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Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS

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Abstract

Diclofenac is a non-steroidal anti-inflammatory drug that is widely used clinically but side effects associated with the administration of the drug have been reported. The apoptotic effect of the drug has been evaluated in human and rat hepatocytes. Apoptosis was observed after exposure to sub-cytotoxic concentrations of the drug, without overlapping with cell necrosis. Flow cytometric analysis revealed a time- and dose-dependent increase of apoptotic nuclei with sub-diploid DNA content. Caspase 8 and 9 mediate the cell-receptor and the mitochondria-initiated apoptotic pathways, respectively. Inhibition of both caspases prevented activation of downstream caspases, thus indicating that diclofenac at least activates caspase 3 and both effector caspases 8 and 9. The hierarchy of caspase activation by diclofenac was investigated. Analysis of kinetics revealed a simultaneous activation of these caspases that was maximal after 12 hr of exposure to the drug. Inhibitors of MPT, prevented the downstream activation of the caspase cascade, thus showing that diclofenac opened the mitochondial pore. On the other hand, antioxidants were able to prevent caspase activation by diclofenac, revealing that oxidative stress at the mitochondrial level is in the root of MPT induction and caspase cascade activation. Caspase activation is not mediated by Bid cleavage, suggesting that the cell-receptor pathway seems not to be involved. However, a dose-dependent release of caspase 8 from the mitochondria was observed, indicating that caspase 8 can be processed independently of cell death receptors. Caspases 8 and 9 are very likely the apical caspases in diclofenac-induced apoptosis. In addition, an early dose-dependent increase of $bclX_L$ expression parallel to the generation of reactive oxygen species in the mitochondria was found. In conclusion, the mitochondrial pathway is very likely the only pathway involved in diclofenac-induced apoptosis, which was related to CYP-mediated metabolism of diclofenac, with the highest apoptotic effect produced by the metabolite 5OH-diclofenac.

Keywords: $BclX_L$; Caspases 3, 8 and 9; Bid; Diclofenac; DNA fragmentation; MPT; Reactive oxygen species

1. Introduction

Diclofenac, an arylacetic non-steroidal anti-inflammatory drug (NSAID), is frequently prescribed in treating rheumatic diseases and as an analgesic [1]. The use of diclofenac has been associated with occasional hepatic toxicity [2–4]. While in some case reports, the adverse hepatic effects of diclofenac showed features compatible with a drug hypersensitivity reaction [5,6], the clinical findings in other patients appear to be more consistent with a direct toxic effect of the drug or a drug metabolite [1,2].

Early studies in animals and man [7,8] showed that diclofenac undergoes an extensive hepatic metabolism involving aromatic hydroxylations and conjugations. It has been recently described how CYP-mediated metabolic activation of the drug and the formation of reactive metabolite(s) by drug oxidation is related to diclofenac hepatotoxicity [9–12]. The two major urine metabolites in rat and man are 4′OH-diclofenac together with 5OH-diclofenac [7,12] (Fig. 1). *In vitro* toxicity is associated with the oxidative metabolism of the drug and correlates with the formation by hepatocytes of 5OH-diclofenac and, in particular, *N*,5$(OH)_2$-diclofenac, a minor metabolite derived

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Abbreviations: Cs A, cyclosporine A; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethylsulfoxide; DUBQ, decylubiquinone; DMTU, *N*,*N*-dimethylthiourea; LDH, lactate dehydrogenase; MNTC, maximal non-toxic concentration; ROS, reactive oxygen species; SOD, superoxide dismutase.

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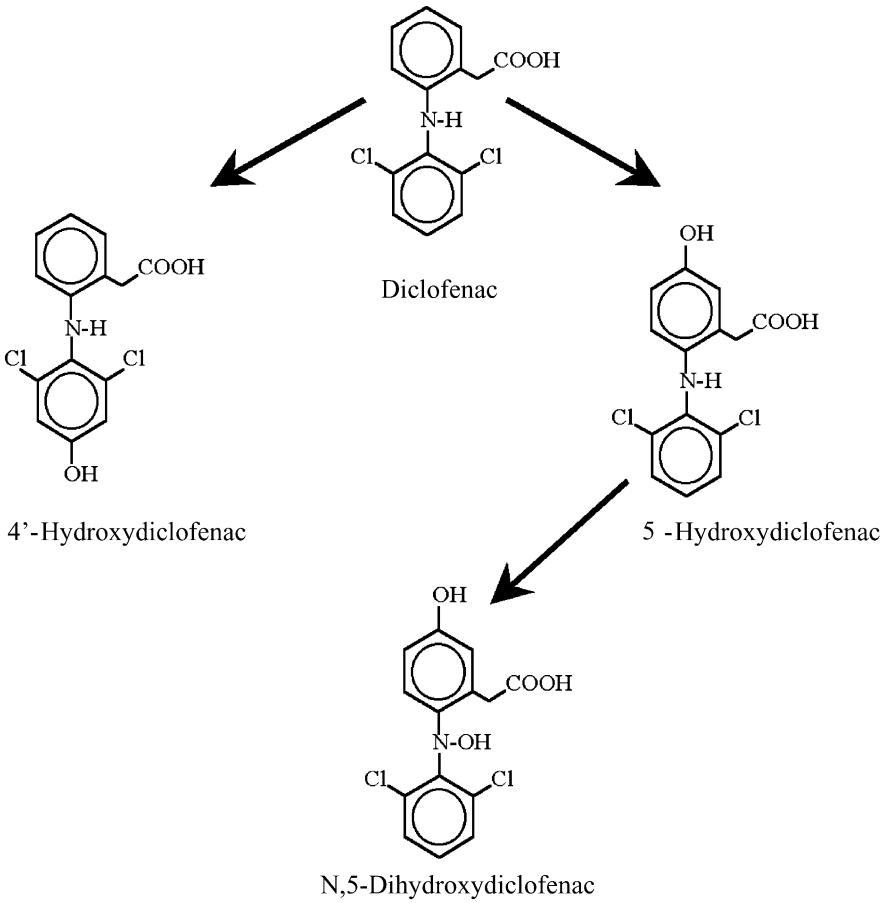

Fig. 1. Main metabolic route of diclofenac.

from 5OH-diclofenac, first characterized by Bort and coworkers [14]. It was also shown that hepatocyte injury was preceded by a decrease in ATP levels, indicating that the inability of mitochondria to produce ATP is the cause of drug toxicity [12,14].

