An Integrative Overview on the Mechanisms Underlying the Renal Tubular Cytotoxicity of Gentamicin

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Gentamicin is an aminoglycoside antibiotic widely used against infections by Gram-negative microorganisms. Nephrotoxicity is the main limitation to its therapeutic efficacy. Gentamicin nephrotoxicity occurs in 10–20% of therapeutic regimes. A central aspect of gentamicin nephrotoxicity is its tubular effect, which may range from a mere loss of the brush border in epithelial cells to an overt tubular necrosis. Tubular cytotoxicity is the consequence of many interconnected actions, triggered by drug accumulation in epithelial tubular cells. Accumulation results from the presence of the endocytic receptor complex formed by megalin and cubulin, which transports proteins and organic cations inside the cells. Gentamicin then accesses and accumulates in the endosomal compartment, the Golgi and endoplasmic reticulum (ER), causes ER stress, and unleashes the unfolded protein response. An excessive concentration of the drug over an undefined threshold destabilizes intracellular membranes and the drug redistributes through the cytosol. It then acts on mitochondria to unleash the intrinsic pathway of apoptosis. In addition, lysosomal cathepsins lose confinement and, depending on their new cytosolic concentration, they contribute to the activation of apoptosis or produce a massive proteolysis. However, other effects of gentamicin have also been linked to cell death, such as phospholipolysis, oxidative stress, extracellular calcium-sensing receptor stimulation, and energetic catastrophe. Besides, indirect effects of gentamicin, such as reduced renal blood flow and inflammation, may also contribute or amplify its cytotoxicity. The purpose of this review was to critically integrate all these effects and discuss their relative contribution to tubular cell death.

Key Words: gentamicin; aminoglycoside antibiotics; cytotoxicity; apoptosis; necrosis.

Nephrotoxicity is one of the main side effects of the aminoglycoside antibiotics, especially of gentamicin, and also one of its main therapeutic limitations. Gentamicin accumulates in the renal cortex (see next section) and induces renal morphological changes and an overall syndrome very similar in humans and experimental animals (Laft et al., 1977). However, the precise characterization of the pathophysiological and molecular mechanisms underlying gentamicin’s nephrotoxicity at the organism, tissue, cell, and molecular levels has been mostly obtained in animal and cellular experimental models. Gentamicin nephrotoxicity is typically characterized by tubular damage arising from tubular epithelial cell cytotoxicity. Treatment of experimental animals with gentamicin produces apoptosis (Li et al., 2009a) as well as necrosis (Edwards et al., 2007) of tubular epithelial cells in vivo and also in cultured cells (Pessoa et al., 2009). For other toxins, such as chemotherapeutic agents (Edinger and Thompson, 2004) and H₂O₂ (Saito et al., 2006), a relationship also exists between toxin concentration and death phenotype. Low concentrations cause apoptosis, whereas high ones cause necrosis. The death phenotype strongly depends on the cell energy status and adenosine triphosphate (ATP) reserve. Apoptosis requires ATP, at least for the initial steps. At such, other circumstances different from drug concentration may modulate the death mode. For example, a severely diminished renal blood flow (RBF) may lower oxygen availability in some areas of the kidneys and limit respiration and ATP pool. In these circumstances, cell death may lose the typical characteristics of apoptosis and acquires those of necrosis (Chiarugi, 2005). Still, the most commonly observed phenotype in vitro is apoptosis, an observation that is in agreement with the fact that high concentrations of the drug (>1–2 mg/ml) are necessary to induce a modest cytotoxic effect in cultured cells (Pessoa et al., 2009; Servais et al., 2006).
ACCUMULATION OF GENTAMICIN IN TUBULAR CELLS

