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Report

Nitric oxide synthase inhibitor suppresses the ototoxic side effect of cisplatin in guinea pigs

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Cisplatin is known to cause inner ear damage (ototoxicity). The role of inducible nitric oxide synthase (iNOS) in the cochlea of guinea pigs after injections of cisplatin or a combination of cisplatin and NOS inhibitor (N^G-nitro-L-arginine methyl ester, L-NAME) i.p. was examined electro- and immunohistochemically. The auditory brain stem responses (ABR) were measured prior to injection and 3 days after the injection. Three days after injection, the cochleas were examined immunohistochemically for iNOS. We found that iNOS was expressed in the cisplatin- and L-NAME/cisplatin-treated cochlea. The threshold shift of ABR was significant in the cisplatin group, whereas it was decreased in the L-NAME/cisplatin group. iNOS catalyzed high NO levels lead to inner ear dysfunction. Our results indicate that iNOS mediates the ototoxicity of cisplatin. [© 2000 Lippincott Williams & Wilkins.]

Key words: Anti-NOS drug, cisplatin, hearing disturbance, inducible nitric oxide synthase, ototoxicity.

Introduction

The anticancer drug cisplatin shows severe side effects, e.g. renal toxicity, myelosuppression and ototoxicity, which limit its administration. The combined administration of mannitol, saline and high amounts of fluid reduces the renal toxicity. However, details of ototoxicity have not yet been clarified. Recent reports indicate that cisplatin increases the activity of inducible nitric oxide synthase (iNOS/NOS II) in the kidney and liver.[1] iNOS produces large amounts of NO, which reacts with free radical species. In the cisplatin-treated inner ear, free radical species are detected,[2] but an explanation is lacking for how they cause problems.

NO is known to have various biological activities. NO is catalyzed from L-arginine by the action of NOS. Among NOS, three isoforms were detected and two of these are constitutive, i.e. Ca^{2+}-dependent endothelial NOS (eNOS/NOS III) and brain NOS (bNOS/NOS I). Both constitutive NOS release small amounts of NO to maintain homeostasis under physiological conditions. In the cardiovascular system, NO acts as an endothelium-derived relaxing factor (ERDF), regulating blood flow and pressure through its action on the smooth muscle cells of blood vessels. bNOS is detected mainly in the central nervous system as a neurotransmitter.[3] By contrast, Ca^{2+}-independent iNOS catalyzes 100- to 1000-fold higher amounts of NO. In the vestibular system, iNOS expression accompanied with reduced caloric response is reported after transtympanic injection of endotoxin.[4] In the cochlea, iNOS activities are also detected under pathological conditions, e.g. inflammation, noise exposure or endolymphatic hydrops.[5] These reports suggest that inadequate quantities of NO are associated with various pathological conditions of the inner ear. Data on the effect of NO production are missing. In kidney and gut, the toxicity of cisplatin is markedly reduced by NOS inhibitor.[1] The application of cisplatin causes the expression of iNOS in the vestibule.[6] The purpose of our study was to examine whether the blockade of NOS activity would lead to a decrease in ototoxicity after cisplatin application.

Materials and methods

Twenty-four guinea pigs weighing between 250 and 350 g were used in this study. All animals

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were confirmed to have a positive Preyer's reflex and were microscopically examined to be free from otitis media. Animals were anesthetized adequately with 5% (w/v) ketaminhydrochloride (50 mg/kg body weight) and 2% (w/v) xylazinhydrochloride (10 mg/kg body weight) before all procedures. The animals were divided into four groups: (i) cisplatin, (ii) NOS inhibitor [N^G-nitro-L-arginine methyl ester (L-NAME)]+cisplatin (L-NAME/cisplatin), (iii) L-NAME and (iv) control (NaCl 0.9%, w/v).

