Attenuation of streptomycin ototoxicity by tetramethylpyrazine and its effect on K⁺ channels in the outer hair cells of guinea pig cochlea

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Abstract: In order to elucidate the mechanism underlying the attenuation of streptomycin ototoxicity by tetramethylpyrazine (TMP), the present study investigated the effect of TMP on the outward K⁺ current in the outer hair cells of guinea pig cochlea. Sixty guinea pigs were divided into 6 groups randomly. Auditory brainstem response (ABR) was used to observe the change in thresholds and to evaluate ototoxicity induced by streptomycin. Whole-cell patch-clamp technique was used to observe the effect of TMP on outward K⁺ current in isolated outer hair cells. The results showed that TMP attenuated the threshold shift caused by streptomycin and increased the amplitudes of Ca²⁺-sensitive K⁺ current [I_{K(Ca)}] in the outer hair cells. The present data suggest that TMP displays anti-ototoxicity induced by streptomycin. The augmented amplitudes of I_{K(Ca)} of the outer hair cells induced by TMP may be one of the mechanisms underlying its ototoxicity-attenuating effect.

Key words: tetramethylpyrazine; outer hair cells; streptomycin; K⁺ channel

The aminoglycoside antibiotics (AmAn), such as streptomycin (SM), were discovered more than half a century ago (Schatz et al., 1944), and since then had been widely used in clinical treatments. AmAn are the most commonly used antibiotics worldwide for its high efficacy in the treatment of serious Gram-negative bacterial infections as well as its low price. However, the clinical usage of AmAn had been limited since SM was found to be ototoxic, impairing hearing ability. In the past few years, SM has drawn more attention because tuberculosis (TB) is found to have a trend to spread again around the world and SM is still the major and most effective medicine. In China, many reports have discussed the possible prevention methods for ototoxicity induced by SM. Tetramethylpyrazine (TMP) has been
shown to significantly attenuate the augmented activity of superoxide dismutases and the increased concentration of malondialdehyde induced by kanamycin, and the impaired hearing was improved as a consequence\(^3\). Meanwhile, many actions of TMP have been found, including capillary dilation, organ blood volume augmentation, microcirculation improvement and protection against radicals\(^4,5\). However, it is not known whether TMP's anti-ototoxicity effect is related to modulation of hair cell ion channels since ion channels, especially K\(^+\) channels, have been shown to be involved in cell proliferation and anti-apoptotic activity\(^6\). The present study investigated the effect of TMP on the auditory brainstem response (ABR) threshold of guinea pigs treated with SM and on the K\(^+\) channels in the outer hair cells (OHCs).

1 MATERIALS AND METHODS

1.1 Grouping
Adult guinea pigs (~2 months, 200-260 g) with sensitive pryer's reflexes were randomly divided into 6 groups, 10 animals per group. Control group: saline 2.5 mL/kg, i.m.; SM group: SM 450 mg/kg, i.m.; Low concentration of TMP group: TMP 12 mg/kg, i.p.; High concentration of TMP group: TMP 60 mg/kg, i.p.; Low concentration of TMP + SM group: TMP 12 mg/kg, i.p., SM 450 mg/kg, i.m.; High concentration of TMP + SM group: TMP 60 mg/kg, i.p., SM 450 mg/kg, i.m. Animals were properly labeled and given daily injection of the above drugs for 10 d. For feeding, all animals from different groups were mixed. TMP was obtained from Qiqihaer Pharmaceutical Company, and SM from Dalian Pharmaceutical Company.

1.2 ABR test and ototoxic animal model
Experimental guinea pigs were anesthetized with 2% sodium pentobarbital, i.p. The animals were placed in electrical shielding room for ABR recording. Briefly, recording needle was placed subcutaneously in the scalp, a ground needle was placed subcutaneously in mastoid, and a control needle was placed in other mastoid subcutaneously. The click (duration of 0.1 ms) stimulation of 4 kHz primary frequency was triggered by sound stimulator (Danac-7), via the TDH-39 earphone to the ear. The signal was collected by biological recording system (ATAC-450) with analysis duration of 20 ms, 200 times averagely, the stimulus interval was 90 ms, and filter band-pass frequency was 100-3 000 Hz. Hearing threshold was calculated from 95 dB SPL, with 10 dB step decrease after the clear ABR wave was observed. The stimulation wave intensity changed to 5 dB step decrease when it was close to the hearing threshold till the P3 wave diminished. Then, the sound intensity was regarded as hearing threshold. The ototoxic animals were determined when their hearing threshold was increased over 20 dB.

