Sulfhydryl compounds and antioxidants inhibit cytotoxicity to outer hair cells of a gentamicin metabolite in vitro

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Abstract

Aminoglycoside antibiotics such as gentamicin have long been known to destroy cochlear and vestibular hair cells in vivo. In the cochlea outer hair cells are preferentially affected. In contrast, gentamicin will not damage outer hair cells in vitro unless it has been enzymatically converted to a cytotoxic metabolite. Several potential inhibitors of this enzymatic reaction were tested in an in vitro assay against outer hair cells isolated from the guinea pig cochlea. Viability of hair cells (viable cells as per cent of total number of cells observed) averaged about 70% under control conditions. Addition of metabolized gentamicin significantly reduced viability to less than 50% in one hour. Sulfhydryl compounds (glutathione, dithioerythritol) and antioxidants (vitamin C, phenylene diamine, trolox) prevented the cytotoxic actions of the gentamicin metabolite. Inhibitors of amine oxidases and compounds reportedly protective against renal and acute lethal toxicity of aminoglycosides (poly-L-aspartate and pyridoxal phosphate, respectively) were ineffective as protectants. The results reinforce the hypothesis that gentamicin is enzymatically converted to a cytotoxin and imply the participation of sulfhydryl-sensitive groups or free radicals in this reaction. Alternatively or additionally, sulfhydryl compounds or antioxidants may participate in detoxification reactions.

Key words: Aminoglycosides; Ototoxicity; Protection; Glutathione; Free radicals; Scavengers

I. Introduction

Aminoglycoside antibiotics are essential drugs in therapy against gram-negative infections. The major side effects associated with aminoglycoside treatment both in patients and experimental animals are impairment of kidney and inner ear function. Histologically, drug-induced damage is manifested as progressive and irreversible destruction of the sensory cells of the vestibular and auditory systems whereby the primary site of insult depends on the type of aminoglycoside. In the mammalian cochlea, outer hair cell death occurs first in the basal turn and progresses toward the apex. Correlating with the morphological damage, the sensorineural hearing loss begins in the higher frequencies, advances into the speech range and is most often permanent (for reviews see Hawkins, 1976; Garetz and Schacht, 1994).

Contrasting with the well-documented morphological and physiological pathology, attempts to establish a biochemical mechanism of the toxic action of these drugs have had limited success (Schacht, 1993; Garetz and Schacht, 1994). It was believed that aminoglycoside antibiotics did not require metabolism to exert either their therapeutic or deleterious effects. This notion was based largely on studies showing that the drugs were excreted in the urine apparently without being metabolized (Schentag and Jusko, 1977). However, it has been recently shown in our laboratory that gentamicin does not of itself damage outer hair cells in vitro (Dulon et al., 1989). It is rendered acutely cytotoxic when first incubated with an enzyme preparation from liver (Huang and Schacht, 1990; Crann et al., 1992) suggesting that an enzymatic reaction precedes the ototoxicity. This theory has opened new investigations into the characterization of the enzyme(s) involved, the reaction mechanism, the structure of the metabolite...
and its mechanism of action. Furthermore, it prompted
the search for agents capable of blocking the cytotoxic
mechanism at the enzymatic level (Garetz and Schacht,
1992; Garetz et al., 1993).

The current study reports on the efficacy of several
compounds to interfere with hair cell destruction by
the metabolite of gentamicin (tentatively termed
'gentatoxin') in vitro. Drugs were chosen based on
suspected properties of the enzymatic reaction and the
toxic metabolite, and on previous attempts to amelio-
rate aminoglycoside ototoxicity (Federspil, 1979;
Schacht, 1993) or drug-induced nephrotoxicity (Kishore
et al., 1992; Jones et al., 1992). In particular, we
investigated sulfhydryl compounds, antioxidants and
free-radical scavengers for their capacity to protect
isolated outer hair cells from the acute cytotoxicity of
gentotoxin.

