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Review Article

Neurotransmission of the Peripheral Chemoreflex in the Nucleus Tractus Solitarii in Unanesthetized Experimental Models

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Abstract

Peripheral chemoreflex activation with potassium cyanide (KCN) in awake rats, or in the working heart brainstem preparation (WHBP) produces: (a) a pressor response and sympathoexcitation; (b) bradycardia; and (c) tachypnea and an increase in the frequency of phrenic nerve activity (PNA). During the last few years in our laboratory, we have studied the neurotransmission of the sympathoexcitatory components of the chemoreflex within the nucleus tractus solitary (NTS) in unanesthetized experimental models. Recently, we verified that simultaneous antagonism of ionotropic glutamate receptors and purinergic P2 receptors by sequential microinjections of kynurenic acid (a non selective antagonist of ionotropic glutamate receptors) and PPADS (a non-selective antagonist of P2 receptors) into the NTS elicited a significant reduction in the pressor and bradycardic responses but no changes in the tachypneic response to chemoreflex activation in awake rats. Microinjection of kynurenic acid and PPADS into the caudal commissural NTS of the WHBP almost abolished the increase in thoracic sympathetic activity and the bradycardic response but produced no change in the increase of the frequency of PNA. The data indicate that combined microinjections of PPADS and kynurenic acid into the NTS are required to block the sympathoexcitatory response to peripheral chemoreflex activation, suggesting an interaction of L-glutamate and ATP in this neurotransmission. In this review, I explain why we are using unanesthetized models in our experiments and also present the perspectives for a better understanding of the complex interaction of glutamatergic and purinergic mechanisms involved in the processing of the sympathoexcitatory component of the chemoreflex at the NTS level. [*Tzu Chi Med J* 2009; 21(1):6–11]

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1. Introduction

The question as to why we need to study the neurotransmission of the peripheral chemoreflex in the nucleus tractus solitary (NTS) of unanesthetized experimental models is an interesting one. I have been asking myself this for the last 15 years and each time I receive criticisms from reviewers about manuscripts submitted from our laboratory, I try to convince myself that I am heading in the right direction. In this mini-review, I summarize the reasons and the main findings from our laboratory (1) and also include some perspectives for the experiments using animal models without anesthetic effects.

2. Awake rats

Considering that most of my scientific training has been in integrative physiology, I have a natural tendency to become involved in experiments using unanesthetized models. When my colleagues and I started our studies on the neurotransmission in the NTS, the initial idea was to explore the nucleus for possible changes in the pharmacological profile of L-glutamate ionotropic receptors after sinoaortic deafferentation in rats. For this purpose, we used a method described by Michelini and Bonagamba (2), which allowed microinjections to be performed in the NTS of awake rats. However, we verified that microinjections of L-glutamate into the NTS of unanesthetized rats produced pressor responses, while microinjections of the same dose of L-glutamate in the same animals under urethane or chloralose anesthesia produced depressor responses (3). These findings were important for several reasons: (a) L-glutamate elicited a pattern of cardiovascular responses different from those expected for the putative neurotransmitter of the baroreflex, i.e. hypotension; and (b) the pressor responses observed in the unanesthetized group but not in the anesthetized group suggested a possible predominance of the activation of the chemoreflex pathways by L-glutamate. Therefore, the initial finding that L-glutamate might activate the chemoreflex pathways in unanesthetized rats but not in the anesthetized rats opened an interesting opportunity for us to explore the possible role of L-glutamate in the neurotransmission of the pressor (sympathoexcitatory) component of the chemoreflex in the NTS of awake rats, because the pressor responses to chemoreflex activation were abolished in anesthetized rats (4,5).