In the kidneys, aminoglycosides distinctively accumulate in epithelial cells of the proximal tubule (PTECs). This has been verified both in humans and in experimental animals (Luft et al., 1977). However, the mechanism of accumulation has been mostly studied in animals. This specific accumulation is because of the existence in these cells of a membrane endocytic complex involving the proteins megalin and cubilin (Cui et al., 1996; Moestrup et al., 1995; Nagai et al., 2002, 2006), which has also been described as an endocytic receptor in human proximal tubules (Lee et al., 2009). This complex transports cations present in the ultrafiltrate, such as a vast variety of proteins and certain xenobiotics, as for example aminoglycoside antibiotics (Schmitz et al., 2002; Fig. 1). Accumulation of aminoglycosides inside the PTECs alters the function of several organelles and processes that are crucial for cell viability. Moreover, gentamicin activates the extracellular calcium–sensing receptor (CaSR), a membrane receptor sensitive to the amount of extracellular calcium, which has also been associated with tubular cell death.

It has been demonstrated in animal models and cultured cells that, quantitatively, most gentamicin enters tubular cells via endocytosis mediated by the megalin/cubilin complex. This process requires the electrostatic binding of gentamicin to the negative charges of membrane phospholipids (Frommer et al., 1983; Lipsky et al., 1980). Gentamicin then passes via pinocytosis to the endosomal compartment. The drug mostly accumulates in the lysosomes, travels retrograde through the secretory pathway to the Golgi and endoplasmic reticulum (ER; Sandoval and Molitoris, 2004; Silverblatt, 1982; Silverblatt and Kuehn, 1979) and alters vesicular traffic (Giurgea-Marion et al., 1986; Jones and Wessling-Resnick, 1998). In the lysosomes, gentamicin produces membrane destabilization, lysosomal aggregation (De Broe et al., 1984), alteration of lipid metabolism, and phospholipidosis, which have been associated with cell death (see below). It also generates multilamelar structures known as myeloid bodies (Edwards et al., 1976; Houghton et al., 1978; Silverblatt, 1982), whose pathophysiological role is uncertain.

ER STRESS AND UNFOLDED PROTEIN RESPONSE

Accumulation of gentamicin in the ER may originate ER stress (Fig. 2). ER stress activates the unfolded protein response (UPR) and cell cycle arrest (Zhang et al., 2006). Under circumstances of UPR overload, the cell undergoes apoptosis (Fribley et al., 2009), which is mediated by the classical route of calpains and caspase 12 (maybe caspase 4 in humans) activated by the release of Ca from the ER; UPR-activated apoptosis also involves Jun kinase and C/EBP homologous protein transcription factor (Kim et al., 2008; Lai et al., 2007; Peyrou and Cribb, 2007; Peyrou et al., 2007). In this line, a calpain inhibitor reduces the cytotoxicity of gentamicin in cultured auditory hair cells (Shimizu et al., 2003). Once activated, these enzymes promote the proteolytic activation of executor caspases and unleash the mitochondrial pathway of apoptosis (Kerbiriou et al., 2009; Peyrou et al., 2007). In fact, gentamicin joins calreticulin and inhibits its necessary chaperon activity for a correct posttranslational protein folding (Horibe et al., 2004). It is well known that the bactericidal effect of gentamicin is related to its capacity to bind the small subunit of the ribosome and skew protein translation (Recht et al., 1999). However, it is not yet well characterized whether gentamicin exerts similar effects in mammalian cells, which could be the cause or participate in cell death. Recht et al. (1999) reported that the minimum inhibitory concentration of gentamicin for the eukaryotic 16S
ribosomal ribonucleic acid (rRNA) was 0.23 mM, 128 times higher than that for the prokaryotic rRNA. Despite this, different reports have suggested that aminoglycosides alter ribosomal accuracy (Buchanan et al., 1987) and inhibit protein synthesis (Bennett et al., 1988; Monteil et al., 1993; Sundin et al., 2001). Protein synthesis is reduced by 50% before gross cellular morphological alterations appear (Sundin et al., 2001). The implications of these effects need to be further clarified.

