Small intestinal ulceration is a frequent and potentially serious condition associated with nonselective cyclooxygenase 1/2 inhibitors (nonsteroidal anti-inflammatory drugs, NSAIDs) including diclofenac (DCF). An initial topical effect involving mitochondria has been implicated in the pathogenesis, but the exact mechanisms of NSAID-induced enteropathy are unknown. We aimed at investigating whether DCF caused enterocyte demise via the mitochondrial permeability transition (mPT) and whether inhibition of critical mPT regulators might protect the mucosa from DCF injury. Cultured enterocytes (IEC-6) exposed to DCF readily underwent mPT-mediated cell death. We then targeted mitochondrial cyclophilin D (CypD), a key regulator of the mPT, in a mouse model of NSAID enteropathy. C57BL/6J mice were treated with an ulcerogenic dose of DCF (60 mg/kg, ip), followed (+ 1 h) by a non-cholestatic dose (10 mg/kg, ip) of the CypD inhibitor, cyclosporin A (CsA). CsA greatly reduced the extent of small intestinal ulceration. To avoid potential calcineurin-mediated effects, we used the non-immunosuppressive cyclosporin analog, D-MeAla3-EtVal4-cyclosporin (Debio 025). Debio 025 similarly protected the mucosa from DCF injury. To exclude drug-drug interactions, we exposed mice genetically deficient in mitochondrial CypD (peptidyl-prolyl cis-trans isomerase F [Ppif2−/−]) to DCF. Ppif-null mice were largely protected from the ulcerogenic effects of DCF, whereas their wild-type littermates developed typical enteropathy. Enterocyte injury was preceded by upregulation of the proapoptotic transcription factor C/EBP homologous protein (Chop). Chop-null mice were refractory to DCF enteropathy, suggesting a critical role of endoplasmic reticulum stress induced by DCF. In conclusion, mitochondrial CypD plays a key role in NSAID-induced enteropathy, lending itself as a potentially new therapeutic target for cytoprotective intervention.

Key Words: Chop; cyclosporin A; Debio 025; diclofenac; enteropathy; mitochondrial permeability transition; Ppif-null knockout mice.

With the advent of the wireless video capsule endoscopy technique, it has recently become evident that small intestinal lesions in patients who are treated with nonsteroidal anti-inflammatory drugs (NSAIDs) occur much more frequently than believed previously (Iddan et al., 2000). For example, damage to the mucosa of the lower intestinal tract was observed in 71% of patients with rheumatoid arthritis, osteoarthritis, or nonselective arthritides who were chronic NSAID users (vs. 10% in non-NSAID users) (Graham et al., 2005). Some of the lesions may be subclinical and asymptomatic, but there is evidence that other complications include ulcers, mucosal bleeding, and even perforations, which are clinically more relevant and even potentially life threatening. Furthermore, small intestinal inflammation and ulceration do not only develop after chronic NSAID use but may also occur after short-term (7 days) therapy (Bjornsson et al., 2008).

Unfortunately, the underlying mechanisms of NSAID enteropathy are not clear, and, in contrast to NSAID-associated gastropathy, there are no effective and clinically approved therapeutic intervention strategies (Higuchi et al., 2009). One way to circumvent the intestinal liability is the use of the more selective cyclooxygenase (COX)-2 inhibitors, which have a lower potential for inducing enteropathy than the traditional nonselective COX-1/2 inhibitors. However, because many of the COX-2 inhibitors have been associated with cardiovascular liability, there is an increasing trend toward re-prescribing classical NSAIDs. In addition, the pathogenesis seems to be multifactorial, further complicating therapeutic approaches. First, combined inhibition of both forms of COX (COX-1 and COX-2) contributes to the pathogenesis (Sigthorsson et al., 2002; Tanaka et al., 2002). Second, topical, off target effects are also involved; they include enterohepatic circulation and delivery via bile of the conjugated NSAIDs to the distal parts of the small intestine, where the drug conjugates are cleaved by intestinal bacteria and the released parent drug taken up by enterocytes (Seitz and Boelsterli, 1998; Treinen-Moslen and Kanz, 2006). Finally, there is also an inflammatory component; increased intestinal permeability
leads to increased release of luminal bacteria–derived lipopolysaccharide that results in activation of toll-like receptor 4 and generation of proinflammatory cytokines that aggravate the damage (Watanabe et al., 2008).

