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FORUM MINIREVIEW

The Non-neuronal Cholinergic System

The Non-neuronal Cholinergic System in the Endothelium: Evidence and Possible Pathobiological Significance

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ABSTRACT—An increasing body of knowledge indicates that the cholinergic system is not confined to the nervous system, but is practically ubiquitous. The present paper will address the question of the non-neuronal cholinergic system in vascular endothelial cells (EC). In tissue sections of human skin, immunohistochemical studies using confocal laser scanning microscopy showed ChAT (choline acetyltransferase) activity in the EC of dermal blood vessels. Positive ChAT immunoreactivity was also demonstrated in monolayer cultures of human umbilical vein EC (HUVEC) and a human angiosarcoma EC line (HAEND). That the synthesizing enzyme is not only present in EC, but also active was shown by measuring ChAT activity. Thus, in HUVEC cultures, ChAT activity amounted to 0.78 ± 0.15 nmol · mg protein^{-1} · h^{-1} (n = 3), but was only partially (about 50%) inhibited by the ChAT inhibitor bromoacetylcholine (30 μM). In HPLC measurements, a concentration of 22 ± 2 pmol acetylcholine (ACh) per 10^6 cells was found (n = 6). However, using a cholinesterase-packed analytical column to check the identity of the acetylcholine peak, the peak height was found to be reduced, although a significant peak still remained, indicating the existence of a compound closely related to ACh. Further immunocytochemical experiments indicated that EC in vitro also express the vesicular acetylcholine transporter (VAChT) system. Preliminary immunoelectron microscopic studies suggest a topographical association of VAChT with endothelial endocytotic vesicles. The presented experiments clearly demonstrate the existence of essential elements of the cholinergic system (ChAT, VAChT, ACh) in the human endothelium. The biological functions of ACh synthesized by endothelial cells are the focus of ongoing research activity.

Keywords: Non-neuronal cholinergic system, Endothelium, Signal transduction

It is becoming increasingly apparent that acetylcholine is far from being exclusively the realm of the nervous system. Thus, non-neuronal acetylcholine (ACh) has been found not only in the major classes of the animal kingdom but also in primitive plants. This has led to the concept of the "non-neuronal cholinergic system" (1, 2), the essential elements of which have been demonstrated in various human tissues, including immunocompetent cells such as lymphocytes (3), respiratory epithelial cells (4, 5) and keratinocytes (6). The ubiquity of the endothelium in the body (with the exception of, for example, cartilage) leads to the interesting question of whether the non-neuronal cholinergic system is expressed in this cell type. In the following sections, we will discuss the state of current knowledge from published data, as well as present new data from our research group, principally on the basis of experiments with endothelial cells (EC) in tissue culture.

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Overview of published data

It is well known that acetylcholine mediates multiple effects on the vascular endothelium, for example, the generation of the endothelium-derived relaxing factor (7). Therefore it appears of great cell biological interest whether EC are endowed with the biochemical apparatus to synthesize, transport, store and secrete ACh by themselves to control endothelial cell functions by local regulatory loops. The following will present the literature evidence for such a

system, followed by novel data from our own laboratories supporting this thesis. Parnavelas and co-workers (8) used ultrastructural immunocytochemistry to localize the synthesizing enzyme ChAT (choline acetyltransferase; EC 2.3.1.6) in EC of rat brain capillaries. These authors suggested that the release of ACh from EC as a result of ischemic damage could be protective against further hypoxia via the vasodilatory activity of ACh. Following isolation of rat brain cortex capillaries, a ChAT specific activity of 0.264 nmol · mg protein^{-1} · min^{-1} in the dissociated EC has been reported (9). However, in human fetal brain microvessels in culture, other authors failed to detect ChAT activity (10). A very significant finding was provided by Kawashima and colleagues, who studied ACh synthesis and release in bovine carotid artery EC in vitro (11). Analytical measurements by radioimmunoassay and HPLC indicated that the EC synthesize ACh and that the synthesized ACh was rapidly released from the cells (11).

