International Immunopharmacology 29 (2015) 185–188

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Future perspectives of a cardiac non-neuronal acetylcholine system targeting cardiovascular diseases as an adjunctive tool for metabolic intervention

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ARTICLE INFO

Article history:
Received 8 February 2015
Received in revised form 20 April 2015
Accepted 18 May 2015
Available online 28 May 2015

Keywords:
Cardiomyocytes
Non-neuronal ACh
Energy metabolism
Angiogenesis
Ischemia resistance

ABSTRACT

It has been several years since the function of the non-neuronal cholinergic system was independently reported in cardiomyocytes by several research groups. Although these findings initially seemed to be negligible and insignificant, extraordinary findings about cardiomyocytes were subsequently reported in studies involving the knockdown of the non-neuronal cholinergic system. These studies provide the evidence that this system may be indispensable for maintaining principal cardiac functions. Despite the absence of an appropriate and reliable technology to detect cellular ACh in real time in cardiomyocytes, studies of this system have progressed, albeit very slowly, to gradually consolidate the significance of this system. Based on the many significant findings regarding this system, these will be critical to develop adjunctive intervention therapy against cardiovascular diseases, including peripheral artery disease and heart failure. In this study, previous studies focusing on the non-neuronal cholinergic system are reviewed along with our studies, both indicating the biologically significant roles of the cardiac non-neuronal acetylcholine system from a clinical perspective.

1. Intervention of the cholinergic system through vagal nerve stimulation

Recent substantial studies regarding the cholinergic system in the cardiovascular field have accumulated evidence of significant roles of the system. One of the outstanding studies for a possible intervention modality to the cholinergic system includes vagal nerve stimulation (VS). Application of VS to patients or animal models with heart failure has been performed to provide convincing results, i.e., VS treats arrhythmia [1] and improves clinical symptoms in patients with chronic heart failure [2], suggesting that VS application would be another adjunctive therapeutic modality against heart failure. However, those clinical trials had a drawback of small-sized enrollment. Therefore, larger clinical trial tests have been waited to be accomplished, although several trials have been ongoing [3,4]. Indeed, application of VS to clinical fields has been gradually and extensively executed, however, the molecular mechanisms remain to be fully understood; rather it would be complicated, because varied factors with many features are simultaneously involved; and moreover, effects of VS stimulation are transmitted simultaneously through efferent fibers of vagal nerve to the heart as well as the afferent fibers to the brain. Recent studies disclose that such afferent effects of VS would contribute to one of the underlying beneficial mechanisms of VS [5]. Therefore, sole and simple factor may not account for all features of VS.

1.1. The non-neuronal cholinergic system as another part of the cholinergic system

The cholinergic system classically means the parasympathetic nerve system, which plays a counterpart of the sympathetic nerve system. For several decades, it has been gradually advocated that rather than the parasympathetic nerve system, i.e., neuronal cells equipped with acetylcholine (ACh) synthesis machinery, even non-neuronal cells can synthesize ACh, i.e., a non-neuronal ACh (NNA). It has been reported that the local ACh, however, not from nerve endings of the parasympathetic nerve system, plays a role in modulating biological signals in the microenvironment, where ACh is synthesized, with an autocrine or paracrine fashion [6]. NNA had been already found in many types of cells including immune cells, endothelial cells, epithelial cells, e.g., keratinocytes and bronchial cells; however, the reports regarding NNA in muscle-derived cells have, thereafter, appeared including cardiomyocytes from several research groups [7–10] and skeletal myoblasts from the other group [11].

These intriguing reports strongly suggest that the heart, composed of cardiomyocytes, possesses potency to synthesize and produce ACh by their own exertions, particularly meaning the non-neuronal cardiac cholinergic system or cardiac NNA. Therefore, based on these epoch-making findings, the cholinergic system is considered to be composed of two systems involving the classic cholinergic system, i.e., the parasympathetic nerve system, and the novel local system, i.e., the non-neuronal cholinergic system or NNA. Specifically, in the heart the cholinergic system is constructed by vagal nerve and NNA.

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http://dx.doi.org/10.1016/j.intimp.2015.05.029
1567-5769/

1.2. The biological significance of cardiac NNA

The reason why the heart possesses such two cholinergic systems remains to be concluded. However, some anatomical alteration in the nerve innervation should be considered between the sympathetic nerve and vagal nerve, because the distribution mode between the two nerve endings is quite different. Particularly, the sympathetic nerve dominates the whole cardiac ventricles; in contrast, vagal nerve dominates the cardiac atria and conduction system alone, not the whole ventricles [12,13]. It is hypothesized that the anatomical deficit of the vagal nerve endings in the cardiac ventricle, compared with the sympathetic nerve system, may be compensated by NNA in the heart.

Why is it speculated like that? The speculation has been confirmed by our previous studies. When NNA of a murine cardiomyocyte cell line HL-1 cells was knocked down through downregulation of choline acetyltransferase (ChAT) mRNA, the knockdown HL-1 cells vigorously produced reactive oxygen species (ROS), detected by a molecular probe [14]; and therefore, the ChAT downregulated HL-1 cells were more exposed to ROS than the cells equipped with the intact ACh synthesis, showing more susceptible to apoptosis through activation of caspase-3 due to ROS production [14]. These results suggest that NNA in the heart or cardiomyocytes negatively regulates production of ROS, in other words, NNA suppresses overshooting of ROS production through negatively regulating cellular energy metabolism. Actually ACh treated cells suppressed its oxygen consumption; in contrast, ChAT knockdown cells enhanced oxygen consumption. Therefore, these results indicate that one of the biological significance of NNA is first to negatively regulate cellular energy metabolism.