One of the early upstream events induced by NSAIDs in the small intestinal mucosa has been postulated to be mitochondrial damage because of uncoupling (Somasundaram et al., 2000) or endoplasmic reticulum (ER) stress (Somasundaram et al., 1997; Tsutsumi et al., 2004) in enterocytes, leading to activation of mitochondria-mediated cell death. A key mode in mitochondria-mediated apoptosis or necrosis is permeabilization of the outer mitochondrial membrane, leading to mitochondrial release of proapoptotic factors into the cytosol, which can occur through a number of distinct mechanisms. One way this is accomplished is the opening of the mitochondrial permeability transition (mPT) pore, which is primarily an event of the inner mitochondrial membrane. Although the mPT as a phenomenon has been well studied in vitro, the actual molecular nature of the pore is still poorly understood (Halestrap, 2009; Zorzov et al., 2009). A critical regulator of the mPT is cyclophilin D (CypD), a peptidyl-prolyl cis-trans isomerase (PPIase) and molecular chaperone located in the mitochondrial matrix. Chemical inhibition of CypD by specific ligands (e.g., cyclosporin A, CsA) that bind to CypD and prevent it from associating with other pore regulatory components has been shown to protect cells from mPT induction (Halestrap and Davidson, 1990). We hypothesized that if the mPT was indeed a key mechanism in NSAID-induced enteropathy, inhibition of the mPT could afford cytoprotection from drug-induced enterocyte damage. The specific aim of this study was to explore the use of selectively targeting mitochondrial CypD with cyclophilin inhibitors and to assess the degree of mucosal protection from drug enteropathy in a previously characterized mouse model of diclofenac (DCF)-induced enteropathy (Ramirez-Alcantara et al., 2009). We found that inhibition of mitochondrial permeabilization by chemical CypD inhibitors or by targeted deletion of the mitochondrial CypD in peptidyl-prolyl cis-trans isomerase F (Ppif)–null mice significantly protected from DCF-induced small intestinal ulceration, underscoring the mechanistic role of mitochondria in the early phase of the pathogenesis of DCF enteropathy.

MATERIALS AND METHODS

Chemicals. DCF sodium and CsA were obtained from Sigma (St Louis, MO). D-MeAla3-EtVal4-cyclosporin (Debio 025) was kindly provided by DebioPharm (Lausanne, Switzerland). Solutol HS-15 was obtained from BASF (Ludwigshafen, Germany).

Cell culture. IEC-6 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in 75-cm² tissue culture flasks in high glucose and phenol red–free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (ATCC) and 0.1 U/ml of bovine insulin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 10% CO₂ in air because DMEM contains 3.7 g/l of sodium bicarbonate. Cells were passaged every 6 days at a density of 1 × 10⁶ cells per flask. Cell passages 34–38 were used. IEC-6 cells were harvested by trypsinization and seeded at a density of 3 × 10⁶ cells per well on black 96-well plates with clear bottom (BD Biosciences; Franklin Lakes, NJ) or 3 × 10⁵ cells per dish on six-well plates, both thinly coated with Matrigel (Engelbreth-Holm-Swarm mouse sarcoma basement membrane; BD Biosciences).

Assessment of enterocyte injury. Three days after seeding, cells were exposed to serum-free culture medium containing the drugs. DCF or CsA were dissolved in dimethyl sulfoxide (DMSO) and then added to the culture medium to obtain a final DMSO concentration of 0.1% (vol/vol). Lactate dehydrogenase (LDH) release was used as an indicator of cytotoxicity (activity in the culture medium as percentage of total intra- and extracellular activity). LDH activity was determined with a CytoTox-ONE Homogenous Membrane Integrity Assay kit (Promega, Madison, WI).

Measurement of mPT. To measure the mPT, we used the fluorescent marker calcine whose fluorescence can be quenched by a number of metal ions including cobalt (Poncet et al., 2003). The assay is based on the principle that calcine acetoxymethyl ester (AM) is readily taken up by cells and distributes in all subcellular compartments including mitochondria, where it is enzymatically cleaved to release free calcine. Because added Co²⁺ is taken up into the cytosol but cannot be transported across the intact inner mitochondrial membrane, the mitochondrial matrix will remain highly fluorescent. Upon induction of the mPT, Co²⁺ influx will quench the mitochondrial fluorescence, which is taken as an index of mitochondrial inner membrane permeabilization. IEC-6 cells exposed to DCF or solvent were washed with Hank’s balanced salt solution (HBSS) and incubated with 1μM calcine AM and 1mM CoCl₂ for 20 min at 37°C. Cells were then washed and replenished with HBSS. Fluorescence was measured in a Tecan multiwell plate reader at 488 and 520 nm excitation and emission, respectively.