**CYTOSOLIC REDISTRIBUTION AND MITOCHONDRIAL TARGETING**

Recent studies with cultured cells have shown that a critical aspect of gentamicin’s tubular cytotoxicity is its cytosolic concentration and not, as previously thought, its accumulation in lysosomes (Servais et al., 2006, 2008; Fig. 3). In comparison, a small amount of gentamicin directly enters the cytosol and nucleus independently from the endocytosis mediated by the megalin/cubilin complex (Myrdal et al., 2005). Very recently, it has been demonstrated that gentamicin also enters cultured tubule cells through an unspecific cation channel, namely the transient receptor potential vanilloid type 4 (Karasawa et al., 2008) channel. However, this channel is expressed in epithelial cells of the distal tubule but not in the proximal tubule (Karasawa et al., 2008). Besides, the relative contribution of this entry mechanism is probably small.

The most important effect occurs when the concentration of gentamicin inside the lysosomes, the Golgi, and ER exceeds a threshold and destabilizes their membrane (Ngaha and Ogunleye, 1983; Regec et al., 1989; Fig. 3). The accumulated gentamicin is released into the cytosol from where it acts on mitochondria and activates the mitochondrial pathway of apoptosis, produces oxidative stress, and reduces the ATP reserve (Morales et al., 2010; Simmons et al., 1980). On the other hand, the rupture of lysosomes causes the release of proteases into the cytosol, such as L, B, D, and other cathepsins, which intervene in the induction of cell death (Schnellmann and Williams, 1998). Cathepsins catalyze the proteolytic activation of executor caspases 3 and 7 and activate the mitochondrial pathway of apoptosis through the activation of Bid (Chwieralski et al., 2006; Yin, 2006). In the absence of ATP, cathepsins in the cytosol produce a massive proteolysis that leads to necrotic cell death (Golstein and Kroemer, 2007).

In cell cultures, cytosolic gentamicin acts on mitochondria and triggers the translocation of cytochrome c and other proapoptotic proteins, such as apoptosis-inducing factor (AIF). In the cytosol, cytochrome c activates caspase 9 and, finally, the executor caspases 3 and 7, which result in cellular death by apoptosis (Servais et al., 2008). The effect of gentamicin on mitochondria is produced in a direct and also in an indirect fashion. The mechanism of the direct action is unknown. However, it has been demonstrated that incubation of isolated mitochondria with gentamicin induces the release of proapoptotic proteins from the intermembrane space (Mather and Rottenberg, 2001), a requisite for the activation of the intrinsic
pathway of apoptosis. The indirect action is mediated by Bax, and it is inhibited by overexpression of Bcl-2. In this sense, gentamicin binds the proteasome (Horibe et al., 2004), which might affect the degradation of Bax and increase its cellular levels (Servais et al., 2006).

CELL ENERGY STATUS IMPAIRMENT

Studies carried out in rats and mice demonstrate that peroxisome proliferator–activated receptor alpha (PPAR-α) activation (1) maintains ATP production by sustaining fatty acid oxidation; (2) prevents the increase in reactive oxygen species (ROS) and oxidative stress; and (3) reduces apoptosis of tubule cells, both in vitro and in vivo, during the acute kidney injury induced by ischemia and a variety of drugs, including cisplatin (Li et al., 2004, 2009b), doxorubicin (Lin et al., 2007), and gentamicin (Hsu et al., 2008). Indeed, these drugs reduce the level of PPAR-α in tubular cells through ubiquitination-dependent degradation, which has been shown to be crucial for their tubular toxicity (Lopez-Hernandez and Lopez-Novoa, 2009). The inhibition of cell membrane transporters might also contribute to an undetermined extent to the cytotoxicity of gentamicin. Indeed, both glucose intake inhibition and reduced Na+ efflux can theoretically lead to decreased cellular ATP levels and cell swelling. Glucose transport in proximal tubule cells depends on the sodium gradient generated by adenosin triphosphatases (ATPases). Deficient sodium extrusion caused by gentamicin may (1) indirectly reduce intracellular glucose availability and contribute to ATP pool reduction and (2) lead to sodium and, consequently, water accumulation, cell swelling, and necrotic death. Na–K ATPase is a key component of cell volume homeostasis, and deregulated swelling may lead to necrosis (DiBona and Powell, 1980; Lieberthal and Levine, 1996). In experiments carried out with cultured cells or membrane vesicles from tubular cells, it has been shown that gentamicin inhibits a variety of cell membrane transporters (reviewed in Mingeot-Leclercq and Tulkens, 1999) of both the brush border and the basolateral membrane, such as the Na–Pi cotransporter and Na–H exchanger (Levi and Cronin, 1990), brush-border dipeptide transporters (Skopicki et al., 1996), electrogenic Na transport (Todd et al., 1992), and the Na–K ATPase (Fukuda et al., 1991; Lipsky et al., 1980). Figure 4 schematically represents the cellular events activated by gentamicin that lead to ATP exhaustion.

