Major Pathways of Polymyxin-Induced Apoptosis in Rat Kidney Proximal Tubular Cells

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Identifying the pathways involved in the apoptotic cell death that is associated with polymyxin-induced nephrotoxicity is crucial for the development of strategies to ameliorate this dose-limiting side effect and for the development of novel safer polymyxins. The primary aim of this study was to identify the major pathways which lead to polymyxin-induced apoptosis in cultured rat kidney proximal tubular cells (NRK-52E). Caspase-3, -8, and -9 were activated by polymyxin B treatment in a concentration-dependent manner. Concentration- and time-dependent expression of FasL and deformation of mitochondrial morphology were revealed following polymyxin B treatment. The proportion of cells with filamentous mitochondria (regular morphology) following an 8-h treatment with 1.0 mM polymyxin B was 56.2% ± 9.7% (n = 3). This was decreased to 30.7% ± 7.5% when the polymyxin B concentration was increased to 2.0 mM. The mitochondrial membrane potential (Δψm) decreased to 14.1% ± 2.9% in the cells treated with 1.0 mM polymyxin B for 24 h (n = 3) compared to that in the untreated control group. Concomitantly, concentration- and time-dependent production of mitochondrial superoxide was also observed. This study is the first to have demonstrated that polymyxin-induced apoptosis is mediated through both the death receptor and mitochondrial pathways in cultured renal tubular cells. It provides key information not only for the amelioration of polymyxin-induced nephrotoxicity but also for the discovery of novel safer polymyxin-like antibiotics against Gram-negative “superbugs.”

Gram-negative “superbugs” that are resistant to almost all current antibiotics have become a major global medical challenge. In particular, Pseudomonas aeruginosa, Acinetobacter baumannii, and Klebsiella pneumoniae are common Gram-negative pathogens causing life-threatening infections in critically ill and immunocompromised patients (1). Most worrying, many clinical isolates have become resistant to all antibiotics except polymyxin B and polymyxin E (colistin) (2). Unfortunately, there are virtually no new antibiotics against these difficult-to-treat Gram-negative pathogens in the drug discovery pipeline (3, 4). Therefore, polymyxin B and colistin are a precious therapeutic resource in the uphill battle against these very problematic human pathogens.

Notwithstanding their excellent antibacterial activity, the Achilles’ heel of the polymyxins is their dose-limiting nephrotoxicity. Pharmacological data generated by our group indicate that plasma concentrations achieved with the currently recommended dosage regimens are suboptimal in many patients; potentially leading to poor clinical outcome and emergence of resistance (5–7). Simply increasing the daily dose of polymyxins is not an option because of nephrotoxicity, which occurs in up to ~60% of patients (5, 8). The renal handling mechanisms of polymyxins almost certainly contribute to their propensity for causing kidney tubular cell damage. We have demonstrated that colistin and polymyxin B undergo very avid tubular reabsorption (6, 9, 10). Polymyxin-induced nephrotoxicity presents as acute apoptotic tubular cell death, cell cycle arrest, decreased urine output, and increased serum creatinine concentration (11–13). In a recent clinical study, polymyxin-induced tubular cell toxicity was suggested from both the microscopic analysis of urine samples and the time course of renal recovery (14). Importantly, substantial intracellular accumulation of polymyxins has been discovered by employing synchrotron X-ray fluorescence microscopy and it is highly likely that this accumulation leads to cellular toxicity (15).

The accumulation of polymyxins in kidney tubular cells may be mediated through the endocytic receptor megalin and other transporters (10, 16). In many mammalian cells, drug-induced apoptotic cell death is regulated by the death receptor- and mitochondrion-mediated pathways (17–19). The present study aimed to identify the major apoptotic pathways involved in polymyxin-induced apoptotic death in rat kidney proximal tubular cells. The data presented advance our understanding of polymyxin-induced nephrotoxicity.

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MATERIALS AND METHODS

Chemicals. Polymyxin B (catalogue number 81334) (≥6,500 IU/mg) and staurosporine were purchased from Sigma-Aldrich (Australia). A stock solution of 40 mM polymyxin B in Milli-Q water was prepared and stabilized by the use of a 0.22-μm-pore-size syringe filter (Milllex-GV;Millipore). Staurosporine stock solution (1.0 mM) was prepared in sterile dimethyl sulfoxide (DMSO) (American Type Culture Collection [ATCC], USA).