Animals. The study design and all protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Male C57BL/6j mice were obtained from Jackson Laboratory (Bar Harbor, ME) and acclimatized for at least 1 week prior to experimental use. Heterozygous breeder pairs of CypD-knockout mice (Ppiftm1Mmos/J) were obtained from the Jackson Laboratory and a breeding colony was established in our animal facilities (originally, the mice had been kept on a mixed C57BL/6J × 129X1/SvJ background and bred to C57BL/6J for one generation). C/EBP homologous protein (Chop) mouse (B6.129S-Ddit3tm1Dron/J) and their wild-type controls were purchased from Jackson Laboratory. All mice were kept on a 14:10 h light/dark cycle and under controlled environmental conditions. They received mouse chow (Tekland Global Rodent Diet, Harlan Laboratories, Boston, MA) and water ad libitum. The animals were 10–14 weeks old at the time of experimentation.

Genotyping. Male homozygous Ppiftm1Mmos mice and their wild-type littermates were selected for the study. When the mice were 3 weeks old, a 1-mm sample of the tail was cut and DNA was extracted using 1M NaOH. The tails were heated to 94°C, and 1M Tris-HCl was added to neutralize the reaction. PCR analysis was performed, and the DNA was loaded onto agarose gels according to standard procedures. The three primer sequences were AAACCTTCAGTCCAGCTTGCCTCCT, TCTTACACAGTGCATAGGGCTCTCTG, and GCTTTGTATCCACGCTGGCGC.

Treatment. DCF was dissolved in 10% (in PBS) Solutol HS-15 solution and administered ip (60 mg/kg) in a volume of 10 μl/g body weight. All animals were treated at 5 h before the start of the dark cycle. CsA or Debio 025 were dissolved in 10% (in PBS) Solutol HS-15 solution and administered ip (10 mg/kg) 1 h post-DCF administration to minimize drug-drug interactions. Debio 025 is effective in mice after both peroral and ip injection (Tiepolo et al., 2009).

Assessment of small intestinal ulceration. Enteropathy was quantitatively and qualitatively analyzed as described previously (Ramirez-Alcantara et al., 2009). Briefly, mice were sacrificed by CO₂ inhalation. A midline incision was
made, and blood was obtained via cardiac puncture. Blood was transferred to a serum separator tube and allowed to clot; serum was prepared and frozen at −80°C until use for analysis. The entire small intestine (from the gastroduodenal junction to the ileocecal junction) was removed and opened longitudinally along the anti-mesenteric side. The tissue was rinsed in cold PBS and incubated for 15 min in 1mM nitroblue tetrazolium solution containing 16mM HEPESS-NaOH, 125mM NaCl buffer, 3.5mM KCl, and 10mM glucose. Next, the tissues were fixed in 10% zinc formalin for 24 h, washed, and transferred to 70% ethanol. The intestine was metrically divided into four quadrants of equal length and evaluated under ×10 magnification for quantitative and qualitative analyses of ulcers, and the lesions were assigned to the respective quadrants. The following scoring system was used: 0, no apparent lesions; 1, small erosions or ulcers (< 0.1 mm); 2, medium ulcers (0.1–0.8 mm); and 3, large confluent ulcers (> 0.8 mm). The size of the lesions was quantified by ImageJ software.

**Bile flow measurements.** Mice were anesthetized using ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip). An incision was made along the *linea alba* to expose the gallbladder. The animals were kept at constant temperature of 37°C (rectal thermometer) with the use of a heat lamp. A 3-0 silk ligature was tied distal to the gallbladder, which was then punctured with PE10 tubing (Manautou and Chen, 2004). The silk ligature was used to hold the cannula in place. Bile was collected in 0.6 ml preweighed Eppendorf tubes at 10-min intervals for 70 min to determine bile volume and flow (assuming a specific weight of bile of 1.0). A bolus dose of DCF was injected through the tail vein, and CsA was injected ip.

**Clinical chemistry.** Serum activity of alkaline phosphatase (ALP) was measured with a kinetic colorimetric kit (BioAssay Systems, Hayward, CA). Serum total bile salts were quantitatively assessed with a colorimetric method (Diazyme, San Diego, CA).

