

## NOX3, a Superoxide-generating NADPH Oxidase of the Inner Ear\*<sup>§</sup>

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Reactive oxygen species (ROS) play a major role in drug-, noise-, and age-dependent hearing loss, but the source of ROS in the inner ear remains largely unknown. Herein, we demonstrate that NADPH oxidase (NOX) 3, a member of the NOX/dual domain oxidase family of NADPH oxidases, is highly expressed in specific portions of the inner ear. As assessed by real-time PCR, NOX3 mRNA expression in the inner ear is at least 50-fold higher than in any other tissues where its expression has been observed (e.g. fetal kidney, brain, skull). Microdissection and *in situ* hybridization studies demonstrated that NOX3 is localized to the vestibular and cochlear sensory epithelia and to the spiral ganglions. Transfection of human embryonic kidney 293 cells with NOX3 revealed that it generates low levels of ROS on its own but produces high levels of ROS upon co-expression with cytoplasmic NOX subunits. NOX3-dependent superoxide production required a stimulus in the absence of subunits and upon co-expression with phagocyte NADPH oxidase subunits p47<sup>phox</sup> and p67<sup>phox</sup>, but it was stimulus-independent upon co-expression with colon NADPH oxidase subunits NOX organizer 1 and NOX activator 1. Pre-incubation of NOX3-transfected human embryonic kidney 293 cells with the ototoxic drug cisplatin markedly enhanced superoxide production, in both the presence and the absence of subunits. Our data suggest that NOX3 is a relevant source of ROS generation in the cochlear and vestibular systems and that NOX3-dependent ROS generation might contribute to hearing loss and balance problems in response to ototoxic drugs.

The inner ear is a highly complex structure involved in hearing and balancing. The conversion of sound into electrical signals occurs within the cochlea, in the organ of Corti, and the electrical signals are conducted by the axons of spiral ganglion

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1S.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY573239 (rat NOX3) and AY573240 (mouse NOX3).

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neurons to the brain. The linear movement of the head is sensed by the otolith organs (utricle and saccule) and the rotation movements by the ampulla of the semicircular canals. The signals generated in the vestibular system are transmitted by the vestibular ganglion neurons to the central nervous system.

Hearing impairment caused by loss of cochlear function occurs frequently, if not invariably, over a lifetime. Noise and ototoxic chemicals may lead to a precocious, rapid hearing loss, whereas aging leads to a more insidious, chronic loss of hearing. Research over the last decades has identified reactive oxygen species (ROS)<sup>1</sup> as the major factor mediating hearing loss (1). ROS is generated within the cochlea after exposure to ototoxic drugs (e.g. cisplatin (2, 3), aminoglycoside antibiotics (3)) or to noise (4). Signs of oxidative stress, such as DNA damage and lipid peroxidation, have been documented *in vivo* in response to those challenges (5, 6), as well as in cochlear aging (7). The vestibular system is also damaged by ototoxic drugs (8, 9) in a process that includes excessive ROS production (10, 11).

Although the role of oxidative stress in inner ear damage is well established, its source is poorly understood. Non-enzymatic generation of ROS by the ototoxic gentamicin-Fe<sup>2+</sup> complex has been suggested. However, an enzymatic source of superoxide was needed to convert inactive gentamicin-Fe<sup>3+</sup> into redox-active gentamicin-Fe<sup>2+</sup> complex (12). Thus, the presence of a superoxide-producing enzyme in the inner ear and its involvement in drug ototoxicity could be anticipated.

Over the last decade, it has been proven that the expression of superoxide-generating NADPH oxidases (NOX) is not restricted to phagocytes. Besides the well known catalytic subunit of the phagocyte NADPH oxidase, gp91<sup>phox</sup>/NOX2 (for review, see Ref. 13), six other superoxide-producing enzymes have been identified in mammals (14, 15). For most NOX and dual domain oxidase (DUOX) enzymes, a predominant tissue localization has been described (e.g. colon epithelium for NOX1 (16, 17), kidney cortex for NOX4 (18, 19), lymphoid organs and testis for NOX5 (20), and the thyroid gland for DUOX1 and DUOX2 (21, 22)). The tissue distribution of NOX3 was systematically investigated in two studies (23, 24) in which the authors found only very low levels of NOX3 mRNA expression in embryonic kidney.

Our knowledge of the activation mechanisms of the members of NOX/DUOX family varies considerably among individual enzymes. NOX1 and gp91<sup>phox</sup>/NOX2 are subunit-dependent en-

<sup>1</sup> The abbreviations used are: ROS, reactive oxygen species; NOX, NADPH oxidase; NOXA1, NOX activator 1; NOXO1, NOX organizer 1; gp91<sup>phox</sup>, 91-kDa glycoprotein subunit of the phagocyte NADPH oxidase; DUOX, dual domain oxidase; HEK, human embryonic kidney; RT, reverse transcription; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C.

zymes that need to assemble with an activator subunit (NOXA1 and p67<sup>phox</sup>, respectively) and an organizer subunit (NOXO1 and p47<sup>phox</sup>, respectively) to generate superoxide (25–28). NOX5, DUOX1, and DUOX2, on the other hand, have N-terminal Ca<sup>2+</sup>-binding motifs (EF-hand domains) and, so far, one of them, NOX5, has been shown to be activated by increased Ca<sup>2+</sup> concentration (29). The mechanism of NOX4 activation is less clear; there are indications that it might be a constitutively active enzyme (18, 19). Virtually nothing is known about NOX3 activation. Indeed, it has not been established whether NOX3 is a superoxide-producing enzyme at all.

Herein, we demonstrate that NOX3 is highly expressed in the inner ear. We show that it generates low levels of ROS in a subunit-independent manner and high levels of ROS in the presence of activator and organizer subunits. We also demonstrate that an ototoxic drug, cisplatin, markedly increases NOX3-dependent superoxide production.

