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Detection and characterization of nitric oxide synthase in the mammalian cochlea

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Abstract

The messenger molecule nitric oxide (NO) is involved in blood flow regulation, cytotoxicity, and neural signalling, processes that are important in the physiology and pathophysiology of the mammalian cochlea. However, neither the presence of NO nor its synthetic enzyme, NO synthase, has been established in the peripheral auditory system. NO synthase activity, measured as the enzymatic conversion of radioactive arginine to citrulline, was predominantly soluble in the auditory nerve, lateral wall, vestibule and cochlear neuroepithelium. *N*-methyl-L-arginine and trifluoperazine inhibited NO synthase activity in the lateral wall and auditory nerve. Histochemical staining by NADPH-diaphorase localized NOS activity to the lateral wall and the neuronal elements of the organ of Corti. Based on these results, the predominant NO synthase isoform in the cochlea is the neuronal type-I isoform.

Keywords: Nitric oxide; Cochlea; NADPH-diaphorase; Outer hair cell; NMMA

1. Introduction

The chemical messenger nitric oxide (NO) has been implicated in a wide variety of biological phenomena including neurotransmission, cytotoxicity, and blood flow regulation. NO is a gas and thus can cross membranes readily and affect neighboring cells. It exerts many of its effects by activating the soluble isoform of guanylate cyclase [5], an enzyme which converts GTP to cGMP. The cGMP, in turn, affects cellular physiology through the activation of cGMP-dependent protein kinases [27]. Proteins whose activities are modulated by this kinase include a plasma-membrane Ca^{2+}-ATPase and a Na^+/Ca^{2+} exchanger [14,33]. NO also affects cellular physiology independent of the cGMP system. NO stimulates the ADP-ribosylation of enzymes such as glyceraldehyde-3-phosphate dehydrogenase [21], thus resulting in a modulation of activity. In addition, NO can directly inhibit enzymes involved in the mitochondrial electron transport chain [15]. Finally, as a free radical, NO participates in radical-mediated cell death by reacting with superoxide to produce the highly cytotoxic compound, peroxynitrite [2].

NO is synthesized by the enzyme nitric oxide synthase (NOS) which catalyzes the oxidation of the terminal guanidinium nitrogen in the amino acid L-arginine to form NO and L-citrulline [20]. Three distinct isoforms of this enzyme have been characterized which vary in Ca^{2+}/calmodulin dependency, subcellular localization, mode of induction, and tissue expression. Type-I NOS activity is Ca^{2+}/calmodulin-dependent, cytosolic and constitutively expressed [7]. First localized in brain, this isozyme has since been found in many locations including the pancreas [28], corpus cavernosum [8], and neurons innervating the gastrointestinal lining of the gut [34]. In these tissues, NO participates in insulin secretion, penile erection, and peristalsis, respectively. In the brain, NO synthesized by this isozyme may act as a neuromodulator in glutamate neurotransmission [5]. Moreover, a role for NO in long-term potentiation has been proposed [23]. In contrast to the neuronal type-I NOS, type-II NOS is not

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constitutively expressed but can be induced in many tissues such as smooth muscle, macrophages, glia, and liver [22]. In macrophages, type-II NOS is induced in response to a bacterial infection, and the NO produced can kill the invading cells. However, the function of type-II NOS in other cell types is unknown. Type-III NOS is distinctly localized to the endothelial lining of blood vessels where NO acts as a vasodilator [24] via relaxation of the smooth muscle surrounding the vessel. Similar to type-I NOS, this isozyme is constitutively expressed and Ca^{2+}/calmodulin dependent [18,25]. However, type-III NOS is unique among the three isoforms in that it is localized to the plasma membrane due to posttranslational *N*-myristoylation [29]. The vasodilatory action of several hormones including bradykinin and acetylcholine is thought to be mediated by type-III NOS in the endothelium [16].