In EC isolated from porcine cerebral microvessels, both HPLC and radioimmunoassay methods were used to measure ACh content (12). As well as clearly demonstrating the ability of EC to synthesize and release ACh, they also investigated possible inductive pathways for the synthesis. Phorbol ester was found to up-regulate ACh synthesis via a protein kinase C-independent pathway (12). However, studies with the ChAT inhibitor, bromoacetylcholine, suggested that ACh synthesis occurred independently of ChAT (12); i.e., an enzyme different from ChAT mediated the synthesis of ACh. Further studies by the same group investigated choline uptake in porcine brain EC and its utilization for both phospholipid and ACh synthesis (13). Recently, an immortalized rat brain EC (RBE4) has been used to engineer stable EC lines producing ACh (14). This has been achieved by employing an expression vector construct to transfect ChAT. Further studies have been carried out on EC transfected with the vesicular ACh transporter (VAChT) gene. It is to be expected that such genetically modified EC types may help unravel some of the regulatory pathways involved in ACh synthesis, storage, transport and release.

As already outlined above, the ability of the endothelium to respond to ACh has been well documented. This response depends on the expression of ACh receptors (AChR), which are of either muscarinic (mAChR) or nicotinic (nAChR) type. The pioneering work of Furchgott and Zawadzki (7) demonstrated that following interaction of ACh and mAChR, the endothelium produced a vasodilator, endothelium-derived relaxing factor (EDRF), now accepted as nitric oxide. Utilizing an in situ hybridization technique, it was demonstrated previously that cultured EC from the human aorta express nAChR of the alpha 3, alpha 5, beta 2 and beta 4 subtypes (15). Patch clamp experiments confirmed the functionality of these receptors. On the endothelium of human pulmonary veins, M_1 muscarinic receptors have been demonstrated (16).

The breakdown of ACh is mediated by the enzyme acetylcholinesterase (AChE), a ubiquitously distributed enzyme, which is also present in the endothelium. Over the years, data on AChE have been collected from studies in various species. Thus, AChE was demonstrated by histochemistry in newt brain capillaries (17) and at the ultrastructural level, in the external cuneate nucleus of gerbils (18). AChE activity, which decreased with prolonged culture, has also been found in human brain cortex EC cultures (19).

New data on the endothelial cholinergic system

As can be seen from the above review of the literature with respect to the non-neuronal cholinergic system in the endothelium, most of the experimental work has been performed on non-human tissue, especially from rodents. Our interest during the past years has been the use of human

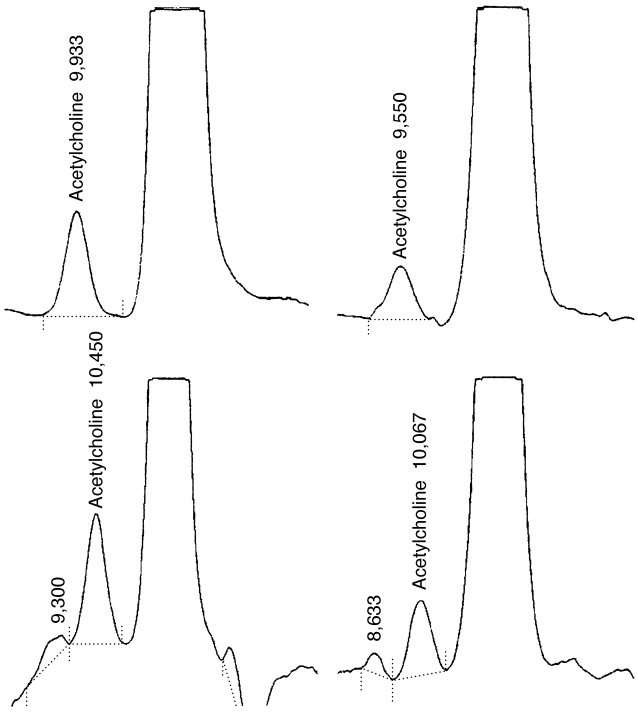

Fig. 1. ACh production in cultured HUVEC (upper two traces) and HAEND cells (lower two traces). The analysis was performed by HPLC using an bioreactor (column packed with choine-oxidase and cholinesterase and placed behind the analytical column) and electrochemical detection. The right column shows the trace after elution over an analytical column packed with cholinesterase to prove the identity of the ACh-peaks. For both HUVEC and HAEND, the ACh peak is clearly reduced in size, but not removed entirely, indicating that the peaks consist of both ACh and an ACh-related compound not sensitive to cholinesterase. In control expremiments with ACh-standards, the ACh-peak was eliminated when the cholinesterase packed analytical column was used. The second peak corresponds to choline.

