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Diclofenac induces apoptosis in hepatocytes

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Abstract

Hepatotoxicity is one of the side effects associated with the administration of diclofenac, a non-steroidal anti-inflammatory drug widely used clinically. The effect of diclofenac on the early events that trigger apoptosis cascade have been evaluated in rat hepatocytes. To do this, early and late apoptotic markers, associated with the pivotal steps of the execution phase, have been evaluated after incubation with the drug. The results show that the apoptotic effect of diclofenac occurs after exposure to sub-cytotoxic concentrations of the drug (maximal non toxic concentration, MNTC, after 24-h treatment was 450 μM), without overlapping with cell necrosis (LDH leakage evaluation). Flow cytometric analysis revealed a time- and dose-dependent increase of apoptotic nuclei with sub-diploid DNA content. Caspase 3 activation (3–5-fold control) was maximal after 12 h of exposure to 350 μM of the drug. The involvement of the mitochondrial permeability transition (MPT) in diclofenac-induced apoptosis was investigated. Cyclosporine A and decylubiquinone, MPT specific inhibitor, prevented the activation of caspase 3, thus showing that diclofenac opened the MPT pore. Treatment of hepatocytes with antioxidants (α-tocopherol, *N*,*N*-dimethylthiourea, superoxide dismutase) were able to prevent caspase cascade activation by diclofenac, revealing that oxidative stress at the mitochondrial level is involved in MPT induction. Finally, the differential cytotoxic and apoptotic effect produced in hepatocytes and non-metabolizing hepatoma cells suggest that CYP-mediated metabolism of diclofenac apoptosis may be related to the apoptotic effect of the drug.

Keywords: Apoptosis; Caspase 3; Diclofenac; Hepatocytes; DNA fragmentation

1. Introduction

Diclofenac, an arylacetic non-steroidal anti-inflammatory drug (NSAID), is frequently prescribed in treating rheumatic diseases and as an analgesic. The use of diclofenac has been associated with a mild to severe hepatotoxicity in a reduced but significant number of patients, suggesting that diclofenac-associated hepatitis may be more common than previously realised (Purcell et al., 1991; Scully et al., 1993). 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 (Raffray and Cohen, 1997; Gill and Dive, 2000). A better understanding of the fundamental mechanisms involved in apoptosis has led to the acceptance of its real significance in pharmacology and toxicology. In fact, it is now believed that apoptosis could be the major form of chemically induced cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury (Alison and Sarraf, 1995; Raffray and Cohen, 1997; Gill and Dive, 2000).

According to recent work, CYP-mediated metabolic activation of the drug and the formation of reactive metabolite(s) is related to diclofenac hepatotoxicity in animals and man (Schmitz et al., 1992; Jurima-Romet et al., 1994; Bort et al., 1999a,b). Since it is acknowledged that apoptosis in the liver plays a central role in the toxicity of many xenobiotics and CYP-generated metabolites (Feldmann, 1997; Gómez-Lechón et al., 2002) the investigation of the potential apoptotic effect of diclofenac in hepatocytes is of toxico-pharmacological interest. Moreover, NSAIDs have been shown to cause apoptosis in several cell lines (Kim et al., 2001; Zhou et

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al., 2001; Ashton and Hanson, 2002; Berman et al., 2002). Therefore, the effect of diclofenac on the early events that trigger apoptosis cascade were evaluated in rat hepatocytes after exposure to sub-cytotoxic concentrations of diclofenac.

Early and late apoptotic markers associated with the pivotal steps of the execution phase have been evaluated in diclofenac-induced apoptosis. The involvement of the mitochondrial permeability transition (MPT), oxidative stress at the mitochondrial level and participation of initiator caspases 8 and 9 in activation of caspase cacade in diclofenac-induced apoptosis have been investigated.