During a series of subsequent studies, we tested the hypothesis that L-glutamate acting on ionotropic receptors might be the neurotransmitter of the sympathoexcitatory component of the chemoreflex in the NTS of awake rats. These experiments were performed even though very important studies in rats under anesthesia were published at that time, which documented that the pressor responses to chemoreflex activation were almost abolished by antagonism of the ionotropic receptors of L-glutamate (6,7). Initially, we used AP-5, an antagonist of NMDA receptors, and we verified that the bradycardic, but not the pressor response, was reduced in a dose-dependent manner, indicating that the pressor/sympathoexcitatory component of the chemoreflex was not mediated by the NMDA receptors (8). Although we did not record the sympathetic nerve activity in awake rats, we were convinced that the pressor responses to chemoreflex activation were mediated by the sympathetic activity because the increase in pressure was blocked by prazosin (iv), an $\alpha 1$ adrenoceptor antagonist (8). A subsequent experiment with antagonism of non-NMDA receptors was mandatory and, for this purpose, we used DNQX, a selective non-NMDA receptor antagonist. The data showed that the pressor responses after DNQX administration into the NTS were reduced but we also observed a significant increase in the baseline MAP, which could affect and reduce the magnitude of the pressor responses to chemoreflex activation. Despite the increase in baseline MAP, we verified that the pressor responses to chemoreflex activation were not blocked by DNQX, even at a range of doses that were not selective to non-NMDA receptor subtypes. Considering that the pressor responses were not blocked by selective antagonism of non-NMDA receptors, we used kynurenic acid, a non-selective antagonist of ionotropic receptor of L-glutamate and, surprisingly, we were unable to block the pressor responses of the chemoreflex (9). The manipulation of the NTS with kynurenic acid or DNQX produced large increases in the baseline MAP in intact animal models due to the possible blockade of the sympathoinhibitory component of the baroreflex, which may mask the net effect of these antagonists on the pressor responses to chemoreflex activation. Therefore, the next step was to normalize the large increases in the baseline MAP observed after microinjections of kynurenic acid into the NTS and, for this reason, we used intravenous infusion of sodium nitroprusside. Under this experimental condition, the chemoreflex was activated again and we observed that the magnitude of the pressor responses, when the baseline MAP had reverted to normal levels, was similar to the control responses. This indicated that in whole animal models the antagonism of L-glutamate ionotropic receptors in the NTS produced no effects on the pressor responses to chemoreflex activation (10). Despite these findings, the data were not sufficient to exclude the involvement of L-glutamate in the neurotransmission of the sympathoexcitatory component of the chemoreflex in the NTS. However, at the same time, we started to consider other potential neurotransmitters, focusing on ATP because of important evidence about the role of

this purine on chemosensory transduction in the central nervous system [11,12] and in the NTS [13,14].

Microinjections of ATP into the caudal aspect of the commissural NTS of unanesthetized rats produced increases in MAP similar to the pressor responses to chemoreflex activation [15,16], suggesting the involvement of ATP in the processing of the sympathoexcitatory component of the chemoreflex in the NTS. A possible interaction between ATP and L-glutamate in the neurotransmission of the sympathoexcitatory component of the chemoreflex at the NTS level was also considered. However, in an experimental protocol involving microinjection of ATP into the NTS before and after local microinjection of kynurenic, the pressor responses to ATP were not affected by the antagonism of L-glutamate ionotropic receptors [17]. To test the direct involvement of purinergic receptors in the processing of the chemoreflex in the NTS, we activated this reflex before and after bilateral microinjection of PPADS into the caudal commissural NTS, but no changes in the magnitude of the pressor responses were observed [1]. Based on the experimental evidence that microinjections of L-glutamate or ATP microinjected into the NTS produced pressor responses similar to those produced by chemoreflex activation, but that the selective antagonism of the ionotropic/metabotropic receptors of L-glutamate or the antagonism of P2X receptors of ATP produced no changes in the magnitude of the pressor responses to chemoreflex activation, we considered the possibility that both neurotransmitters might be interacting in the processing of the sympathoexcitatory component at the NTS. Accordingly, we performed simultaneous blockade of both groups of receptors [1].