For many years it was assumed that chemically induced injury and death occurred primarily by necrosis, now, however, it is recognized that cell death may also be the result of an apoptotic process [15]. Moreover, it is believed that apoptosis could be the major form of chemically induced cell death and that necrosis is associated only with circumstances of severe cell injury, a fact of great significance from the pharmaco-toxicological point of view [15–18].

The two pathways of apoptosis include the cell receptor pathway (i.e. Fas), that mediates transduction of the death signal and the mitochondria-initiated pathway, that involves membrane permeabilization and release of several mitochondrial proteins, such as cytochrome *c* and procaspases, which commits hepatocytes to apoptosis [19–21]. The most important apoptotic effectors are the caspases that are activated at the execution stage of apoptosis. Progression of the caspase cascade ends with the activation of caspase 3 that occurs in early apoptosis, long before DNA-fragmentation appears [22]. It has been proposed that drug-induced apoptosis is mainly mediated by the mitochondrial pathway, which involves MPT, a non-selective inner membrane permeabilization, considered as a common mechanism that may precede necrotic and apoptotic cell death [20,21].

Evidence showing that NSAIDs cause apoptosis in several cell lines have been recently reported [23–26]. Moreover, diclofenac has been described to induce DNA fragmentation in cultured gastric mucosa cells which were inhibited by caspase inhibitors [27]. In addition, the key role of MPT and the decrease of ATP in the pathogenesis of diclofenac-induced hepatocyte injury have been recently reported [28]. On the basis of these previous findings, the investigation of the potential apoptotic effect of diclofenac on liver cells is of great pharmacological relevance. Whether or not apoptosis is related to diclofenac metabolism, the mechanism that triggers apoptosis and the hierarchy of activation of downstream effector caspases in apoptosis caused by diclofenac could be established.

2. Material and methods

2.1. Reagents

Diclofenac sodium salt, Cs A, DUBQ, propidium iodide, SOD, α-tocopherol, DCFH-DA and DMTU were purchased from Sigma Chemical Co. 5OH-Diclofenac and

4′OH-diclofenac were synthesized as described (Bort, 1996). Collagenase was from Roche. Ac-DEVD-AMC caspase 3 and Ac-IETD-AFC caspase 8 fluorogenic substrates were from PharMingen. Ac-LEHD-AFC caspase-9 fluorogenic substrate, and IETD-CHO and LEHD-CHO (caspase-8 and caspase-9 cell permeable inhibitors, respectively) were purchased from Calbiochem. Anti-Bid polyclonal antibody was from R&D Systems. Anti-Fas monoclonal antibody CH-11 was purchased from MBL. Anti-caspase 8 polyclonal antibody was from Neo Markers. Culture media (Ham's F-12, Lebovitz L-15) and DNase I Amplification Grade were from Gibco-BRL. All other chemicals were of analytical grade.

2.2. Isolation and culture of rat and human hepatocytes

Rat hepatocytes were obtained from 200 to 300 g Sprague–Dawley male rats by perfusion of the liver with collagenase and cultured as described in detail elsewhere [17]. Surgical human liver biopsies were obtained in conformity with the rules of the Hospital's Ethics Committee and after informed consent from patients undergoing programmed cholecystectomy. Human hepatocytes were isolated using a two-step perfusion technique and cultured as described [29]. Cellular viability was assessed by the trypan blue dye exclusion test. Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 μg/cm^2) at a density of 8×10^4 viable cells/cm^2 and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% new-born calf serum, 50 mU/mL penicillin, 50 μg/mL streptomycin, 0.2% bovine serum albumin and 10^{-8} M insulin. One hour later the medium was changed, and after 24 hr the cells were shifted to serum-free hormone-supplemented medium (10^{-8} M dexamethasone and insulin).

2.3. Preparation of stock solutions for treatment of cultures

Stock solutions of 6 mM diclofenac, 50 mg/mL Cs A, 500 mM DMTU and 10 U/mL SOD in PBS, 20 mM 5OH-diclofenac and 20 mM 4′OH-diclofenac in methanol, 20 mM α-tocopherol in ethanol, and 5 mM DUBQ in DMSO were prepared and diluted with culture medium to obtain the appropriate final concentrations in culture. A 400 mM stock solution of IETD-CHO and LEHD-CHO (caspase-8 and caspase-9 cell permeable inhibitors, respectively) were prepared in DMSO and added to hepatocytes at a final concentration of 100 μM. In all cases the concentration of solvent in culture medium did not exceed 0.5% (v/v).

2.4. Cytotoxicity assay

Increasing concentrations of the drug in PBS were added to cultures after medium renewal and cells were incubated for a 12 or 24-hr period. Cytotoxicity was assessed by measuring the intracellular LDH content [30]. The maximal concentration not causing a significant decrease in intracellular LDH, the MNTC, was determined after several assays.

2.5. Flow cytometric analysis of DNA fragmentation

Cell monolayers were covered with hypotonic lysis solution containin propidium iodide (50 μg/mL, final concentration) for fluorescent staining of DNA and the nuclei with a DNA content lower than the diploid (2C) was analyzed by flow cytometry [17].

2.6. Measurement of intracellular reactive oxygen species

ROS production was monitored by flow cytometry using the stable nonpolar dye DCFH-DA that readily diffuse into cells. Once inside the cells, the acetate groups of DCFH-DA are cleaved from the molecule by intracellular esterases to yield DCFH, which is trapped within the cells. Intracellular H_2O_2 or low-molecular-weight peroxides, in the presence of peroxidases, oxidize DCFH to the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxides produced by the cells. Hepatocytes were seeded in 96-well plates and were incubated in the absence or presence of increasing concentrations of diclofenac for different periods of time (1–6 hr). Thirty minutes before ending the incubation, cells were incubated with 10 μM DCFH-DA at 37°, then washed twice with PBS and finally the cellular fluorescence intensity was measured in a microplate-reader fluorimeter (excitation 488 nm and emission 525 nm for DCFH).

2.7. Caspases 3, 8 and 9 activity

Detached cells collected by centrifugation at 2500 *g* for 3 min and attached cells scraped off, were pooled and lysed at 4° as described [17]. Caspases 3, 8 and 9 activity was measured using the specific fluorogenic substrates Ac-DEVD-AMC, Ac-IETD-AFC and Ac-LEDHD-AFC, respectively, as previously described in detail [31]. Cellular protein was determined as described [32].