2. Material and methods

2.1. Reagents

Diclofenac sodium salt, superoxide dismutase (SOD), α-tocopherol, cyclosporine A (Cs A) and *N*,*N*-dimethylthiourea (DMTU) were purchased from Sigma (MO, USA). Collagenase was from Roche (Mannheim, Germany). Ac-DEVD-AMC caspase 3 substrate was from PharMingen (San Diego, CA). Caspase-8 and caspase-9 cell permeable inhibitors (IETD-CHO and LEHD-CHO, respectively) were purchased from Calbiochem (Nottingham, UK). Culture media (Ham's F-12, Lebovitz L-15) were from Gibco (Paisley, UK). All other chemicals were of analytical grade.

2.2. Cell cultures

HepG2 cells were cultured in DMEM supplemented with 10% fetal calf serum and containing 50 μg streptomycin/ml and 50 mU penicillin/ml. Cells were routinely seeded in 3.5 cm diameter plates at a density of 14×10^3 cells in 0.1 ml medium per well and used 24 h later (75% monolayer confluence).

2.3. Isolation and culture of rat hepatocytes

Rat hepatocytes were obtained from 200 to 300 g Sprague–Dawley male rats by perfusion of the liver with collagenase as described in detail elsewhere (Gómez-Lechón et al., 2002). Cellular viability of cell suspension was assessed by the trypan blue dye exclusion test, being higher than 90%. 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% BSA and 10^{-8} M insulin. One hour later the medium was changed, and after 24 h the cells were shifted to serum-free hormone-supplemented medium (10^{-8} M dexamethasone and insulin).

2.4. Preparation of stock solutions for treatment of cultures

Stock solutions of 6 mM diclofenac; 50 mg/ml of Cs A, and 500 mM DMTU in phosphate buffered saline; 20 mM α-tocopherol in ethanol; 10.000 U/ml SOD in water, and 5 mM dodecylubiquinone (DBUQ) in DMSO were prepared and diluted with culture medium to obtain the appropriate final concentrations in culture. A 400 mM stock solution of caspase 8 and 9 inhibitors was prepared in DMSO and added to hepatocytes at a final concentration of 100 μM (concentration of solvent in culture medium did not exceeded 0.5%, v/v). Treatment of rat hepatocytes with the compounds were started, after medium renewal, 1 h after cell plating in rat hepatocytes.

2.5. Cytotoxicity assay

Increasing concentrations of the drug in PBS were added to cultures after medium renewal and cells were incubated for a 24-h period. Cytotoxicity was assessed by the LDH release to the culture medium (Gómez-Lechón et al., 2002). The maximal concentration not causing a significant decrease in intracellular LDH (maximal non toxic concentration, MNTC) was determined after several incubations.

2.6. Flow cytometric analysis of DNA

Cell monolayers were kept frozen at −20 °C until the time of DNA fragmentation analysis. Then monolayers were thawed and covered with hypotonic lysis solution (Gómez-Lechón et al., 2002) and kept overnight at 4 °C in order to release nuclei. Propidium iodide (50 μg/ml, final concentration) was added to the nuclei suspension for fluorescent staining of DNA. Nuclei suspensions were incubated for 30 min at room temperature in the dark, prior to the analysis of the nuclei with a DNA content lower than the diploid (2n) in the flow cytometer (Gómez-Lechón et al., 2002).

2.7. Caspase 3 activity

Detached cells were collected by centrifugation at 2500*g* for 3 min and attached cells scrapped off, pooled, and lysed at 4 °C. Then, caspase 3 like activity was measured using the fluorogenic substrate Ac-DEVD-AMC, as described elsewhere with some modifications (Gómez-Lechón et al., 2002). Cellular protein was determined as described (Lowry et al., 1951).

2.8. Statistical analysis

Each experiment was done with at least three different cultures, and the results shown are the mean value ± S.D.

Student *t*-test was performed to analyse the statistitical significance of the results.

3. Results

3.1. Cytotoxicity of diclofenac on cultured hepatocytes and HepG2 hepatoma cell line

Cells were incubated for 24 h with increasing concentrations of diclofenac, and intracellular LDH release, as a result of the breakdown of the plasma membrane and the alteration of its permeability, was evaluated as a cytotoxicity end-point. The maximal concentration of the drug not causing cytotoxic effect (MNTC) was 450 μM.