1.3 Preparation of isolated OHCs
The isolation procedure was essentially the same as described by Ashmore et al\(^6\). Briefly, an adult guinea pig was killed by rapid cervical dislocation, both bullae were removed and the cochlea was exposed. The whole cochlea was immediately cut and placed in a Ca\(^{2+}\)-free external solution with papain (0.5 mg/mL, Wako Pure Chemical Industries, Ltd.) for 30 min at room temperature. The enzyme was rinsed from the specimen by superfusion with the standard external solution three times. The OHCs were then manually teased from the basilar membrane with fine tungsten wire microprobes. After isolation, the cells were transferred into recording chamber and the single OHCs were dipped into the bath through for 20 min until the cells attached to the slide under bath through and then were recorded.

1.4 Patch-clamp recordings
Patch pipettes were pulled from glass capillaries with two-step technique (Model PP-830, Narishige, Japan). The electrode tip diameter was about 1 µm and had a resistance about 3-7 MΩ in the standard external solution, which contained (in mmol/L): NaCl 137, KCl 5.4, MgCl\(_2\) 1.8, CaCl\(_2\) 1.3, HEPES 5.0, glucose 5.0, and pH was adjusted to 7.4 with NaOH. Ca\(^{2+}\)-free external solution was the same as the above solution without CaCl\(_2\). The pipette filling solution contained (in mmol/L): KC\(_1\) 135, CaCl\(_2\) 0.1, HEPES 10, glucose 10, and pH was adjusted to 7.3-7.4 with KOH. Chamber with isolated OHCs was perfused with the standard external solution by pump (DDB-320, Zhejiang Xiangshan Shipu Electronic Company) at a speed of 2.5 mL/min. Whole-cell patch-clamp technique was used. Recordings were done using an Axo-patch 1D patch-clamp amplifier interfaced with a personal computer equipped with Clamp 5.1.1 special-purpose software (Axon Instrument, USA). Currents were filtered at 2 kHz and sampled at 10 kHz. Capacitance and series resistance were compensated. Data analyses were performed using the Clamp-fit software. Data were presented as mean±SD. Significant differences between groups were analyzed using student’s t-test. \(P<0.05\) was considered significant. Tetraethylammonium (TEA), a blocker of K\(^+\) channel, was used to confirm the involvement of K\(^+\) channels.
2 RESULTS

2.1 Effect of TMP on SM-induced ABR thresholds
The ABR data in all groups were collected by using click stimulation of 4 kHz primary frequency and the results were shown in Table 1 and Fig.1. TMP alone did not significantly affect the wave and thresholds of guinea pig, excluding possible effect of TMP by itself. On the contrary, SM significantly enhanced the ABR thresholds of the animals. However, the ABR thresholds of the animals treated with SM and high or low concentrations of TMP were significantly decreased compared to that in SM group, indicating anti-ototoxicity effect of TMP.

Table 1. Auditory brainstem response (ABR) thresholds of guinea pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Threshold (dB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>8.50±2.90</td>
</tr>
<tr>
<td>SM</td>
<td>10</td>
<td>38.21±3.12</td>
</tr>
<tr>
<td>TMP (12 mg/kg)</td>
<td>10</td>
<td>8.35±2.87</td>
</tr>
<tr>
<td>TMP (60 mg/kg)</td>
<td>10</td>
<td>8.46±3.12</td>
</tr>
<tr>
<td>TMP (12 mg/kg) + SM</td>
<td>10</td>
<td>28.23±2.66</td>
</tr>
<tr>
<td>TMP (60 mg/kg) + SM</td>
<td>10</td>
<td>20.93±3.97</td>
</tr>
</tbody>
</table>

mean±SD. *P<0.01 vs control, †P<0.05 vs SM, ‡P<0.05 vs TMP (12 mg/kg).

2.2 Effects of TMP on \( I_{\text{K(Ca)}} \) in OHCs
At holding potential of -50 mV, the command potentials (-30 to +80 mV), with duration of 60 ms, elicited whole-cell currents with similar characteristic similar to \( I_{\text{K(Ca)}} \) currents (Fig.2A). The current-voltage relationship was shown in Fig.2D, showing a reversal potential towards the K⁺ equilibrium potential. The current could be inhibited by TEA, a blocker of the \( I_{\text{K(Ca)}} \) channels. At command potential of +80 mV, the current magnitudes of \( I_{\text{K(Ca)}} \) at different concentrations of TMP were summarized in Table 2 and Fig.3.