In the cisplatin group (n=6) 10 mg/kg body weight of cisplatin (0.5 mg/ml; ASTA Medica, Frankfurt, Germany) dissolved in physiological saline (NaCl 0.9%, w/v) was injected i.p. In the L-NAME/cisplatin group (n=6) 50 mg/kg body weight of L-NAME (5 mg/ml, Lot 33H0653; Sigma, St Louis, MO) dissolved in physiological saline (NaCl 0.9%, w/v) was injected 1 h before the injection of 10 mg/kg body weight cisplatin. In the L-NAME group (n=6) 50 mg/kg body weight of L-NAME dissolved in physiological saline (NaCl 0.9%, w/v) was injected. In the control group (n=6), only physiological saline (10 ml/kg, NaCl 0.9%, w/v) was injected. This investigation was approved by the Ethical Committee of Animal Experimentation (Bezirksregierung Köln/Germany, permit 23.203.2 K42 3/98).

Immunohistochemical examination

All animals were sacrificed 3 days after the injection of each solution. The tissues were fixed via cardiac perfusion with 4% (w/v) paraformaldehyde (pH 7.4) after flushing out the blood with 0.1 M PBS. The cochleas were incubated in the same fixative overnight. Decalcification was performed with 10% (w/v) EDTA solution in Tris at pH 7.4 for 5 days. Subsequently, the tissues were embedded in paraffin (Citadel 1000, Shandon, Germany). Each paraffin-embedded specimen was sectioned at a thickness of 8 μm with a microtome (HM360; Microm, Walldorf, Germany). The paraffin was removed by immersion in graded series of ethanol. Then the sections were immersed in 3% (v/v) H_2O_2 for 20 min, followed by 0.25% (v/v) Triton X for 10 min. Subsequently they were incubated with the first antibody to iNOS at 1:1000 dilution (rabbit polyclonal antibody, SA200; Biomol, Hamburg, Germany) overnight. After rinsing with 0.1% (w/v) Tris–PBS solution (pH 7.4) and treatment with 3% (v/v) normal goat serum, the sections were incubated with the second antibody at 1:400 dilution (anti-rabbit; Dako, Glostrup, Denmark) for accentuation. The reaction was developed with a horseradish peroxidase (HRP) complex at 1:100 dilution for 1 h (Amersham Life Science, Little Chalfont, UK) and a nickel-enhanced DAB (Sigma).

