Role of nitric oxide on purinergic signalling in the cochlea

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Abstract In the inner ear, there is considerable evidence that extracellular adenosine 5'-triphosphate (ATP) plays an important role in auditory neurotransmission as a neurotransmitter or a neuromodulator, although the potential role of adenosine signalling in the modulation of auditory neurotransmission has also been reported. The activation of ligand-gated ionotropic P2X receptors and G protein-coupled metabotropic P2Y receptors has been reported to induce an increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in inner hair cells (IHCs), outer hair cells (OHCs), spiral ganglion neurons (SGNs), and supporting cells in the cochlea. ATP may participate in auditory neurotransmission by modulating $[\text{Ca}^{2+}]_i$ in the cochlear cells. Recent studies showed that extracellular ATP induced nitric oxide (NO) production in IHCs, OHCs, and SGNs, which affects the ATP-induced Ca^{2+} response via the NO-cGMP-PKG pathway in those cells by a feedback mechanism. A cross-talk between NO and ATP may therefore exist in the auditory signal transduction. In the present article, I review the role of NO on the ATP-induced Ca^{2+} signalling in IHCs and OHCs. I also consider the possible role of NO in the ATP-induced Ca^{2+} signalling in SGNs and supporting cells.

Keywords Cochlea · Nitric oxide · P2 receptors · Feedback

Introduction

Previous studies revealed evidence that extracellular adenosine 5'-triphosphate (ATP) acts as either a neurotransmitter or a neuromodulator in the peripheral and central nervous

system [1–3]. ATP has been described as a neurotransmitter or a neuromodulator in auditory neurotransmission. ATP has been reported to induce an increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in outer hair cells (OHCs) [4, 5], inner hair cells (IHCs) [6–8], spiral ganglion neurons (SGNs) [9, 10], and supporting cells [11–13]. These previous studies suggested that ATP may affect auditory neurotransmission by modulating $[\text{Ca}^{2+}]_i$ in the cochlear cells. It has been shown that OHCs and IHCs possess both ligand-gated ionotropic P2X receptors and G protein-coupled metabotropic P2Y receptors [5, 6, 14]. The extensive distribution of P2X₂ receptor subunit expression in the guinea pig cochlea provides evidence for divergent roles for extracellular ATP acting via ATP-gated ion channels [14]. Therefore, ATP plays an important role in auditory neurotransmission as a neurotransmitter or a neuromodulator [15].

Nitric oxide (NO), a gaseous membrane permeate messenger, has been thought to play an important role in signal processing as a neurotransmitter or a neuromodulator in the olfactory system [16], visual system [17], and central neural system [18]. NO is synthesized by three isoforms of NO synthase (NOS). The neuronal isoform (nNOS) and the endothelial isoform (eNOS) of NOS are constitutive and Ca^{2+} -dependent, whereas the inducible NOS is Ca^{2+} -independent [19]. In the peripheral auditory organ, Ca^{2+} -dependent constitutive NOS, nNOS, and eNOS have been demonstrated by means of immunocytochemistry [20–22]. Both nNOS and eNOS were localized in several types of cells in the cochlea, such as IHCs, OHCs, SGNs, and supporting cells [20–22]. Recent studies using the NO-sensitive dye, 4,5-diaminofluorescein (DAF-2), showed NO production to occur in several types of cells in the guinea pig cochlea [7, 8, 10, 23–25]. These results suggest that NO may play an important role in the inner ear [25]. The soluble guanylate cyclase (sGC) is the target enzyme of NO. It

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raises the intracellular cGMP levels and subsequently activates cGMP-dependent protein kinase (PKG). The effects of NO in cellular signaling are related to its ability to regulate Ca^{2+} homeostasis through the activation of the NO-cGMP-PKG pathway [26]. There is also increasing evidence that NO influences the mechanism of cellular Ca^{2+} homeostasis in several cell types by either a positive or negative feedback mechanism [27, 28]. It has been suggested that the effects of NO are involved in the regulation of $[\text{Ca}^{2+}]_i$ through the NO-cGMP-PKG signaling pathway in the sensory system of olfaction [29] and vision [30]. Morphological studies have shown this pathway to also exist in the cochlea [31]. The role of the NO-cGMP-PKG pathway in auditory neurotransmission has been suggested [30, 32–34].