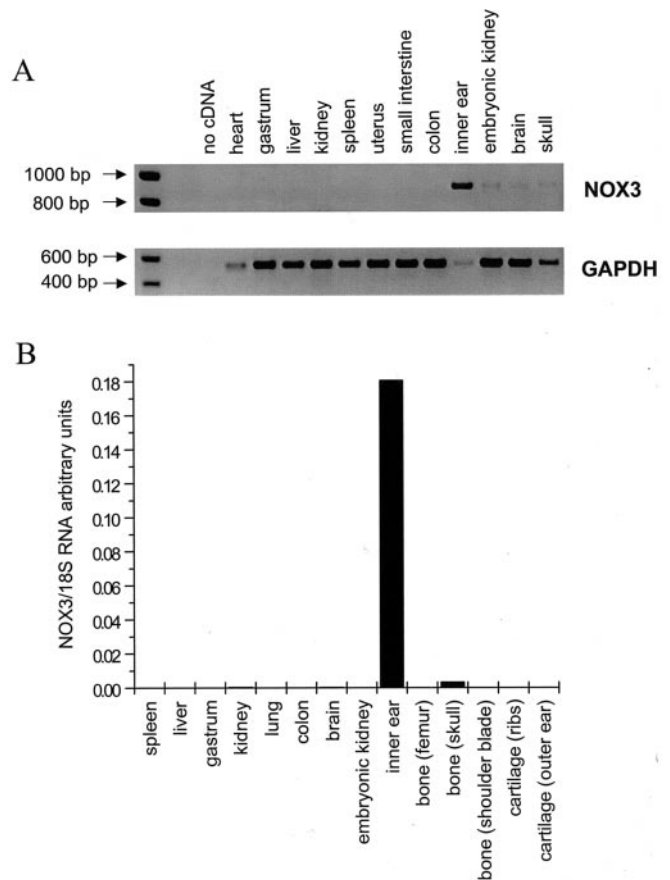
#### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—HEK293 cells were maintained in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 that was supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 4 mM L-glutamine. NOX3-, NOXO1, NOXA1, p47<sup>phox</sup>, and p67<sup>phox</sup> cDNAs were subcloned into pcDNA3.1 (Invitrogen) and transfected into HEK293 cells with the Effectene transfection system (Qiagen). To obtain stable clones, NOX3-, NOXO1-, and NOXA1-transfected HEK293 cells were selected with 400 µg/ml G418 starting on the second day after transfection. After 14 days of selection, 24 surviving clones were tested for superoxide production. The positive clones were verified to express NOX3, NOXO1, and NOXA1 RNA by RT-PCR.

**Cloning of Mouse and Rat NOX3 cDNA**—The first and last exons of mouse and rat NOX3 genes were identified based on their homology with the human NOX3 gene using the Ensembl Genome Browser (www.ensembl.org). Inner ear samples of mouse (strain C57Bl/6) and rat (strain Sprague-Dawley) were isolated, and total RNA was purified as described previously (30). Primers were designed and used to amplify the full length of coding sequences (mouse NOX3: forward, 5'-atg ccg gtc tgc tgg att ctg aac-3'; reverse, 5'-cta gaa gtt ttc ctt gtt gta ata gaa-3'; rat NOX3: forward, 5'-gtg ttg gta gta aga gaa gtc tca tg-3'; reverse, 5'-c tag aag ttt tcc ttg ttg taa tag-3') with TaqDNA polymerase (Qiagen) under standard conditions. PCR products were subcloned into pcDNA3.1 vector (Invitrogen) and verified by sequencing.

**Tissue Distribution of NOX3**—Total RNA was isolated from different organs of rat and mouse and from specific regions of the rat inner ear using the TRIzol reagent. Except for RNA purified from parts of the inner ear, samples were treated with DNase and then further purified with the use of an RNeasy kit (Qiagen). 2 µg total RNA from each tissue was reverse-transcribed using Superscript reverse transcriptase (Invitrogen). PCR was carried out with TaqDNA polymerase using the following primers: mouse NOX3: forward, 5'-gtg ata aca ggc tta aag cag aag gc-3'; reverse, 5'-cca ctt tcc cct act tga ctt tag-3'; rat NOX3: forward, 5'-gcg tgt gct gta gag gac cgt gga g-3'; reverse, 5'-gag cct gtc cct ctg ctc caa atg c-3'; mouse GAPDH: forward, 5'-ggg tgt gaa cca cga gaa at-3'; reverse, 5'-gtc atg agc cct tcc aca at-3'; rat GAPDH: forward, 5'-cgg tgt caa cgg att tgg ccg tat t-3'; reverse, 5'-act gtg gtc atg agc cct tcc acg a-3'; rat NOXO1: forward, 5'-acc caa acc tct gga tct gga gcc c-3'; reverse, 5'-gga tgg cac tca tac agg ggc gag t-3'; rat NOXA1: forward, 5'-tac tgg ccg tag cac ggc aag act g-3'; reverse, 5'-gga cct ccc agg ctt gca gtt tga a-3'; rat p47<sup>phox</sup>: forward, 5'-gca gca cct gtc gga gaa ggt ggt c-3'; reverse, 5'-tct gtc gct ggg cct ggg tta tct c-3'; rat p67<sup>phox</sup>: forward, 5'-aag cag aag agc agt tag cat tgg c-3'; reverse, 5'-gga gtg cct tcc aaa ttc ttg gct g-3'; and rat gp91<sup>phox</sup>: forward, 5'-tga tct tgc tgc cag tgt gtc gga a-3'; reverse, 5'-tcg atg gtt ttg aaa ggc gtc g-3'. Standard PCR conditions were used, and the number of PCR cycles was 30 (Figs. 1 and 2) or 28 (Fig. 3) for the amplification of GAPDH and 35 for all other amplifications unless stated otherwise.

**Quantitative PCR**—Quantitative PCR was carried out using ABI Prism 7900HT sequence detection system with standard temperature protocol and 2× SYBR Green PCR Master Mix reagent (Applied Biosystems, Warrington, UK) in 25 µl volume, in triplicates. 300 nM concentrations of the following primer pairs were used for the reactions: mouse 18 S: forward, 5'-aca tcc aag gaa ggc agc ag-3'; reverse, 5'-ttt tgc tca cta cct ccc cg-3' and mouse NOX3: forward, 5'-cga cga att caa gca gat gc-3'; reverse 5'-aag agt ctt tga cat ggc ttt gg-3'. All amplifications were