3. Antagonism of L-glutamate and ATP receptors

Double antagonism of L-glutamate and ATP receptors in the NTS of awake rats produced large increases in the baseline MAP and, for the reasons mentioned above, we used sodium nitroprusside infusion to normalize MAP. Under this experimental condition, the chemoreflex was activated and we verified that the double antagonism largely abolished the pressor responses to chemoreflex activation, an antagonism which was reversible, considering that, 30 minutes after the microinjection, the patterns of the pressor responses were back to the control level [1]. Although the pressor responses to chemoreflex activation were almost abolished by double antagonism in the NTS of awake rats, the data were not enough to prove that the sympathetic activity was really affected because, as part of several regulatory mechanisms in whole animal models, the arterial blood pressure parameter may be affected by different neural and hormonal components, which explains the large increases in the baseline MAP. The undesirable effects may counterbalance the changes elicited by manipulations in the NTS, masking a clear evaluation of the neurotransmission of a specific reflex such as the sympathoexcitatory component of the chemoreflex. Given that recording the sympathetic nerve activity in awake rats while placing microinjections into the correct spots in the NTS is not a simple task, we decided to use working heart-brainstem preparations (WHBP) developed by Paton [18] to confirm these findings.

4. Working-heart brainstem preparation

To help us to evaluate the roles of L-glutamate and ATP receptors in the neurotransmission of the sympathoexcitatory component of the chemoreflex, we used the WHBP. This was done for several important experimental reasons: (a) similar to awake rats, it is free of anesthetic despite losing forebrain structures; (b) accessing and recording the thoracic sympathetic chain and the phrenic nerve are relatively easy compared with the same approach in awake rats; (c) the success of correctly targeting the commissural NTS with microinjections is greatly improved; (d) the perfusion pressure is not affected by NTS microinjections of antagonists, thereby avoiding activation of the arterial baroreceptors; and (e) activation of the chemoreflex in the WHBP with KCN presents large increases in the sympathetic and phrenic nerve activities, in addition to bradycardic responses similar to the pattern of responses observed in awake rats [16,19].

The first approach in the WHBP was to verify the effects of bilateral microinjections of PPADS into the commissural NTS on the autonomic and respiratory responses to chemoreflex activation. The data showed that the chemoreflex responses were not affected by bilateral microinjection of PPADS, an antagonist of P2X receptors. For the next experimental protocols in the WHBP, we used double antagonism with kynurenic acid and PPADS. This combination, similar to the findings in awake rats, was effective in blocking the sympathoexcitatory response to chemoreflex activation [1]. Therefore, the data obtained in the WHBP not only confirmed the previous observations in awake rats but extended it to the concept that the effective antagonism of the sympathoexcitatory component of the chemoreflex was possible only when we combined the antagonism of L-glutamate and ATP receptors in the caudal commissural NTS.

The findings obtained in awake rats and in the WHBP showing that only the combined antagonism of L-glutamate and ATP receptors were effective in the blockade of the sympathoexcitatory response of the chemoreflex are really important; this is especially

true when one considers that our previous NTS microinjections studies in awake rats and in the WHBP all failed to block the pressor/sympathoexcitatory responses to chemoreflex activation. In these previous studies we used microinjections of L-glutamate ionotropic and metabotropic receptor antagonists [8–10,20], GABA agonists [21,22], glycine [23], adenosine receptor antagonists [15] and a substance P receptor antagonist [24]. Based on these negative findings, we proposed that an additional neurotransmitter in the NTS may take part in mediating the sympathoexcitatory component of the chemoreflex. Therefore, our recent studies were based on the notion that P2X and ionotropic L-glutamate receptors in the NTS participate in the expression of the sympathoexcitatory component of the chemoreflex. Moreover, our findings that the pressor/sympathoexcitatory response to chemoreflex activation was almost abolished by this combined antagonism in the commissural NTS in awake rats and in the WHBP strongly support the concept that these two neurotransmitters play a pivotal role in processing the sympathoexcitatory component of this reflex.

5. Explanation of why only double antagonism was effective in blocking pressor/sympathoexcitatory responses to chemoreflex activation