2.8. $bclX_L$ analysis

After incubation of hepatocytes total RNA was isolated and contaminating genomic DNA was removed by incubation with DNase I Amplification Grade. For quantitative RT–PCR analysis, 1 μg of RNA was reverse transcribed as described [33], and diluted cDNA was amplified with a rapid thermal cycler (LightCycler Instrument, Roche Diagnostics) as previously described in detail [17].

2.9. Western blot analysis of Bid and pro-caspase 8

In all cases, detached and attached cells were collected as described above. To detect Bid and pro-caspase 8 protein

levels, cells were processed as described [31] and subjected to a 12 and 15% SDS–PAGE, respectively. The gels were transferred to a PVDF membrane (Millipore), and the blots were probed either with an anti-Bid or with anti-caspase 8 polyclonal antibodies, and developed using a horseradish peroxidase-coupled anti-rabbit secondary antibody by enhanced chemiluminescence.

2.10. Statistical analysis

Each experiment was done in at least two different cultures. The statistical significance of the experimental data was analyzed by the Student's *t*-test.

3. Results

3.1. Cytotoxicity of diclofenac and its major metabolites on cultured hepatocytes

Cells were incubated with increasing concentrations of diclofenac, 4′OH-diclofenac and 5OH-diclofenac for 12 and 24 hr, and changes in intracellular LDH content was evaluated as a cytotoxicity end-point. The maximal concentration of the compounds not causing cytotoxic effect, the MNTC, was determined at each time point.

Diclofenac, 4′OH-diclofenac and 5OH-diclofenac produced a concentration- and time-dependent decrease in intracellular LDH levels when added to hepatocytes. Diclofenac and 4′OH-diclofenac showed similar MNTCs after 12 and 24 hr of treatment (Table 1). However, 5OH-diclofenac showed a higher toxic potential and the MNTCs were significantly lower (Table 1). Similar MNTC values of diclofenac and its metabolites were obtained in human hepatocyte cultures.

3.2. Flow cytometric analysis of apoptosis

Flow cytometric analysis was performed in cultured rat hepatocytes treated for different periods of time with diclofenac at concentrations which did not cause significant LDH leakage. As Fig. 2A shows, a time-dependent increase of apoptotic nuclei with sub-diploid DNA content was found after exposure to 350 μM diclofenac. The percentage of apoptotic nuclei reached the maximum after 24 hr of exposure to the compounds. The dose-dependence of the percentage of appearance of apoptotic nuclei was then evaluated after 24 hr exposure to increasing concentrations of diclofenac. As seen in Fig. 2B, a clear dose-dependent effect was observed.

Table 1
MNTC of diclofenac and its major metabolites in rat hepatocytes exposed to the drugs

	MNTC (μM)	
	12 hr of treatment	24 hr of treatment
Diclofenac	647 ± 115	455 ± 75
4′OH-diclofenac	635 ± 145	439 ± 110
5OH-diclofenac	360 ± 72	152 ± 46

3.3. Caspase cascade activation

The kinetics of caspase 3 activation was measured in rat hepatocytes treated with 350 μM of diclofenac up to 24 hr. A time-dependent increase in caspase 3 activity was observed up to 12 hr, when activity reached a plateau or decreased slightly (Fig. 3A). A concentration-dependent effect of diclofenac on caspase 3 activation was also obtained by exposing hepatocytes to increasing concentrations of the drug not overlapping cell necrosis, for 12 hr (Fig. 3B).

To analyze the apoptotic pathway involved in the caspase activation cascade by diclofenac, the effect of specific inhibitors of the effector caspases 8 and 9 on activation of caspase 3 was investigated. As Fig. 3C shows, the inhibition of both caspases prevented caspase 3 activation in rat hepatocytes. Similar results were obtained after incubation of human hepatocytes with diclofenac and the effector caspases 8 and 9 inhibitors. The results suggest that both effector caspases are involved in diclofenac-induced apoptosis. Trying to clarify the hierarchy of caspase activation by diclofenac, the time-course of caspase 8 and 9 activation was also assayed in rat hepatocytes treated with diclofenac at time intervals up to 24 hr. A parallel time-dependent increase of caspase 8 and 9 activation, was observed up to 12 hr of incubation, then activity reached a plateau or decreased slightly (Fig. 3A).

3.4. Effects of diclofenac at the mitochondrial level, intracellular generation of reactive oxygen species and prevention of diclofenac-induced apoptosis by antioxidants

The involvement of the MPT in diclofenac-induced apoptosis was investigated in rat and human hepatocytes The effect of Cs A and DUBQ, MPT specific inhibitors that prevent cytochrome *c* release from mitochondria, on caspase cascade activation was evaluated. The results show that both compounds significantly prevented the activation of caspases in hepatocytes, thus suggesting that MPT is deeply involved in diclofenac-induced apoptosis both in human and rat hepatocytes (Fig. 4).

To assess the contribution of oxygen-derived free radicals in diclofenac-induced MPT, cultures were pre-treated for 15 min with DMTU, α-tocopherol and SOD, then diclofenac was added and caspase 3, 8 and 9 activation was evaluated after 12 hr of incubation. DMTU, SOD and α-tocopherol protected hepatocytes from the effect of ROS impairing caspases activation, thus indicating that oxidative damage is involved in the apoptotic process induced by diclofenac (Fig. 5A). After incubation of rat hepatocytes

