Involvement of mitochondrial pathway and Bax in gentamicin (GEN)-induced apoptosis in renal LLC-PK1 cells.

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Background : GEN induces apoptosis in the proximal tubule epithelium of rats treated at low, therapeutically relevant doses[1]. This effect has been reproduced on LLC-PK1 renal cells and is correlated with cell GEN content[2]. Lysosomal permeabilisation appears as a primary event observed as from 2 h following exposure to GEN[3]. We have now examined the potential role of mitochondrial pathway (Bax expression and translocation, release of cytochrome c)

Methods: Cells were treated with GEN 2 mM (to obtain cellular concentrations similar to those observed in proximal tubular cells of animals receiving 10 mg/kg GEN for 10 days). Bax and cytochrome c were detected by western blot and their respective translocations assessed by cell fractionation. Caspase 3 was assayed with Ac-DEVD-AFC.

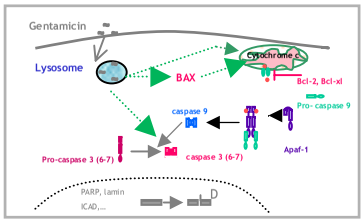
Results: Bax protein level increased from 8 h of incubation with GEN. At 12h, we observed a partial translocation of Bax from cytosol to granular fraction in parallel to the release of cytochrome c from mitochondria. Caspase 3 was activated only after approx. 24 h.

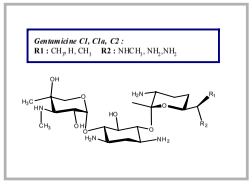
Conclusions: Mitochondria are involved in GEN-induced apoptosis, acting, probably, as a relay between lysosomal permeabilisation and caspase 3 activity. These data may provide a link between GEN lysosomal accumulation in kidney proximal tubular cells and the onset of the cascade of cellular and tissular alterations leading to GEN nephrotoxicity.

INTRODUCTION:

Gentamicin has been shown to induce apoptosis in the LLC-PK1 cell line[2] and several perturbations have been associated with this process, namely (i) a lysosomal destabilization (observed after 2 hours of treatment; lysosomes are the major site of accumulation of this antibiotic), (ii) a decrease of mitochondrial potential (after 10 hours) and (iii) nuclear fragmentation[3].

Mitochondria play a central role in apoptosis which is tightly regulated by the Bcl-2 family of proteins[4]. Some are anti-apoptotic (such as Bcl-2 and Bcl-xl) whereas others are pro-apoptotic (such as Bak and Bax). Activation of the pro-apoptotic Bax appears to involve its subcellular translocation and dimerization[5-6]. Thus, in viable cells a substantial portion of Bax is found either in the cytosol or loosely attached to membranes. Following a death stimulus, cytosolic Bax translocates to the mitochondria where it becomes an integral membrane protein and can trigger the opening of specialised pores with release of intermembrane mitochondria pro-apoptotic proteins such as cytochrome c. Cytochrome c can activate caspase-9 and further caspase-3 through the formation of the "apoptosome".





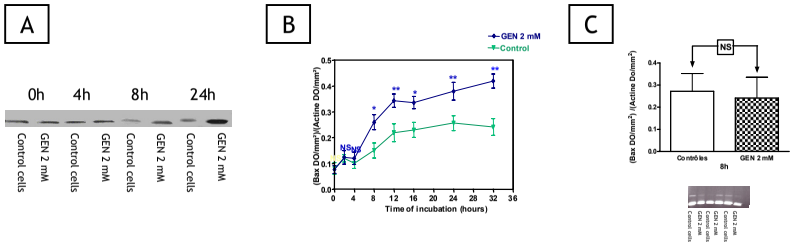
AIM OF THE STUDY:

→ To analyze the involvement of the mitochondrial pathway, focusing on the role of the pro-apoptotic Bax protein and on the release of cytochrome c

→ To study the kinetic relationship between mitochondria and caspase-3 activity.

RESULTS

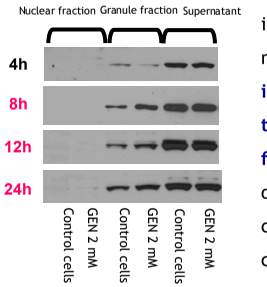
RESULTS (1): Increase of Bax protein level in cell lysates



From 8 hours of incubation with gentamicin, a significant increase of Bax protein level is observed in cell lysates of treated cells as quantified by densitometric analysis. This increase does not result from an increase of Bax expression as shown by RT-PCR analysis.

Fig.1: Panel A: Western blot detection of Bax protein level in cell lysates of control and treated cells with 2 mM of gentamicin. Panel B: Densitometric analysis of western blot band standardized with β-actin. Panel C: RT-PCR Analysis of Bax expression in cell treated with 2 mM of gentamicin in comparison with control cells.