The three best studied effects of NO, modulation of neurotransmission, regulation of blood flow, and induction of cytotoxicity are all involved in the physiology or pathophysiology of the mammalian cochlea. In fact, several key components of the NO/cGMP signal transduction pathway have already been demonstrated in the sensory tissues of the inner ear. Calmodulin [30], cGMP [32], and a potential cGMP-dependent protein kinase substrate [9] are present in the neuroepithelium. Although NOS localization has been attempted in central auditory structures [12] little information concerning the presence or distribution of NOS in the cochlea is known. We assessed the biochemical activity of NOS in the lateral wall, auditory nerve, cochlear neuroepithelium, and vestibule. Using a combination of histochemical and enzymatic methods, we characterized NOS in these tissues and determined the location and nature of the NOS isoforms present in the peripheral auditory system.

2. Materials and Methods

Except where noted, all chemicals were purchased from Sigma chemical company (St. Louis, MO).

NOS was localized in cochlear tissues using NADPH-diaphorase histochemistry. Adult pigmented guinea pigs (Murphy Laboratories, Plainfield, IN) were decapitated and the bullae removed, opened, and placed in 1% paraformaldehyde in phosphate buffered saline (PBS). PBS consisted of 80 mM Na_2HPO_4, 100 mM NaCl with the pH adjusted to 7.5 using HCl. The cochleae were perfused with 1% paraformaldehyde/PBS through the round window and maintained in this solution for 3 h at 4°C. They were then rinsed 3 times in PBS, and the lateral wall was removed and stained as described below. The modiolus containing the sensory epithelium was then removed from the temporal bone and placed in NADPH-diaphorase staining solution consisting of 0.2% Triton X-100, 1 mM nitro blue tetrazolium (NBT), 1 mM NADP, 15 mM malic acid, 1 mM $MnCl_2$ (Mallinckrodt, St. Louis, MO), 50 mM Tris base with the pH adjusted to 8.0 using HCl. After 1 h, the reaction was stopped by placing the modiolus in 10 ml of PBS and dissecting the neuroepithelium. The dissected tissue was mounted onto slides using 50% glycerol (Merck, Rahway, NJ). The tissue was visualized under brightfield microscopy.

The NADPH-diaphorase reaction was also performed on cells isolated from neuroepithelium. The sensory epithelium was dissected from freshly isolated cochleae in Hanks' Balanced Salt Solution (Gibco-BRL, Gaithersburg, MD) consisting of 137 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.5 mM D-glucose, 5 mM *N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffered to pH 7.4 with NaOH and adjusted to 300 ± 2 mOsM using 4 M NaCl. Tissue sections containing Hensen's cells, outer hair cells and Deiters' cells were obtained by microdissection and incubated for 15 min in type IV collagenase diluted to 0.5 mg/ml in Hanks' medium. Cells were then dispersed into a fresh droplet of Hanks' medium. The dissociated cells were maintained in 1% paraformaldehyde/PBS for 2 h at 4°C. The cells were pelleted at $1000 \times g$ for 10 min and placed onto coverslips coated with 0.1% polyethylenimine. They were washed several times with Hanks' medium and then incubated with the NADPH-diaphorase staining solution for 1 h at 37°C.

Tissue was prepared for NOS enzymatic assays using the following procedure. Pigmented guinea pigs were decapitated and the bullae quickly removed and placed in Hanks' medium. The auditory nerve, neuroepithelium, lateral wall, and vestibular tissues were dissected and homogenized in buffer containing 50 mM HEPES, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) and the pH adjusted to 7.4. The neuroepithelium was homogenized in 25 μl of this buffer whereas the other tissues were homogenized in 50 μl. The tissues were homogenized twice on ice using 200 μl glass microhomogenizers (Kontes Life Sciences, Vineland, NJ) and then centrifuged at $10,000 \times g$ for 15 min to pellet cell membranes. In some experiments, the pelleted fraction was resuspended in an equal volume of homogenization buffer and also used for NOS activity determinations. The supernatant fraction was removed and centrifuged at $100,000 \times g$ for 1 h. The $100,000 \times g$ supernatant was used for the NOS assays.