To see whether cytotoxicity was caused by a direct effect of diclofenac or after its metabolization by hepatocytes, cytotoxicity of diclofenac was also evaluated in the hepatoma cell line HepG2. Hepatoma cells were less sensitive to diclofenac at the assayed concentrations after 24 h of treatment not showing any effect up to 800 μM of the drug.

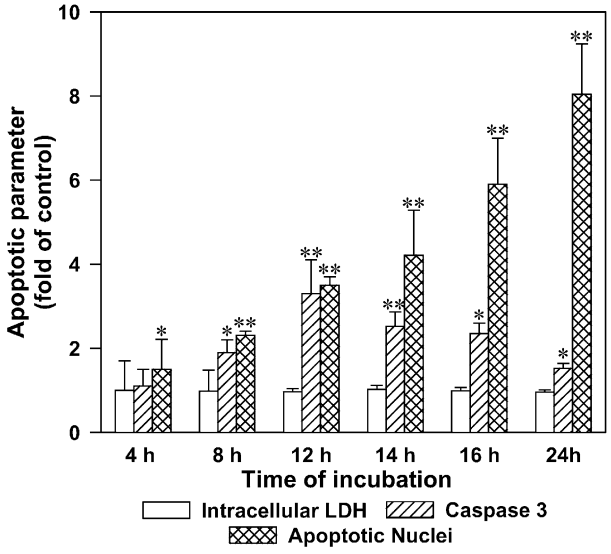

Fig. 1. Evaluation of the time-course of apoptosis in hepatocytes exposed to diclofenac. Cultures were exposed to 350 μM diclofenac and experimental parameters evaluated at time point intervals. The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak as a result of loss of nuclear DNA integrity in a single-parameter histogram of PI fluorescence distribution. Intracellular LDH release, as a result of the breakdown of the plasma membrane and the alteration of its permeability, was evaluated as a cytotoxicity end-point. The time-course of caspase 3 activation was assayed in hepatocytes treated with diclofenac the drug at time intervals up to 24 h, by using the fluorescent substrate Ac-DEVD-AMC. Data are expressed as *n*-fold increases over the control values (hepatocytes non exposed to diclofenac), and represent the mean±S.D. of three different experiments. (*$P<0.5$; **$P<0.01$, comparing results with the respective control at each time of treatment).

3.2. Flow cytometric analysis of apoptosis

Flow cytometric analysis was performed in cultured hepatocytes treated for different periods of time with diclofenac at concentrations which would not cause significant LDH leakage (up to the MNTC). As Fig. 1 shows, a time-dependent increase of apoptotic nuclei with sub-diploid DNA content was found after exposure to 350 μM diclofenac. While up to 8 h of incubation no significant effects were observed, the percentage of apoptotic nuclei increased thereafter, reaching the maximum after 20 h of exposure to the compounds. The dose-dependence of the percentage of apoptotic nuclei was then evaluated after 24 h exposure to increasing concentrations of diclofenac, obtaining a direct relationship in the range 150–450 μM (Fig. 2). Diclofenac did not increase the number of nuclei with sub-diploid DNA content in HepG2 cells exposed at concentrations up to 800 μM of the drug.

3.3. Caspase 3 activation

The caspases are the most important effector molecules in the execution of apoptosis and progression of the caspase activation cascade ends with the activation of caspase 3 in the early apoptosis. Once caspase 3 has been activated the program for cell death is irreversibly activated. Therefore, time-course analysis of caspase 3 activation was examined in hepatocyte cultures treated