MEMBRANE DESTABILIZATION AND PHOSPHOLIPIDOSIS

Another mechanism potentially involved in its cytotoxicity is the accumulation of gentamicin in cell membranes. Because of its polycationic properties, gentamicin binds to phospholipids.
This has been shown to cause cell membrane structure alterations (Forge \textit{et al.}, 1989) and a condition known as phospholipidosis, which has been observed in humans (De Broe \textit{et al.}, 1984) and experimental animals treated with the drug (Giuliano \textit{et al.}, 1984; Nonclercq \textit{et al.}, 1992). Phospholipidosis is derived from (1) the disruption of phosphatidylinositol signalling pathways (Ramsammy \textit{et al.}, 1988), (2) the reduction of phospholipid turnover (Ramsammy \textit{et al.}, 1989a) and phospholipid accumulation in cell membranes (Kacew, 1987; Laurent \textit{et al.}, 1982), (3) the reduction in the available negative charge necessary for the correct function of phospholipases (Mingeot-Leclercq \textit{et al.}, 1995), and (4) the inhibition of calcium-dependent phosphodiesterases by competing with and displacing calcium from the enzyme (van Rooijen and Agranoff, 1985). Binding to plasmalemmal phospholipids and plasma membrane accumulation occurs in other cell types exposed to the drug, in which intracellular accumulation and cell death are comparatively much less significant or absent. This indicates that these effects initiated in the cell membrane might not contribute largely to tubule cell death.

However, because aminoglycosides accumulate in lysosomes, lysosomal phospholipidosis has been more closely linked to cell death. In fact, lysosomal phospholipidosis correlates tightly with the level of toxicity of aminoglycosides (Kaloyanides, 1992; Nonclercq \textit{et al.}, 1992; Tulkens, 1989). Precisely, lysosomal phospholipidosis has been proposed to be the result of (1) the reduction in the available negative charge, which is necessary for the proper function of lysosomal phospholipases (Mingeot-Leclercq \textit{et al.}, 1995) and (2) the direct inhibition of A1, A2, and C1 phospholipases (Abdel-Gayoum \textit{et al.}, 1993; Laurent \textit{et al.}, 1982; Ramsammy \textit{et al.}, 1989a). Support for a role of phospholipidosis in cell death comes from experiments in which rats were treated with polyaspartic acid (PAA), which has been shown to mitigate (Ramsammy \textit{et al.}, 1989b) or to completely prevent the nephrotoxicity of gentamicin (Swan \textit{et al.}, 1991). The effect of PAA has been ascribed to its capacity to bind gentamicin and thus to prevent its union to phospholipids (Ramsammy \textit{et al.}, 1990). However, binding to phospholipids is also a requirement for gentamicin endocytosis (as described in section “Accumulation of Gentamicin in Tubular Cells”), which blurs conclusions. As such, it is not known to what extent (if to any) lysosomal or endosomal phospholipidosis contribute to cell death or to other sublethal alterations.

