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Nephrotoxic cell death by diclofenac and meloxicam

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ABSTRACT

The nephrotoxicity of diclofenac, a non-steroidal anti-inflammatory drug that inhibits both isoforms of cyclooxygenase (COX) has been reported to be fatal to vultures but this was not so with meloxicam which is COX-2 selective. Our study showed that diclofenac was more toxic than meloxicam to both the proximal tubular LLC-PK1 cells and the distal tubular Madin-Darby canine kidney type II (MDCKII) cells, and that LLC-PK1 cells were more susceptible. Exposure of MDCKII cells to meloxicam caused activation of caspase-9/-3 and release of cytochrome *c*. These observations together with a positive annexin V-FITC staining implicate an intrinsic mitochondrial cell death pathway by apoptosis. Diclofenac-treated MDCKII cells on the other hand showed extensive propidium iodide staining, suggestive of cell death by necrosis. The mode of cell death in LLC-PK1 cells was however less well-defined with positive annexin V-FITC staining but minimal increase in caspase-3 activity alluding to a caspase-independent pathway.

Meloxicam and diclofenac are two non-steroidal anti-inflammatory drugs (NSAIDs) which share similar anti-inflammatory, analgesic, and antipyretic properties. These NSAIDs exert many of their effects through the inhibition of cyclooygenase (COX), with meloxicam exhibiting greater COX-2 selectivity while diclofenac was approximately equipotent against both COX I and II isoforms [1]. Although diclofenac is known for its chronic gastrointestinal [2–4] and hepatic toxicity [5], the kidney seems to be an early target [6]. A literature search on diclofenac-induced renal toxicity on humans showed a number of cases, almost all of which were associated with acute renal failure and some were fatal [7–9]. Our previous study also demonstrated that diclofenac was toxic to rat kidney mitochondria, mainly by exerting its effect on the malate–aspartate shuttle and therefore limits the entry of Complex I respiratory substrates [10]. Interestingly, acute renal failure of vultures in India and Pakistan was also linked to the consumption of diclofenac present in life stock. Experimental vultures in the laboratory fed diclofenac exhibited acute renal failure and visceral gout similar to those observed in scavenging vultures in the wild. It was concluded that the residues of veterinary diclofenac were responsible for the drastic decline of the vulture population [11].

This study aims to examine the mechanism of cell death induced by diclofenac and to compare its nephrotoxicity with that of meloxicam which has recently been recommended as an alternative for veterinary use [12]. We have demonstrated that diclofenac was more toxic to renal cells than meloxicam with proximal tubular cell line LLC-PK1 showing greater sensitivity towards drug toxicity than the distal tubular cell line Madin-Darby canine kidney type II (MDCKII). In addition there were distinct morphological changes when both cell lines were exposed to diclofenac and meloxicam alluding to involvement of different modes of cell death. In MDCKII cells, meloxicam elicited a typical apoptotic response while diclofenac caused a mixed type of response with early apoptosis which was later switched to necrosis. On the other hand, both NSAIDs induced a caspase-independent necrosis in LLC-PK1 cells, with diclofenac causing more severe damage than meloxicam.

Materials and methods

Materials. Meloxicam, diclofenac, etoposide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), protease inhibitors and all other common chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Medium 199, trypsin, penicillin–streptomycin and fetal bovine serum (FBS) were obtained from Gibco Life technologies. Annexin V-FITC and propidium iodide (PI) were from Molecular Probes Inc. (Eugene, OR, USA). Caspase-3 substrate (Ac-DEVD-AFC), caspase-9 substrate (Ac-LEHD-AFC) and caspase-8 substrate (Ac-AEVD-AFC) were from Alexis Biochemicals (Lausen, Switzerland). Mouse anti-cytochrome *c* monoclonal antibody was from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA); rabbit anti-actin polyclonal antibody, goat anti-VDAC polyclonal antibody, and the secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. MDCKII and LLC-PK1 cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDCKII cells were cultured in DMEM containing 100 U/ml each of penicillin and streptomycin and 0.25 μg amphotericin B supplemented with 10% FBS. LLC-PK1 cells were maintained in medium 199 supplemented with 3% FBS, 100 U/ml each of penicillin and streptomycin.