2. Materials and methods

Preparation of the gentamicin metabolite

Fresh guinea pig liver (pigmented guinea pigs from
Murphy, Plainfield, NJ) and HBSS (Hanks' Balanced
Salt Solution without bicarbonate from Gibco Labora-
tories, Grand Island, NY; buffered with 5 mM HEPES
to pH 7.40, and osmolality adjusted to 300 mOsM with
NaCl) were mixed 1:3 (w/v) and homogenized with a
Polytron (Brinkman, Westbury, NY) for 10 s. The
suspension was centrifuged for 10 min at 1000 × g to
remove nuclei and gross sediment, and the supernatant
('post-nuclear fraction') used for the incubation with
gentamicin. Alternatively, liver was homogenized in
HBSS in a teflon-glass homogenizer (two passes of the
pestle at 1000 rpm), and a 'cytosolic fraction' was
obtained by centrifugation at 10,000 × g for 20 min
followed by centrifugation of the supernatant at
100,000 × g for 90 min. The enzyme fractions were
incubated for 1 hr at 37°C with or without 10 mM
gentamicin as previously described (Huang and
Schacht, 1990; Crann et al., 1992) and detailed in the
figure legends. Reactions were terminated by heating
at 55°C for 90 s. After 5 min on ice, precipitated
proteins were removed by centrifugation at 10,000 × g
for 10 min and the supernatants assayed directly or
stored at −20°C for no longer than one week prior to
use.

Trolox was purchased from Aldrich Chemical Co
(Milwaukee, WI), other chemicals from Sigma (St.
Louis, MO). MDL 72-527 was a gift from Merrell Dow
Research Laboratories, Cincinnati, OH.

Toxicity assay with isolated outer hair cells

Cochleae were immediately excised from decapi-
tated guinea pigs and outer hair cells isolated essen-
tially as described previously (Zenner et al., 1985; Zajic
and Schacht, 1987). The sensory epithelium from the
three upper turns was removed by microdissection,
transferred into HBSS containing type IV collagenase
(0.5 mg/ml; Sigma, St. Louis, MO) for 15 min after
which cells were mechanically dissociated. For each
assay, approximately 100 isolated hair cells were placed
in a 45-μl droplet of HBSS in a Petri dish with con-
trolled humidity. Five μl of the deproteinated incuba-
tion mixture were added yielding a final drug concen-
tration of 1 mM. After 1 hr at room temperature, outer
hair cells were carefully examined by light microscopy
(20 ×) by a blinded observer. Cells were classified
according to previously established morphological cri-
teria (Zajic and Schacht, 1987) as either healthy (long,
cylindrical cells with smooth cytoplasm and central
nucleus) or damaged (ruptured, swollen, constricted
with a displaced or absent nucleus). The percentage of
healthy cells was the index of cell viability.

3. Results

Controls

Outer hair cells exposed to HBSS alone or incuba-
tion media without gentamicin generally maintained a
viability of 65 to 75% following the isolation procedure
and the subsequent 1-h assay at room temperature
(Figs. 1a, f, g, h; 2 and 3). Likewise, when gentamicin
was added to the hair cell assay only and not to the
preceding enzymatic incubation, cells maintained their
normal appearance. However, when gentamicin was
first metabolized, its addition decreased hair cell viabil-
ity to approximately 40% within 1 h (Figs. 2 and 3).
Both enzyme preparations, post-nuclear fraction and
cytosol, were equally effective in converting gentamicin
to a cytotoxin (Figs. 2 and 3).

Each experiment included a negative control for the
quality and viability of the hair cell preparation and a
positive control (with gentamicin) for the successful
formation of a cytotoxin. A typical experiment addi-
tionally accommodated tests of a potential inhibitor
with and without gentamicin for a total of four to eight

Fig. 1. Effect of metabolized gentamicin on isolated outer hair cells. Gentatoxin was prepared by incubating gentamicin with a cytosolic fraction
(see legend to fig. 3) and assayed against isolated hair cells as described in 'Methods'. The same group of hair cells is seen exposed to toxin for 2
min (panel a), 8 min (b), 14 min (c), 35 min (d) and 45 min (e). Panels f, g and h show representative control cells exposed for 45 min to
incubation medium to which gentamicin was added after incubation and heat precipitation of proteins.
conditions. Five to eleven independent experiments were performed to examine the efficacy of a drug as an inhibitor.