A glimpse of light on this issue was provided by the study of Kishore \textit{et al.} (1990). These authors used three different polyanionic peptides, namely poly-L-Asp with poly-L-Glu and

\begin{figure}[h]
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\caption{Mechanisms contributing to the energetic catastrophe caused by gentamicin. FAO, fatty acid oxidation; PARP, poly (ADP-ribose) polymerase; PARS, poly (ADP-ribose) synthase. $\Psi$, mitochondrial transmembrane potential.}
\end{figure}
poly-D-Glu to inhibit the nephrotoxicity and lysosomal phospholipidosis caused by gentamicin in rats. These peptides showed similar capacity to bind gentamicin, and thus to displace it from phospholipids in wide range of pH, including acidic pH. However, they showed a significantly different degree of hydrolysis in the presence of lysosomal extracts. Interestingly, their capacity to prevent gentamicin-induced phospholipidosis and gentamicin’s nephrotoxicity was inversely proportional to their hydrolysis rate, supporting the hypothesis that their site of action was inside the lysosomes and not at the level of other renal membranes. Clearly, further research is necessary to shed light on this matter.

**CaSR STIMULATION**

CaSR, a member of the family C of cell membrane G-protein–coupled receptors (Trivedi et al., 2008), has also been implicated in gentamicin-induced tubule cell death (Fig. 1). In vitro experiments using HEK-293 cells have shown that gentamicin induces the death of cells expressing CaSR but not of those lacking it (Ward et al., 2005). Moreover, pharmacological antagonism of CaSR prevents the cell death induced by gentamicin in CaSR-expressing cells (Gibbons et al., 2008). However, a number of issues invites to caution when interpreting these results. First, there has been some controversy about the origin and phenotype of HEK-293 cells. Second, the extent of cell death induced by gentamicin in CaSR-expressing cells is low. Finally, in vivo evidence is missing because there are no useful tools to manipulate the CaSR. Moreover, an important pathophysiological role of gentamicin-induced CaSR–mediated tubule cell death odds with the evidence showing that the critical event is its cytosolic concentration, as explained above. In addition, the CaSR has been found in many other cell types outside the kidneys, where gentamicin has no evident cytotoxicity, including bone, brain, colon, parathyroid gland, smooth muscle, endothelial cells, etc. Clearly, more information is necessary to clarify the exact role of CaSR in tubule cell death.

**OXIDATIVE STRESS**

Treatment with gentamicin produces oxidative stress in tubular cells, both in vivo (in rats; Karatas et al., 2004) and in cultured tubular cells (Juan et al., 2007). This oxidative stress is mediated by hydroxyl radicals from hydrogen peroxide and by superoxide anions (Basnakian et al., 2002; Nakajima et al., 1994) from mitochondrial origin (Yang et al., 1995). Gentamicin directly increases the production of mitochondrial ROS from the respiratory chain (Morales et al., 2010). Reduced glutathione (GSH; Ali et al., 1992; Sandhya and Varalakshmi, 1997) and superoxide dismutase (SOD; Nakajima et al., 1994; Kadkhodaei et al., 2007) levels have been found to be low in the kidneys upon treatment with gentamicin.

Oxidative stress plays an important role in the nephrotoxicity of gentamicin (Koyner et al., 2008). Cotreatment of rats with a variety of antioxidants significantly reduces renal dysfunction and tissue damage (Ali, 2003; Cuzzocrea et al., 2002; Koyner et al., 2008; Martínez-Salgado et al., 2002; Morales et al., 2002). However, a note of caution was introduced by the study of Stratta et al. (1994), who demonstrated that GSH administration had no effect on the nephrotoxicity of gentamicin, despite reducing lipid peroxidation and increasing renal GSH content. Interestingly, this absence of effect was observed with a dosage of gentamicin that apparently resulted in an excess of the drug. With a lower dosage, GSH implementation softened renal damage, which curiously correlated with a lower accumulation of gentamicin in the kidneys. It is thus possible that (1) the critical level of highly cytotoxic oxidative stress induced by gentamicin depends on the dose or accumulation of gentamicin, and consequently, that the weight of oxidative stress in the nephrotoxicity of gentamicin depends on the dose of the drug and (2) the mechanism of damage is, at least partially, derived from the prevention of its renal accumulation. These aspects need further investigation.