While there is evidence that ATP acting on P2X receptors may induce glutamate release [25], the specific involvement in the neurotransmission of the autonomic components of the chemoreflex in the NTS is not yet known. Studies performed on unidentified NTS neurons by Kato and Shigetomi [26] showed that ATP increased the spontaneous postsynaptic currents, which were blocked by PPADS, indicating that this excitatory effect was mediated by P2X receptors. Studies by Jin et al [27] showed that activating P2 receptors with ATP or α,β-methylene ATP, a stable ATP analog, in the NTS facilitated presynaptic glutamate release. Shigetomi and Kato [28] also showed that activation of presynaptic P2X receptors with α,β-methylene ATP triggered Ca^{2+}-dependent glutamate release in the NTS. Therefore, it is conceivable that ATP, acting presynaptically, may induce glutamate release in the NTS to mediate the chemoreflex sympathoexcitatory/pressor response. Because ATP can be co-released with other neurotransmitters [29], it is possible that ATP and L-glutamate are both involved in processing the sympathoexcitatory component of the chemoreflex. However, the previous findings about the interaction between ATP and L-glutamate at the same neurons at the presynaptic levels are not consistent with our findings because, in accordance with this interaction at the presynaptic level, only the antagonism of ATP receptors at the presynaptic level or L-glutamate receptors at the post-synaptic level should be enough to block the sympathoexcitatory pathways of the chemoreflex in the NTS. This was, however, not the case in awake rats or in the WHBP. Our findings suggest that both ionotropic glutamate and P2 receptors in the commissural NTS play key roles in the complex neurochemical mechanisms expressing the pattern of cardiovascular responses to chemoreflex activation. Moreover, this suggests redundancy within the NTS because, based on our findings, if one of these systems (glutamatergic or purinergic) fails, the sympathoexcitatory component of the chemoreflex remains functional. To explain this possibility we suggest that NTS neurons projecting to the RVLM might be mediated by L-glutamate, while NTS neurons projecting to the paraventricular nucleus of hypothalamus (PVN) and/or to parabrachial nucleus (PBN), nuclei that are also involved in the sympathoexcitatory component of the chemoreflex, might be mediated by ATP, or vice versa. These possibilities need to be evaluated in further electrophysiological studies.

We propose that in unanesthetized preparations such as awake rats and WHBP, this dual neurotransmitter system for the sympathoexcitatory component of the chemoreflex emphasizes the vital importance of this protective reflex and its importance for survival. However, dysfunction of these complex neuronal and neurochemical systems may affect the modulation of the sympathetic outflow and result in increases in arterial pressure. For this reason, we believe that our findings provide new perspectives for pharmacological therapy for reducing sympathoexcitation during pathophysiological conditions such as neurogenic hypertension, obstructive sleep apnea and heart failure.

6. Perspectives

The findings that only double antagonism of L-glutamate ionotropic receptors and ATP P2 receptors were effective in blocking the sympathoexcitatory component of the chemoreflex open several interesting perspectives for studies aimed at a better understanding of the central mechanisms involved in the generation of sympathetic over-excitation, which may induce hypertension. To verify whether this interaction is restricted to the NTS level, further experiments at the RVLM level must be performed to verify whether similar interactions of these dual systems occur at sites where the sympathetic activity is originally generated. Taking into account that the interaction between ATP and L-glutamate apparently is not restricted to a simple and direct interaction between these two neurotransmitters on their specific receptors, further studies are needed to determine whether another neuromodulatory mechanism such as nitric oxide and/or GABA,

for example, plays a role in this interaction. With respect to the electrophysiological approach, it is critical to determine whether the excitation of NTS neurons projecting to the RVLM are mediated equally by L-glutamate and ATP or whether there are different neurochemical profiles for those neurons of the NTS projecting to RVLM that are directly involved with the sympathoexcitatory pathways of the chemoreflex. Because the modulation of the sympathoexcitatory responses to chemoreflex activation is not restricted to the NTS-RVLM projections, it is essential to investigate the NTS neurons that send projections to the PVN and PBN, which are also integral to the sympathoexcitatory chemoreflex pathways. It is very important to note that, in future cases in which we are able to explore most of the sympathoexcitatory pathways of the chemoreflex in the NTS, it may not be enough to explain this complex interaction of L-glutamate and ATP. On this point, it is essential to consider the role of the ventilatory components in the chemoreflex on this complex interaction system, because the respiratory rhythm is coupled with modulation of the sympathetic activity. Therefore, the integrative systems of the cardiovascular and respiratory functions must be evaluated using only preparations free of anesthetics. This will allow us to better understand this vital, complex and potentially dangerous integrative system, in which L-glutamate and ATP are just two of several important players in the modulation of this sophisticated system essential for our life, which does not include any anesthetic as a natural player.

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