A complication in assessing viability could arise if the toxin would reduce affected cells to debris which then might escape scoring. In this case, the total number of cells observed in gentatoxin-treated incubations should be consistently and significantly lower. This, however, was not observed. Even within a limited number of experiments (Fig. 3), total cells in the absence and presence of toxin were within 10% of each other (3739 vs. 3414).

**Morphology**

Loss of viability was initially seen as granulation of the cell cytoplasm, loss of birefringence and swelling or distortion of the cylindrical shape (Figs. 1 a–c). These changes became evident at different times in individual cells, visible in some after a few minutes (Figs. 1b and c), while other cells appeared normal throughout much of the assay period (Figs. 1 d and e). In the absence of gentatoxin, hair cells survived without apparent alterations of their morphology (Figs. 1f, g and h).

**Effects of sulfhydryl compounds and antioxidants**

The morphology of hair cells remained unaffected by glutathione or dithioerythritol or the antioxidants phenylene diamine, vitamin C or trolox. When these compounds were incubated with the enzymatic fractions in the absence of gentamicin and added to isolated hair cells, viability was maintained at control levels (Figs. 2 and 3). Vitamin C appeared to compromise cell integrity in some of the experiments, but the cumulative observations showed no significant difference from controls. However, each one of these compounds prevented the expected decrease in viability when added to enzymatic incubations with gentamicin. All assays in the additional presence of the protective drugs differed significantly from assays with metabolized gentamicin alone and were not different from assays with the protective agents without gentamicin (Figs. 2 and 3). A quantitative difference between the tested agents could not be inferred from the results since all effects were of similar magnitude and drugs were only studied at a single concentration. Glycine, glutamate and cysteine, the amino acid constituents of glutathione, were tested in combination in two experiments (121 cells) where they did not attenuate gentamicin toxicity.

In a related series of experiments, glutathione or dithioerythritol were absent from the enzymatic incubation with gentamicin but subsequently added to the hair cell assay. The resulting protective effect was similar to that achieved when these agents were included in the enzymatic incubation. Viability was maintained at 63 ± 10% in the presence of glutathione and at 62 ± 14% in the presence of dithioerythritol (observations on 669 and 586 cells, respectively).

**Other agents**

Inhibitors of polyamine and serum diamine oxidases, aminoguanidine and MDL 72-527, were incu-
bate and tested on 326 and 202 cells, respectively. At 1 and 7 mM final concentrations, they were ineffective at preventing the cytotoxic actions of the gentamicin metabolite.

Preliminary experiments also included agents previously tested against other side effects of aminoglycosides. Pyridoxal phosphate can antagonize lethal actions of gentamicin in the rat (Kenniston et al., 19871, and differential effects of poly-L-aspartate, poly-L-glutamate and poly-D-glutamate on renal aminoglycoside toxicity have been reported (Kishore et al., 1992). Pyridoxal phosphate (10 mM) was investigated in six, each poly amino acid (10 mM with respect to amino acid residues) in two experiments. None of these agents altered the toxicity of the gentamicin metabolite.

### 4. Discussion

Sulfhydryl compounds, antioxidants and free-radical scavengers protect isolated guinea pig outer hair cells from the damaging effects of the toxic gentamicin metabolite. At least two mechanisms can account for this effect. Firstly, the formation of the cytotoxin can be inhibited by these agents. In this case, sulfhydryl or disulfide groups (e.g. on enzymes) or free radicals should participate in the reaction converting gentamicin to gentatoxin. This does not imply that the metabolite itself is a free radical. In fact, the ability to recover it from the incubation medium after heating or filtration (Crann et al., 1992) would seem to argue against this. Peroxides or endoperoxides are examples of chemicals which are not radicals but whose formation is susceptible to the influence of radical scavengers and antioxidants. Such compounds can, in turn, generate free radicals and thus, initiate chain reactions that may lead to cell damage or cell death.