NOS activity in inner ear tissues was assessed using a modified procedure of published methods [19]. L-[2,3-^{3}H]arginine from New England Nuclear (36.1 Ci/mmol) was purified before each experiment by anion exchange chromatography. Dowex-1 resin (Cl^- form) was added to warm (~ 70°C) 1 M NaOH and allowed to settle. A 0.5 ml column was poured and washed with H_2O (~ 70°C) until the pH was neutral. [^{3}H]arginine was added to the column and the first peak of radioactivity was collected. This peak contained the purified [^{3}H]arginine. Assays were conducted in a 30 μl volume in 16 mM HEPES, 3 μM calmodulin, 3 mM $CaCl_2$, 1 mM EDTA, 1 mM EGTA and 100 μM NADPH, pH 7.4. The initial [^{3}H]arginine level was 250,000 dpm in all experiments except in the experiments shown in Fig. 3 where 400,000 dpm were added. Assays were run at room temperature for 15 min except in the experiments shown in Fig. 3 where the reaction time was extended to 60 min. Reactions were terminated by the addition of 0.5 ml of buffer containing 50 mM HEPES, 2 mM EDTA with the pH adjusted to 5.5 using HCl. The entire reaction mix was loaded onto a 1 ml AG50WX-8 column (Na^+ form, BioRad, Richmond, CA) and the column was washed with 0.5 ml of the buffer used to terminate the reaction. The column retained [^{3}H]arginine and excluded [^{3}H]citrulline as confirmed by thin layer chromatographic analysis (see below).

Enzyme activities were determined by subtracting the dpm in the citrulline fraction of a control reaction (-NADPH) from the experimental value. NOS specific activity was expressed as dpm of [^{3}H]citrulline formed min^{-1} mg $protein^{-1}$. Protein concentrations were determined using the BioRad protein assay kit (BioRad, Richmond, CA) and bovine serum albumin as standard.

Arginine to citrulline conversion was also determined using thin layer chromatography (TLC). Auditory nerve tissue from 6 cochleae was homogenized as described above and the $10,000 \times g$ supernatant

was used. The reaction volume was increased to 100 μl. At 10 min intervals, 10 μl aliquots of the reaction mix were spotted onto a 20×20 cm silica gel G TLC plate (Uniplate cat# 80013, Newark, DE). Unlabeled L-arginine and L-citrulline (26 and 18 μg respectively) were added as carriers. The spots were dried and the plate was developed in 5:30:2:5 *n*-butanol:acetone:diethylamine:water. The plates were sprayed with 0.1 mg/ml fluorescamine in acetone to visualize the arginine and citrulline bands. These bands were scraped and the amount of [^{3}H]arginine and [^{3}H]citrulline was determined by scintillation counting.

3. Results

We first characterized NOS activity in the soluble fraction of the auditory nerve. [^{3}H]citrulline was produced by the auditory nerve in a linear time-dependent fashion in the presence but not in the absence of the NOS cosubstrate NADPH (Fig. 1). The production of citrulline could be abolished by first heating the enzyme fractions at 100°C for 15 min (Fig. 1 inset).

Enzyme-specific inhibitors confirmed that the observed conversion of arginine to citrulline was catalyzed by NOS. N^G-methyl-L-arginine (NMMA) and the calmodulin antagonist trifluoperazine (TFP) are effective inhibitors of the neuronal type-I isoform [6]. Both of these compounds significantly reduced arginine to citrulline conversion catalyzed by cochlear tissues. In lateral wall, the soluble NOS activity of 2610 dpm min^{-1} mg protein^{-1} was reduced by 73% or 96% in the presence of 50 μM TFP or NMMA, respectively.

