the basal release ± SEM. (ACh was converted to choline prior to measurement as described in the text.) A to E represent the 5 fractions collected from the beginning to the end of the hypoxic period. *$p < 0.05$; **$p < 0.025$; ***$p < 0.01$.

od in 'normoxic' control preparations where substance P levels were 15 ± 20% (n = 6) above the basal level. When the fractional release of substance P per preparation during hypoxia (A to E consecutively) was calculated as a percentage of the mean of all the pre- and post-hypoxic fractions from the same preparation, a histogram of the mean percentage release ± SEM showed a significant release of substance P in fractions B, C and D, $p < 0.05$ (fig. 4A).

ACh release during hypoxia

Perfusates from 6 Langendorff rat hearts exposed to hypoxic conditions were analysed for ACh content, although in only 4 were the levels of ACh detectable (>24 pmol/fraction). In these 4 there was a significant increase in levels of ACh during the hypoxic period (57.6 ± 5.2 pmol/fraction/g heart) compared to basal levels and the recovery period (42.0 ± 4.8 and 39.9 ± 3.5 pmol/fraction/g heart, $p < 0.05$ and 0.025, respectively). The mean paired increase in ACh levels during the hypoxic period (38 ± 9% (n = 4)) was significant ($p < 0.02$). When the fractional release of ACh during hypoxia (A to E) was expressed as the mean percentage of the basal release (as for substance P), the release was significant in fractions B, C and D ($p < 0.05$, $p < 0.01$, $p < 0.025$, respectively) (fig. 4B).

Discussion

The paradox that ACh, a potent vasodilator of arteries in vivo often produced no relaxation or even contraction in isolated preparations in vitro was resolved in 1980 when Furchgott and Zawadzki[20] showed that the endothelial cells of the intimal surface of these vessels were required for vasodilatation; these had been accidentally removed in the in vitro preparations where no vasodilatation occurred. Since then a number of other potent vasodilators including substance P, ATP and serotonin[21,22] have been shown to act by releasing from the endothelium a humoral factor, EDRF, that produces arterial smooth muscle relaxation[10,23].

The results of the present study show firstly that substance P and ChAT are localised in a small percentage of endothelial cells of the rat coronary artery and secondly that substance P and ACh are released into the lumen of the coronary artery during a period of hypoxia when vasodilatation occurs; during recovery from the hypoxic period the levels of substance P and ACh are significantly reduced to the basal level. This suggests that these substances could well be involved with the endothelial-dependent[6] vasodilatory response to hypoxia.

Endothelial cell localisation of substance P and ChAT is not confined to rat coronary arteries. Substance P, and serotonin, have been localised in 5–10% of endothelial cells of rat mesenteric and femoral arteries[12] where it has been postulated that choline is taken up by the endothe-

lial cells and converted in the presence of ChAT to be stored as ACh.

In order that the endothelium may play a role in control of blood flow it must be exposed to the agents which act on it, causing release of EDRF during physiological and/or pathophysiological conditions. It has been suggested that substance P may originate from blood, where plasma levels are sufficient to elicit vasodilatation in vitro [10]. However, it is rapidly broken down by proteases; ACh is also quickly degraded in blood by acetylcholinesterase, therefore these substances must be released locally to be effective on the endothelium. A source of these agents within the endothelial cells themselves would allow such action.

Our studies provide no evidence that substance P and ACh released from the Langendorff heart during hypoxia is from an endothelial source. These neurotransmitters are also found in periarterial nerves at the medial-adventitial border [24]. However, if they are causing vasodilatation via endothelial receptors, it is physiologically unlikely that intramural vascular nerves are their origin, since they would have to pass through the medial muscle coat and elastic lamina to the intraluminal surface of the endothelium to have effect [10].

Mechanical stimulation and shear stress can also bring about vasodilatation which is endothelium-dependent [5, 10]. Damage to endothelial cells could lead to release of their contents which may act locally to cause vasodilatation and so prevent further damage. In some pathophysiological conditions, endothelial cells could become more fragile [25] and hence release vasoactive substances more readily.

Resting release of substance P and ACh and/or choline was detected in the present study, perhaps suggesting that as well as having a pathophysiological role in hypoxia they may be involved, in balance with excitatory neurotransmitters released from perivascular nerves, in normal homeostatic control of local blood flow.

During hypoxia, ATP is released from guinea-pig coronary arteries [26] and serotonin is released from rat coronary arteries where it is found in 50% of endothelial cells [13], a notably higher percentage than those showing substance P and ChAT immunoreactivity. Recent evidence suggests that ATP is the predominant agent acting via the endothelium (Hopwood, unpublished observation). However, the detailed distribution and specific roles of each of the vasoactive substances located in the endothelium are not yet known.

The results of the present study suggest that 2 vasoactive substances released from coronary arteries during vasodilatation caused by hypoxia are also localised in the endothelial cells and an attractive hypothesis is that this is their source to allow local control of blood flow and hence protect the heart from hypoxic damage.

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