Alternatively, the observed protection can arise from a detoxification of the cytotoxin rather than from the prevention of its formation. The fact that sulfhydryl reagents also prevent hair cell destruction when added after the enzymatic incubation makes detoxification a possibility. This sulfhydryl-mediated attenuation of toxicity is intriguing in view of established cellular biochemical defense mechanisms. Glutathione, a thiol-containing tripeptide found in virtually all mammalian cells, plays a key role in a number of reactions related to detoxification of xenobiotics and protection against reactive oxygen species (Jakoby, 1978; Listowsky et al., 1988; Meister, 1991). It is thus possible that glutathione inactivates the gentamicin metabolite in an enzymatic reaction involving glutathione transferase or glutathione peroxidase. This, however, remains speculative as it is currently unknown whether the inactivation proceeds enzymatically or non-enzymatically. Furthermore, it is conceivable that the protective agents act at both the level of formation and detoxification of the gentamicin metabolite.

Hypersensitivity to aminoglycoside-induced hearing loss has recently been linked to a mutation in a specific mitochondrial gene in several patient populations (Hutchin et al., 1993; Prezant et al., 1993). Until the functional consequences of this mutation are known, any association with the formation or inactivation of the proposed metabolite remains speculative. However, oxidative metabolism in mitochondria is the major source of reactive oxygen radicals, and mitochondria are a site of enzymes of detoxification and a major glutathione reserve. Enhanced mitochondrial leakage of reactive oxygen species or defects in detoxification mechanisms are possible links between mitochondrial DNA polymorphism and sensitivity to aminoglycosides.

While the reaction mechanisms of gentamicin metabolism remain speculative, at least one pathway may be ruled out. Aminoglycosides are structurally characterized by the presence of primary and secondary amine groups. Thus, they could serve as substrates for enzymes of amine or polyamine metabolism yielding, for example, reactive aldehydes. The inability of oxidase inhibitors to prevent cytotoxicity makes this possibility unlikely.

In addition to ototoxic side effects, the spectrum of aminoglycoside toxicity includes acute effects and chronic renal impairment. There is evidence to suggest that the underlying mechanisms differ from one another (Schacht, 1993; Garetz and Schacht, 1994). The lack of protection afforded here by pyridoxal phosphate, a blocker of acute lethal toxicity (Kenniston et al., 1987), and poly-L-aspartate, a blocker of nephrotoxicity (Kishore et al., 1992) could be indicative of such a dissociation of acute, nephrotoxic and ototoxic mechanisms. It still remains possible, however, that these compounds exert their effects at different steps in the sequence of aminoglycoside toxicity.

The successful protection of isolated hair cells from gentamicin cytotoxicity in vitro prompts the question of a possible protection against gentamicin ototoxicity in vivo. Attenuation of aminoglycoside ototoxicity by free radical scavengers has been claimed in animal experiments (Pierson and Moller, 1981) but has also met with failure (Bock et al., 1983). Likewise, sulfhydryl compounds have been employed in vivo with varying success and were eventually considered ineffective (reviewed by Federspil, 1979). However, support for an influence of glutathione in the expression of aminoglycoside toxicity has come from experiments with the glutathione-synthesis inhibitor buthionine sulfoximide. Lowering of glutathione levels with this drug in guinea pigs potentiated the ototoxicity caused by the combined administration of the aminoglycoside kanamycin and the diuretic ethacrynic acid (Hoffman et al., 1988). Preliminary experiments suggest that the converse is
true, namely attenuation of ototoxicity by elevating glutathione levels (Garetz et al., 1993, 1994). This renders the current in vitro observations relevant for both the elucidation of mechanisms of gentamicin metabolism and therapeutic intervention in aminoglycoside ototoxicity.

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References