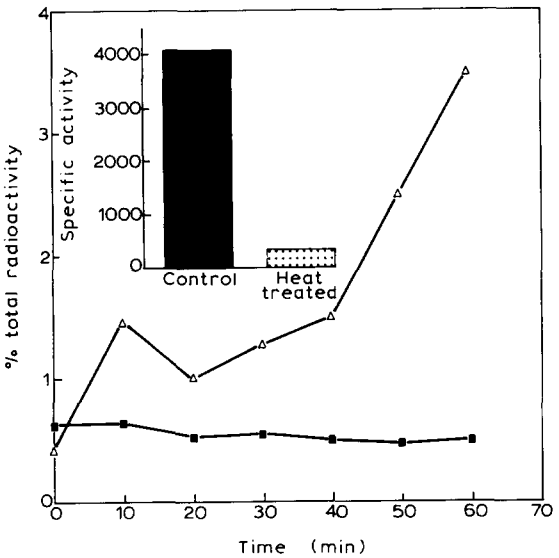


Fig. 1. NOS activity in auditory nerve. [^{3}H]citrulline was separated from [^{3}H]arginine by TLC as described in Methods. Arginine to citrulline conversion was observed in the presence of 10 mM NADPH (triangles) but not in its absence (squares). [^{3}H]citrulline produced is expressed as the percentage of total radioactivity recovered from each time point. Inset: NADPH-dependent citrulline production is abolished by prior incubation of the tissue at 100°C for 15 min. Specific activity is defined in Methods.

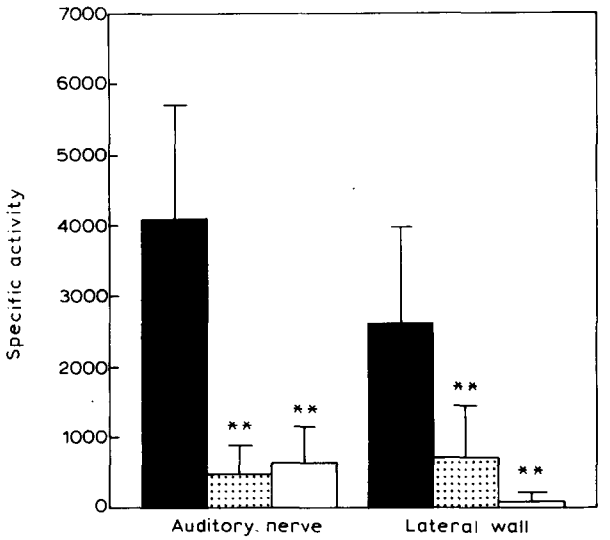


Fig. 2. NOS inhibitors block cochlear NOS activity. NOS activity was assayed as described in Methods. Cytosolic NOS activity (black bars) was inhibited by either 50 μM TFP (stippled bars) or 50 μM NMMA (unfilled bars) in auditory nerve and lateral wall. When TFP was tested, calmodulin was not added to the assay. Each point is the mean ± S.D. of either 17 experiments (controls) or 3–8 experiments (inhibitors). Data are expressed as dpm citrulline produced min^{-1} mg protein^{-1}. Statistical significance of differences between experiments in the absence and presence of inhibitors was determined by Student's *t*-test (** $P < 0.001$).

In auditory nerve, soluble actvitiy of 4090 dpm min^{-1} mg protein^{-1} was reduced by 88% or 84% in the presence of 50 μM TFP or NMMA respectively (Fig. 2).

NOS was present in roughly equal specific activities in the lateral wall, auditory nerve, cochlear neuroepithelium, and pooled utricule and crista ampullaris tissues (Fig. 3). These activities ranged from 1343 to 3092 dpm min^{-1} mg protein^{-1}. Also, the majority of the NOS activity was cytosolic indicating that the soluble NOS isoforms (type-I and type-II) predominate in the inner ear. Consistent with the finding in cochlear tissues, NOS activity in the vestibular tissues also could be completely inhibited by NMMA in 2 independent experiments (data not shown).

NADPH-diaphorase histochemistry was performed to identify the cell types containing NOS in the cochlea. In brain, cells stained by this technique colocalize with cells recognized by NOS immunohistochemistry [10]. NADPH-diaphorase histochemical staining in the cochear neuroepithelium labeled the nerve endings at the base of inner and outer hair cells, tunnel crossing fibers, tunnel and inner spiral bundles, and nerve fibers entering through the habenula perforata (Fig. 4a,b). The finding that NADPH-diaphorase activity was located at the base of outer hair cells was confirmed by experiments on isolated cells (Fig. 4c). In control experiments conducted in the absence of NADP, no