Auditory brain stem response (ABR) measurement

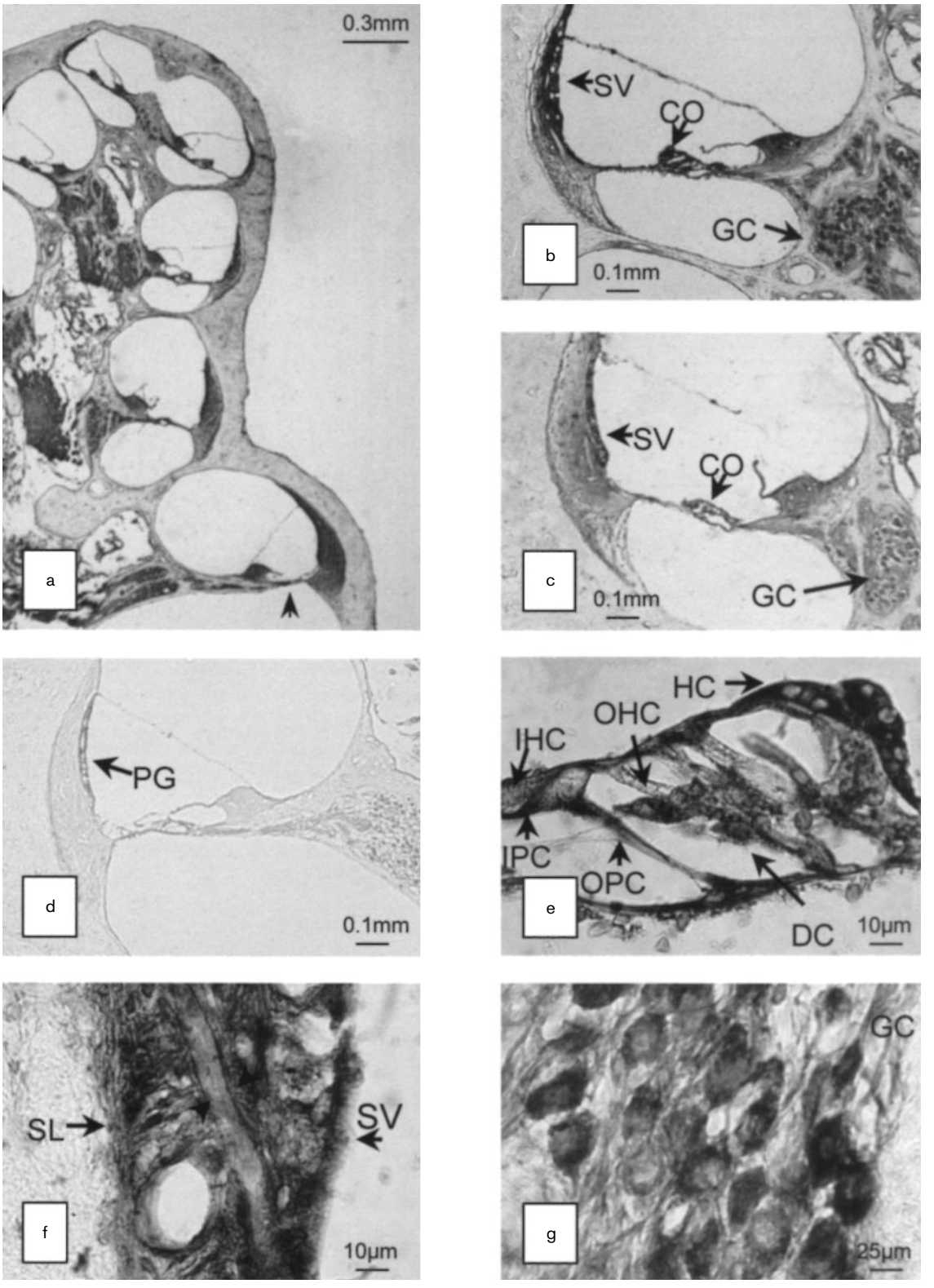
ABR recordings were obtained prior to and 3 days after the injection of each solution by means of an electrodiagnostic system (Pathfinder I; Nicolet Biomedical Instruments). The responses were recorded in the far-field technique. The active electrode was inserted s.c. into the ipsilateral pinna, the reference electrode into the contralateral pinna and the ground electrode into the top of the head, respectively. Acoustic stimuli were delivered by an earphone through a small tube inserted into the external ear meatus in a sound-proof box. The stimuli consisted of click and tone bursts of 2, 4, 6 and 8 kHz (sine wave pulses with a trapezoidal envelope, the total duration was 10 ms, and the rise and fall times were 2 ms). They were presented at a rate of 11.1 s^{-1} and a duration of 0.11 ms. Responses were accumulated 500 times. The levels of stimuli were lowered from 95 to 10 dBSPL by 5 dB steps. The ABR threshold was determined as the minimum sound level giving reproducible waveforms. The recordings were repeated twice at the threshold level and the reproducibility was confirmed.

Results

Immunohistochemical expression of iNOS

The cochleas in the L-NAME and control group did not show immunoreactivity for iNOS (Figure 1).

Figure 1. Paraffin sections of the cochlea, 8 μm. Immunohistochemistry, anti-iNOS. (a) Cisplatin-treated whole cochlea. iNOS is mainly expressed in the organ of Corti (CO), the stria vascularis (SV) and the spiral ganglion cells (GC). These immunoreactivities were observed in all turns of cochlea. The structural change of the organ of Corti was severe in the basal turn (arrow), $\times$8. (b) Cisplatin-treated cochlea. The expression of iNOS was detected, $\times$25. (c) L-NAME/cisplatin-treated cochlea. Immunoreactivity to iNOS was similar to that in the cisplatin-treated group, $\times$25. (d) Control group. The natural



pigmentation of the stria vascularis was observed; however, expression of iNOS was not detected, ×25. (e) The organ of Corti organ. iNOS is positive in Deiter's cells (DC), Hensen's cells (HC), and the inner and outer pillar cells (IPC/OPC). However, there is no apparent immunoreactivity in the inner and outer hair cells (IHC/OHC), ×250. (f) In the lateral wall, the spiral ligament (SL) and the stria vascularis (SV) show reactivity. The wall of the capillaries showed strong immunoreactivity for iNOS (arrow head), ×250. (g) The spiral ganglion cells (GC) have reactivity to iNOS, ×100.