It has been shown that interactions between ATP and NO exist in the gastrointestinal tract [35] and vascular system [36]. ATP induced Ca^{2+} response and release of NO through activation of P2X and P2Y receptors in the gastrointestinal tract [37–39]. Recent studies also showed that NO affects the ATP-induced Ca^{2+} response via the NO-cGMP-PKG pathway in IHCs, OHCs, and SGNs by a feedback mechanism [7, 8, 10, 24]. Therefore, NO may play a crucial role in auditory neurotransmission. A cross-talk between NO and ATP exists in auditory signal transduction.

In the present review, I summarize current knowledge of the possible role of NO on the ATP-induced Ca^{2+} signalling in IHCs, OHCs, SGNs, and supporting cells.

Role of NO on ATP-induced Ca^{2+} signalling in IHCs and OHCs

It has been shown that interactions between ATP and NO exist in the gastrointestinal tract [35], the vascular system [36], and glossopharyngeal neurons [40]. The ATP and NO interactions appear to be involved in the control of body temperature and cardiovascular-respiratory systems [41]. Previous studies also showed that ATP evoked Ca^{2+} response and the release of NO in the gastrointestinal tract through the activation of P2X receptors and P2Y receptors [37–39]. Therefore, the activation of P2X receptors and P2Y receptors may contribute to intracellular NO production. In the cochlea, there is ample evidence demonstrating the expression of ligand-gated ionotropic P2X receptors and G protein-coupled metabotropic P2Y receptors in IHCs and OHCs [14, 15]. In the cochlea, ATP has been reported to elicit an elevation of $[\text{Ca}^{2+}]_i$ in IHCs and OHCs [4–8, 24]. Therefore, ATP plays an important role as a neurotransmitter or neuromodulator in the inner ear [42]. There is clear evidence that Ca^{2+} homeostasis in IHCs and OHCs plays an important role in auditory signal transduction [43].

nNOS and eNOS require an increase in $[\text{Ca}^{2+}]_i$ to obtain their maximal activity. Several agonists that increase $[\text{Ca}^{2+}]_i$ activate constitutively expressed NOS transiently, evoking NO production [44–47]. A rise in $[\text{Ca}^{2+}]_i$ may thus serve to amplify the NO production as previously reported [48]. Recent studies using the fluorescent NO-sensitive dye, DAF-2, have shown intracellular NO production in several types of cells [49–53]. The ATP-induced NO production has been reported in aortic endothelial cells [52, 54] and astrocytes [55]. In the cochlea, ATP also evoked endogenous NO production in IHCs and OHCs [7, 8, 24] (Fig. 1). Those findings are consistent with the notion that the elevation of $[\text{Ca}^{2+}]_i$ was necessary for NO production as seen in other cell types [54–56]. It has been shown that Ca^{2+} influx is the only preferential source for stimulating the NO production in endothelial cells, smooth muscle cells, and pulmonary artery endothelial cells [54, 56, 57]. Therefore, NO production may be due to an increase in the level of $[\text{Ca}^{2+}]_i$ by the Ca^{2+} influx through activated P2X receptors in those cells. In IHCs and OHCs, ATP-induced NO production was abolished in the absence of extracellular Ca^{2+} [8, 24]. The simultaneous measurements of NO production and $[\text{Ca}^{2+}]_i$ changes also showed that the ATP-induced rapid rise of $[\text{Ca}^{2+}]_i$ preceded the increase in endogenous NO production in IHCs and OHCs, suggesting that an increase in the level of $[\text{Ca}^{2+}]_i$ may be due to the Ca^{2+} influx through the activation of P2X receptors in IHCs and OHCs [8, 24].

Both nNOS and eNOS, Ca^{2+} /calmodulin-dependent NOS isoforms, are likely to contribute to the ATP-induced NOS signal. P2Y receptors and nNOS co-localization in the hypothalamus has been observed [58, 59]. The co-localization of nNOS and P2X₂ receptors in the neurons of hypothalamus and brain stem of rat [41] has also been observed. In non-neuronal cells, several studies have shown that the ATP-induced Ca^{2+} influx is the preferential source for eNOS activation in endothelial cells [52, 54]. Both nNOS and eNOS are considered to contribute to the NOS signal in the cochlea since these two isoforms are present in several cell types in the cochlea, including IHCs and OHCs [21, 60].