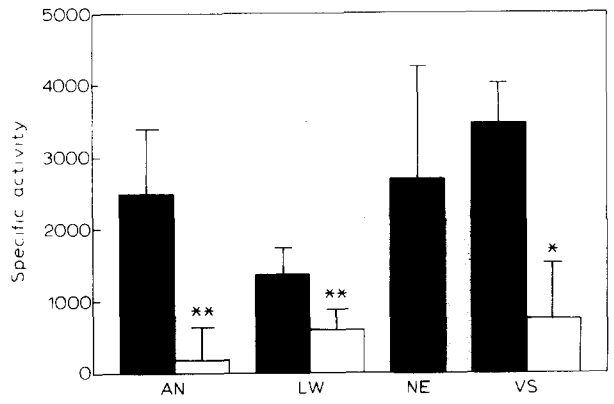


Fig. 3. NOS activity in cochlear tissues. Cytosolic (solid) and particulate (clear) NOS activity is found in auditory nerve (AN), lateral wall (LW), cochlear neuroepithelium (NE), and pooled utricle and cristae ampullaris (VS). No particulate NOS activity could be measured in the cochlear neuroepithelium. Assays were conducted in the presence of 400,000 dpm of [^{3}H]arginine for 60 min. Each point is the mean ± S.D. of 6 independent experiments except for the vestibular tissues which were assayed 3 times. Statistical significance of differences between particulate and cytosolic activities were assessed using Student's t-test (* $0.02 < P < 0.01$, ** $P < 0.001$).

staining was observed in the neuroepithelium (Fig. 4d) or the lateral wall (Fig. 4f). In the lateral wall tissues, the NADPH-diaphorase reaction stained the stria vascularis and the region of the spiral prominence (Fig. 4e). No labeling of the spiral ligament was observed in cross-sections of stained lateral wall tissue (data not shown).

4. Discussion

NOS is present in the peripheral auditory system. NOS activity can be quantitated enzymatically in the auditory nerve, cochlear neuroepithelium, lateral wall, and vestibule. NADPH-diaphorase activity, which has been used to localize NOS in neural tissues [10] shows a distinct staining pattern in both the lateral wall and neuroepithelium.

