Gentamicin Decreases the Abundance of Aquaporin Water Channels in Rat Kidney

JongUn Lee1,3,*, Ki Sup Yoo2,3, Dae Gill Kang1,3, Soo Wan Kim2,3 and Ki Chul Choi2,3

Departments of 1Physiology and 2Internal Medicine, Chonnam National University Medical School, 3Chonnam University Research Institute of Medical Sciences, Kwangju 501-746, Korea

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ABSTRACT—The present study was performed to examine whether the gentamicin-induced urinary concentration defect is related to an altered regulation of aquaporin (AQP) water channels in the kidney. Male Sprague-Dawley rats were subcutaneously injected with gentamicin (20, 50 or 100 mg/kg per day) for 6 days. The protein expression of AQP1 – 3 channels and the catalytic activity of adenylyl cyclase were determined in the kidney. Gentamicin treatment resulted in renal failure associated with decreased tubular free water reabsorption and increased urinary flow rate. The expression of AQP2 proteins was significantly decreased in the kidney, in which the cortex was most susceptible, followed by the outer medulla and inner medulla in order. Gentamicin treatment also decreased the shuttling of AQP2, as evidenced by a decrease of its expression in the membrane fraction in proportion to that in the cytoplasmic fraction. The protein expression of AQP1 as well as that of AQP3 was also decreased in the cortex by treatment with the highest dose of gentamicin. The cAMP generation in response to arginine vasopressin or sodium fluoride was decreased by gentamicin, while that to forskolin was not significantly altered. These findings suggest that the primary impairment in the pathway leading to the generation of cAMP lies at the level of G proteins, resulting in a decreased expression of cAMP-mediated AQP channels. The gentamicin-induced urinary concentration defect may in part be accounted for by a reduced abundance of AQP water channels in the kidney.

Keywords: Gentamicin, Aquaporin channel, cAMP, Urinary concentration defect

Gentamicin has been widely used in the treatment of gram-negative bacterial infections. However, the therapeutic usefulness of gentamicin may be limited by its potential nephrotoxicity (1). In rats receiving gentamicin, the urinary concentration ability is decreased, which is dose-dependent and becomes more pronounced following a more prolonged treatment (2, 3). Previous studies have shown that the gentamicin-induced concentration defect is associated with an inhibited responsiveness of the kidney to arginine vasopressin (AVP) and hence reduced generation of the hormone’s second messenger, cAMP (4, 5). Detailed mechanisms involved in the urinary concentration defect due to gentamicin have not been established.

Recent advances in our understanding of water transport and AVP action in the kidney have been made possible by the discovery of aquaporin (AQP) water channels. Among the multiple isoforms of the AQP family, AQP1 is highly expressed in the proximal tubule and descending thin limb (6). The critical role of AQP1 was confirmed in transgenic mice lacking AQP1 that were unable to concentrate the urine and became severely dehydrated (7). The abundance of AQP2 is highly expressed in the principal cell of the collecting duct, in which it is located in the intracellular vesicle as well as at the apical membrane (8, 9). It is short-term and long-term regulated by the AVP-cAMP pathway to increase the osmotic water reabsorption in the collecting duct. The short-term regulation of AQP2 channels occurs as a result of an exocytic insertion of the intracellular AQP2 vesicles into the apical membrane (10), whereas the long-term effect is to increase the total abundance of AQP2 proteins (11). Water reabsorption across the basolateral membrane of the collecting duct is, on the other hand, mediated by AQP3 and AQP4 (12, 13). Taken together, these AQP water channels play an important role in the urinary concentration through generating the hypertonic medullary interstitium and increasing the collecting duct water permeability.

Indeed, an altered role of AQP channels in the kidney has been observed in several pathophysiological states as-
sociated with a decreased urinary concentration ability such as acute ischemic renal failure (14), urinary tract obstruction (15), lithium-induced nephropathy (16), chronic renal failure induced by surgical renal mass reduction (17) and cisplatin-induced nephropathy (18). Our present study was performed to examine the hypothesis that an altered regulation of AQP channels in the kidney accounts for the gentamicin-induced urinary concentration defect. The expression of AQP1–3 proteins and the catalytic activity of adenylyl cyclase in the kidney were determined in rats treated with gentamicin.

MATERIALS AND METHODS

Animals and urine collection
Male Sprague-Dawley rats (200–220 g) were used. They were kept in accordance with the Institutional Guidelines for Laboratory Animal Care and Use. Gentamicin was subcutaneously injected (Choongwae, Hwaseong, Korea; 20, 50 or 100 mg/kg per day for 6 days). The rats injected with vehicle during the same period served as the control. On the day after the last treatment, the rats were anesthetized with ketamine (50 mg/kg, i.p.). In some rats, the urinary bladder was cannulated to collect urine samples. A 0.5–1-h period was allowed to elapse after the surgical preparation. The urine was then collected for 1 h, at the end of which the arterial blood was taken to determine creatinine clearance and free water clearance. The kidneys of the other rats were removed and kept at −70°C until assayed for the expression of AQP proteins and the catalytic activity of adenylyl cyclase.