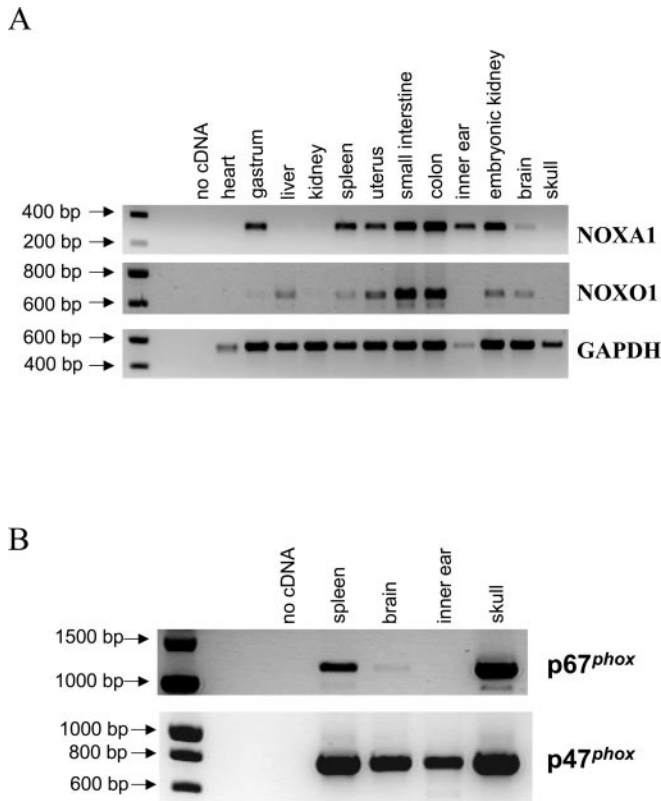


**FIG. 1. Tissue distribution of NOX3 mRNA.** A, NOX3 mRNA expression was evaluated in 12 rat tissues by RT-PCR (top); GAPDH mRNA was used as a reference transcript (bottom). No cDNA represents negative control PCR devoid of added cDNA. The leftmost lanes show DNA size markers. B, quantification of NOX3 mRNA in 14 mouse tissues using real time PCR. NOX3 mRNA expression is shown relative to 18 S rRNA expression. The amounts of NOX3 and 18 S PCR products were measured using SYBR Green.

carried out in MicroAmp optical 96-well reaction plates with optical adhesive covers (Applied Biosystems). The accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye.

**In Situ Hybridization**—For *in situ* hybridization experiments, digoxigenin-labeled antisense and sense (negative control) cRNA probes (nucleotides 560–849 of mNOX3) were generated and used as described previously (20) on decalcified, 7-µm thick inner ear sections.

**Measurement of Reactive Oxygen Species**—ROS generation was measured by the peroxidase-dependent luminol-amplified chemiluminescence technique (referred to as luminol-amplified chemiluminescence) in 96-well microplates using Luminometer Wallac 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences). Measurements were performed in Hanks' balanced salt solution supplemented with 1 mg/ml D-glucose, 1 unit/ml horseradish peroxidase, and 250 µM luminol. In some experiments, phorbol 12-myristate 13-acetate (PMA) was added during the measurements to a final concentration of 100 nM. When the effect of cisplatin or 5-fluorouracil was investigated, these compounds were dissolved in Me<sub>2</sub>SO and pre-incubated with the cells for the indicated time and concentration in cell culture medium. Before ROS measurements, the cell culture medium was exchanged with the assay solution and chemiluminescence or absorption (see below) was measured at 37 °C. After measurements, cells were counted, and the results were normalized to 150,000 cells. Extracellular superoxide production was measured in 96-well microplates at 550 nm as the superoxide dismutase-sensitive reduction of 100 µM ferricytochrome *c* (referred to as cytochrome *c* reduction technique) in the presence or absence of ionomycin (1 µM) or hypoxanthine (40 µM) and xanthine oxidase (20 milliunits/ml). The superoxide production was calculated using an absorption coefficient of 21.1 mm<sup>-1</sup> cm<sup>-1</sup> and was normalized to 10<sup>7</sup> cells (31).



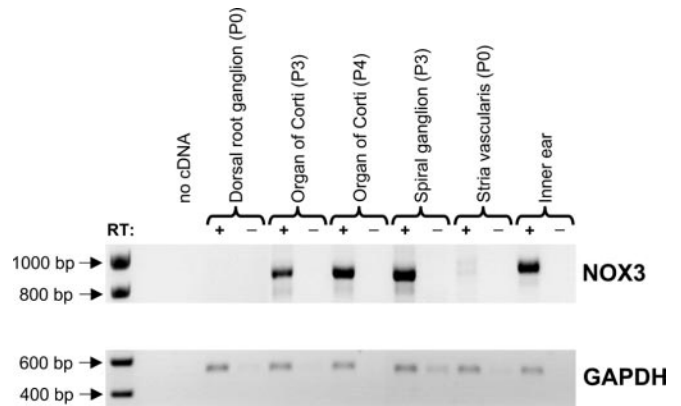
**FIG. 2. PCR detection of cDNAs encoding NOX activator and regulator subunits in the inner ear.** *A*, RT-PCR amplification of NOXA1, NOXO1, and the reference GAPDH cDNA from the indicated rat tissues. *B*, RT-PCR amplification of p67<sup>phox</sup> and p47<sup>phox</sup> cDNA from the indicated rat tissues. The leftmost lanes show DNA size markers.

## RESULTS

**Cloning of Mouse and Rat NOX3 cDNA from Inner Ear**—NOX3 mRNA has been shown to be expressed in human embryonic kidney, but expression levels were very low (23, 24); hence, the physiological relevance was questionable. We reasoned that the physiologically relevant localization of NOX3 might have been missed because previous studies had restricted their analysis to commercially available human RNA sources. To overcome these limitations, we decided to work in mouse and rat and to prepare RNA from tissues that had not yet been investigated. Because hitherto only the human NOX3 sequence was known, we identified mouse and rat NOX3 genes by searching genomic DNA databases and designed, based on these results, mouse and rat NOX3 PCR primers.

We then prepared RNA from a variety of mouse and rat tissues, including bone (femur, skull, shoulder blade), cartilage (joints of ribs, outer ear), and inner ear and analyzed them for NOX3 expression by RT-PCR. As shown on Fig. 1A, high levels of NOX3 transcript were detected only in the rat inner ear sample (despite its relatively low mRNA content demonstrated by the low amount of GAPDH PCR product). Using primer pairs designed from the first and the last exons of the mouse and rat NOX3 gene, respectively, we amplified the complete coding regions of mouse and rat NOX3 cDNA sequences from inner ear samples. The predicted amino acid sequences of both mouse and rat NOX3 showed 81% sequence identity with the human sequence and 93.5% identity with each other (GenBank accession numbers of rat and mouse NOX3: AY573239 and AY573240, respectively).