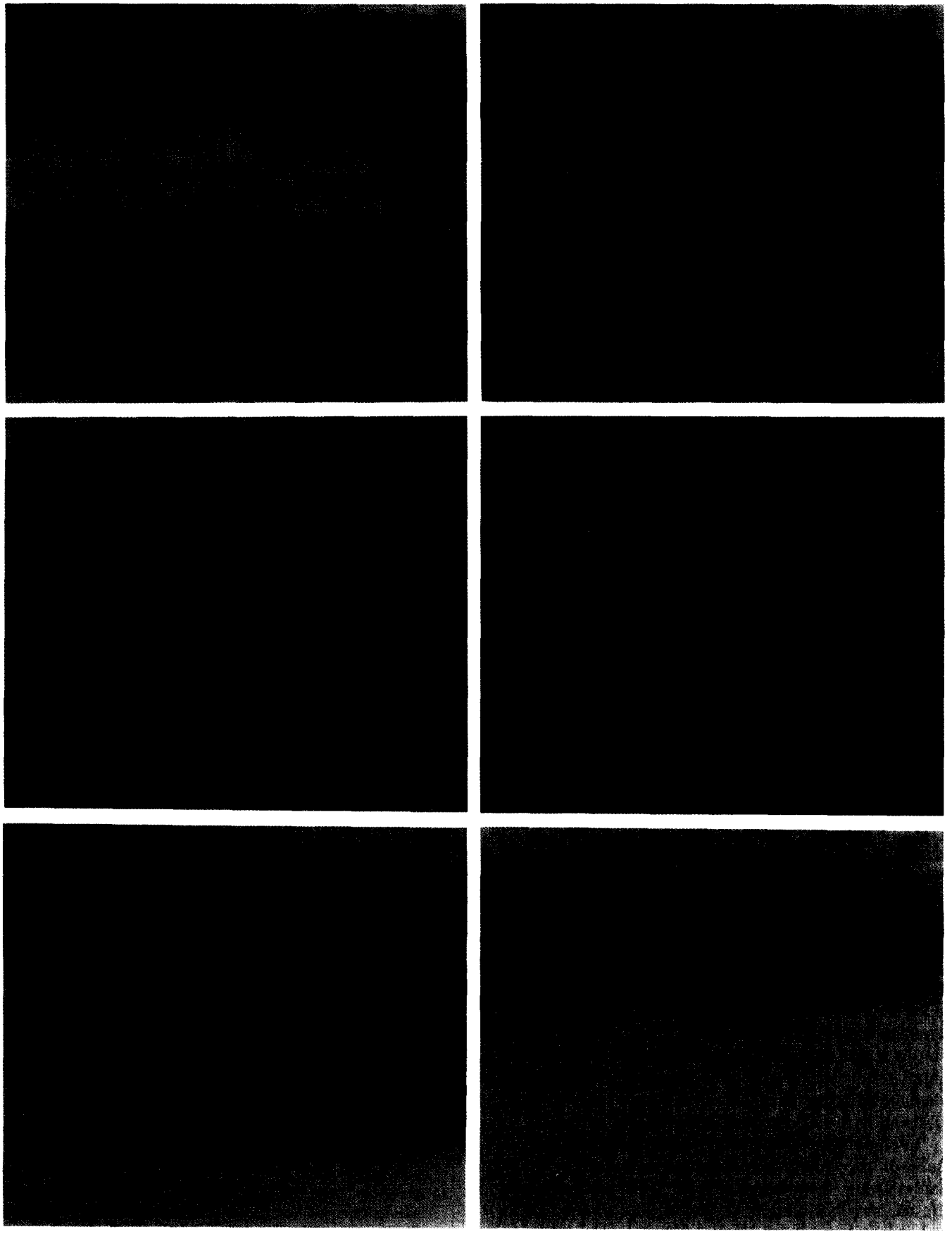
In the auditory nerve, NOS activity is predominantly cytosolic and can be inhibited by both NMMA and TFP, unique characteristics of the neuronal type-I isoform [27]. This finding is in agreement with the neuronal nature of this tissue. In neurons, NO produced from type-I NOS can act as a neuromodulator or even as a neurotransmitter [5]. It is possible that NO produced from type-I NOS may be playing a similar role in the auditory nerve.

Similar to auditory nerve, NOS activity in the lateral wall is predominantly cytosolic and Ca^{2+}/calmodulin-dependent thus indicating type-I NOS activity. NOS can also be localized histochemically by NADPH-diaphorase labeling. Type-III NOS could not be detected in our experiments as indicated by the lack of particulate activity. The absence of type-III NOS activity is unexpected since this isozyme is generally associated with blood vessels and thus may be expected in the highly vascularized lateral wall tissue. Type-III NOS activity in the lateral wall may have been below the detection threshold of our assay, a distinct possibility since type-III NOS has the lowest catalytic rate of the three NOS isoforms [31]. Although NO can affect cochlear blood flow [4], the lack of type-III NOS as well as smooth muscle lining strial vessels indicates that NO blood flow regulation may occur at some other location or through some novel mechanism in the lateral wall.

In the cochlear neuroepithelium, the NADPH-diaphorase reaction labels the neural elements innervating both inner and outer hair cells. However, in some experiments, additional cell types such as interdental cells of the spiral limbus, pillar, and Deiters' cells were stained. It should be noted that the correlation between cells recognized by the NADPH-diaphorase technique and immunocytochemistry using anti-NOS-I antibodies is the strongest in neurons [10]. In non-neuronal cells in the peripheral olfactory tissues, NADPH-diaphorase staining results in labeling of non-NOS-I immunoreactive cells [17]. Thus, NADPH-diaphorase labeling of cochlear supporting cells may not indicate the presence of NOS. In contrast, the combination of NADPH diaphorase staining in the neuronal elements of the cochlear neuroepithelium and enzymatic detection of NOS activity in this tissue strongly suggests that NOS is located in the neurons.