EC culture systems to investigate the pathobiology of the endothelium in various disease states with special emphasis on inflammation, sepsis and tumour extravasation (20 – 23). Using monolayer cultures of human umbilical vein EC (HUVEC), HPLC techniques were able to demonstrate the production of ACh and a closely related compound (Fig. 1). Similar results were found for cultures of a human angiosarcoma cell line (HAEND, ref. 24; see Fig. 1).

Figure 2 presents immunocytochemical evidence of the presence in both HUVEC and HAEND of the ACh synthesizing enzyme ChAT. The distribution pattern at light microscopic level appears diffuse within the cytoplasm. Of great importance is the question of whether such signals can be detected in situ in the endothelium. In tissue sections of human skin, immunohistochemical studies using confocal laser scanning microscopy showed ChAT positivity in the EC of dermal blood vessels (25), thus confirming that the in vitro reactions cannot be ascribed to any artifact of tissue culture.

A further element of the non-neuronal cholinergic system of interest is the vesicular transporter. The lower two images of Fig. 2 show its immunocytochemical localization in the two endothelial culture models. Especially in the case of HUVEC, a marked reactivity was observed, with a distri-

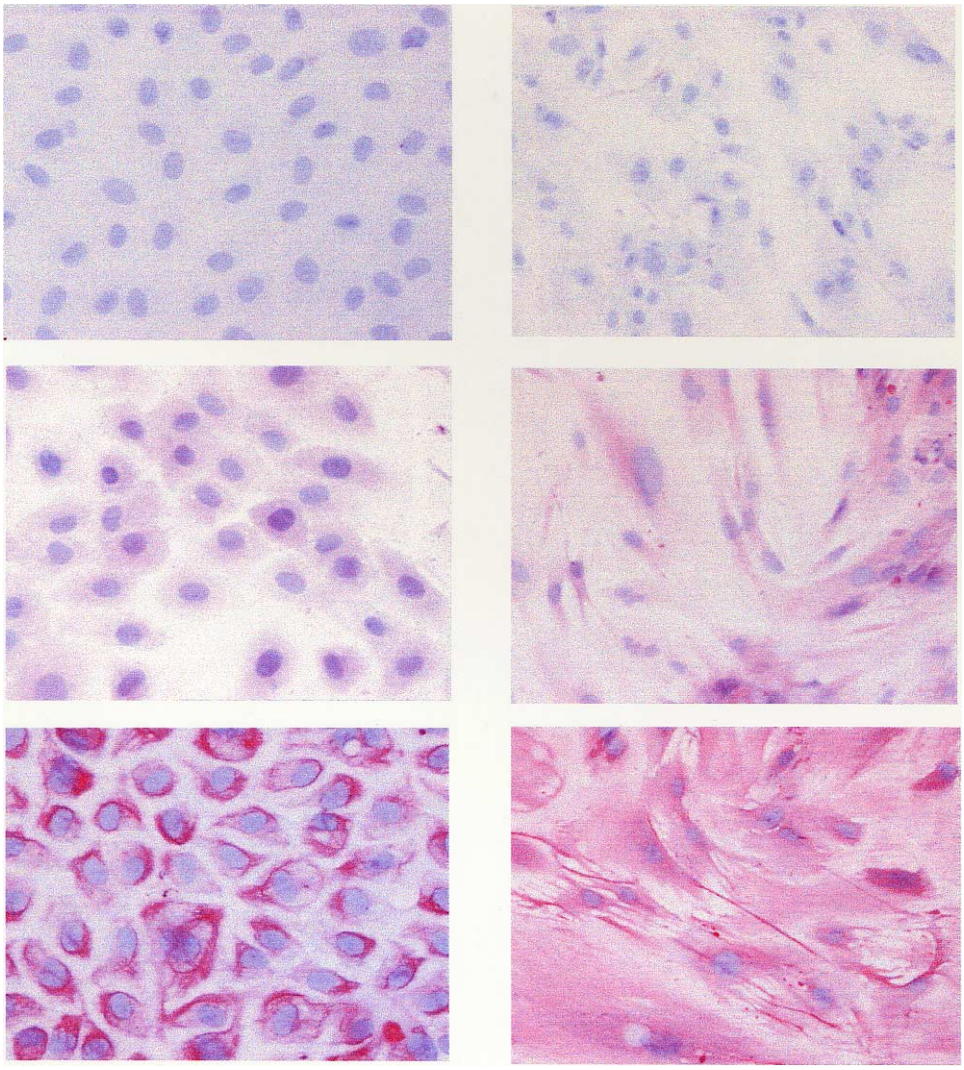

Fig. 2. Immunocytochemical study of parameters of the non-neuronal cholinergic system in cultured HUVEC (left column) from passage 4, and HAEND (right column, magnification 200-fold). The primary antibody was a polyclonal rabbit anti-choline acetyltransferase antibody (middle row) or a polyclonal goat anti-VAChT antibody (low row). Secondary antibody was a goat anti-rabbit IgG antibody-alkaline phosphatase conjugate (middle row) or a biotinylated anti-goat Ig G antibody (lower row). The upper row shows the control reaction in the absence of the primary antibody. The only staining reaction seen is the nuclear counterstain. The middle row shows positive staining in both cell types for