In addition, Gavioli M et al. revealed another role of NNA in the cardiac system, i.e., NNA is responsible for protecting the heart from sympathetic hyperactivation-induced cardiac dysfunction [15]. Therefore, they raised a novel concept that NNA is induced to counteract the enhanced sympathetic effects and prevents the failing heart from further decompensation partly through preserving calcium handling and muscarinic receptor-mediated NO production [15].

1.3. NNA responsible for negatively regulating cellular energy metabolism shifts the energy metabolism predominantly to glucose metabolism

This NNA-induced suppression of the energy metabolism does not mean that NNA decreases cellular activity, but shifts the metabolism predominantly to glucose-dependent metabolism through partially inhibiting mitochondrial function, as reported in our previous study [7,14]. Based on this study, extrinsic ACh decreased cellular oxygen consumption; in contrast, NNA knockdown cells reciprocally accelerated its consumption [7,14]. Moreover, our recent study clearly demonstrates that ChAT-overexpressing cells, i.e., NNA enhanced cells, preferentially incorporate glucose into cells through induction of a glucose transporter Glut1 and a growth factor IGF-1, but not insulin receptor [16]. Consequently, the ChAT-overexpressing cells survived for more prolonged duration despite serum starvation for almost 1 week [16].

1.4. The link between ACh and hypoxia-inducible factor (HIF)-1α induction

Besides, we previously reported that ACh upregulates HIF-1α stability to increase the protein levels even during a normoxic condition [17]. HIF-1α is a specific transcription factor with a helix–loop–helix domain and responsible for cell survival during a hypoxic condition through transcriptional activation of the downstream genes including Glut, an angiogenic factors vascular endothelial growth factor (VEGF), and genes for glycolytic system-related enzymes. Therefore, in conditions where ACh synthesis is activated, even during normoxic conditions, induction of HIF-1α was enhanced to trigger the transcriptional activation of the HIF-1α-related downstream genes. Therefore, it is indicated that ACh-treated cells respond as if they are subjected to real hypoxia, as a result, they decrease oxygen consumption via downregulation of a mitochondrial function. These results, taken together, strongly support our speculation that local ACh synthesized by NNA directly modulates cellular energy metabolism to shift the energy substrate preference rather to glucose, enhances glucose utilization and accelerates glucose metabolism.

1.5. Consolidation of NNA roles revealed in vitro using the heart-specific choline acetyltransferase (ChAT) gene transgenic mice in vivo

These characteristics of NNA are convinced not to be restricted to *in vitro* situations alone. We have developed the ChAT expressing mice (ChAT tgm), specifically, ChAT overexpressed in the cardiac ventricles, not the atrium, to confirm that augmented NNA in the heart suppresses energy metabolism in the heart and upregulates the similar *in vivo* responses to *in vitro* ones [18]. As expected on the basis of the *in vitro* results, the survival rate of ChAT tgm was superior to that of WT mice after myocardial infarction (92.2% vs. 47%, $P < 0.01$). Resistance to myocardial infarction-induced cell death in the heart resulted in more survived cardiomyocytes left even after the myocardial infarction and enhancement of tolerance to hypoxia of cultured cardiomyocytes from ChAT tgm, compared with wild type cardiomyocytes.

The underlying mechanisms for the resistance may be contributed mainly to a decrease in oxygen consumption of cardiomyocytes because ChAT tgm-derived cardiomyocytes sustained the cellular metabolism much lower than WT mice-derived cardiomyocytes either with or without adequate oxygen [18]. Another speculated mechanism responsible for the resistance may be accelerated angiogenesis in the heart of ChAT tgm. Cardiomyocyte-derived ACh production, which was remarkably augmented in the heart of ChAT tgm, accelerated angiogenesis to increase capillary density in the ventricle, particularly when they were subjected to myocardial infarction. The angiogenesis acceleration in the ventricle was evident both in a non-ischemic and non-infarcted condition.

1.6. The ChAT tgm heart continues to beat for a longer duration in ischemic conditions and more rapidly recovers its functional beating

To functionally evaluate the resistance of the heart to ischemia, the excised hearts from ChAT tgm or WT mice connected to Langendorf apparatus were evaluated. During global ischemia by cessation of Langendorf perfusion, the excised heart from ChAT tgm sustained beating for more prolonged time than that from WT mice; and moreover, when perfusion was started, the ChAT tgm heart vigorously resumed spontaneous beating sooner than that of WT mice. This result clearly demonstrated that the transgenic heart was definitely resistant to ischemia and sustained the function even during the ischemic or hypoxic events [18].

1.7. Anti-inflammatory effects of the cholinergic system including NNA implicated in cardiovascular diseases

Other than the specific role of NNA modulating cellular energy metabolism, it has been well known and established that the cholinergic system negatively regulates inflammatory responses, i.e., an anti-inflammatory effect, since the study by Tracey KJ et al. was reported, because vagal nerve stimulation inhibited the inflammatory responses induced by endotoxin and sustained blood pressure, which was decreased by endotoxin [19]. Other than the direct evidence of vagal nerve stimulation as an anti-inflammatory factor in immunological fields, it has been also addressed in a cardiovascular field that modification of the cholinergic system shows beneficial roles through an anti-inflammatory effect. For example, an acetylcholinesterase inhibitor donepezil and physostigmine were reported to attenuate atherosclerotic changes in vasculatures in mice fed with high-fat diet [20], suggesting that cholinergic modification plays a role in inhibiting atherosclerotic responses through attenuating inflammatory responses. Additionally,