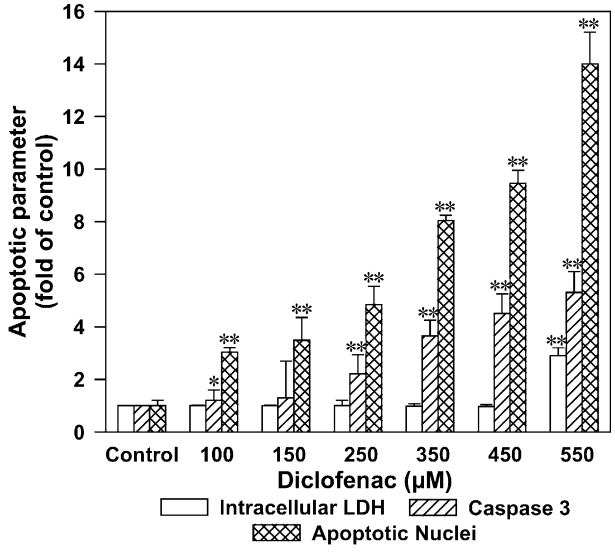

Fig. 2. Dose-dependent effect of diclofenac on apoptosis to hepatocytes. Cultured hepatocytes were exposed to increasing sub-cytotoxic concentrations of diclofenac, for a 12-h period to determine caspase 3 activation and a 24-h period to evaluate cytotoxicity (LDH leakage) and the percentage of apoptotic nuclei with sub-diploid DNA content evaluated by flow cytometry. Data are expressed as *n*-fold increases over the control values (hepatocytes non exposed to diclofenac), and represent the mean±S.D. of three different experiments. (*$P<0.5$; **$P<0.01$, comparing results with the untreated control).

with 350 μM of diclofenac. A time-dependent increase in caspase 3 like activity was observed up to 12 h, then activity reached a plateau or decreased slightly (Fig. 1). A clear dose-dependence of caspase 3 activation in hepatocytes exposed for 12 h to increasing sub-cytotoxic concentrations of diclofenac was observed (Fig. 2). No activation of caspase 3 was found in Hep G2 cells exposed to diclofenac at concentrations up to 800 μM.

Caspase 8 and 9 mediate the cell-receptor and the mitochondria-initiated apoptotic pathways, respectively. To analyse 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 also investigated. To this end hepatocytes were simultaneously treated with diclofenac and 100 μM of caspases 8 and 9 cell permeable inhibitors. As Fig. 3 shows, the inhibition of caspases 8 and 9 resulted in a nearly complete inhibition of caspase 3 activation. The results suggest that both effector caspases are fully involved in the pathway of diclofenac-induced apoptosis.

3.4. Effects of diclofenac at the mitochondrial level

The involvement of the MPT in diclofenac-induced apoptosis was investigated. 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 prevented the activation of caspase 3, suggesting that MPT is involved in diclofenac-induced apoptosis (Fig. 4A).

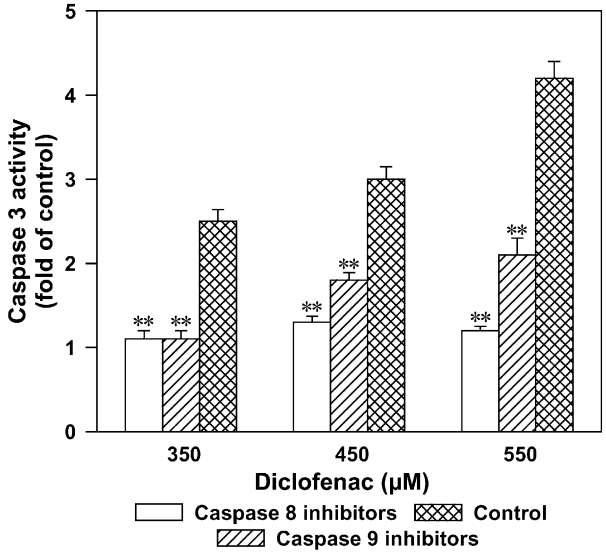

Fig. 3. Effect of inhibitors of effector caspases on the caspase cascade activation by diclofenac. Hepatocytes were exposed simultaneously to 350 μM diclofenac in the presence of 100 μM cell permeable caspases inhibitors of the caspases 8 and 9. The role of the effector caspase inhibitors on caspase 3 activation was evaluated after 12 h of treatment. Data are expressed as *n*-fold increases over the control values (hepatocytes not exposed to diclofenac), and correspond to a representative experiment. (**$P<0.01$, comparing results with the cells non-treated with caspase inhibitors).