In the cisplatin group, the immunoreactivity for iNOS was detected after 3 days in the lateral wall, the organ of Corti and spiral ganglion cells. These iNOS expressions were observed in all turns of the cochlea. Within the organ of Corti, supporting cells such as Hensen's cells, Deiter's cells, inner and outer pillar cells, and marginal cells showed iNOS immunoreactivity. By contrast, sensory cells [inner and outer hair cells (IHC/OHC)] were not stained. iNOS expression in the nerve fibers in the organ of Corti was not evident under light microscopy. iNOS expression was observed in the lateral wall (stria vascularis and spiral ligament). The walls of capillaries showed strong iNOS reactivity. In the modiolus, nerve fibers and some perikarya of the spiral ganglion cells showed iNOS reactivities.

In the basal turn, the morphological change of the organ of Corti was more severe than that in the other turns. The structure was not maintained because of the degeneration of supporting cells and loss of outer hair cells.

The cochleas in the cisplatin/L-NAME group showed immunoreactivity to iNOS in the lateral wall (the stria vascularis and spiral ligaments), the organ of Corti and the spiral ganglion cells

Threshold shifts of ABR

The threshold shifts of the ABR before and 3 days after the injection are shown in Figure 2. The threshold shift of the ABR was elevated significantly after 3 days only in the cisplatin group with the stimuli of the 8 kHz tone burst (ANOVA, $p<0.0001^{*}$). In the L-NAME/cisplatin group, there were tendencies of threshold shifts for all kinds of stimuli; however, these changes were not significant. Threshold shifts were not apparent in the L-NAME and control group.

Discussion

The hearing loss caused by cisplatin in patients is usually bilateral and dominant at 4 and 8 kHz, and progresses to lower frequencies.[7] In animal models,