Treatment of cells with drugs. Cells were seeded onto either 96-well plates (1.5×10^4 cells in 200 μl of medium/well), 24-well plates (2×10^5 cells in 1 ml of medium/well), or T25 cell culture flasks (1.5×10^6 cells in 5 ml of medium/flask) depending on the experimental requirements, and incubated overnight until a

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monolayer was formed. They were then exposed to etoposide, meloxicam, or diclofenac. These drugs were dissolved in serum-free cell culture medium to exclude binding with serum proteins. Etoposide, an inducer of apoptosis via DNA damage was included as a positive control for comparison. Following exposure to the drugs for various time periods, the cells were gently washed once with warm PBS and used accordingly.

Assessment of cell viability. The cell viability after drug treatment was determined using the MTT method as described [13].

Measurement of activities of caspase-3, -8, and -9. Cells were harvested and lysed by the addition of lysis buffer containing a cocktail of protease inhibitors and incubated on ice for 10 min. Supernatants were collected by centrifugation at 20,000*g* at 4 °C for 10 min to remove cellular debris. The cell lysate was then added to a reaction buffer containing 10 mM Hepes, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 4 mM DTT and a cocktail of protease inhibitors. Each of the following fluorogenic substrates Ac-DEVD-AFC, Ac-AEVD-AFC, and Ac-LEHD-AFC, respectively, for caspase-3, -8, and -9, was added and the fluorescence was determined at Ex/Em of 400/505 nm for 1 h at 5 min intervals using a microplate reader (Molecular Devices, Gemini XS). Caspase activity was expressed as Relative Fluorescence Units (RFU)/min/mg protein.

Annexin V-FITC/PI double staining. PS externalization was examined with a two-color analysis of FITC-labeled annexin V binding and PI uptake using flow cytometry (BD Facs Vantage SE from Becton Dickinson, Franklin Lakes, NJ, USA). Annexin V labels externalized PS, which indicates apoptotic cell membrane disruption, while staining with PI represents necrotic cells with cell membrane damage. For this analysis, 1×10^6 cells were stained according to the manufacturer's instructions (Vybrant Apoptosis assay kit from Molecular Probes). A total of 10,000 cells were analyzed per sample. Cells labeled with annexin V-FITC or PI were used to adjust the compensation. Data acquisition and analysis were performed by the WinMDI 2.8 software program. The fluorescence distribution was displayed as dot plot analysis, and the percentage of fluorescent cells in each quadrant was determined. This method was used to discriminate between intact living cells ($FITC^-/PI^-$), apoptotic cells ($FITC^+/PI^-$) and late apoptotic/necrotic cells ($FITC^+/PI^+$) [14,15].

Protein extraction and immunoblot assay. Cytosolic protein extracts for determining cytochrome *c* release were prepared as described [16]. Sample buffer was then added to the cytosolic fractions, which were subsequently boiled for 5 min and subjected to electrophoresis on a 15% SDS–polyacrylamide gel (25 μg protein/lane). Proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes and immunoblotted with anti-cytochrome *c*, anti-VDAC or anti-actin antibodies (each at 1:1000 dilution). Detection was performed with their appropriate HRP-conjugated secondary antibodies (1:10,000 dilution) and an enhanced chemiluminescence reagent (Amersham).

Statistical analysis. The significance of the differences between drug-treated and their respective controls was determined using the Student's *t*-test. Values were expressed as means ± standard deviation (SD) and were calculated from three independent experiments. A value of $p < 0.05$ was considered statistically significant and represented by asterisks: $^{*}p < 0.05$; $^{**}p < 0.01$.

Results

Cell viability

Cell viability, measured by the MTT reduction assay, was minimally affected at 10 and 50 μM of meloxicam and diclofenac but was compromised when MDCKII and LLC-PK1 cells were exposed to 250 and 500 μM of meloxicam (Fig. 1A and C) and diclofenac (Fig. 1B and D) for 12 and 24 h. Diclofenac was more toxic than meloxicam to both cell lines and the effects of both drugs were more pronounced in LLC-PK1 than in MDCKII cells as shown by their respective LC_{50} values.