In cochlear surface preparations, dense punctate staining is observed at the base of both inner and outer hair cells. Studies on isolated outer hair cells confirm the presence of labeling at the base of the cell and also demonstrate the lack of stain in the cytoplasm. The labeling at the base of the outer hair cells is connected to the tunnel crossing fibers which also are identified by NADPH-diaphorase histochemistry. The staining pattern of the tunnel crossing fibers is very similar to

Fig. 4. Localization of NOS in cochlear tissues using NADPH-diaphorase histochemistry. Cochlear neuroepithelium and lateral wall tissues were stained as described in Methods. A: in the cochlear neuroepithelium, nerve endings at the base of outer (arrow) and inner hair cells (arrowhead) are stained. B: the same area as in A at a different focal plane shows labeling in tunnel crossing fibers (CF), the inner spiral bundle (ISB), the tunnel spiral bundle (TSB) and fibers entering through the habenula perforata (arrow). C: nerve endings of isolated outer hair cells are stained (arrow). D: control. No staining is observed in the neuroepithelium in the absence of NADP. E: in the lateral wall, the region of the stria vascularis (SV) and the spiral prominence (SP) is labeled. F: control labeling experiments as in D do not label the lateral wall. Scale bar = 10 μm.



the pattern observed using anti-choline:acetyltransferase immunocytochemistry which selectively stains efferent neurons in the organ of Corti [1]. Thus it is likely that NOS is located in efferent neurons innervating the outer hair cells. Punctate NADPH-diaphorase staining near inner hair cells is also observed and spiral ganglion cells can be labeled (data not shown) in agreement with a previous report [35]. Thus, NOS may also be located in type-I afferent neurons. In contrast, no NADPH-diaphorase labeling occurs in the cytoplasm of inner or outer hair cells.

Most documented NO-mediated processes involve paracrine signalling whereby NO produced from one cell type diffuses to a neighboring cell type and affects its physiology. In the cochlea, a similar situation may exist whereby NO produced from nerve endings could diffuse to the hair cells and affect their physiology directly.

In this fashion, NO could conceivably affect many hair cell processes. NO can lower the cytosolic free $[Ca^{2+}]$ in smooth muscle which leads to a change in cell shape [3]. By analogy, NO produced from efferent nerve endings could lower the cytosolic $[Ca^{2+}]$ in outer hair cells and affect their length which is Ca^{2+}-dependent [13]. Thus, outer hair cell length and the micromechanics of the basilar membrane could be modulated by NO-mediated efferent stimulation. Regulation of the cytosolic $[Ca^{2+}]$ could also affect other inner and outer hair cell processes such as neurotransmitter release. Release of neurotransmitters is dependent on an increase in intracellular $[Ca^{2+}]$. By lowering calcium levels in hair cells, NO could downregulate the release of neurotransmitter and affect cochlear sound transduction.

NO is also a cytotoxin. Overstimulation of NOS-containing neurons by glutamate agonists leads to overproduction of NO and the death of surrounding neurons [11]. In the cochlea, excessive acoustic stimulation damages the afferent neurons innervating the inner hair cell. Perfusion of the glutamate receptor agonist AMPA into the cochlea mimics the excitotoxic damage [26] suggesting that overstimulaton of glutamate receptors is associated with this damage. Overstimulation of NOS located in the afferent nerve endings either through acoustic or glutamate stimulation could produce excessive amounts of NO which could in turn damage the surrounding tissue. The presence of NOS in this region supports this hypothesis. Pharmacological intervention with NOS inhibitors should reduce this damage and thus may have some clinical benefits in the prevention of noise-induced hearing loss.

In summary, NOS activity is present in several tissues of the inner ear including the auditory nerve, lateral wall, vestibule and cochlear neuroepithelium. Based on subcellular fractionation and pharmacological characterization of this activity, the prominent isoform in the cochlea is the neuronal type-I isozyme. NOS is localized to the stria vascularis as well as neurons synapsing on the inner and outer hair cells. Due to the wide range of effects attributed to NO in other systems, it is likely that NO is an important signalling molecule in the auditory system as well.

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