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Cell culture. Rat kidney proximal tubular cells (NRK-52E; ATCC, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All components of the growth medium were purchased from Invitrogen (Life Technologies, Australia). NRK-52E cells (0.5 × 10^5 cells/ml) were seeded onto 8-well chamber slides with a glass coverslip–like bottom (SARSTEDT AG and Co., Germany) in DMEM at 37°C. Incubation occurred in a humidified atmosphere containing 5% CO2 for 48 h. The medium was then discarded by aspiration, and the cells were washed two times with phosphate-buffered saline (PBS) (Invitrogen) (pH 7.4). The treatments described in the following section were conducted in DMEM supplemented with 0.1% FBS.

Activation of caspase-3, -8, and -9. NRK-52E cells on chamber slides were treated with 0.25, 0.5, and 1.0 mM polymyxin B for 24 h. At the end of incubation, medium containing polymyxin B was replaced by fresh DMEM without phenol red to assess the caspase-3, -8, and -9 activities. Cell membrane-permeable fluorogenic caspase-specific substrates were used according to the manufacturer’s instructions (CaspGLOW red active caspase-3, -8, and -9 assay kit; BioVision, USA) as follows: sulforhodamine-Asp-Glu-Val-Asp-fmk (Red-DEVDFmK; caspase-3 specific), sulforhodamine-Leu-Ile-Thr-Asp-fluoromethyl ketone (Red-IETDFmK; caspase-8 specific), and sulforhodamine-Leu-Glu-His-Asp-fluoromethyl ketone (Red-LEHD-fmk; caspase-9 specific), respectively (20). Control cells and cells treated with growth medium only. As a positive control, cells were incubated with 1 μM staurosporine for 24 h. As a negative control, cells were exposed to 1.0 mM polymyxin B for 24 h and treated with the caspase inhibitor Z-VAD-fmk (BioVision, USA) before incubation with the fluorogenic substrates. Hoechst 33342 (Invitrogen) (2 μg/ml) was used as a nuclear stain. The fluorescence was quantified by laser scanning microscopy (LSM 780 microscope equipped with Plan-Apochromat 20×/0.8 M27 objective and ZEN 2011 software; Carl Zeiss, Germany). For Hoechst 33342, an excitation wavelength of 405 nm and an emission wavelength of 410 to 551 nm (Ex/Em 405/410 to 551) were used; for the fluorogenic caspases, an excitation wavelength of 561 nm and an emission wavelength of 566 to 685 nm were used (20). The quantification of background-subtracted average integrated fluorescence intensity per cell was performed after counting the Hoechst 33342 (Invitrogen)-positive nuclei using NIH ImageJ (21). Counts were obtained from three random locations with at least 50 cells.

Measurement of the expression of FasL. Polymyxin-induced FasL expression in NRK-52E cells was determined using a previously reported flow cytometry method (22). Briefly, NRK-52E cells cultured on 12-well plates (Corning, USA) were treated with 0.5, 1.0, and 2.0 mM polymyxin B for 24 h at 37°C in a humidified atmosphere with 5% CO2 for measuring the concentration-dependent effect induced by polymyxin B. For assessment of the time-dependent effect, cells were treated with 1.0 and 2.0 mM polymyxin B for 0, 4, 8, and 24 h. After treatment, cells were washed and detached from plates by trypsinization followed by neutralization with DMEM. The cell suspension was centrifuged and the supernatant was discarded. Cell pellets were resuspended and washed with PBS. Cells were incubated with Alexa Fluor 647-conjugated anti-CD178 (FasL) monoclonal antibody (MAB) (hamster anti-mouse CD178) (Alexa Fluor 647; AbD Serotec, Germany) for 45 min at 20°C. After incubation, cells were centrifuged and washed with PBS. FasL expression in NRK-52E cells was measured by fluorescence-activated cell sorter (FACS) analysis (Ex/Em 650/668 nm) (FACSCanto II; Becton-Dickson, USA).

Evaluation of mitochondrial morphology changes. NRK-52E cells grown on chamber slides were treated with polymyxin B (1.0 and 2.0 mM) for 4, 8, and 24 h. Polymyxin B-induced mitochondrial morphology changes were assessed after staining with MitoTracker Green FM mitochondrial-specific dye (MTG FM; Invitrogen) (100 nM). Mitochondrial morphology was observed using an LSM780 confocal microscope (Zeiss) equipped with a 63× objective at Ex/Em 488/499 to 674 nm. Propidium iodide (PI; Invitrogen) (1.5 μM) (Ex/Em 561/569 to 691 nm) was used to assess the plasma membrane integrity. NIH ImageJ software was used to count the cells with filamentous (normal morphology) and/or fragmented (stressed morphology) mitochondria, and counts were expressed as a percentage of the total cell count. At least 50 cells were counted for each treatment (21, 23).