**NOX3 Is Predominantly Expressed in the Inner Ear**—Based on the cDNA sequence of mouse NOX3, we designed primers for real-time PCR to study quantitative expression of NOX3

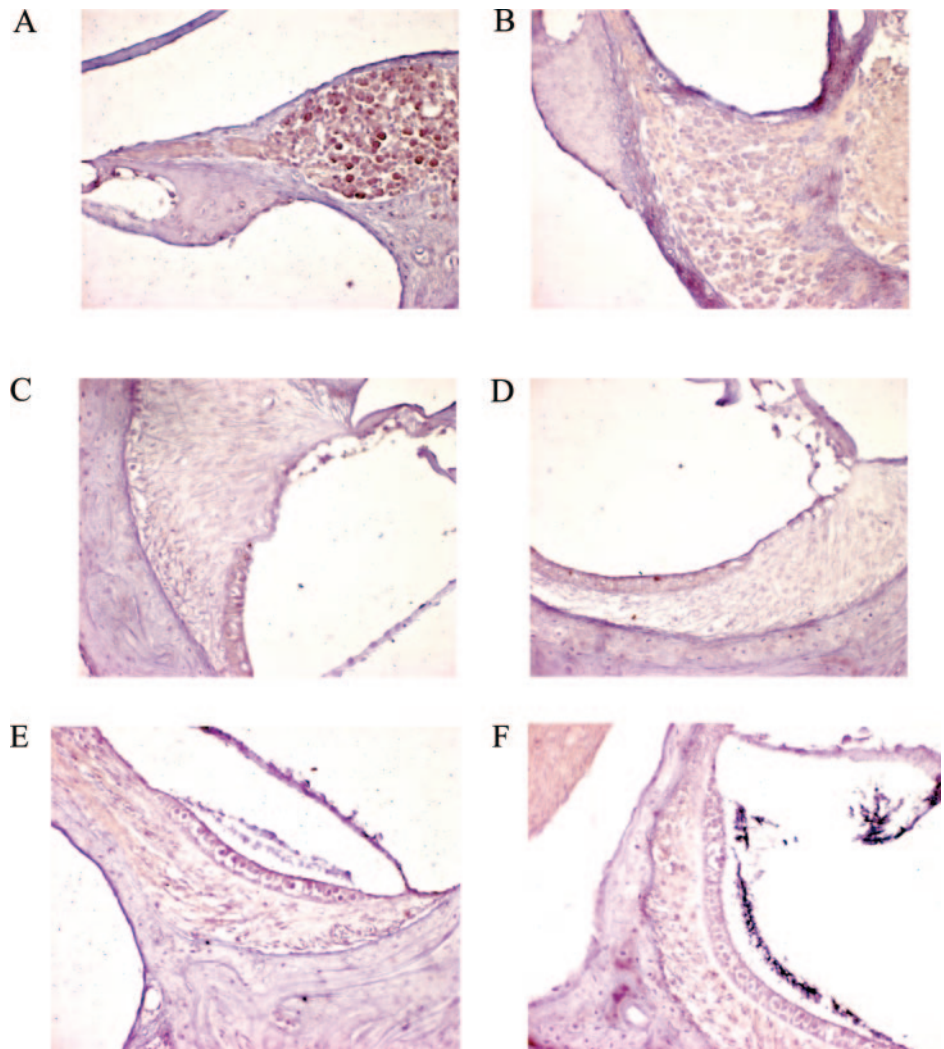


**FIG. 3. Expression of NOX3 mRNA in specific regions of cochlea.** Indicated regions of rat inner ear were obtained by microdissection, and NOX3 (*top*) and GAPDH (*bottom*) expressions were assessed by RT-PCR. +, samples that were reverse-transcribed; -, samples that were not reverse-transcribed (RT-negative). P0, P3, and P4 indicate the postnatal days when samples were taken. Positive control inner ear sample was isolated from adult rat. The faint GAPDH PCR product observed in the spiral ganglion RT-negative sample indicates the amplification of GAPDH pseudogenes.

mRNA in different mouse tissues. 18 S RNA was used as a reference gene. The results of real-time PCR demonstrated that NOX3 was predominantly expressed in the inner ear (Fig. 1B). Low amounts of NOX3 mRNA could also be detected in skull, brain, and embryonic kidney. However, inner ear contained 50- and 870-fold greater NOX3 content compared with skull and embryonic kidney, respectively (Fig. 1B).

**Expression of Cytoplasmic NOX Subunits in the Inner Ear**—NOX1 and gp91<sup>phox</sup>/NOX2 require cytoplasmic organizer subunits (NOXO1, p47<sup>phox</sup>) and activator subunits (NOXA1, p67<sup>phox</sup>) to form a functional enzyme. Because NOX3 shows a high degree of homology with NOX1 and gp91<sup>phox</sup>/NOX2 (32), we considered that it might also be a subunit-dependent enzyme. We therefore investigated expression of cytoplasmic NOX subunits in the inner ear. RT-PCR analysis (using 35 PCR cycles) showed that mRNA of the activator subunit NOXA1, as well as mRNA of the organizer subunit p47<sup>phox</sup>, were expressed in the inner ear (Fig. 2, A and B). mRNA of the activator subunit p67<sup>phox</sup> and the organizer subunit NOXO1 could be detected only at very high cycle numbers (40 PCR cycles; data not shown). Because p47<sup>phox</sup> mRNA is expressed in phagocytic cells, its detection is probably a result of the leukocyte content of the tissue sample. Confirming that hypothesis, gp91<sup>phox</sup>/NOX2 mRNA, typically expressed in phagocytes, could also be detected from inner ear by RT-PCR (supplemental Fig. 1S). In contrast, NOXA1 is not expressed in blood cells (26) or in tissues neighboring the inner ear (Fig. 2A); thus, it is most probably expressed within cells of the inner ear.