ROS, mainly superoxide anions and hydroxyl radicals, cause cellular damage and death through diverse mechanisms, including the following (Cuzzocrea et al., 2004; Morgan et al., 2007; Ott et al., 2007; Ryter et al., 2007): (1) inhibition of the electron transport chain and suppression of cellular respiration and ATP production; (2) stimulation of the release of cytochrome c, AIF, etc. from the mitochondrial intermembrane space; (3) DNA damage, which triggers an increase in poly ADP ribose synthase activity, a decrease in the cell’s ATP reserve, and cell cycle arrest; (4) lipid peroxidation, destabilization of the cellular membrane, activation of death receptors (Fas, etc.) by alteration of lipid rafts, and generation of proapoptotic lipid metabolites, such as 4-hydroxynonenal and ceramide; (5) stress on different organelles and cellular structures, such as the ER (Yokouchi et al., 2008; Santos et al., 2009) and (6) inhibition of transmembrane sodium flow, by oxidative inhibition of the Na+/K+ ATPase pump and of sodium channels, which originates cellular swelling, loss of membrane integrity, and necrosis.

However, there is little information on the ability of antioxidants to modulate the direct cytotoxic effect of gentamicin on cultured tubule cells. To our knowledge, only Juan et al. (2007) have reported a protective effect in this sense. In their article, tetramethylpyrazine (TTP) reduces ROS accumulation and apoptotic events in rat renal NRK-52E cells. However, the effect of TTP on cell viability is not reported. Because there are many apoptotic and necrotic pathways leading to cell death as a consequence of gentamicin action, and because their redundancy and hierarchical organization are not well understood, the magnitude of the direct cytoprotection afforded by ROS inhibition is unknown. The question that remains to be solved is, is the increment in ROS production the consequence of the mitochondrial injury directly and indirectly exerted by gentamicin?
Or are ROS increased by gentamicin previously to or independently from its mitochondrial proapoptotic effects, which in turn the trigger apoptosis? Speculatively, oxidative stress can be viewed at least as an amplification factor.

**INTEGRATIVE OVERVIEW OF TUBULAR CELL DEATH**

From the information presented above, it can be concluded that gentamicin needs to accumulate inside the cells to a significant level in order to induce cell death. CaSR stimulation (from the outside) has also been shown to induce some degree of cell death in tubule cells and might participate in mesangial and tubular cell death (Martínez-Salgado et al., 2007). However, this is proportionally small compared with the cytotoxicity caused by intracellular accumulation, and might show cell type dependency, because many other CaSR-expressing cells do not die when exposed to gentamicin.