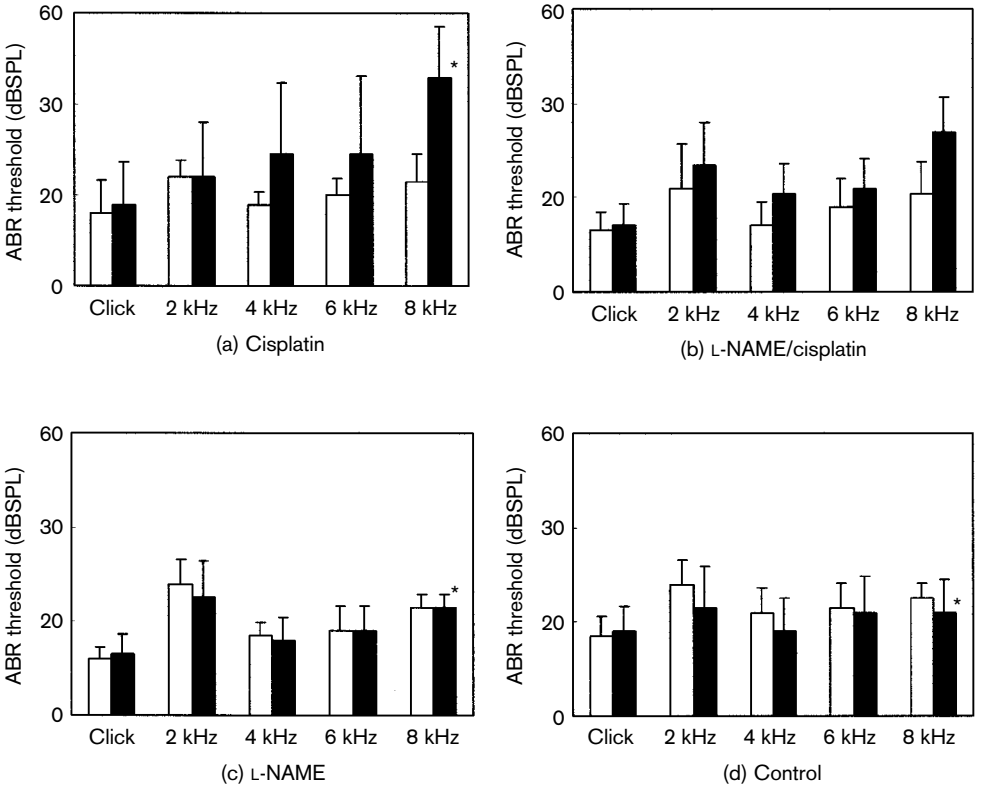


Figure 2. Threshold shift of the ABR. The mean ± SD of the threshold shift of the ABR (white columns; before injection, black columns; after injection). There was a significant elevation of the ABR threshold after 3 days in the cisplatin-treated group compared to the L-NAME and control groups (ANOVA, $p<0.0001^{*}$). In the L-NAME/cisplatin-treated group, the ABR thresholds tendered to be worse; however, they were not significant. No apparent changes existed in the L-NAME and control group.

the hearing disturbance also starts from higher frequencies.[7–9] In our study, the threshold shift of ABR was observed only at 8 kHz, 3 days after the injection of cisplatin. This supports the results of previous reports.

Cisplatin reacts with DNA at the *N*-7 bisguanine position and causes cytotoxicity by inducing rapid intra-strand cross-linking.[10] Reactive oxygen species are detected after cisplatin application in the cochlea.[11] High amounts of NO are catalyzed by iNOS and may react with free radical species which have direct cytotoxicity. In the kidney, cisplatin elevates iNOS activity,[1] and induces the generation of free radical species and subsequent lipid peroxidation.[12] Ranjan *et al*.[11] reported that macrophages treated with cisplatin and cytokine produced larger amounts of NO than those treated with cisplatin or cytokine only. From these reports, it is supposed that part of the cytotoxicity of cisplatin is due to the superoxide species via the NO pathway mediated by iNOS.

Our immunohistochemical results showed that iNOS could be detected in the cochlea after cisplatin administration, particularly in the stria vascularis, spiral ligament and supporting cells, but not in the sensory cells (IHC/OHC). The cochlear nerve and spiral ganglion cells exhibited immunoreactivity to iNOS.

NO is catalyzed from L-arginine.[13] L-NAME is an L-arginine analog, it acts as a competitive inhibitor to NOS and inhibits unspecifically both constitutive NOS (cNOS) and iNOS activity. L-NAME decreases the blood flow of the cochlea and this would decrease the ototoxic effect of cisplatin. However, in the experiment of transtympanic injection of lipopolysaccharide (LPS), L-NAME also decreased the ototoxicity of LPS.[14] Furthermore, iNOS produces 100- to 1000-fold more NO than cNOS. These high amounts of NO and free radical species thus seem responsible for the cytotoxic effect. In our experimental design, the threshold measured by ABR shifted, 23 ± 9.8 dBSPL, 3 days after cisplatin administration as the expected sign of ototoxicity. In rat kidney, L-NAME markedly reduced the renal damage caused by cisplatin.[1] Similarly, after L-NAME injection prior to cisplatin application, we observed no hearing threshold shift in the guinea pig. Under pathological conditions, it is supposed that L-NAME acts on the iNOS pathway and this leads to the suppression of ototoxicity of cisplatin.

We observed that the morphological change in the basal turn of the cochlea was more severe than that in the other turns. Komune *et al*.[8] and Laurell *et al*.[15] reported that the endocochlear potential, which is generated from stria vascularis, was decreased after a single i.v. injection of cisplatin. It is known that the degeneration of supporting cells precedes OHC damage[16] leading to morphological and functional depletion. Histologically, severe damage is observed mainly in the basal turn of the cochlea and explained by the higher metabolic rate of OHC.[17]

Conclusion

iNOS was expressed in the organ of Corti, the stria vascularis and the spiral ganglion cells of the cisplatin and L-NAME/cisplatin groups. The threshold shift of ABR became significant in the cisplatin group, whereas it was decreased in the L-NAME/cisplatin group. Our results suggest, in accordance with immunohistological observations, that iNOS expression and therefore unphysiological NO levels contribute to the ototoxic effect of cisplatin.

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