**Expression of NOX3 in Different Parts of the Cochlea**—To identify regions of the inner ear that express NOX3, we isolated distinct parts of rat cochlea, such as organ of Corti, stria vascularis, and spiral ganglia, from newborn rats (postnatal day 0 to 4) as described previously (33). As a control tissue, we used dorsal root ganglia. Total RNA was extracted from these tissues and tested for NOX3 and GAPDH housekeeping gene expression by RT-PCR. Results showed that NOX3 was expressed in spiral ganglia and in the organ of Corti, whereas stria vascularis and dorsal root ganglia were devoid of NOX3 mRNA (Fig. 3). Our experiments demonstrated that 1) NOX3 is expressed only in selected structures of the cochlea (*i.e.* organ of Corti and spiral ganglia) and 2) its expression is not a general property of the peripheral nervous system (*i.e.* it was absent from dorsal root ganglia).



**FIG. 4. Localization of NOX3 mRNA in inner ear by *in situ* hybridization.** Mouse inner ear sections hybridized with digoxigenin-labeled antisense (A, C, and E) and sense (B, D, and F) probes of NOX3, shown at 20 $\times$  magnifications. A, the antisense probe hybridized with the RNA of spiral ganglion neurons. B, the sense probe yielded only a weak, uniform signal and no labeling of spiral ganglion neurons. C and D, hybridization of antisense NOX3 probe (C) or sense NOX3 probe (D) with stria vascularis yielded no specific signal. E, antisense NOX3 probe hybridized with the sensory epithelial cell layer of the saccule. F, only a weak uniform signal was observed with the sense NOX3 probe.

*Localization of NOX3 mRNA by in Situ Hybridization*—To further define the sites of NOX3 expression, we performed *in situ* hybridizations of adult mouse inner ear sections. The antisense NOX3 probe labeled spiral ganglion neurons (Fig. 4A) but not the stria vascularis (Fig. 4C). The sense probe gave only a weak, uniform background signal demonstrating the specificity of the antisense hybridization (Fig. 4, B and D). The cellular structures within the organ of Corti were not sufficiently well preserved to verify NOX3 expression by *in situ* hybridization. Specific labeling for NOX3 was also observed in the vestibular system, in the sensory epithelial cell layer of the saccule (Fig. 4, E and F).

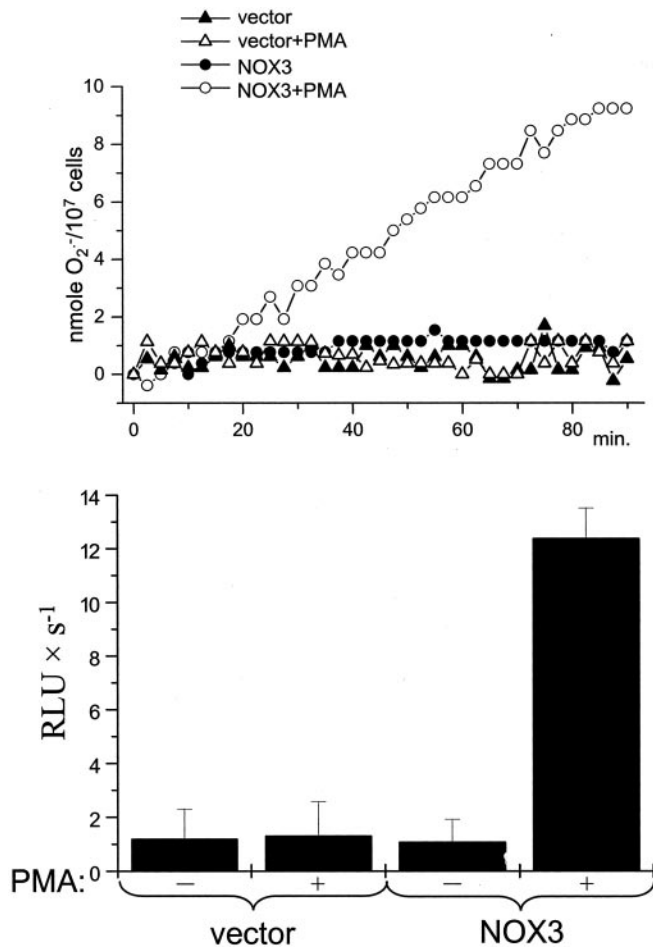
*NOX3-dependent Superoxide Generation in the Absence of Subunits*—To investigate its molecular function, we transiently expressed NOX3 in HEK293 cells, which do not express this enzyme endogenously. Superoxide production was measured with cytochrome *c* reduction technique and with luminol-amplified chemiluminescence. Using either technique, NOX3-transfected cells generated low amounts of superoxide, but only in the presence of a PKC activator (PMA) (Fig. 5). Because both NOX1 and gp91<sup>phox</sup>/NOX2 have an obligatory subunit requirement, the stimulus-dependent and subunit-independent activity of NOX3 is a unique and distinguishing feature of this NOX isoform.

*Regulation of NOX3 by the Organizer and Activator Subunits of NOX1 and gp91<sup>phox</sup>/NOX2*—Because expression of NOX regulator and activator subunits was detected in the inner ear (see above, Fig. 2), we reasoned that they might influence NOX3 activity. Thus, we investigated superoxide generation by NOX3

upon co-transfection with cytoplasmic subunits. In the first series of experiments, NOX3 was co-transfected with the cytosolic subunits of the phagocyte NADPH oxidase, p67<sup>phox</sup> and p47<sup>phox</sup>. In these transfectants, the NOX3-dependent superoxide generation was markedly increased, even without an added stimulus (Fig. 6A). The addition of PMA, however, led to a strong enhancement of NOX3 activity (Fig. 6A). HEK293 cells, transfected with p47<sup>phox</sup> and p67<sup>phox</sup> but devoid of NOX3, produced no superoxide (data not shown). It is interesting that p67<sup>phox</sup> in the absence of p47<sup>phox</sup> was sufficient to double the PMA-induced superoxide generation of NOX3, whereas p47<sup>phox</sup> in the absence of p67<sup>phox</sup> did not modify NOX3 activity (compare Fig. 5 and Fig. 6A).

We next investigated whether NOX3 could be regulated by the NOXO1 and NOXA1 subunits, which are associated with NOX1 in the colon. Co-transfection of NOX3 with NOXO1 and NOXA1 resulted in a massive increase of superoxide production (Fig. 6B). The NOXO1/NOXA1-enhanced superoxide generation was insensitive to PMA (Fig. 6B). The co-expression of NOXA1 with NOX3, in the absence of NOXO1, had an enhancing effect on PMA-stimulated NOX3 activity. NOXO1 alone, however, did not influence NOX3-dependent superoxide production (Fig. 6B, bottom).