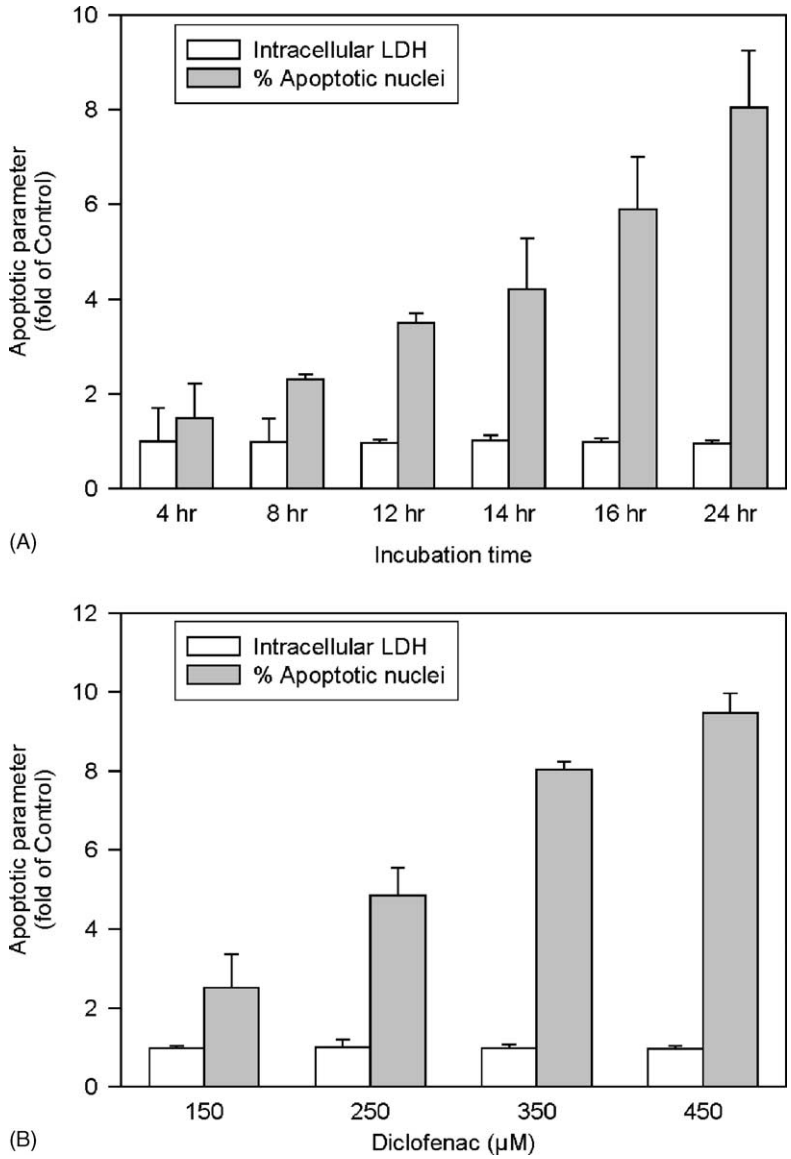

Fig. 2. Flow cytometric analysis of apoptosis. The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak in rat hepatocytes. Changes in intracellular LDH content was evaluated as a cytotoxicity end-point. (A) The kinetics of apoptosis was examined in rat hepatocyte cultures exposed to 350 μM diclofenac and percentage of apoptotic nuclei evaluated at time point intervals. (B) The dose-dependence of the percentage of appearance of apoptotic nuclei was evaluated after 24 hr exposure to increasing concentrations of diclofenac (control value 3.2 ± 1.80%). Data are expressed as fold increase the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of triplicate dishes from a representative experiment.

with diclofenac and DCFH-DA as a probe for increasing periods of time up to 6 hr, production of ROS was evaluated fluorimetrically. The results show a significant increase of intracellular ROS, reaching the maximum after 5 hr of incubation with diclofenac (Fig. 5B).

3.5. Analysis of Bid and mitochondrial protein levels of pro-caspase 8

As caspase 8 cleaves Bid, a proapoptotic member of the Bcl-2 family [34], we decided to analyze the decrease of the pro-form of Bid protein. No changes of uncleaved Bid were observed in the cytosol up to 12 hr of treatment of rat hepatocytes with diclofenac, when activation of caspases 8 and 9 was maximal. Thus, indicating that caspase 9 activation is not mediated by Bid activation (Fig. 6A).

We investigated the mitochondrial source of pro-caspase 8 available for activation by diclofenac. The results show a dose-dependent decrease of pro-caspase 8 in the mitochondria that correlated with caspase 8 activation observed in the cytosol, thus indicating the very likely mitochondrial origin of caspase 8 (Fig. 6B).

3.6. $bclX_L$ expression

mRNA levels of $bclX_L$ were analyzed in cultured hepatocytes treated with diclofenac at concentrations that did not cause any observable LDH leakage. Hepatocytes were