Protein preparation and Western blot analysis
The cortex, outer medulla and inner medulla were dissected from the frozen kidney, and homogenized at 3,000 rpm in a solution containing 250 mM sucrose, 1 mM ethylendiamine tetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Tris-HCl buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low speed centrifugations in succession. The resulting pellet was used as the membrane fraction, of which the protein contents were 20, 10 and 2 mg for the cortex, outer medulla and inner medulla, respectively. AQP shuttling was assessed by comparing the magnitudes of its expression in the membrane-enriched fraction and in the cytoplasmic fraction (19). The kidney homogenate was centrifuged at low-speed (1,000 × g for 10 min) to remove cell debris and nuclear fragments. It was then centrifuged at 17,000 × g for 20 min to yield membrane-enriched pellets (high-density fraction, HD). The supernatant was centrifuged again at 100,000 × g for 1 h to obtain a cytoplasmic pellet (low-density fraction, LD). An altered shuttling of AQP2 channels was determined by the ratio of HD/LD, where a diminished ratio implies a decrease.

Differential centrifugation
AQP2 shuttling was assessed by comparing the magnitudes of its expression in the membrane-enriched fraction and in the cytoplasmic fraction (19). The kidney homogenate was centrifuged at low-speed (1,000 × g for 10 min) to remove cell debris and nuclear fragments. It was then centrifuged at 17,000 × g for 20 min to yield membrane-enriched pellets (high-density fraction, HD). The supernatant was centrifuged again at 100,000 × g for 1 h to obtain a cytoplasmic pellet (low-density fraction, LD). An altered shuttling of AQP2 channels was determined by the ratio of HD/LD, where a diminished ratio implies a decrease.

Membrane preparation and adenylyl cyclase activity
The cortex, outer medulla and inner medulla were separately homogenized in ice-cold buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM PMSF and 250 mM sucrose) and centrifuged at 1,000 × g and 100,000 × g in succession. The resulting pellet was used as the membrane preparation. Protein concentrations were measured by a bicinchoninic acid assay kit (BioRad, Hercules, CA, USA).

Adenylyl cyclase activity was assayed as described previously (14). Adenylyl cyclase consists of three major parts: receptor, G protein and catalytic unit (20). The G protein acts as a transducer and sends a signal from the hormone-occupied receptor to the catalytic unit (21). The catalytic unit then induces enzymatic activity responsible for ATP hydrolysis to yield cAMP. To examine these parts separately, AVP was used to activate V2 receptor, sodium fluoride was to stimulate adenylyl cyclase in a receptor-independent but G-protein-dependent manner, and forskolin was to probe the catalytic unit of the adenylyl cyclase complex. The reaction was started by adding the membrane fraction, of which the protein contents were 20, 10 and 10 μg for the cortex, outer medulla and inner medulla, respectively, in 100 μl working solution (50 mM Tris-HCl, pH 7.6, containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine and 0.02 mM GTP). The reaction was stopped after 15 min by application of cold solution consisting of 50 mM sodium acetate, pH 5.0, and centrifuged at 1,000 × g for 10 min at 4°C.

cAMP in the supernatant was measured by equilibrated radioimmunoassay. Iodinated 2′-O-monomethyladenosine
3',5'-cyclic monophosphate tyrosyl methyl ester (\(^{125}\)I-cAMP) was prepared as described by previous investigators (22). Standards or samples were taken up in a final volume of 100 \(\mu\)l of 50 mM sodium acetate buffer (pH 4.8). One hundred microliters of dilute cAMP antiserum (Calbiochem-Novabiochem, San Diego, CA, USA) and \(^{125}\)I-cAMP (10,000 cpm/100 \(\mu\)l) were added and incubated at 4°C for 15 h. The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in a gamma counter (Packard, Meriden, CT, USA). All samples in one experiment were analyzed in a single assay. Non-specific binding was <2.0%. The 50% intercept was at 16.5 ± 0.8 fmol/tube (n = 10). The intra- and inter-assay coefficients of variation were 5.0 ± 1.2% (n = 10) and 9.6 ± 1.9% (n = 10), respectively. Results were expressed as moles of cAMP generated per mg protein per min.

Drugs and statistical analyses

Drugs were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise. Results are expressed as the mean ± S.E.M. The statistical significance of differences between the groups was determined using the unpaired t-test.