We and others had previously shown that, at least on a biochemical level, there is promiscuity among the organizer and regulator subunits: NOXO1 is able to function with p67<sup>phox</sup> and NOXA1 is able to function with p47<sup>phox</sup> (26–28). Therefore, we investigated which combinations of organizer and activator



**FIG. 5. NOX3-dependent superoxide production in the absence of other NOX subunits.** HEK293 cells were transfected with either pcDNA3.1 vector or NOX3, and superoxide generation was measured as cytochrome *c* reduction (*top*) or as luminol-amplified chemiluminescence (*bottom*) in the presence or absence of 100 nM PMA, as indicated. *Top*, result of a single experiment representative of three independent studies. *Bottom*, statistical analysis of peak superoxide production. Chemiluminescence signals were measured with relative light units (RLU) and normalized to 1 s and 150,000 cells.

subunits are capable of regulating NOX3 and what kind of properties those complexes might have. Expression of NOXO1, p67<sup>phox</sup>, and NOX3 in HEK293 cells led to spontaneous superoxide generation that could not be further enhanced by PMA (Fig. 6C). However, when p47<sup>phox</sup>, NOXA1, and NOX3 were expressed, superoxide production by HEK293 cells was largely PMA-dependent (Fig. 6C). Thus, the organizer subunit (p47<sup>phox</sup> versus NOXO1) determines whether NOX3 activity is PKC-dependent or -independent.

**Increased Superoxide Production after Cisplatin Treatment**—Cisplatin is an ototoxic drug that exerts its toxic effect, at least in part, through induction of ROS generation in the inner ear (2). To investigate the effect of cisplatin on NOX3-dependent superoxide production, we generated HEK293 clones stably expressing NOX3, NOXO1, and NOXA1 subunits. These clones produced superoxide constitutively and spontaneously as observed in the transient transfectants (Fig. 7A); upon incubation with cisplatin (20  $\mu$ M, 12 h), a marked increase of superoxide production was detected by the luminol-amplified chemiluminescence (Fig. 7, A and B), and also by cytochrome *c* reduction (Fig. 7E). Vector-transfected cells did not generate superoxide in either the presence or the absence of cisplatin (Fig. 7A). Superoxide generation of cisplatin-treated NOX3/NOXO1/NOXA1-transfected cells was insensitive to PMA (Fig.

7B) and could be abolished by an NADPH oxidase inhibitor, diphenylene iodonium (Fig. 7A). Likewise, after 12-hour incubation with cisplatin (20  $\mu$ M), increased superoxide production was measured using HEK293 cells co-transfected with NOX3, p47<sup>phox</sup>, and p67<sup>phox</sup> (Fig. 7B) or using HEK293 cells transfected with NOX3 alone (data not shown). As control, we investigated the effect of another chemotherapeutic drug, 5-fluorouracil, which is devoid of ototoxicity. Incubation of NOX3/NOXO1/NOXA1-expressing cells with 5-fluorouracil (100  $\mu$ M, 17 h) did not influence superoxide production (data not shown).

We next investigated the range of cisplatin concentrations that affect superoxide production of NOX3/NOXO1/NOXA1-transfected cells. After incubating the cells with various concentrations of cisplatin for 12 h, superoxide production was measured (Fig. 7C). Cisplatin caused an increase of NOX3-dependent ROS generation at 1  $\mu$ M concentration, and 20  $\mu$ M cisplatin had a nearly maximal effect (Fig. 7C; EC<sub>50</sub> = 3.6  $\pm$  1.4  $\mu$ M).

To examine the time course of the effect of cisplatin on superoxide production, NOX3/NOXO1/NOXA1-transfected cells were incubated with 20  $\mu$ M cisplatin for various periods of time. Superoxide generation was enhanced already after 5 h of cisplatin treatment and reached its maximal effect after around 17 h (Fig. 7D; *t*<sub>50</sub> = 11.5  $\pm$  1.7 h).