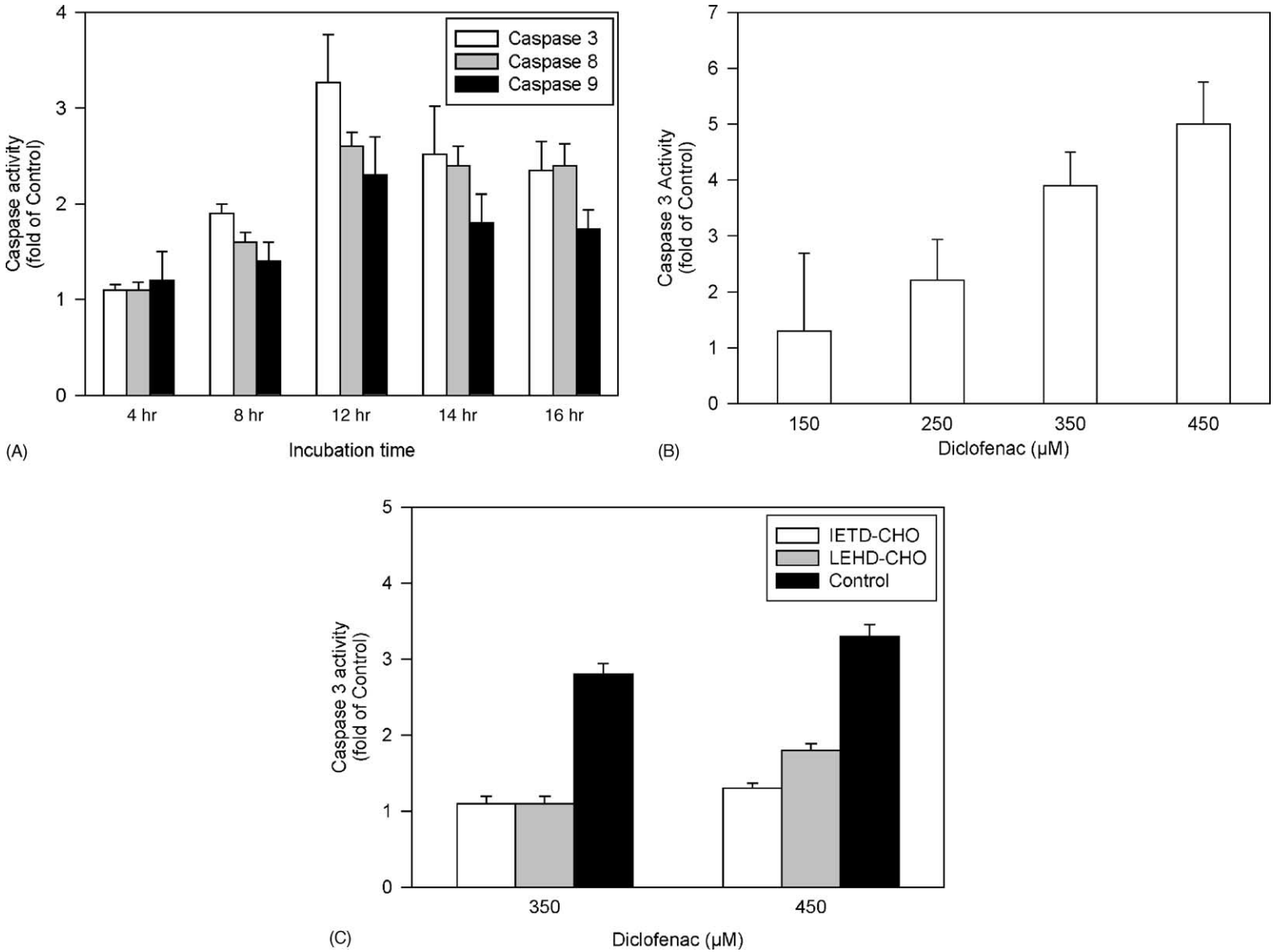

Fig. 3. Caspase cascade activation by diclofenac and effect of inhibitors of effector caspases on the caspase cascade activation. (A) The time-course of caspase 3, 8 and 9 activation by 350 μM diclofenac was assayed in rat hepatocytes treated with the drug at time intervals up to 24 hr, by using the fluorescent substrates Ac-DEVD-AMC, Ac-IETD-AFC and Ac-LEHD-AFC, respectively. (B) Concentration-dependent activation of caspase 3 by diclofenac. Hepatocytes were treated for 12 hr to increasing concentrations of the drug not overlapping necrosis (control value 108 ± 14 mU/min × mg cell protein). (C) Hepatocytes were exposed simultaneously to diclofenac in the presence of 100 μM cell permeable caspase inhibitors of the caspases 8 (IETD-CHO) and 9 (LEHD-CHO). The role of the effector caspase inhibitors on caspase 3 activation was evaluated after 12 hr of treatment (control value 92 ± 10 mU/min × mg cell protein). Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac) and correspond to the mean ± SD of triplicate dishes from a representative experiment.

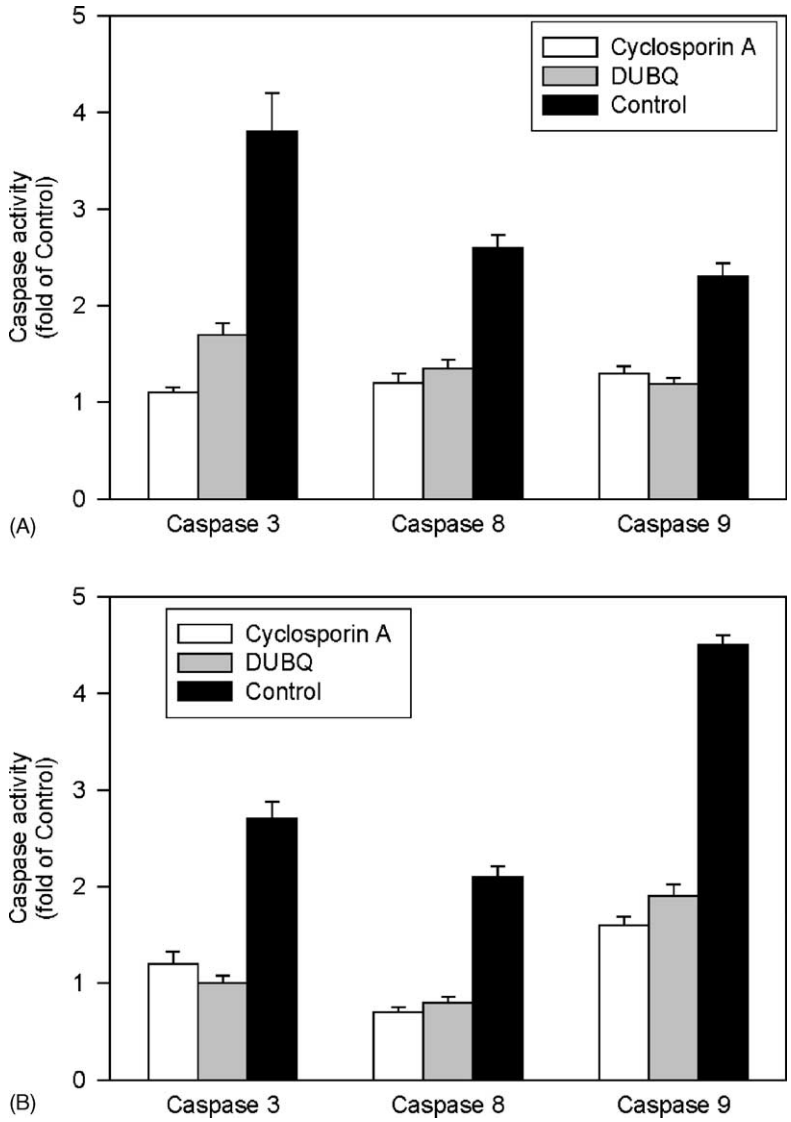

Fig. 4. Effects of diclofenac at the mitochondrial level. The involvement of the MPT in diclofenac-induced apoptosis was investigated by evaluating the effect of 5 μM Cs A and 5 μM DUBQ, MPT specific inhibitors, on caspase cascade activation after 12 hr of treatment. (A) Inhibitory effect of MTP blockers on caspases 3, 8 and 9 activation by 350 μM diclofenac in rat hepatocytes (control values: 108 ± 14 mU/min × mg for caspase 3; 25 ± 3 mU/min × mg for caspase 8; 23.5 ± 5 mU/min × mg for caspase 9) and (B) in human hepatocytes (control values: 76 ± 9 mU/min × mg for caspase 3; 17 ± 3 mU/min × mg for caspase 8; 14 ± 3 mU/min × mg for caspase 9). Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of triplicate dishes from a representative experiment.

exposed up to 8 hr and $bclX_L$ mRNA was measured by real-time quantitative RT–PCR. Early and significant increases of $bclX_L$ mRNA levels were produced by diclofenac, reaching the maximum after 8 hr of treatment. Dose-dependent effects were significant above 200 μM diclofenac (Fig. 7).