ChAT, seen as a light red coloration of the cytoplasm. The lower row demonstrates the marked positive reaction for VAChT, which in the case of HUVEC appears to have a cytoskeletal distribution. The human astrocytoma cell line U373 was used as a positive control for the expression of ChAT (images not shown).

bution pattern reminiscent of the cytoskeleton. Preliminary studies at ultrastructural level using immunoelectron microscopy (IEM) provide the first evidence that VAChT epitopes can be found within endothelial intracellular vesicles (C.J. Kirkpatrick et al., 2000; unpublished results).

Future developments

Whilst we and others have demonstrated essential elements of the non-neuronal cholinergic system in the endothelium, it must be stressed that we are standing at the beginning of this fascinating chapter. There are many unanswered questions. One of the important tasks is to further define the subcellular localization of the synthesizing enzyme ChAT, as well as the vesicular transporter VAChT. The preliminary indications from our immunoelectron microscopical study that VAChT can be detected in vesicles of the endothelial cytoplasm and the cytoskeletal-like distribution demonstrated by light microscopic immunochemistry require further investigation. This could be achieved by co-localisation studies with antibodies recognizing characteristic gene products of the cytoskeletal and vesicular systems. Also of biological interest is the problem of possible heterogeneity of expression within the various vascular segments and even microcirculatory beds. This question could be adequately addressed using cultures of different EC types, as well as immunohistochemical studies in situ.

The central question in this field of research must however remain the delineation of the functional significance of endogenous endothelial ACh. Thus, the possible role in endothelial cytokine expression, induction and regulation of cell adhesion molecules, proliferation, angiogenesis and hemostatic control should be advanced during the next years.