7-NI, a selective nNOS inhibitor, inhibited the ATP-induced NO production in IHCs and OHCs [8, 24]. The effects of 7NI and L-NAME, a non-selective NOS inhibitor, did not cause any significant differences in the ATP-induced NO production. It is clearly indicated that the main isoform of NOS on the ATP-induced NO may be nNOS rather than eNOS in IHCs and OHCs. The ATP-gated ion channels composed mainly of P2X₂ receptor subunits are predominant in cells lining the endolymphatic compartment, including IHCs and OHCs [14, 61–64]. Previous studies showed the localization of the P2X₂ receptors in the apical region of IHCs and OHCs [14, 64]. Immunofluorescent staining of nNOS and P2X₂ receptors

Fig. 1 ATP-induced NO production in cochlear inner hair cells (IHCs) and outer hair cells (OHCs). **a, e** A bright field image of single IHC (**a**) and OHC (**e**). Fluorescence images of NO production induced by 100 μ M ATP at pre-stimulation (**b**) and 10 min (**c**) after ATP stimulation in single IHC. Fluorescence images of NO production induced by 100 μ M ATP at pre-stimulation (**f**) and 15 min (**g**) after ATP stimulation in single OHC. Scale bar, 10 μ m. The calibration bar on the right shows the DAF-2 fluorescence intensity in pseudo-color. **d, h** Summary histogram of the effects of NO on ATP-induced $[Ca^{2+}]_i$ increase in IHCs and OHCs. Pretreatment of the IHCs with 100 μ M

SNAP inhibited the ATP-induced $[Ca^{2+}]_i$ increase (**d**), while 100 μ M SNAP enhanced it in OHCs (**h**). L-NAME, 200 μ M, enhanced the ATP-induced $[Ca^{2+}]_i$ increase in IHCs (**d**), while 200 μ M L-NAME inhibited the ATP-induced $[Ca^{2+}]_i$ increase in OHCs (**h**). Values of the second Ca^{2+} response to ATP plus agent are normalized to the control (first) response. The numbers of cells tested are given in parentheses. Error bar shows SD * $p < 0.01$ vs. ATP-induced Ca^{2+} response as a control (one-way analysis of variance). Reproduced from [7, 24] with permission from Elsevier

in isolated IHCs and OHCs showed the co-localization of nNOS and P2X₂ receptor in the apical region of IHCs and OHCs [8, 24]. These results indicate that the ATP-induced Ca^{2+} influx via a direct action of P2X receptors may be the source for nNOS activity in the apical region of IHCs and OHCs. It has been shown that the apical region of IHCs and OHCs, including stereocilia and the cuticular plate, is abundant in calmodulin [65], P2X receptors [14], and plasma membrane Ca^{2+} -ATPase 2a subunit [66]. Calmodulin binding to nNOS is reported to be essential for the nNOS activity, by which electron transfer is triggered [67]. Calmidazolium, a calmodulin antagonist, inhibited the ATP-induced NO production in IHCs and OHCs [8, 24]. P2X receptors are implicated in the ATP-induced Ca^{2+} influx, while the plasma membrane Ca^{2+} -ATPase contributes to Ca^{2+} homeostasis by extruding Ca^{2+} from the cytoplasm.

Only Ca^{2+} influx through the *N*-methyl-D-aspartate (NMDA) receptor has been reported to efficiently activate nNOS in cerebellar granule cells [68]. It has been also suggested that nNOS may bind to the NMDA receptor through their PDZ domain interaction [69], thus determin-

ing its upstream and downstream signaling specificity [70]. A recent study revealed evidence that the PDZ domain, the important scaffolding for organizing proteins into signaling complexes such as nNOS complex, was found in IHCs and OHCs [71].

Therefore, these proteins co-localized in the apical region of OHCs might be involved in the upstream and downstream signaling for nNOS, thereby accounting for the activation and functions of nNOS in IHCs and OHCs.