3.7. Apoptotic potential of diclofenac and its metabolites

The dose-dependence of the appearance of apoptotic nuclei and the activation of caspase 3 by diclofenac and its two major metabolites was studied. Rat hepatocytes were exposed to increasing sub-cytotoxic concentrations (250–450 μM) of diclofenac, 5OH-diclofenac and 4′OH-diclofenac, and percentage of sub-diploid nuclei and caspase 3 activity were measured after 12 and 24 hr, respectively. As shown in Fig. 8, diclofenac, 5OH-diclofenac and 4′OH-diclofenac produced a concentration-dependence of both apoptotic parameters, but, at equimolar concentration, the highest activation was produced by 5OH-diclofenac.

4. Discussion

We have previously shown that, despite the fact that diclofenac itself is effective, hepatotoxicity is associated with the oxidative metabolism of the drug and that 4′OH-diclofenac together with 5OH-diclofenac are the major urine metabolites in rat and man [7,12,13]. The inability of mitochondria to produce ATP has been described to be the

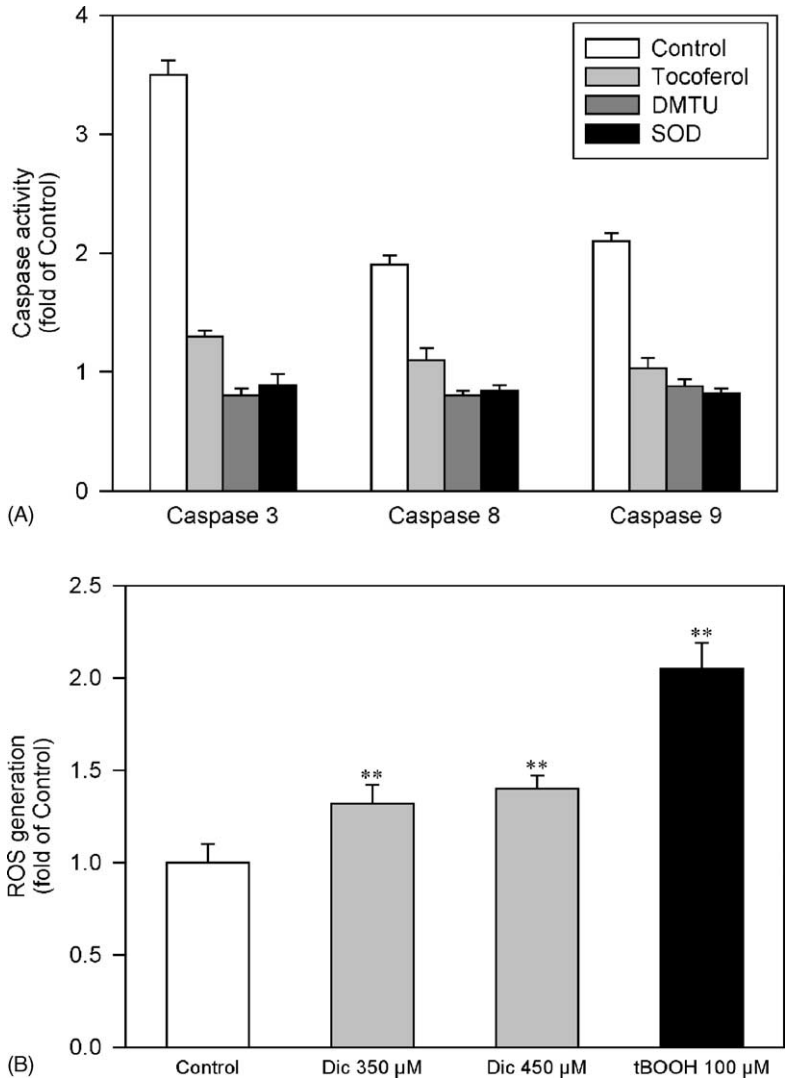

Fig. 5. Effects of antioxidants in diclofenac-induced MPT and intracellular generation of ROS. The involvement of oxidative damage in diclofenac-induced MPT was investigated. (A) Prevention of caspases 3, 8 and 9 activation by antioxidants in response to 350 μM diclofenac. Rat hepatocytes were pre-treated with antioxidants 10 mM DMTU, 250 μM α-tocopherol, and 300 U/mL SOD, before exposure to the drug and activation of caspases was evaluated 12 hr after incubation (control values: 58 ± 4 mU/min × mg for caspase 3; 10.5 ± 3 mU/min × mg for caspase 8; 11 ± 3 mU/min × mg for caspase 9). (B) Intracellular ROS generation was analyzed fluorimetrically by using the oxidation sensitive probe DCFH-DA. Thirty minutes before ending the incubation, cells were incubated with 10 μM DCFH-DA at 37°, then washed twice with PBS and finally the cellular fluorescence intensity was measured in a microplate-reader fluorimeter (excitation 488 nm and emission 525 nm for DCFH). Significant increase of intracellular ROS was observed after 5 hr incubation with diclofenac. *tert*-Butyl hydroperoxide (*t*BOOH) was used as positive control. Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of triplicate dishes (A) and ten wells (B) from representative experiments; $^{**}P < 0.01$, Student's *t*-test.

major cause of diclofenac-induced toxicity [12,14]. Moreover, the key role of mitochondrial dysfunction in the pathogenesis of diclofenac-induced hepatocyte injury, as a result of the decrease of ATP and MPT, has also been recently reported [28]. Since MPT is considered a major common mechanism for drug-induced hepatocyte necrosis and apoptosis [36,37], it is very likely that apoptosis is involved in the adverse effect of diclofenac. In the current study we have used human and rat hepatocytes to investigate how oxidative stress is involved and the mechanism leading to mitochondrial alteration triggering apoptosis, at concentrations not overlapping necrosis. Therefore, we have investigated the potential apoptotic effects of diclofenac at a concentration close to the MNTC (455 ± 75 μM; Table 1). However, at 50 μM diclofenac could be already found caspase-3 activation and of sub-diploid DNA nuclei increase (data not shown).