3.5. Antioxidants prevent apoptosis caused by diclofenac

The contribution of oxygen-derived free radicals in diclofenac-induced MPT was assessed. Hepatocytes were pre-treated for 15 min with DMTU, a small and permeable scavenger of hydrogen peroxyde and the hydroxyradical, α-tocopherol, and SOD. Then diclofenac was added and caspase 3 activation was evaluated

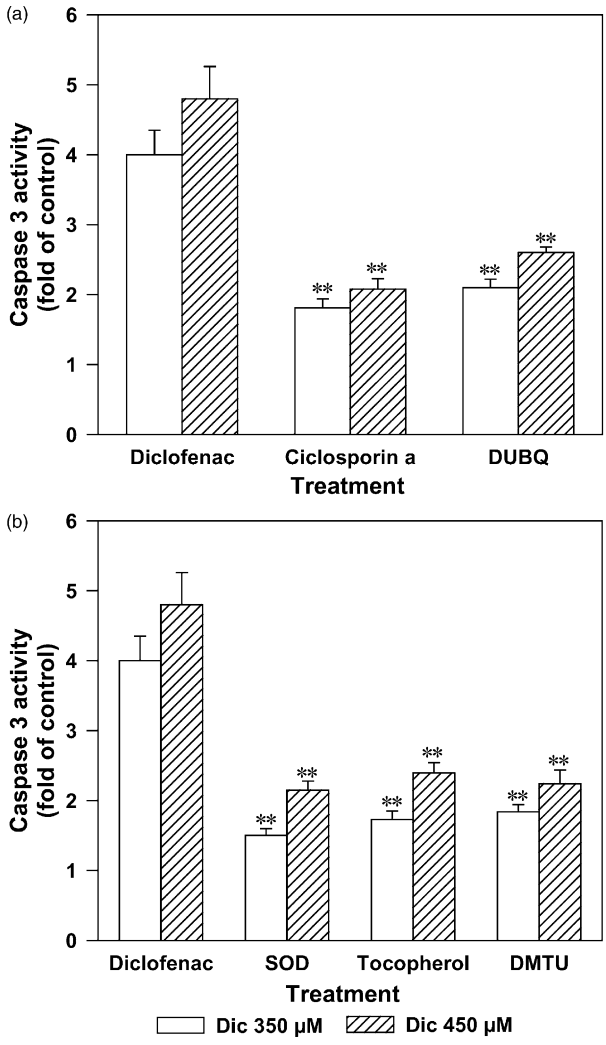

Fig. 4. Effects of diclofenac at the mitochondrial level. (A) The involvement of the MPT in diclofenac-induced apoptosis by evaluating the effect of 5 μM Cs A and 5 μM DUBQ, MPT specific inhibitors, on caspase 3 activation was evaluated. (**$P<0.01$, comparing results with the cells non-treated with MPT specific inhibitors). (B) The effect of antioxidants in diclofenac-induced MPT, was investigated by pretreating hepatocytes with 10 mM DMTU, 3 μM α-tocopherol, and 300 U/ml SOD, then diclofenac was added and caspase 3 activation was evaluated after 12 h of incubation. Data are expressed as *n*-fold increases over the control values, and represent the mean±S.D. of three different experiments. (**$P<0.01$, comparing results with the cells non-treated with antioxidants).

after 12 h of incubation. DMTU, SOD and α-tocopherol protected hepatocytes from the effect of oxygen-derived free radicals impairing caspase 3 activation, thus indicating that oxidative damage is involved in the apoptotic process induced by diclofenac (Fig. 4B).

4. Discussion

NSAIDs have been shown to cause apoptosis in several cell lines (Kim et al., 2001; Zhou et al., 2001; Ashton and Hanson, 2002; Berman et al., 2002), and in particular diclofenac has been described to induce DNA fragmentation in cultured gastric mucosa cells inhibited by caspase inhibitors (Kusuhara et al., 1998). At present apoptosis is considered the major form of chemically induced cell death while necrosis seems to occur in circumstances of severe cell injury (Alison and Sarraf, 1995; Raffray and Cohen, 1997; Gill and Dive, 2000).