The results about distribution of eNOS and nNOS in IHCs and OHCs were varied in the previous studies. The light microscopic study by Michel et al. could detect neither eNOS nor nNOS in IHCs and OHCs [34], while other studies showed evidence of the distribution of eNOS and nNOS in IHCs and OHCs [20, 21, 72]. These different results may be due to the histochemical techniques applied in their studies. A recent study showed a quantitative immunoelectron microscopic analysis to reveal cellular differences in the degree of nNOS and eNOS expression in IHCs and OHCs, while these two NOS were located in the apical region of IHCs and OHCs [22]. These two constitutive NOS (nNOS and eNOS) might be located at

different subcellular sites and might be regulated by a different Ca^{2+} response. Previous studies also showed that nNOS and eNOS exhibited different subcellular distributions, which are associated with the different upstream and downstream signaling molecules [70, 73, 74]. They suggested that different distributions of nNOS and eNOS may respond to the different extracellular signals, thus resulting in different cellular specific functions. It thus seems likely that nNOS and eNOS may differentially function as cellular signal molecules in IHCs and OHCs.

It has been shown that NO production induced by glutamate in cultured retinal ganglion neurons was considered to be mainly associated with nNOS [75]. eNOS did not appear to play a major role in NO production because the NO production was inhibited by 7-NI, while immunohistochemically, both nNOS and eNOS were expressed in retinal ganglion neurons [75]. It is suggested that the synthesis of NO by eNOS may represent a compensatory mechanism in the absence of nNOS. Therefore, the different roles of eNOS and nNOS may also be associated with different cellular upstream and downstream signaling molecules, which respond to different extracellular signals and are responsible for different cellular functions in IHCs and OHCs.

In OHCs, active hair bundle motion may have an important role in cochlear amplification in mammals [76]. Interaction between nNOS and P2X_2 in the apical region of OHCs, including cilia and hair bundle, may therefore participate in the control of amplification by active hair bundle motion. The functional significance and role of nNOS in active hair bundle motion of OHCs should be explored in future study.

NO acts as intracellular messenger via cGMP and its downstream pathways. The soluble guanylate cyclase (sGC) is the target enzyme of NO. It raises the intracellular cGMP levels and subsequently activates cGMP-dependent protein kinase (PKG). The effects of NO in cellular signaling are related to its ability to regulate Ca^{2+} homeostasis through the activation of the NO-cGMP-PKG pathway [26]. There is ample evidence demonstrating that NO either enhances or inhibits intracellular Ca^{2+} signaling through the NO-cGMP-PKG pathway by a feedback mechanism [27, 28]. A cross-talk between NO and $[\text{Ca}^{2+}]_i$ has been recently reported. Several studies using fura-2 techniques suggest that the $[\text{Ca}^{2+}]_i$ increase induced by ATP may activate NO production, which thus affects Ca^{2+} signaling by a positive feedback mechanism [77, 78]. Since the localization of soluble guanylate cyclase has been identified in IHCs and OHCs [31], it might be possible that the NO-cGMP-PKG pathway also plays a role in auditory neurotransmission.

Interestingly, NO inhibits the ATP-induced Ca^{2+} influx via the NO-cGMP-PKG pathway in IHCs by a negative feedback mechanism, while NO enhances the ATP-induced

Ca^{2+} influx via the NO-cGMP-PKG pathway in OHCs by a positive feedback mechanism [8, 24]. NO thus plays a role not only in afferent modulation but also in efferent modulation of the cochlea. Regarding the opposite effects of NO on ATP-induced Ca^{2+} signalling in OHCs and IHCs (Fig. 1), they could be relevant to the roles of these two classes of sensory cells for hearing in the cochlea.

Under noise and ischemic conditions, the release of ATP into scala media results in a decrease in cochlear partition resistance [79–81] associated with the decline in endocochlear potential. The ATP-induced depolarization of the OHC membrane potential would limit the driving force of forward and reverse transduction across the membrane of OHCs, uncoupling the active hearing process mediated by OHCs, thereby contributing to temporary threshold elevations in hearing which serve a protective role in response to cochlear stressors. Under noise and ischemic conditions, the increase of ATP may thus enhance NO production in OHCs. As a result, NO may subsequently enhance the ATP-induced depolarization of the OHC membrane potential by a feedback auto-regulatory loop. Therefore, NO may enhance the protective effect of OHCs under noise condition.