We first showed by flow cytometry a time and concentration-dependent increase of apoptotic nuclei with sub-diploid DNA content after exposure to diclofenac. Then we investigated caspase 3 activation by diclofenac and we found a clear time- and concentration-dependent activation. To analyze the apoptotic pathway involved in caspase activation, the effect of specific inhibitors of the initiator caspases 8 and 9 on caspase 3 activation were analyzed. The results clearly suggest that both caspases 8 and 9 are

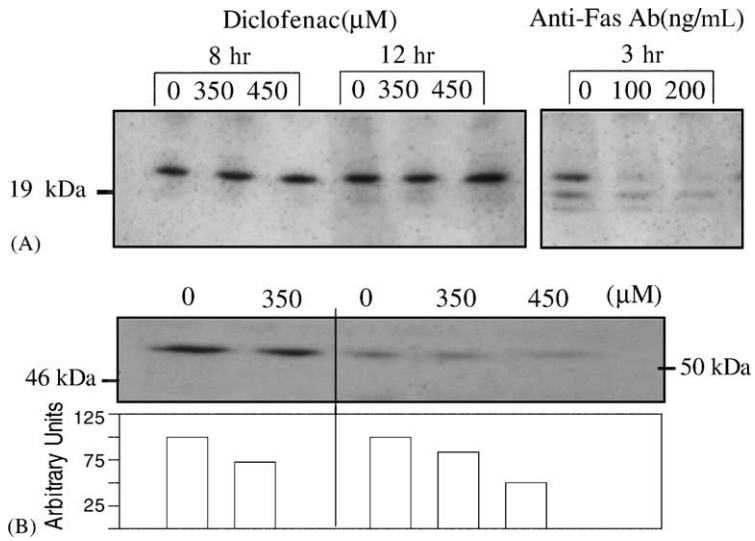

Fig. 6. Analysis of Bid and pro-caspase 8 protein levels. Uncleaved Bid was analyzed in the cytosolic fraction and pro-caspase 8 in the mithochondrial fraction by SDS–PAGE, after treatment of rat hepatocytes with diclofenac. The gels were transferred to PVDF membranes, and the blots were probed either with anti-Bid or anti-pro caspase 8 polyclonal antibodies, and developed using a horseradish peroxidase-coupled secondary antibody. (A) Levels of the uncleaved Bid protein in the cytosol after 8 and 12 hr of treatment with diclofenac (left side). Jurkat cells treated for 3 hr with anti-Fas monoclonal antibody were used as positive control for Bid cleavage (right side). (B) Dose-dependent effect of diclofenac (12 hr) on pro-caspase 8 levels in the mitochondrial extracts (two representative experiments). Bars represent the quantitative dosimetric analysis of detected bands.

fully involved in the pathway of diclofenac-induced apoptosis, since activation of caspase 3 was totally blocked by the inhibitors of both caspases.

During the early phase of apoptosis, mitochondria undergo a MPT which commits hepatocytes to apoptosis leading to the release of several mitochondrial proteins into cytoplasm, after which the apoptotic events become irreversible [37–39]. However, inhibiting the MPT ameliorates caspase activation and apoptosis in several cellular systems [40,41]. According to this, our results show that activation of caspases 3, 8 and 9 is totally inhibited by specific blockers of MPT [42,43], which confirms that diclofenac-induced apoptosis is MPT-dependent both in human and rat hepatocytes.

It has been described elsewhere how MPT is not a consequence of the opening of a pre-formed pore, but the consequence of oxidative damage to pre-existing membrane proteins [44]. Hepatic microsomes both oxidize 5OH-diclofenac to *N*,5(OH)$_2$-diclofenac, a minor metabolite first characterized in our laboratory, and back reduce the latter to 5OH-diclofenac entering a futile cycle that results in NADPH oxidation by O_2 [12]. As a result oxidative injury to the mitochondria could be expected. Our results show that simultaneous incubation of diclofenac with antioxidants largely prevented caspase cascade activation and apoptosis, suggesting that oxidative stress mediates the MPT and is a crucial event in diclofenac-induced apoptosis to hepatocytes, as has been recently

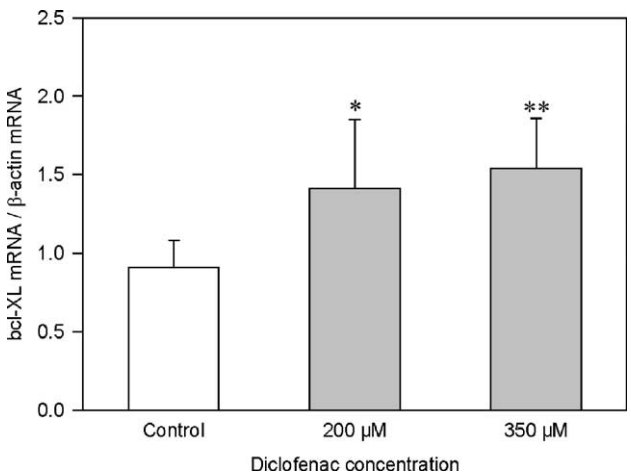

Fig. 7. Analysis of bclX$_L$ expression. Rat hepatocytes were exposed up to 8 hr to diclofenac and bclX$_L$ mRNA was measured by real-time quantitative RT–PCR. A dose-dependent increase of bclX$_L$ mRNA levels was observed. Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of three different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