Table 2. Effects of different concentrations of TMP on \( I_{\text{K(Ca)}} \) in guinea pig outer hair cells

<table>
<thead>
<tr>
<th>Concentration (μmol/L)</th>
<th>n</th>
<th>Control (nA)</th>
<th>TMP (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1.72±0.28</td>
<td>1.91±0.34*</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>1.08±0.21</td>
<td>1.26±0.25**</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>1.48±0.43</td>
<td>1.81±0.38**</td>
</tr>
<tr>
<td>1000</td>
<td>7</td>
<td>1.03±0.19</td>
<td>1.38±0.26**</td>
</tr>
</tbody>
</table>

mean±SD. *P<0.05, **P<0.01 vs control.
DISCUSSION

TMP is the main active compound isolated from Chuanxiong. TMP has a variety of pharmacological function with little toxicity and side effects, and consequently, it has been widely used clinically including heart and brain blood vessel diseases and some liver diseases. In the past few years, some researchers have also used TMP to treat deaf patients. Xu et al.[8] have used combined Chinese traditional medicines including Chuanxiong, Salvia miltiorrhiza Bunge and leech to treat gentamicin ototoxicity in deaf guinea pigs and found that it significantly attenuated the ABR threshold of animals. They believed that the active compound responsible for the beneficial effect was TMP. Indeed, TMP attenuates the ototoxicity induced by gentamicin through blood vessel relaxation, improvement of microcirculation, protection of blood vessel endothelial cells (see review in this Issue by Zhu and Chan). Liu et al.[9] reported that TMP was used to treat 72 sudden deaf patients and it significantly improved the hearing of these patients. The researchers believed that TMP improved the cochlear blood microcirculation. However, Zhang et al. reported that TMP could reduce oxygen radicals[5], indicating possible alternative mechanism(s) underlying the beneficial effect of TMP on antibiotics-induced ototoxicity. Indeed, many previous reports have suggested that AmAn induced damages to the cochlear hair cells and caused deafness by metabolism inhibition, inner ear endolymph overload, Ca\(^{2+}\) modulation disturbance and oxygen radical overproduction[1,2].

In the present study, we have observed the effects of TMP on SM-induced ototoxicity in guinea pig hearing in vivo. The results indicated that TMP significantly decreased the ABR threshold of SM-treated animals, confirming its effect in attenuating the hearing impairment induced by SM and thus improving the hearing of guinea pigs. We further examined the effect of TMP on K\(^{+}\) channels in the OHCs and the results suggest possible involvement of the Ca\(^{2+}\)-activated K\(^{+}\) channel. Gu et al.[10] have reported that extracellular SM caused significant reduction in the outward currents of the OHCs, suggesting impaired ion channel function could be responsible for SM-induced ototoxicity. Interestingly, the present study has shown that TMP improved the hearing of SM-treated animals and enhanced outward currents of the OHCs. These results suggest that the anti-ototoxic effect of TMP may be mediated by the activation of the Ca\(^{2+}\)-activated K\(^{+}\) channels. It should be noted that TMP has recently been demonstrated to induce an increase in intracellular Ca\(^{2+}\) in colonic epithelial cells (see review in this Issue by Zhu and Chan). If TMP works in the same way in the OHCs, it explains how TMP activates the Ca\(^{2+}\)-activated K\(^{+}\) channels observed in the present study. Since K\(^{+}\) channels have been reported to play a role in cell proliferation and apoptosis[41], activation of K\(^{+}\) channels may exert a protective action on the OHCs similar to those reported in other tissues, i.e., K\(^{+}\) channel agonists can protect myocardial cells[42]. TMP is also known to alter cellular cAMP production (see review in this Issue by Zhu and Chan) and we have previously reported possible involvement of PKA in mediating the effect of TMP in the OHCs[43]. Therefore, the anti-otoxic effect of TMP may be mediated by both cAMP and Ca\(^{2+}\) signaling pathways, similar to its stimulatory effect on colonic anion secretion (see review in this Issue by Zhu and Chan). However, detailed mechanisms, by which TMP protects the OHCs from SM-induced damage, require further investigation.

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TANG Hao (female) received her Bachelor’s and Master’s degrees, both in Clinical Medicine, from China Medical University in 1967 and 1981, respectively. From 1985 to 1987, she was a visiting scholar at the Department of Otolaryngology of the University of Tsukuba. TANG Hao is now a Professor of Physiology at the Institute of Basic Medical Science of China Medical University (CMU). Among her many honors, she is the President of Liaoning Province’s Physiological Society and a Recipient of the Special Government Allowance. TANG Hao had previously served as the Associate Dean of the Institute of Basic Medical Science, Chair of the Physiology Department, and Director of the Hearing Research Laboratory of CMU. Her main research interests are in hearing physiology. Now her research is focused on the control mechanism of hearing. In particular, she is interested in the mechanism, prevention and treatment of ototoxicity caused by the aminoglycoside antibiotics.