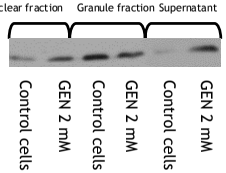
RESULTS (2): Translocation of Bax from supernatant to the Granule fraction



From 8 hours of incubation with 2 mM gentamicin, an increase of Bax in the granule fraction is observed in comparison to control cells.

Fig.2: Western blot detection of Bax in the different fraction (Nuclear, granule fractions and supernatant) obtained by centrifugation of an homogenate from gentamicin-treated cells (2 mM) in comparison to control cells

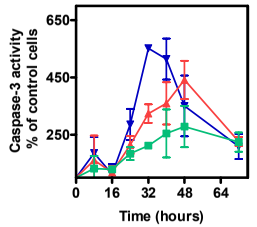
RESULTS (3): Cytochrome c release from mitochondria



An increase of cytochrome c is detected in the supernatant but also in the nuclear fraction after 12 hours of treatment with gentamicin.

Fig.3: Cytochrome c detection by immunoprecipitation and western blot in different fractions obtained by centrifugation of an homogenate from control or cells treated with 2 mM of gentamicin.

RESULTS (4): Caspase-3 activity



Caspase-3 activity is significantly increased from 24 hours of incubation with gentamicin. We observe a decrease of this activity after 48-72 hours

Fig 4: Caspase-3 activity measured with flurogenic substrate Ac-DEVD-AFC.

CONCLUSIONS:

Gentamicin induces an increase in the amount of the pro-apoptotic protein Bax associated with its translocation from the cytosol to the mitochondria. This is followed by the release of cytochrome c from mitochondria. The data suggests that mitochondria activation is involved in gentamicin-induced apoptosis.

MATERIALS AND METHODS:

Cell fractionation: A granule fraction, containing the bulk of mitochondria (together with lysosomes, peroxysomes, and mitochondria), and a cell supernate were prepared by differential centrifugation of homogenates of LLC-PK1 using the general procedures[7]. In brief, cells collected by gentle scraping in 0.25 M sucrose - 20 mM Tricine pH 7.4 - 1 mM EDTA pH 7.4) and homogenized in an all-glass Dounce tissue grinder. The resulting homogenate was subjected to 3 successive low speed centrifugations (600 g for 10 min) to obtain a nuclear fraction, followed by a high speed centrifugation (145,000 g for 30 min) to yield the granule fraction and a final supernate.

Western Blot and immunoprecipitation of cytochrome c: Samples were incubated overnight at 4°C in the presence of anti-cytochrome c antibody in a volume of 1 mL. The next day, samples were mixed with 30 µL Protein-A-agarose for 2 hours at 4°C, then centrifugated at 16,000 x g for 5 minutes at room temperature, and the pellets washed at 4°C with PBS, 0.5 M NaCl, and again twice with PBS. The final pellets were resuspended in Laemmli buffer, mechanically disrupted by forcing them 5-fold through a 20 G-needle, and subjected to separation by electrophoresis in 18 % polyacrylamide gels. Proteins were transferred to 6 x 8 cm nitrocellulose membranes (Hybond C) . The membranes were blocked for 1 h and incubated overnight at 4°C with polyclonal anti-cytochrome c antibodies diluted in TBS-T containing 5% of BSA. Membranes were incubated secondary antibody labeled with HRP, then visualized with SuperSignal® West Pico Chemiluminescence.

Immunodetection of Bax: Cells were resuspended in Laemmli buffer. Detection of Bax was performed by Western blot using the same general technique as for cytochrome c, using a rabbit anti-Bax polyclonal antibody (Calbiochem).

RT-PCR: ARN extraction was performed with Trizol reagent and the reverse transcription were realized with Reverse transcription system from Promega. PCR was performed using polymerase from Biotools, using this pair of primer: 5'-CAGCTCTGAGCAGATCATGAAGACA-3'(forward) and 5'-GCCCATCTTCTTCCAGATGGTGAGC-3' (reverse)(product of 538 pb) . 25 cycles were performed with the following sequence: 95°C for 45 sec, 60°C for 45 sec, 72°C for 2 min and to finish 1 cycle at 72°C for 7 min. Densitometric analysis of the gels was made by scanning them with a commercial scanner and analyzing the images with a public software (Image J v. 1.31, National Institutes of Health, Bethesda, MD; http://rsb.info.nig.gov/ij).

Caspase-3 activity: Activity was determined in the supernatants by fluorimetric assay using the substrate DEVD-AFC (Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarine). The released fluorochrome was measured in a Packard Fluorocount Microplate Fluorometer with an excitation wavelength of 380 nm and an emission wavelength of 520 nm.

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