When glutamate is released from IHCs, it binds to NMDA receptors located on the terminals of the SGNs. Large concentrations of glutamate are released in response to loud noise, and toxic concentrations of this excitatory amino acid lead to the swelling and the destruction of dendrites of the primary auditory neurons under IHCs [82, 83]. In IHCs, enhancement of NO by the increase of ATP may suppress the ATP-induced depolarization of the IHC membrane potential by a feedback auto-regulatory loop under noise condition, which may reduce the release of glutamate from IHCs. Therefore, NO may suppress the excitotoxicity by glutamate under noise condition since the augmentation of the NMDA-induced Ca^{2+} influx has been shown through calmodulin to activate nNOS, leading to release of excessive levels of NO and neuronal death in the brain [84, 85]. These two hypothesis need to be further investigated. The interaction of ATP and NO in cochlear IHCs and OHCs indicates a regulatory loop for the NO-mediated auditory information processing in the cochlea. nNOS may also play a vital role in the ATP-mediated Ca^{2+} homeostasis of IHCs and OHCs by a feedback auto-regulatory loop.

Effects of glucocorticoids on the ATP-induced NO and Ca^{2+} signalling in SGNs

The spiral ganglion is a primary center of the mammalian auditory system that forwards a signal from hair cells to the auditory nuclei in the central nervous system. Type I SGNs (90–95% of SGNs) innervate IHCs and transmit signals

from the cochlea to the cochlear nucleus, while type II SGNs (5–10% of SGNs) innervate OHCs. ATP has been reported to induce an elevation of the $[Ca^{2+}]_i$ in IHCs and type I SGNs [6–9]. ATP thus acts as a hair cell-afferent neurotransmitter or neuromodulator in the cochlea.

Extracellular ATP also evoked NO production in type I SGNs (Fig. 2). The ATP-induced NO production is mainly due to the Ca^{2+} influx through the activation of P2X receptor [10]. It has been shown that dexamethasone, a synthetic glucocorticoid hormone, rapidly and non-genomically enhances the ATP-induced $[Ca^{2+}]_i$ increase in type I SGNs which results in the augmentation of NO production [10] (Fig. 2). The ATP-induced NO production enhances a $[Ca^{2+}]_i$ increase in type I SGNs by a positive feedback mechanism [10]. This indicates that glucocorticoids may rapidly affect auditory neurotransmission. Some interactions may exist between NO and glucocorticoids on the ATP-induced Ca^{2+} signalling in type I SGNs. Glucocorti-

coids have been thought to pass through cell membranes by passive diffusion thus combining with intracellular cytoplasmic or nuclear receptors and thereby influencing gene expression [86, 87]. These slow genomic effects of glucocorticoids have been well studied in neural functions or systems [88, 89].

In contrast to these genomic effects, glucocorticoids rapidly affect membrane properties and alter neural function [90, 91]. The rapid, non-genomic effects on the cell membrane by glucocorticoids are considered to modulate several types of membrane receptors such as acetylcholine, kappa opioid, and GABA receptors in neuronal cells [92]. Glucocorticoids have been reported to rapidly modulate calcium channels and intracellular Ca^{2+} mobilization in several types of cells. The opposite non-genomic effects of glucocorticoids on $[Ca^{2+}]_i$ have been reported. Glucocorticoids suppressed $[Ca^{2+}]_i$ in hippocampal CA1 neurons [93], porcine adrenal chromaffin cells [94]. A recent study also showed that corticosterone rapidly inhibits the high K^+ -induced $[Ca^{2+}]_i$ increase in dorsal root ganglion neurons [95]. In contrast, glucocorticoids also increased $[Ca^{2+}]_i$ in cortical collecting duct cells [96], smooth muscle cells [97], and colonic epithelial cells [98]. A previous study showed that corticosterone could facilitate the Ca^{2+} influx through the voltage-dependent calcium channels in cultured hippocampal neurons [99]. A recent study also showed that corticosterone acutely prolonged NMDA-induced Ca^{2+} elevation in cultured hippocampal neurons [100]. These different responses of the rapid non-genomic effects of glucocorticoids suggest the involvement of different receptors and cellular signaling pathways in each cell type.

In the inner ear, glucocorticoid receptors have been previously detected in the cochlea of the guinea pig, rat, and humans, including SGNs [101–103]. The plasma concentrations of glucocorticoids were elevated under noise conditions in the rat [104].