Evaluation of mitochondrial membrane potential and mitochondrial superoxide formation. NRK-52E cells were treated with 0.25, 0.5, and 1.0 mM polymyxin B for 24 h. For evaluation of time-dependent superoxide formation, cells were treated with 1.0 mM polymyxin B for 0, 4, 8, and 24 h. After treatment, the cells were washed and changes in the mitochondrial membrane potential (ΔΨ) were evaluated by incubating the cells in the dark with 50 nM tetramethylrhodamine ethyl ester (TMRE; Invitrogen) for 30 min at 37°C (24). TMRE fluorescence was detected at Ex/Em 561/568 to 690 nm. To assess the formation of mitochondrial superoxides, the cells were incubated with 5.0 μM MitoSOX Red dye (Invitrogen, Australia) (Ex/Em 514/531 to 622 nm) for 45 min at 37°C (25). Nuclei were stained with 2 μg/ml of Hoechst 33342 (Invitrogen) (Ex/Em 405/410 to 551 nm) to count the cell number. The fluorescence intensity was quantified by laser scanning microscopy (LSM 780 microscope equipped with Plan-Apochromat 20×/0.8 M27 objective and ZEN 2011 software; Carl Zeiss, Germany). The average fluorescence intensity per cell for each treatment was calculated using ImageJ (21, 26). Averages of at least 75 cells per field and 3 fields per condition were analyzed.

All experiments were conducted as three replicates, and results are presented as means ± standard deviations (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey’s test with a significance level (P) of <0.05 using GraphPad Prism 6.

RESULTS
Polymyxin-induced activation of caspase-3, -8, and -9. Activation of the caspase cascade is a key event during apoptosis (23). Concentration-dependent activation of caspase-3, -8, and -9 was observed following polymyxin B treatment for 24 h (Fig. 1). With 1.0 mM polymyxin B, activation of caspase-3 and -8 was increased approximately 4.5- and 3.6-fold, respectively, compared to the untreated control results, whereas the increase of caspase-9 activation was only 1.9-fold.

Polymyxin-induced expression of FasL. A key component in the death receptor-mediated apoptosis pathway is the expression of plasma membrane-anchored FasL (18). Our results show that polymyxin B treatment caused concentration- and time-dependent expression of FasL (Fig. 2). The proportion of FasL-positive cells increased to 33.7% ± 6.6% following treatment with 1.0 mM polymyxin B for 24 h, whereas treatment with 2.0 mM polymyxin B for 24 h increased FasL expression to 91.7% ± 9.0% (Fig. 2a). The proportions of FasL-positive cells after treatment with 1.0 and 2.0 mM polymyxin B for 4 h were 8.6% ± 1.6% and 16.5% ± 0.8%, respectively; these proportions increased to 51.0% ± 13.1% and 92.9% ± 7.8% at 24 h. In addition, we examined the time course (0, 4, 8, and 24 h) of FasL expression in response to treatment with 1.0 and 2.0 mM polymyxin B (Fig. 2b). The percentage of FasL-positive cells increased over time and was most pronounced at the 24-h time point. The levels of FasL expression were also significantly greater at the 8- and 24-h time points for the higher-concentration (2.0 mM) polymyxin B treatment than for the lower-concentration (1.0 mM) treatment (Fig. 2b).

Polymyxin-induced mitochondrial morphology fragmentation. Changes in mitochondrial morphology are highly correlated with the functional state of cells and have been shown to be a key apoptotic event (27, 28). Polymyxin B induced a transition of mitochondrial morphology from filamentous (normal morphology) to fragmented (stressed morphology) in a concentration- and time-dependent manner (Fig. 3). In a concentration-depen-
dent manner at a fixed time point, decreases in the numbers of cells with filamentous mitochondria (56.2% ± 9.7% for 1.0 mM polymyxin B and 30.7% ± 7.5% for 2.0 mM polymyxin B after 8 h) were observed following polymyxin B treatment. In a time-dependent manner at a fixed polymyxin B concentration, the proportion of cells with fragmented mitochondria after treatment with 1.0 mM polymyxin B was 24.9% ± 5.0% at 8 h, whereas this proportion increased to 55.8% ± 9.3% at 24 h (Fig. 3c).