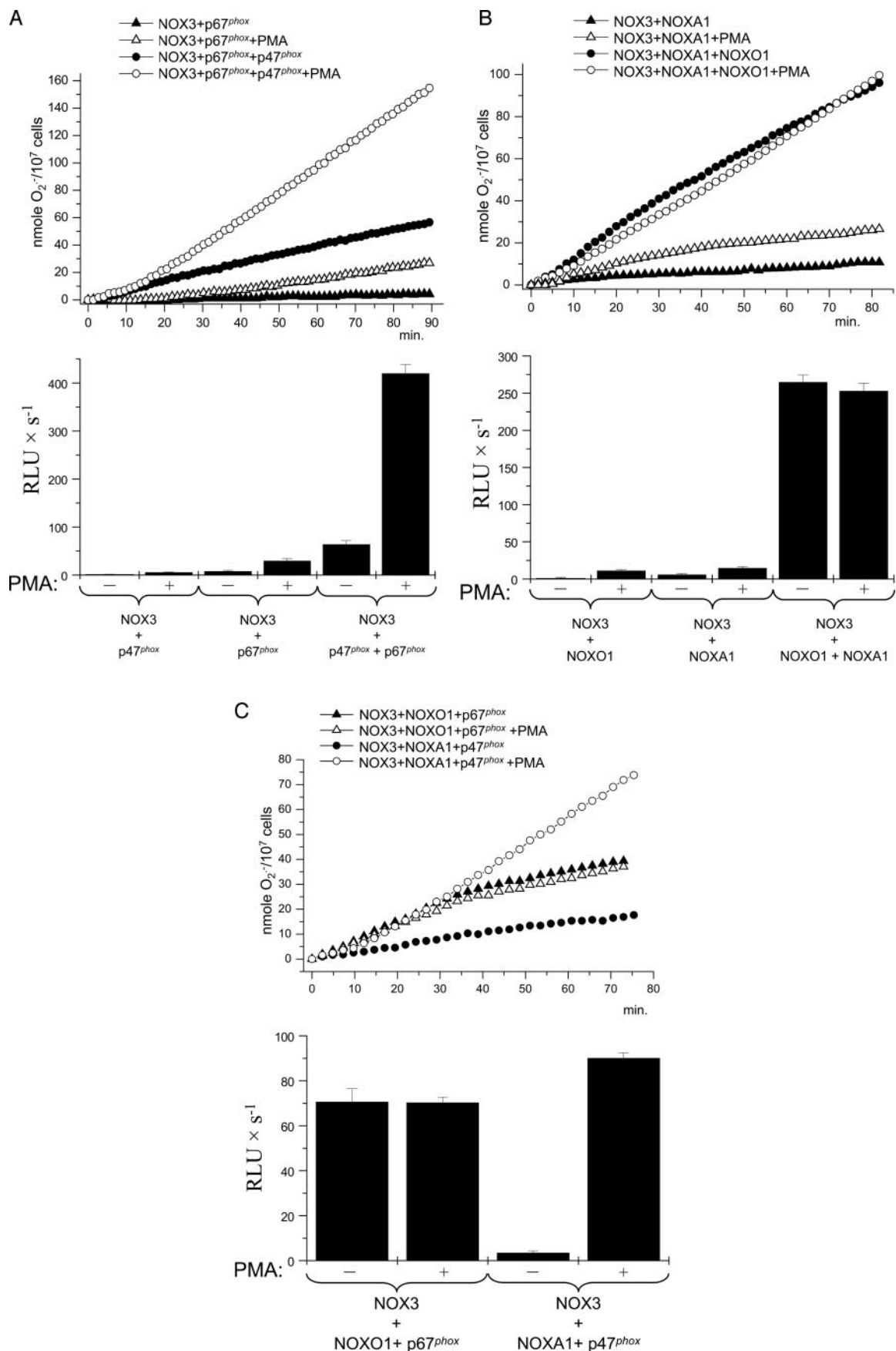
Until this point our results were compatible with two possible modes of action of cisplatin: 1) it might increase NOX3 enzyme activity or 2) cisplatin or its derivatives might produce superoxide themselves in the presence of another superoxide generating system, as has been described for the gentamicin-Fe<sup>2+</sup> complex (12) and for lucigenin (34). In the latter case, cisplatin would increase superoxide production regardless of its source. Therefore, we compared the superoxide production of three different superoxide-producing enzymes (NOX3, NOX5, and xanthine oxidase) with or without cisplatin pre-incubation using cytochrome *c* reduction assay. NOX3/NOXO1/NOXA1-transfected, NOX5-transfected, and control transfected HEK293 cells were treated with 20  $\mu$ M cisplatin for 24 h. Superoxide production was measured in the absence of stimulus (NOX3/NOXO1/NOXA1 transfectants), in the presence of 1  $\mu$ M ionomycin (NOX5 transfectants), or in the presence of 40  $\mu$ M hypoxanthine and 20 milliumits/ml xanthine oxidase (control transfected cells). Cisplatin enhanced NOX3-, NOX5-, and xanthine oxidase-dependent superoxide productions, indicating that its effect was not restricted to NOX3 (Fig. 7E). Thus, cisplatin may amplify superoxide production downstream of the oxidase.

## DISCUSSION

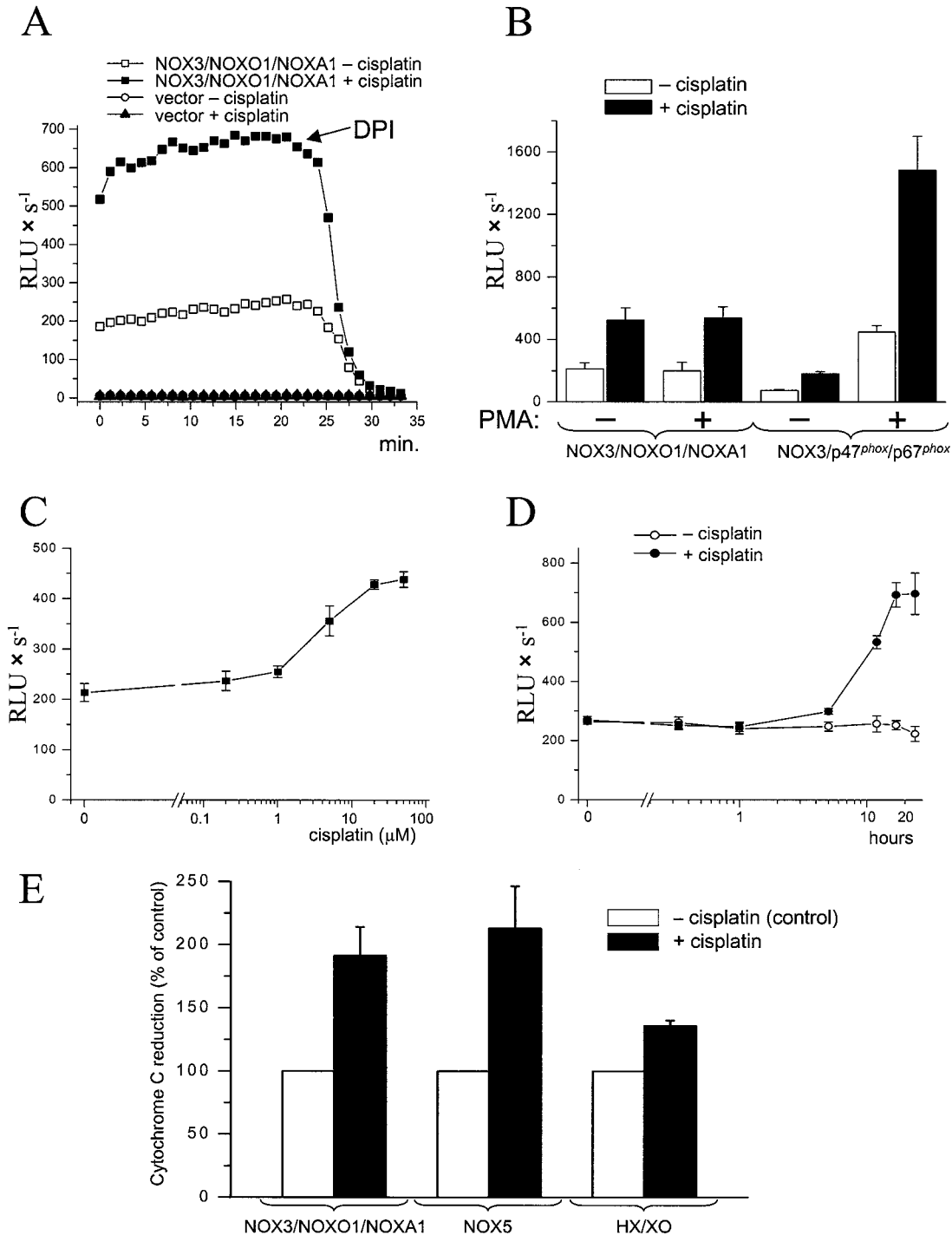
In this study, we demonstrated high-level expression of the NADPH oxidase NOX3 in the inner ear. We also demonstrated that NOX3 is capable of generating superoxide, with a pattern of subunit and stimulus dependence that is distinct from other known NOX family NADPH oxidases. Finally, we showed that an ototoxic drug, cisplatin, could enhance NOX3-dependent superoxide production.