Inside the cells, a critical factor appears to be its cytosolic concentration rather than its accumulation in endosomal structures. Cytosolic gentamicin directly and indirectly attacks mitochondria, inhibits respiration and ATP production, and produces oxidative stress (Morales et al., 2010), all of which activate the intrinsic pathway of apoptosis. These data indicate that cytosolic gentamicin has the ability to trigger apoptosis. However, they do not discard contributions from other damaged structures or signalling pathways. In fact, the cytosolic redistribution of gentamicin probably coincides with the leakage from the ER, permeabilization of lysosomes, and the release of lysosomal proteases (i.e., cathepsins) into the cytosol, which may add a redundant mediation toward cell death. Gentamicin also induces stress of other cellular structures, such as the ER, including protein synthesis inhibition, which, depending on the intensity, can affect cell viability. Unresolved and persistent stress also unleashes apoptosis from the damaged structures. Because the route to cytosolic accumulation goes through accumulation in intracellular membrane structures, including ER, it is difficult to imagine how gentamicin can accumulate in the cytosol without inducing some degree of ER stress. As such, we propose that besides mitochondrial damage, gentamicin also activates other pathways of cell death resulting from stress to other structures and organelles, which add an unknown level of redundancy. Probably, the predominance of some over the others, as well as the phenotype of cell death (highly dependent on energy status), might be a matter of concentration of the drug to which the cell is exposed. It can be hypothesized that low concentrations of the drug would traffic through the endocytic pathway and leak through the ER into the cytosol to a sufficient amount to activate mitochondrial apoptosis, without inducing a significant injury to the ER and without causing lysosomal breakage or energetic catastrophe. High concentrations would cause further leakage through the ER, significant ER stress and protein synthesis inhibition, lysosomal rupture, and redundant apoptotic stimulation. In extreme cases of drug accumulation, massive and rapid cathepsin-driven proteolysis and ATP exhaustion may abort the execution of apoptosis and cause necrotic-like cell death. Also, as a result of accumulation in endosomal vesicles and lysosomes, phospholipidosis may also contribute to an undetermined extent to tubular cell death. A challenge for the coming future is to elucidate the relative contribution of all these mechanisms of cytotoxicity to the different cell death phenotypes, under a range of drug concentrations. This will unravel the key targets for the pharmacological prevention of the tubular cytotoxicity of aminoglycosides, which cannot be achieved with the present level of knowledge.

**INDIRECT DETERMINANTS OF CYTOTOXICITY**

In general, cultured tubular cells exhibit a significant resistance to cell death by exposure to gentamicin. Only very high concentrations of the drug (≥1–3mM), over long periods of time (≥1–4 days), cause a mild degree of cell death (<20%), only in determined cell lines (El Mouedden et al., 2000; Pessoa et al., 2009; Servais et al., 2006; Wu et al., 2009). As such, other factors independent from a direct cytotoxic action of gentamicin might exist, which would amplify deadly stimulation, and which are present in vivo and absent in cultured cells. One hypothesis (Fig. 5) is that inflammation and ischemia may be two of those amplification factors. Alternatively, it might be speculated that tubule cells in culture lose their capacity to efficiently accumulate gentamicin (Servais et al., 2006). This topic is of special interest because therapeutic targets to prevent gentamicin-induced tubular cell death should be sought out of target cells if additional factors are essential for an extensive tubular necrosis.

Gentamicin causes a reduction in RBF in experimental animals (Hishida et al., 1994; Morales et al., 2002), which has been associated to tubular damage (Moran et al., 1992). Although the mechanisms linking reduced RBF to tubular cell death are not well understood, it is hypothesized that limitation in O2 and glucose supply lead to a diminished ATP production, all of which causes or sensitizes to cell death (Jeong et al., 2003; Sato et al., 2010; Seppet et al., 2009). In fact, hypoxia activates inducible nitric oxide synthase (iNOS) expression, which leads to cell death by inducing oxidative stress, inhibiting ATP synthesis, and activating the mitochondrial pathway of apoptosis (Kiang and Tsen, 2006). Platelet-activating factor (PAF) and thromboxane A2 (TXA2) are two mediators of gentamicin-induced renal vasoconstriction (López-Novoa, 1999; Martínez-Salgado et al., 2007). PAF and TXA2 inhibitors improve RBF and lessen tubular damage (Dos Santos et al., 1991; Papanikolau et al., 1992; Rodríguez-Barbero et al., 1992). Gentamicin is also known to impair vascular smooth muscle relaxing capacity, contributing to the reduced RBF (Seçilmiş et al., 2005). Coadministration of
l-arginine normalizes vascular relaxation and softens tubular injury (Seçilmiş et al., 2005). However, results from Hishida et al. (1994) contradict this notion. These authors found that cotreatment with desoxycorticosterone acetate or SOD normalized gentamicin-induced RBF decline but did not reduce the severity of tubular necrosis. Moreover, cotreatment with dimethylthiourea, a hydroxyl radical scavenger, attenuated tubular necrosis but did not ameliorate the reduction in RBF. These data break the link between tubular necrosis and reduced RBF but, interestingly, indicate that intervention on different ROS species may have preferential vascular or tubular effects in gentamicin’s nephrotoxicity. Clearly, more investigation is necessary.