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REFERENCES

1 Wessler I, Kirkpatrick CJ and Racké K: Non-neuronal acetylcholine, a locally acting molecule widely distributed in biological systems: expression and function in humans. Pharmacol Ther **77,** 59 – 79 (1998)
2 Wessler I, Kirkpatrick CJ and Racké K: The cholinergic "pitfall": acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol **26**, 198 – 205 (1999)
3 Kawashima K and Fujii T: Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther **86**, 29 – 48 (2000)
4 Reinheimer T, Bernedo P, Klapproth H, Oelert H, Zeiske B, Racké K and Wessler I: Acetylcholine in isolated airways of rat, guinea-pig, and humans: Species differences in the role of airway mucosa. Am J Physiol **270**, L722 – L728 (1996)
5 Klapproth H, Reinheimer T, Metzen J, Münch M, Bittinger F, Kirkpatrick CJ, Höhle K-D, Schemann M, Racké K and Wessler I: Non-neuronal acetylcholine, a signalling molecule synthesized by surface cells of rat and man. Naunyn Schmiedebergs Arch Pharmacol **355**, 515 – 523 (1997)
6 Grando SA, Kist DA, Qi M and Dahl MV: Human keratinocytes synthesize, secrete, and degrade acetylcholine. J Invest Dermatol **101,** 32 – 36 (1993)
7 Furchtgott RF and Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature **288**, 373 – 376 (1980)
8 Parnavelas JG, Kelly W and Burnstock G: Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature **316**, 724 – 725 (1985)
9 González JL and Santos-Benito FF: Synthesis of acetylcholine by endothelial cells isolated from rat brain cortex capillaries. Brain Res **412**, 148 – 150 (1987)
10 Kasa P, Pakaski M, Joo F and Lajtha A: Endothelial cells from human fetal brain microvessels may be cholinoceptive, but do not synthesize acetylcholine. J Neurochem **56**, 2143 – 2146 (1991)
11 Kawashima K, Watanabe N, Oohata H, Fujimoto K, Suzuki T, Ishizaki Y, Morita I and Murota S: Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett **119**, 156 – 158 (1990)
12 Ikeda C, Morita I, Mori A, Fujimoto K, Suzuki T, Kawashima K and Murota S: Phorbol ester stimulates acetylcholine synthesis in cultured endothelial cells isolated from porcine cerebral microvessels. Brain Res **655**, 147 – 152 (1994)
13 Suzuki T, Ikeda C, Fujii T, Fujimoto K, Morita I, Murota S and Kawashima K: Phorbol ester enhances choline uptake in cultured porcine brain endothelial cells. Biogenic Amines **12**, 49 – 54 (1996)
14 Malo M, Diebler MF, Prado de Carvalho L, Meunier FM, Dunant Y, Bloc A, Stinnakre J, Tomasi M, Tchelingerian J, Couraud PO and Israel M: Evoked acetylcholine release by immortalized brain endothelial cells genetically modified to express choline acetyltransferase and/or the vesicular acetylcholine transporter. J Neurochem **73**, 1483 – 1491 (1999)
15 Maclin KD, Maus AD, Pereira EF, Albuquerque EX and Conti-Fine BM: Human vascular endothelial cells express functional nicotinic acetylcholine receptors. J Pharmacol Exp Ther **287**, 435 – 439 (1998)
16 Walch L, Gascard J-P, Dulmet E, Brink C and Norel X: Evidence for a M1-muscarinic receptor on the endothelium of human pulmonary veins. Br J Pharmacol **130**, 73 – 78 (2000)
17 Ciani F and Franceschini V: Ultrastructural study and cholinesterase activity of paired capillaries in the newt brain. J Hirnforsch **25**, 11 – 20 (1984)
18 Lan CT, Shieh JY, Wen CY, Tan CK and Ling EA: Ultrastructural localization of acetylcholinesterase and choline acetyltransferase in oligodendrocytes, glioblasts and vascular endothelial cells in the external cuneate nucleus of the gerbil. Anat Embryol (Berl) **194**, 177 – 185 (1996)
19 Pakaski M and Kasa P: Glial cells in coculture can increase the acetylcholinesterase activity in human brain endothelial cells. Neurochem Int **21**, 129 – 133 (1992)
20 Offner FA, Wirtz HC, Schiefer J, Bigalke I, Klosterhalfen B, Bittinger F, Mittermayer C and Kirkpatrick CJ: Interaction of human malignant melanoma (ST-ML-12) tumor spheroids with endothelial cell monolayers: damage to endothelium by oxygen-

derived free radicals. Am J Pathol **141**, 601 – 610 (1992)

21 Kirkpatrick CJ, Wagner M, Hermanns I, Klein CL, Köhler H, Otto M, van Kooten T and Bittinger F: Physiology and cell biology of the endothelium: a dynamic interface for cell communication. Int J Microcirc **17**, 231 – 240 (1997)

22 Wagner M, Hermanns I, Bittinger F and Kirkpatrick CJ: Induction of stress proteins in human endothelial cells by heavy metal ions and heat shock. Am J Physiol (Lung Cell Mol Physiol) **277**, L1026 – L1033 (1999)

23 Lehr H-A, Bittinger F and Kirkpatrick CJ: Microcirculatory dysfunction in sepsis: a pathogenetic basis for therapy? J Pathol **190**, 373 – 386 (2000)

24 Hoover ML, Vetvicka V, Hoffpauir JM and Tamburro CH: Human endothelial cell line from an angiosarcoma. In Vitro Cell Dev Biol **29A**, 199 – 202 (1993)

25 Wessler I, Bittinger F, VanKooten T, Kirkpatrick CJ and Kilbinger H: Expression of choline acetyltransferase in glial cells, mast cells and placenta in the human. Naunyn Schmiedebergs Arch Pharmacol, Suppl **359,** R22 (1999)