RESULTS

Functional parameters

Table 1 summarizes the renal functional data. Treatment with gentamicin of 20 mg/kg per day significantly affected none of the parameters examined. With higher doses, however, gentamicin resulted in a renal failure as evidenced by the increase of serum creatinine concentration along with the decrease of its renal clearance. Accordingly, the urine volume was increased, the degree of which was more pronounced with 100 mg/kg than with 50 mg/kg. The tubular free water reabsorption was decreased, and the urine osmolality was decreased.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Gentamicin (mg/kg per day)</th>
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<tbody>
<tr>
<td></td>
<td>20 (n = 6)</td>
<td>50 (n = 7)</td>
</tr>
<tr>
<td>Scr (mg/dl)</td>
<td>0.32 ± 0.02</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
<td>1.38 ± 0.14</td>
<td>1.12 ± 0.25</td>
</tr>
<tr>
<td>UV ((\mu)l \cdot min(^{-1}) \cdot kg(^{-1}))</td>
<td>16.5 ± 1.8</td>
<td>21.5 ± 4.0</td>
</tr>
<tr>
<td>Posm (mOsm/kg H(_2)O)</td>
<td>308 ± 7</td>
<td>310 ± 7</td>
</tr>
<tr>
<td>Uosm (mOsm/kg H(_2)O)</td>
<td>1734 ± 151</td>
<td>1267 ± 195</td>
</tr>
<tr>
<td>(U/P)osm</td>
<td>5.63 ± 0.3</td>
<td>4.08 ± 0.6</td>
</tr>
<tr>
<td>T(_2)H(_2)O ((\mu)l \cdot min(^{-1}) \cdot kg(^{-1}))</td>
<td>83.4 ± 8.1</td>
<td>66.7 ± 23.3</td>
</tr>
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</table>

Values are the mean ± S.E.M. n = number of experiments. Scr, serum creatinine; Ccr, creatinine clearance; UV, urinary volume; Posm, plasma osmolality; Uosm, urinary osmolality; (U/P)osm, urine-to-plasma ratio of osmolality; T\(_2\)H\(_2\)O, free water reabsorption. *P<0.05, **P<0.01, ***P<0.001, compared with the control.
affected the three regions of the kidney. With 20 mg/kg per day, the AQP2 expression was decreased only in the cortex, but not in the outer medulla or inner medulla. With 50 mg/kg per day, the AQP2 expression was decreased in the cortex and outer medulla, but not in the inner medulla. With 100 mg/kg per day, the expression of AQP2 was decreased in the three regions of the kidney. The ratio of the expression in the membrane fraction to that in the cytoplasmic fraction was significantly decreased following the treatment with gentamicin (Fig. 4).

The anti-AQP1 antibody recognized 29-kDa and 35–50-kDa bands, corresponding to nonglycosylated and glycosylated AQP1, respectively. Following the treatment with gentamicin (100 mg/kg per day), the expression of AQP1 was significantly decreased in the cortex, but not in the outer medulla or inner medulla (Fig. 5).

The anti-AQP3 antibody recognized 27-kDa and 33–
Fig. 5. Expression of AQP1 water channels in the cortex (C), outer medulla (OM) and inner medulla (IM) of the kidney. Representative immunoblots of AQP1 and densitometric data are shown. Open columns, control; hatched columns, gentamicin-treated (100 mg/kg). Each column shows the mean ± S.E.M. of 6 rats. *P<0.05, compared with the control.

Fig. 6. Expression of AQP3 water channels in the cortex (C), outer medulla (OM) and inner medulla (IM) of the kidney. Representative immunoblots of AQP3 and densitometric data are shown. Open columns, control; hatched columns, gentamicin-treated (100 mg/kg). Each column shows the mean ± S.E.M. of 6 rats. *P<0.05, compared with the control.

Fig. 7. cAMP production provoked by AVP in the cortex, outer medulla and inner medulla. The experimental group was treated with gentamicin (100 mg/kg per day). Each point represents the mean ± S.E.M. of 6 experiments. *P<0.05, **P<0.01, ***P<0.001, compared with the control.
40-kDa bands, corresponding to nonglycosylated and glycosylated AQP3, respectively. Following the treatment with gentamicin (100 mg/kg per day), the expression of AQP3 was decreased in the cortex, but not in the outer medulla or inner medulla (Fig. 6).

Adenylyl cyclase activity

Adenylyl cyclase was provoked by AVP, sodium fluoride or forskolin. Following the treatment with gentamicin, the cAMP generation in response to AVP was decreased in the cortex, outer medulla, and inner medulla (Fig. 7). The cAMP generation stimulated by sodium fluoride was also significantly blunted by gentamicin (Fig. 8), while that provoked by forskolin was not affected (Fig. 9).