Polymyxin-induced loss of mitochondrial membrane potential and formation of mitochondrial superoxide. Loss of mitochondrial membrane potential and oxidative stress are important indicators of mitochondrial stress during apoptosis (28). Polymyxin B treatment led to a concentration-dependent loss of mitochondrial membrane potential as evidenced by a decrease in TMRE fluorescence intensity (14.1% ± 2.9% with 1.0 mM polymyxin B compared to the untreated control results) (Fig. 4a). At the same polymyxin B concentration, there was a concomitant increase of mitochondrial superoxide formation as evidenced by a significant increase of MitoSOX red fluorescence intensity (345% ± 21% relative to the control cell results) (Fig. 4b). We also followed the time course (0, 4, 8, and 24 h) of MitoSOX red fluorescence in response to treatment with 1.0 mM polymyxin B (Fig. 4c). After polymyxin B treatment for 4 h, the fluorescence intensity was 123.6% ± 21.2% (relative to the control cell results) whereas the intensity increased to 333.0% ± 13.8% after 24 h of treatment.

**DISCUSSION**

Polymyxin-induced nephrotoxicity is the major dose-limiting factor hindering its optimal clinical use. We have previously shown that polymyxin-induced nephrotoxicity is associated with apoptosis in kidney tubular cells (29–31). Therefore, rat kidney proximal tubular cells (NRK-52E) were employed in the present study. Apoptosis, also known as programmed cell death, is generally mediated through the activation of the caspase-dependent cascade, which is triggered via extrinsic and/or intrinsic stimuli (32). With extrinsic stimuli, activation of the death receptor pathway triggers apoptotic death (33–35), whereas, with intrinsic stimuli, the mitochondrial pathway leads to the release of apoptotic factors into the cytoplasm which then trigger downstream events leading to apoptosis (36). Both of these pathways culminate in a final cellular insult, namely, caspase-dependent DNA fragmentation, which eventuates as apoptotic cell death (37). The aim of the present study was to investigate the involvement of these key apoptotic signaling pathways in polymyxin-induced apoptosis of kidney tubular cells.

As a first step, we proceeded to confirm that polymyxin B induces activation of caspase-3, -8, and -9 in a concentration-depen-
dent manner (Fig. 1). Activation of caspase-3 (4.5-fold increase compared to control results) and caspase-8 (3.6-fold increase) was more pronounced than that of caspase-9 (1.9-fold increase). Activation of caspase-8, one of the initiator caspases, is triggered by the extrinsic death receptor pathway (17). Caspase-9, the other initiator caspase, is mainly activated through proapoptotic proteins released via the intrinsic mitochondrial pathway (38). The activation of these initiator caspases culminates in the activation of the downstream caspase-3, which is the major executioner caspase during the demolition phase of apoptosis (37). Thus, our results point to the involvement of both the death receptor and mitochondrial pathways in polymyxin-induced apoptotic kidney tubular cell death. The more pronounced activation of caspase-8 suggests a primary involvement of the death receptor pathway as the apoptotic trigger due to polymyxin treatment. However, this does not discount the involvement of the mitochondrial pathway,

FIG 2 FasL expression in rat kidney proximal tubular cells (NRK-52E) treated with polymyxin B. Cells were treated with 0.5, 1.0, and 2.0 mM polymyxin B, and treatment periods were 0, 4, 8, and 24 h. FasL expression was detected by flow cytometry using Anti-FasL antibody CD178 conjugated with Alexa Fluor 647. Data represent concentration-dependent (a) and time-dependent (b) FasL expression following polymyxin B treatment. The profiles are representative of the results of three independent experiments. The percentages of FasL-positive cells are presented as means ± SD (n = 3). *, P < 0.05; ***, P < 0.001 (compared with control samples).

FIG 3 Polymyxin B-induced mitochondrial morphology changes in rat kidney proximal tubular cells (NRK-52E). (a and b) Representative confocal images exemplifying filamentous (a) and fragmented (b) mitochondrial morphology observed following treatment with polymyxin B (1.0 and 2.0 mM) for 0 to 24 h. Cells were stained with MitoTracker Green FM and PI (a and b). Scale bar, 5 μm. (c) Quantification of the percentages of cells with filamentous or fragmented mitochondrial morphology. Clear bars, cells with filamentous mitochondria; black bars, cells with fragmented mitochondria; gray bars, PI-positive cells. Data are presented as means ± SD (n = 3; at least 100 cells were analyzed per treatment). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (compared with the samples at 0 h).
as it should be noted that the death receptor-activated caspase, caspase-8, can also induce the release of the mitochondrial pro-apoptotic proteins that activate caspase-9 (39, 40).