In the guinea pig cochlea, a downregulation of glucocorticoid receptors induced by acoustic overstimulation has been recently reported [105]. Glucocorticoids may thus directly affect the inner ear function under physiological and pathological conditions.

It has been shown that concentrations of dexamethasone, which can activate glucocorticoid receptors, ranged from 100 nM to 1 μ M under normal and stress conditions [106–108]. In the rat inner ear, the plasma concentrations of glucocorticoids were elevated up to 1 μ M under noise conditions [104]. The circulating glucocorticoid levels were elevated under restraint stress, and the protective effect was present if acoustic injury occurred at short post-stress periods accompanied by elevation of circulating glucocorticoids [109]. Therefore, the glucocorticoid levels might be an important factor for reducing acoustic injury. An elevation of ATP concentrations in the endolymphatic and the perilymphatic

Fig. 2 ATP-induced NO production in type I spiral ganglion neurons (SGNs). Fluorescence images of NO production induced by 100 μ M ATP in type I SGN at pre-stimulation (**b**) and 5 min (**c**) after ATP stimulation. The calibration bar on the right shows the DAF-2 fluorescence intensity in pseudo-color. Left (**a**) shows DIC image of the SGN with a neuritic process. Scale bar=20 μ m. Effect of dexamethasone (Dex) on the ATP-induced NO production in SGNs. A histogram summarized the normalized maximum average for various treatments. Dex, 1 μ M, enhanced the ATP (100 μ M)-induced NO production. The effect of Dex was abolished in the presence of 1 μ M RU38486 or 200 μ M L-NAME. The number of the cells tested is shown in parentheses. Error bar showed SD * p <0.01 compared to the ATP-induced NO production as a control (Student's t test for unpaired observations). NS not significant. Reproduced from [10] with permission from Elsevier

space under acute noise exposure and hypoxia plays an important role in preventing cochlea damage [80, 81]. The non-genomic effects of glucocorticoids on the ATP-induced Ca^{2+} response and NO production may thus play an important role in preventing inner ear dysfunctions.

The pharmacological concentrations of dexamethasone (10 μM [110]) can still rapidly enhance the ATP-induced Ca^{2+} response in SGNs [10]. Clinically, dexamethasone is used as therapy for such as sudden sensorineural hearing loss by the systemic or intratympanic administration of dexamethasone [111, 112]. It has been reported that in some acute clinical situations, the additional benefit of the use of high doses of glucocorticoids is due to their non-genomic effects [113]. The non-genomic effects of dexamethasone on the ATP-induced Ca^{2+} signalling and the Cl^- secretion in cultured human bronchial epithelial cell monolayers have been also reported [114]. The rapid non-genomic effects of glucocorticoids on human bronchial epithelia may have important clinical implications in the treatment of airway infections such as rhinitis, asthma, and cystic fibrosis [114]. A recent study showed the novel non-genomic role of glucocorticoids on NO production in acute myocardial ischemic injury [115]. The rapid activation of eNOS expression was thus suggested to represent an important cardiovascular protective effect of acute high-dose corticosteroid therapy. In addition, the use of glucocorticoids in acute myocardial ischemic injury is limited because of its adverse and genomic effects when administrated chronically. The same seemingly holds true for the use of glucocorticoids in inner ear disorders because most recovery occurs within the first 2 weeks after onset of sudden sensorineural hearing loss. Recovery by glucocorticoid therapy worsens after longer than 2 weeks. The novel non-genomic effects of glucocorticoids may therefore contribute to protection and recovery for inner ear disorder. The functional role of glucocorticoids and NO on the ATP-mediated Ca^{2+} signalling in SGNs remains to be elucidated.

Role of NO on the ATP-induced Ca^{2+} signalling in supporting cells

The activation of ligand-gated ionotropic P2X receptors and G protein-coupled metabotropic P2Y receptors has been reported to induce an increase of $[\text{Ca}^{2+}]_i$ in Hensen's cells in the cochlea [6, 11, 13]. It has been shown that the NO-cGMP-PKG pathway participates in the ATP-induced Ca^{2+} signalling in supporting cells, such as Deiters' cells and Hensen's cells [116]. NO attenuated an ATP-evoked $[\text{Ca}^{2+}]_i$ increase in Hensen's cells, using fura-2 techniques [116]. The effects of NO in cellular signaling are also likely to regulate the ATP-mediated Ca^{2+} signalling through the activation of the NO-cGMP-PKG pathway in Hensen's cells.