DISCUSSION

Following the treatment with gentamicin, serum creatinine concentration was increased along with the decrease of its renal clearance, indicating a renal failure. Accordingly, the urine volume was increased and its osmolality was decreased, in which the polyuria was dose-dependent. Furthermore, the polyuria was with or without derangements in other renal functional parameters. This finding is in line with the previous notion that an actual increase in urinary volume due to a renal concentration defect precedes a fall in creatinine clearance in gentamicin-induced nephro-
It has been known that the gentamicin-induced urinary concentration defect is associated with an inhibited generation of cAMP in the kidney (5). We also observed in the present study that the cAMP generation in response to AVP was blunted following the treatment with gentamicin. Therefore, to further determine the primary site of gentamicin-induced injury, we separately examined the different parts of the adenyl cyclase complex using different pharmacological tools. The adenyl cyclase activity in a receptor-independent but G-protein-dependent manner (24), was attenuated following the gentamicin treatment. In contrast, the cAMP generation stimulated by forskolin, which directly activates the catalytic unit of adenyl cyclase (25), was not affected, indicating that the catalytic unit of adenyl cyclase is unlikely to be affected by gentamicin. Taken together, G protein may be the site primarily injured by gentamicin, which may then result in a failure to adequately stimulate the catalytic unit of the adenyl cyclase and hence to generate cAMP (Fig. 10).

Among the multiple isoforms of the AQP family, it has been known that AQP2 water channels in the collecting duct are short-term and long-term regulated by the AVP/cAMP pathway (10, 11). The present study showed that both short-term and long-term regulation of AQP2 was inhibited by gentamicin, which may well be attributed to an impaired activity of the AVP/cAMP pathway (Fig. 10). These findings appear contradictory to those showing a preserved shuttling despite the reduced abundance of AQP2 in several acquired nephrogenic diabetes insipidus syndromes such as acute ischemic renal failure (14), urinary tract obstruction (15), lithium-induced nephropathy (16), and chronic renal failure induced by surgical renal mass reduction (17). The shuttling of AQP2 may be transiently impaired during the early period following the gentamicin treatment, and a reduction of total abundance may prevail in the long run.

Furthermore, different doses of gentamicin differentially affected the three regions of the kidney. The AQP2 expression was decreased only in the cortex, but not in the outer medulla or inner medulla, by the treatment with 20 mg/kg per day. However, increasing doses of gentamicin also reduced the AQP2 expression in the medulla. With 50 mg/kg per day, the expression was decreased in the cortex and outer medulla, but not in the inner medulla. With 100 mg/kg per day, the expression was significantly decreased not only in the cortex and outer medulla but also in the inner medulla. It has been known that gentamicin is highly concentrated within the cortex (26, 27). Histopathological features of gentamicin-induced renal injury have been also primarily limited to the tubulointerstitial structures of the cortex (28). Taken together, the highest vulnerability of AQP2 channels to gentamicin in the cortex may be attributed to a high concentration of the drug in this region of the kidney. However, the higher doses of gentamicin are, the more distal regions of the kidney may also be affected.

A role of AQP3 in the urinary concentration has been revealed in mice lacking AQP3 water channels. The AQP3-null mice had normal perinatal survival and postnatal growth but were remarkably polyuric and polydipsic (29). The unique form of nephrogenic diabetes insipidus in the AQP3-null mice indicates that the AQP3-mediated water transport across the basolateral membrane of the collecting duct epithelium may play an important role in concentrating the urine. The decreased AQP3 expression may also be related to the urinary concentration defect due to gentamicin. Furthermore, recent studies demonstrated a long-term regulation of AQP3 with a marked increase in its expression in the collecting duct in response to water restriction or AVP infusion, but not that of AQP1 or AQP4 (30). This finding suggests that the AVP/cAMP pathway also plays a role in the long-term regulation of AQP3 as well as of AQP2. In this context, the decreased expression of AQP3 due to gentamicin may also at least in part be attributed to the impaired activity of the AVP/cAMP pathway, which, however, remains to be further determined.

On the other hand, AQP1 appears to provide the chief route for water reabsorption in the proximal nephron. It is extremely abundant in the proximal tubule and descending thin limb, and AQP1 gene knockout mice have demonstrated 80–90% reduction of osmotic water permeability therein (7, 31). The present study showed that the AQP1 expression was also significantly reduced in the cortex following the gentamicin treatment. The decreased abundance of AQP1 may also contribute to the urinary concentration
defect in gentamicin-induced nephropathy.

In summary, the expression of AQP1 – 3 water channels was decreased by gentamicin, in association with the urinary concentration defect. The gentamicin-induced impairment in urinary concentration may be causally related to a reduced expression of AQP water channels in the kidney.

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