The nephrotoxicity of gentamicin has been shown to involve an inflammatory response in experimental animals (Bledsoe et al., 2006; Kalayarasan et al., 2009; Kourilsky et al., 1982). An exaggerated or pathologically skewed inflammatory response seems to be involved in tubular injury and contribute to renal damage progression (Karkar, 2008). In fact, strategies that protect from gentamicin-induced renal damage usually inhibit the inflammatory response (Bledsoe et al., 2006; Sue et al., 2009). An increased or unbalanced ROS production and oxidative stress mediate the inflammatory response unleashed by gentamicin (Kadkhodae et al., 2005; Maldonado et al., 2003; Morales et al., 2002; Fig. 5). Superoxide anion (Schreck et al., 1991) and hydrogen peroxide (Meyer et al., 1993; Lu et al., 2010) activate nuclear factor κB (NFκB), a key mediator of several inflammatory pathways. Indeed, NFκB inhibitors protect the kidney against gentamicin-induced damage (Tugcu et al., 2006). NFκB induces the expression of proinflammatory cytokines (Sánchez-López et al., 2009) and iNOS (Xie et al., 1994). Endothelial NOS-derived NO, at low levels, mediates physiological vasodilatation, whereas excessive NO production because of the overexpression of iNOS can cause cytotoxic effects in surrounding cells. Excessive iNOS-derived NO can react with superoxide anion and produce peroxinitrite, a highly reactive radical that contributes to cell damage (Pedraza-Chaverri et al., 2004) and reduced vascular relaxation ( Förstermann, 2010; Fig. 5). Inflammatory cytokines, such as tumor necrosis factor alpha, can activate tubular apoptosis, especially in the pathological environment (Justo et al., 2006).

**FIG. 5.** Indirect mediators of gentamicin’s cytotoxicity: inflammation and reduced RBF. Ang II, angiotensin II; ET-1, endothelin-1; ILs, interleukins; INFs, interferons; TLRs, toll-like receptors; TNF-α, tumor necrosis factor alpha.
PERSPECTIVES

Many cellular effects of gentamicin have the capacity to cause cell death or contribute significantly to it, including activation of the mitochondrial pathway of apoptosis, ER stress, and onset of an UPR and phospholipidosis. Others have an uncertain capacity to lead directly to cell death, such as oxidative stress and ATP-depleting mechanisms. However, besides the relative contribution of these pathways considered individually, the hierarchic relation among them is still unknown. For example, can gentamicin pass through the endosomal vesicles (endosomes, lysosomes, Golgi, etc.) toward the cytosol without producing ER stress leading to cell death? If mitochondrial effects were inhibited; would other mechanisms lead the way to cell death? To what extent are some of these mechanisms redundant? Does the participation of each individual mechanism vary depending on the level of stimulation (i.e., gentamicin dosage)? After reviewing the existing information on gentamicin tubular cytotoxicity, it must be concluded that these questions remain incompletely answered. These are important aspects of future research, which will yield critical information on the key mechanisms that should be targeted for the pharmacological prevention of gentamicin’s undesired renal side effects. Selective inhibition of specific mediators of individual mechanisms will lead further light on these issues.

A potential limitation to progression in this line is the uncertainty on the reliability of the available tubular cell lines and primary cultures at reproducing the effects of gentamicin in tubular cells in vivo. The relative resistance of cultured cells to gentamicin cytotoxicity might be the result of an experimental artifact or it might reflect the real nature of tubular cells in their tissue environment. If this is the case, indirect mechanisms of cytotoxicity, as those addressed in the previous section (i.e., reduced RBF, inflammation, and the immune response), will need to be invoked to fully explain tubular necrosis and may gain a central role in therapeutics.

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