We have previously shown that diclofenac-induced hepatotoxicity was associated with the oxidative metabolism of the drug and 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 (Ponsoda et al., 1995; Bort et al., 1999a,b). Similar results showing the key role of MPT and the decrease of ATP in the pathogenesis of diclofenac-induced hepatocyte injury have been recently reported (Masubuchi et al., 2002). MPT is recently focused as a major mechanism for drug-induced hepatocyte necrosis and apoptosis (Lemasters et al., 2002). Therefore, DNA fragmentation, mitochondrial alteration, involvement of oxidative damage, and caspase cascade activation in diclofenac-induced apoptosis, at concentrations not overlapping necrosis, have been investigated in cultured hepatocytes.

We first showed by flow cytometry a time and concentration-dependent increase of apoptotic nuclei with sub-diploid DNA content after exposure to concentrations of diclofenac which did not cause significant LDH leakage. The two pathways of apoptosis include the cell receptor pathway (i.e. Fas), that recruits the effector caspase 8 and mediates transduction of the death signal and the mitochondria-initiated pathway that involves activation of the effector caspase 9 (Maeda, 2000). 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 (Cain, 2000). Once caspase 3 has been activated, there is no way back to normal viability; the program for cell death is irreversibly activated. Our results show a clear time- and concentration-dependent activation of caspase 3 by concentrations of diclofenac not overlapping necrosis (not causing LDH leakage). Trying to clarify the hierarchy of caspase activation by diclofenac, the effect on caspase 3 activation produced by specific inhibitors of the effector caspases 8 and 9 were analysed. The results clearly suggest that both caspases 8 and 9 are fully involved in the pathway of diclofenac-induced apoptosis, since activation of caspase 3 was totally blocked by both inhibitors to a similar extent.

Mitochondria are deeply involved in the regulation of cell death and during the early phase of apoptosis undergo a membrane permeability transition which commits hepatocytes to apoptosis (Pessayre et al., 1999; Higuchi et al., 2001; Pourahmad et al., 2001; Jaeschke et al., 2002). As a consequence, several mitochondrial proteins are released into cytoplasm, after which the apoptotic events become irreversible (Cain, 2000). Moreover, it has been described that inhibiting the MPT ameliorates caspase activation and apoptosis in several cellular systems (Kroemer and Reed, 2000; Kroemer, 2002). Our results show that caspase activation could be inhibited by cyclosporin A and dodecylubiquinone, typical specific blockers of MPT (Karpinich et al., 2002; Waldmeier et al., 2002), which confirms the involvement of MPT in diclofenac-induced apoptosis (Fig. 4a). The results also show that oxygen-derived free radicals largely contribute to the diclofenac-induced apoptosis. In fact, simultaneous incubation of diclofenac with the antioxidants was able to prevent caspase cascade activation by diclofenac (Fig. 4b). Therefore, the mechanism of diclofenac-induced mitochondrial injury seems to involve generation of reactive oxygen species causing oxidant stress to hepatocytes, as has been recently proposed by other authors (Sokol et al., 2001; Masubuchi et al., 2002).

Despite the fact that diclofenac itself is effective in altering mitochondrial function and impairing ATP synthesis, we previously found evidence that toxicity was related to drug metabolism (Ponsoda et al., 1995; Bort et al., 1999a,b). The results of this study are consistent with those previous findings and reveal significant differences in diclofenac apoptotic effects observed in primary cultured hepatocytes and HepG2 cells. These results suggest that these differences may related to the different ability of these cells to metabolize diclofenac, although this hypothesis should be confirmed in further studies.

In conclusion, the results presented in this work indicate that diclofenac induces apoptosis at concentrations not overlapping cell necrosis, apoptosis is likely related to CYP-mediated metabolism by microsomes, oxidative stress at the mitochondrial level seems to be involved in MPT induction and the drug activates at least caspase 3, 8 and 9.

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