Vestibular and cochlear sensory epithelia develop from a common ectodermal thickening at the head region, called placode (35). The otic placode also gives rise to the neurons that will form the inner ear ganglia (36). Our data suggest that the expression of NOX3 mRNA may follow this pattern.

We demonstrated in this study that NOX3 is a superoxide-generating enzyme. NOX3, as opposed to NOX1 and NOX2, produces low levels of superoxide upon PKC activation without the need for subunits. Although the activation of phagocyte NADPH oxidase is thought to occur through PKC-dependent phosphorylation of p47<sup>phox</sup> (13), this obviously cannot be the mechanism of the subunit-independent activation of NOX3. At



**FIG. 6. Subunit regulation of NOX3 activity.** A–C. HEK293 cells were transfected with different combinations of NOX3, p67<sup>phox</sup>, p47<sup>phox</sup>, NOXA1, and NOXO1 as indicated. Superoxide generation was measured as superoxide dismutase-sensitive cytochrome *c* reduction (*lines and symbols*) or as luminol-amplified chemiluminescence (*bar graphs*) in the presence or absence of PMA (100 nM) as indicated. *Lines and symbols* show typical experiments, representative of at least three independent studies. *Bar graphs* show statistical analysis of peak superoxide production. Chemiluminescence signals were measured with relative light units (RLU) and normalized to 1 s and 150,000 cells.



**FIG. 7. Effect of cisplatin on NOX3-dependent superoxide production.** Superoxide production of transfected HEK293 cells was measured as either luminol-amplified chemiluminescence (A–D) or as superoxide dismutase-sensitive cytochrome *c* reduction (E). Cells were pre-incubated in the presence or absence of 20  $\mu\text{M}$  cisplatin for 12 h (A–C) or for 24 h (E). A, HEK293 cells were transfected with NOX3/NOXO1/NOXA1 or control vector (pcDNA3.1) and incubated with or without cisplatin before superoxide measurement. 5  $\mu\text{M}$  diphenylene iodonium (DPI) was added as indicated. *Traces* represent a typical experiment, representative of three independent studies. B, statistical analysis of peak superoxide production of HEK293 cells transfected with the indicated constructs and pre-incubated with or without cisplatin. The measurements were carried out in the absence or presence of 100 nM PMA, as indicated. C, superoxide production of an HEK293 cell clone stably transfected with NOX3/NOXO1/NOXA1 after incubation with various concentrations of cisplatin. D, superoxide production of a HEK293 cell clone stably transfected with NOX3/NOXO1/NOXA1 after incubation in the presence or absence of cisplatin for the indicated periods. E, HEK293 cells transfected with NOX3/NOXO1/NOXA1, NOX5, or pcDNA3.1 vector were pre-incubated with or without cisplatin. Superoxide production was measured with the cytochrome *c* reduction assay after addition of 1  $\mu\text{M}$  ionomycin to NOX5 transfectants, or hypoxanthine (HX, 40  $\mu\text{M}$ ) and xanthine oxidase (XO, 20 milliunits/ml) to vector-transfected cells. The *white bars* (control, 100%) represent cytochrome *c* reduction in the absence of cisplatin treatment; the *black bars* represent cytochrome *c* reduction after 24 h of cisplatin treatment.

this point, PKC might activate NOX3 by numerous possible pathways (e.g. direct phosphorylation of NOX3, activation of the small GTPase protein Rac1, or changes in the lipid envi-

ronment). However, because the subunit-independent ROS generation by NOX3 is of low level in the transfected cells, its ability to exert biological effect *in vivo* remains to be clarified.

NOX3 activity is massively enhanced by NOX organizer and regulator subunits. Searches of genomic databases suggest that there are no close homologues of p47<sup>phox</sup> and p67<sup>phox</sup> other than NOXO1 and NOXA1, respectively. Thus, if NOX3 functions in a subunit-dependent manner *in vivo*, it would have to use the subunits of other NOX enzymes. Based on our PCR data (Fig. 2), NOX3 could potentially interact with NOXA1, whereas the detection of p47<sup>phox</sup> is probably the result of the leukocyte content of the inner ear. It is possible, however, that in a very limited number of cells, other cytosolic NOX subunits are also expressed in the inner ear.

It is interesting that although there are a considerable number of studies on the pathological effect of excessive ROS production in the inner ear (for reviews see Refs. 1 and 4), there is only one very recent study, which suggests a physiological role of ROS in the inner ear. During the preparation of this article, Paffenholz *et al.* (37) reported that *het* (head tilt) mice, which are characterized by impaired balance and by the lack of otoconia formation in the utricle and saccule, carry mutations in the NOX3 gene. The *het* mice do not have gross hearing deficits, suggesting no overt physiological role of NOX3 in hearing. However, a pathological role for NOX3 is possible not only in the vestibular system but also in the cochlea. Ototoxic drugs (such as platinum derivatives or aminoglycoside antibiotics) lead to accumulation of ROS both in the cochlea (3) and in the vestibular system (8, 11, 38), and noise trauma has been demonstrated to be a prominent cause of ROS production in the cochlea (39). A permanent increase of ROS concentration, in turn, leads primarily to the death of sensory epithelial cells, and, to a lesser extent, to the death of innervating neurons (1). Cisplatin is able to increase the amount of superoxide generated by three different enzyme systems including NOX3, NOX5, and xanthine oxidase (Fig. 7E). However, because of its tissue localization, NOX3 is the most likely to be involved in ROS-mediated cisplatin ototoxicity. The time course and dose response of the cisplatin effect on superoxide generation of NOX3 transfectants is compatible with the time course (2) and dose-response (40) of cisplatin toxicity to inner ear sensory cells.

Taken together, our results suggest that NOX3 is a superoxide-generating enzyme in the inner ear. NOX3 seems to be required for otoconia formation under physiological circumstances, but it is also likely to be involved in ROS-mediated cisplatin ototoxicity.

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