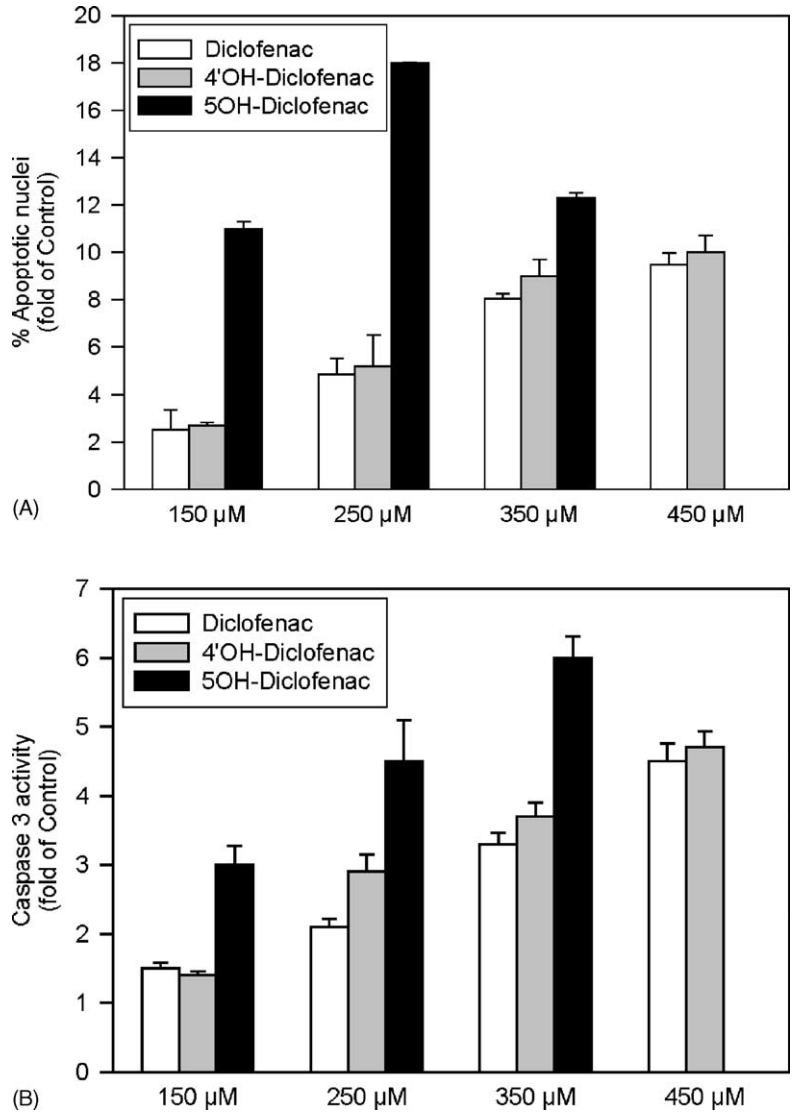

Fig. 8. Dose-dependence of activation of caspase 3 by diclofenac and its major metabolites. Rat hepatocytes were exposed to increasing sub-cytotoxic concentrations of diclofenac, and its major metabolites, 5OH-diclofenac and 4′OH-diclofenac and apoptotic parameters were evaluated. (A) Effect on the percentage of sub-diploid nuclei after 24 hr of treatment with the drugs (control value: $4 \pm 1.3\%$). (B) Caspase 3 activity was measured after 12 hr of exposure to the drugs (control value 82 ± 12 mU/min × mg cell protein). Data are expressed as fold increases over the control values (hepatocytes not exposed to the compounds) and correspond to the mean ± SD of triplicate dishes from a representative experiment.

proposed [28,45,46]. This hypothesis was confirmed by the early and significant increase of ROS observed in hepatocytes treated with diclofenac. An association between generation of ROS and NSAID-induced apoptosis has been reported in gastric epithelial cells [27].

We tried to establish the sequence of events leading to apoptotic cell death with the following results: (a) diclofenac elicits not only activation of the effector caspase 3 but also the key initiators caspases 8 and caspase 9; (b) the time-course of all caspase processing is very similar, with maximal activation after 12 hr, preceeding apoptotic cell death; (c) as previously reported [46,47], MPT is an upstream event that regulates caspase activity; and (d) caspase 8 is clearly activated independently of the receptor pathway. The recruitment and cleavage of pro-caspase 8 to produce the active form of caspase 8, is a critical event in death receptor-mediated apoptosis [48]. In turn it cleaves Bid, a bcl-2 family protein, to a truncated form that translocates to mitochondria and initiates mitochondrial apoptotic pathway. However, in our study we have not found Bid cleavage after treatment with diclofenac (Fig. 6A), thus indicating that caspase 8 can be processed by diclofenac independently of cell death receptors, as has been recently reported for anticancer drugs [49]. Since caspase 8 activation was inhibited by MPT blockers, its mitochondrial origin was, therefore, investigated. A diclofenac concentration-dependent decrease of the intramitochondrial levels of pro-caspase 8 was observed. This finding is supported by previous reports showing pro-caspase 8 predominantly colocalized with cytochrome *c* in mitochondria which is released upon apoptotic stimulation through a MPT-sensitive mechanism [35]. Moreover,

it has been reported that cytochrome *c* release is caspase-independent and is not mediated by activation of Bid in chemical-induced apoptosis [50,51].

An interesting finding was the early increase of the expression of $bclX_L$ coincident with the increase of ROS in hepatocytes treated with diclofenac. Recent evidence suggests that ROS may act as signal transduction messengers involved in the regulation of gene expression and, in particular, NF-κB has been reported to be activated by oxidant stress in mitochondria [52,53]. Stimuli that activate endogenous NF-κB directly activates the expression of $bclX_L$ in hepatocytes [54], as probably occurs in diclofenac-treated hepatocytes.

The proposed mechanism for apoptosis induced by diclofenac can be summarized as follows: Generation ROS would increase as a consequence of the oxidative metabolism of diclofenac, very likely acting as an early signal triggering apoptosis and activating endogenous NF-κB. MPT and mitochondrial dysfunction seems to be a direct and immediate consequence that would lead to a release of pro-caspase 8 and cytochrome *c* from mitochondria, which would contribute to the activation of caspase 9/caspase 3. Cytochrome *c* release seems to be caspase-independent and not mediated by activation of Bid. Caspase 3 could be responsible for processing other caspases including caspase 8.

The link between drug metabolism and toxicity to hepatocytes has been clearly established and the CYPs involved in the production of each metabolite in man have been identified [13,55]. The participation of CYP2C19, a CYP isozyme showing strong variability due to its genetic polymorphism, in the formation of 5OH-diclofenac and, indirectly, of *N*,5$(OH)_2$-diclofenac may explain the variability of the hepatotoxicity exerted by diclofenac in man. It has been clearly shown that 5OH-diclofenac has a higher apoptotic potential than diclofenac itself or 4′OH-diclofenac and, although it is produced to a lesser extent in man than in rat, the results presented here suggest that the variation in the expression of the CYP2C19 may lead to an increase of apoptotic damage to cells, probably caused by a higher production of reactive species in man, leading to a significant increase of ROS generation.

Some key issues should be addressed for *in vitro*/*in vivo* extrapolations, such as the portal vein concentration of the compound after an oral administration and the metabolism of the drug. It should be considered that due to genetic polymorphism of CYPs involved in diclofenac metabolism, the concentrations of diclofenac and its metabolites may be particularly high in some individuals, increasing the risk of cell toxicity [12,13,55]. The concentration of diclofenac required to cause apoptosis in cultured hepatocytes is about 10 times higher than the human peak concentrations after a single administration of a therapeutic dose of the drug [56,57], and than the concentration eliciting apoptotic effects to neural stem cells [58]. However, the ultimate goal of *in vitro* studies is identifying compounds that are potentially toxic to man to assess the risk of toxicity and drug safety [59].

In conclusion, the results presented in this work indicate that diclofenac induces apoptosis at concentrations not overlapping cell necrosis, this is related to CYP-mediated metabolism. Oxidative injury at the mitochondrial level is involved in MPT induction, which allows the release of mitochondrial proteins, which in turn activate caspase 9 and caspase 3.

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