Having definitively established a link between polymyxin-induced kidney tubular cell death and apoptosis, we proceeded to investigate the involvement of the extrinsic (death receptor) and intrinsic (mitochondrial) apoptotic trigger pathways. In the extrinsic death receptor pathway, Fas (syn., death receptor), a 37-kDa type-I transmembrane glycoprotein, acts as a module for interaction with FasL (18). Binding of FasL to Fas triggers the activation of the initiator caspase-8, which subsequently activates the executioner caspase-3, leading to apoptotic cell death (33). FasL expression has been shown to be involved in drug-induced apoptosis in several tissues, including the kidney (41). Notably, treatment of patients with cisplatin, the nephrotoxic anticancer drug, induced the expression of FasL in kidney tubular cells (42).

In the present study, FACS analysis revealed that polymyxin B induced the expression of FasL in rat kidney tubular cells in a concentration- and time-dependent manner (Fig. 2). Taken together, our results clearly demonstrate the association between the activation of caspase-8 and the death receptor pathway during polymyxin-induced apoptosis.

In the intrinsic mitochondrial pathway, the stimuli triggers cytochrome c release from the mitochondrial intermembrane space (40). After release, cytochrome c binds to apoptosis protease activating factor 1 (APAF-1) and pro-caspase-9 to form apoptosome, which promotes the activation of caspase-9, which in turn activates the executioner caspase-3 (43). Our results indicated that polymyxin-induced kidney tubular apoptotic cell death is associated with caspase-9 activation, pointing to the involvement of the mitochondrial pathway. It has been shown that mitochondrial stress during apoptosis not only involves cytochrome c release but also is associated with a massive remodelling of the mitochondrial ultrastructure (27, 44). In healthy kidney tubular cells, mitochondria predominantly adopt a filamentous morphology (23, 45), whereas, in cells undergoing apoptotic cell death, mitochondria adopt a fragmented morphology (23, 45). Polymyxin B induced a concentration- and time-dependent transition of the mitochondrial morphology from filamentous (normal) to fragmented (stressed) (Fig. 3). Mitochondrial morphology changes are associated with a loss of mitochondrial membrane potential (ΔΨm), an indication of mitochondrial dysfunction (45). Polymyxin B also induced a concentration-dependent loss of ΔΨm (Fig. 4). Furthermore, we observed the generation of reactive oxygen species (ROS) in polymyxin-treated kidney tubular cells, a phenomenon that has been associated with apoptotic mitochondrial morphology changes (46). These findings are in line with our previous studies which showed that antioxidants can act to attenuate polymyxin-induced nephrotoxicity in rats (30, 31, 47). The present results support the notion of a need for evaluation of the possible role of antioxidants to ameliorate polymyxin-induced nephrotoxicity in patients.

**FIG 4** Loss of mitochondrial membrane potential and production of mitochondrial superoxide in rat kidney proximal tubular cells (NRK-52E) treated with polymyxin B (0.25, 0.5, and 1.0 mM) for 0 to 24 h. (a) Loss of mitochondrial membrane potential detected using TMRE dye. (b and c) Concentration-dependent (b) and time-dependent (c) production of mitochondrial superoxide was detected using MitoSox Red dye. Scale bar, 20 μm. Results are presented as means ± SD (n = 3). *, P < 0.05; ****, P < 0.0001 (compared with control samples).
To the best of our knowledge, this is the first study to have revealed that polymyxin-induced apoptosis in cultured kidney tubular cells is mediated by both the extrinsic death receptor and intrinsic mitochondrial pathways. We present a putative model for polymyxin-induced apoptosis in kidney proximal tubular cells (Fig. 5). Similar to the findings from a mouse study (31), our cell culture results also suggest that there are interactions between the death receptor and mitochondrial pathways in NRK-52E cells, as activation of both caspase-8 and -9 was observed in the polymyxin-treated cells. This could partly be explained by the capacity of activated caspase-8 (using the death receptor pathway) to cleave Bid, a mitochondrial interacting Bcl-2 protein, to produce tBid, which triggers the mitochondrial apoptotic pathway (39). Very recently, activation of tBid was reported in mice treated with 7.5 and 15 mg of colistin/kg of body weight/day for 7 days (31). To conclude, our findings shed new light on the cellular death pathways that mediate polymyxin-induced nephrotoxicity and may benefit development of novel strategies for the effective and safe clinical use of this important last-line class of antibiotics.

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