Activities of caspase-3, -8, and -9

Caspases, a family of cysteine proteases, are well-known molecular executioners of apoptosis as they bring about most of the morphological and biochemical characteristics of apoptotic cell death

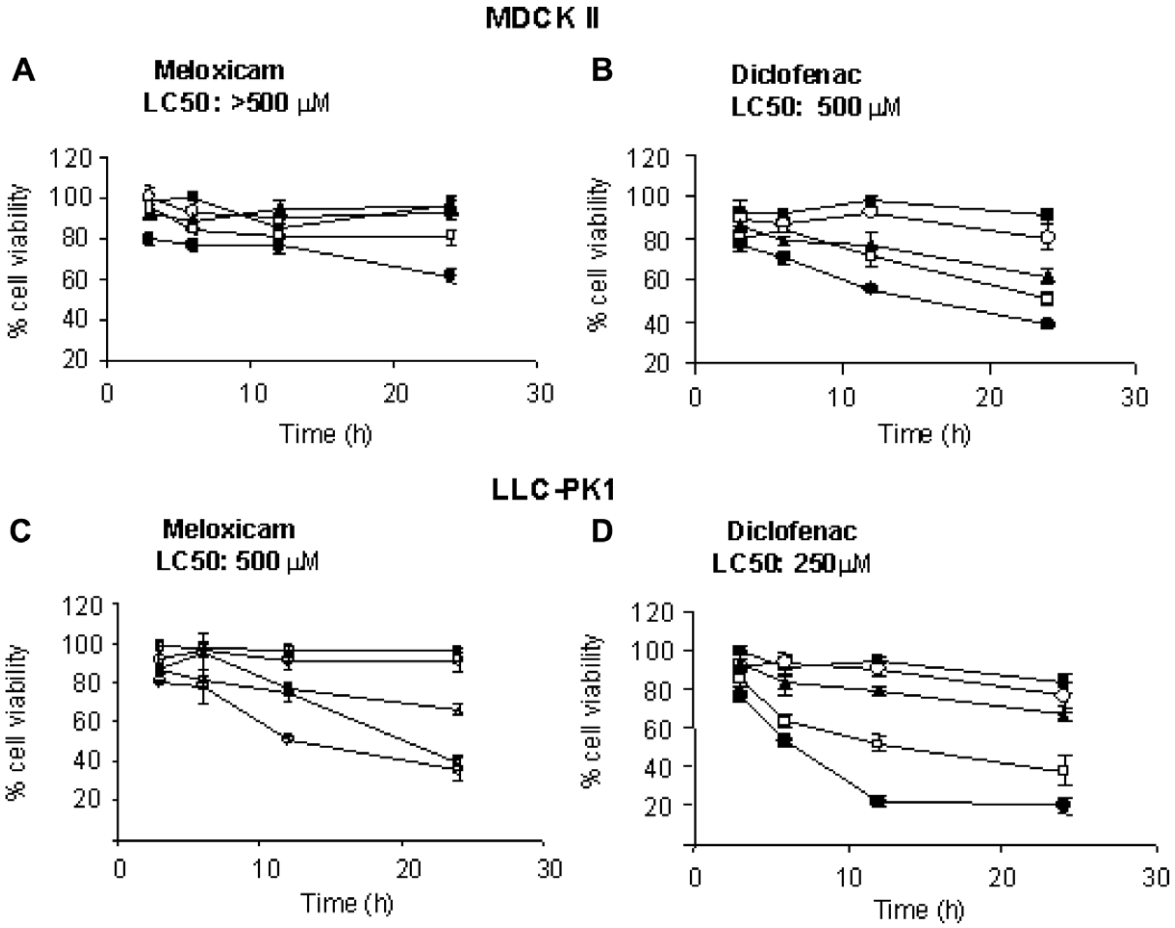

Fig. 1. Effect of meloxicam and diclofenac on cell viability. MDCKII (A,B) and LLC-PK1 (C,D) cells were exposed to different concentrations of meloxicam and diclofenac and cell viability was assessed by the MTT assay. Data points are means ± SD of triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to untreated controls. The concentrations of the drugs were (■) 10 μM; (○) 50 μM; (▲) 100 μM; (□) 250 μM; (●) 500 μM.