Although both nNOS and eNOS are found in Hensen's cells, it is not known which is the dominant isoform [21, 22, 60]. To my knowledge, the ATP-induced NO production in Hensen's cells has not been demonstrated.

The supporting cells of the cochlea, Deiters' cells and Hensen's cells, that surround the sensory hair cells are joined through gap junctions [117, 118]. The Hensen's cells contact the basilar membrane and are highly permeable to K^+ [119], the principal ion flowing through the mechanosensory transduction channels of the hair cells. Hensen's cells are considered to be responsible for buffering K^+ , collaborating in the maintenance of the cochlear fluid homeostasis [120]. Cochlear gap junction channels between Hensen's cells have been thought to participate in buffering and recycling of K^+ following mechanotransduction by the sensory hair cells [121–123].

In many tissues, stimulation of a single cell can initiate a propagated Ca^{2+} wave traveling from cell to cell. Intercellular Ca^{2+} waves play an important role in regulation of ciliary beat in airway epithelia [124], modulation of synaptic transmission by retinal astrocytes and Müller cells [125], coordination of metabolism by glial cells [126], control of vascular system by endothelial cells [127], and

Fig. 3 Simultaneous measurement of $[\text{Ca}^{2+}]_i$ changes and NO production in Hensen's cells. Pseudo-color displays of the 2-MeSATP-induced $[\text{Ca}^{2+}]_i$ increase (*middle panel*) in parallel with the 2-MeSATP-induced NO production (*bottom panel*) in connected four Hensen's cells. The fura-2 and DAF-2 fluorescence signals were alternately excited at 340 nm, 380 nm for fura-2, and 480–490 nm for DAF-2, respectively. Focal application of 2-MeSATP to connected Hensen's cells induced the propagation of Ca^{2+} waves, which was accompanied by NO production. 2-MeSATP, 10 μM , was locally applied to the one (1) of four connected Hensen's cells by puff pipettes (*top panel*). Arrow indicates a direction of solution flow. The 2-MeSATP-induced Ca^{2+} propagation was subsequently blocked by 1 mM octanol, an inhibitor of gap junction in these cells (not shown)

ossification by osteoblasts [128]. An extracellular propagation mode involving ATP as messenger has been identified [129, 130]. Binding of extracellular ATP to P2Y receptors results in the production of intracellular inositol triphosphate (IP₃) and, consequently, an increase in [Ca²⁺]_i of contiguous and noncontiguous cells. In the cochlea, the ATP-dependent propagation of Ca²⁺ waves through Hensen's cells has been reported [13]. It has been shown that metabotropic P2Y₂ and P2Y₄ receptors predominantly act via the PLC-IP₃ signal transduction pathway for Ca²⁺ waves in Hensen's cells, while P2Y₁ are either absent or play a negligible role [131].

It has been shown that the ATP-mediated Ca²⁺ waves occurred by activation of two functionally distinct subtypes of the P2Y₁ and P2Y₂ receptors [132–134]. There is clear evidence that P2Y₁ receptors are also specifically involved in propagation of Ca²⁺ waves in several types of cells [132, 135]. Recently, I investigated the role of NO on putative P2Y₁-mediated Ca²⁺ waves in Hensen's cells. 2-MeSATP, a selective P2Y₁ receptor agonist, induced NO production in the Ca²⁺-free medium, which was accompanied with Ca²⁺ propagation in isolated paired Hensen's cells (Fig. 3). 2-MeSATP-induced NO production was blocked by the selective P2Y₁ antagonists, MRS2179 and A3P5P in Hensen's cells.

In conclusion, the ability of cochlear cells to produce NO in considerable amounts might be of clinical importance in situations associated with the physiological and pathophysiological conditions. The role of NO in the regulation of cell function in the cochlea still remains to be elucidated, but experiments with several reagents such as blockers for NOS and P2 receptors in cochlear dysfunction would be of great interest, both in terms of the pathology of inner ear disease and of the development of more specific therapies for dysfunction of the auditory signaling pathway.

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