**Western Blotting.** Equal amounts of protein (40 μg) from cell lysates were reduced, denatured, and separated on 10% SDS-PAGE gels. The resolved proteins were transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA) and probed with anti-Chop antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution) at 4°C. The bound antigen-antibody complexes were visualized after incubation with horseradish peroxidase–conjugated secondary antibody using enhanced chemiluminescence (Millipore, Billerica, MA). β-Actin was used to control for equal protein loading.

**Statistical analysis.** Ulcer/erosion numbers and ALP activity were expressed as mean ± SEM with a *p* value of ≤ 0.05 to indicate statistical significance. Under normal distribution, a standard ANOVA was used followed by Dunnett’s test for multiple comparisons versus the control group. When normality failed, a Kruskal-Wallis one-way ANOVA on ranks was used followed by Dunn’s test for multiple comparison versus the control group.

**RESULTS**

**DCF Induces Mitochondrial Permeabilization in Cultured Murine Enterocytes**

To determine whether the mPT might be involved as an initial mechanism leading to cell death in DCF-exposed enterocytes, we first used an established in *vitro* approach, as mPT pore opening is difficult to monitor in intact animals. Cultured murine enterocytes (IEC-6 cells) were exposed to various concentrations of DCF for up to 48 h. We found that 100μM DCF caused significantly increased LDH release as compared with solvent controls at 24 h, but not at earlier time points (Fig. 1A). Similar to other cell types, DCF-induced cytotoxicity in enterocytes was prevented by low concentrations (1μM) of CsA, suggesting a role of the mPT. We then assessed the extent of mitochondrial inner membrane permeabilization with the calcein/Co2+ fluorescence imaging method (Poncet et al., 2003). We found that after DCF exposure for 6 h, the overall calcein fluorescence was decreased as compared with solvent controls (Fig. 1B). This was likely a consequence of enhanced quenching of the fluorescence by Co2+ following mitochondrial inner membrane permeabilization that allowed access of Co2+ to the matrix. Addition of CsA fully prevented fluorescence quenching, suggesting an involvement of the CsA-sensitive mode of the mPT in enterocyte injury.

**Low, Non-Cholestatic Doses of CsA Protect from DCF-Induced Enteropathy in Mice**

To determine whether the mPT is involved in DCF-induced enteropathy in *vivo*, mice were injected with a single ulcerogenic dose of DCF (60 mg/kg, ip) (Ramirez-Alcantara et al., 2009) in the presence or absence of CsA (10 mg/kg, ip). CsA was given 60 min post-DCF to minimize interference with

**FIG. 1.** Mitochondrial effects of DCF in cultured murine enterocytes (IEC-6). (A) Time course of LDH release following exposure of cells to 100μM DCF. Data are mean ± SEM (*n* = 3 independent experiments with four to nine wells for each treatment group). *p < 0.05 versus solvent control. *p < 0.05 versus DCF alone (24 h). (B) Opening of the mPT pore. Cells were exposed to 100μM DCF in the presence or absence of 1μM CsA and loaded with calcein/Co2+ as described in the Materials and Methods section. Fluorescence (488 excitation and 520 nm emission) was recorded, and data were normalized. Data are mean ± SEM (*n* = 6–12 wells) and represent one experiment typical of two. *p < 0.05 versus solvent control, *p < 0.05 versus DCF alone.
uptake and disposition of DCF. DCF is rapidly taken up and excreted in mice; in fact, peak drug plasma concentrations in mice following an oral dose of 5 mg/kg DCF were attained 15 min post-administration (Lagas et al., 2010). Similarly, in rats, peak bile levels of parent and conjugated DCF were attained within 1 h (Reuter and Wallace, 1999). In addition, because CsA has been shown to be cholestatic when given at high doses (Bouis et al., 1990; Kukongviriyapan and Stacey, 1991) and because enterohepatic circulation plays an important role in the pathogenesis of enteropathy, it was critical to first ascertain that the selected dose of CsA was not inhibiting bile flow in mice. Therefore, bile was collected in gallbladder-cannulated mice at 10-min intervals for a total duration of 70 min. We found that there was no significant difference in bile flow between the CsA-treated mice and the vehicle control group (Fig. 2A). To further ascertain whether CsA had any inhibitory effects on the hepatobiliary excretion of cholephilic compounds, we quantitated total serum bile acids, a sensitive indicator of cholestasis (Azer and Stacey, 1994). We found that at 6 h post-CsA injection, there was no significant difference in bile acid levels between the two groups (Fig. 2B). Collectively, these data confirm that CsA at a dose of 10 mg/kg was not cholestatic.

A single dose of DCF induced small intestinal ulceration (Fig. 3A), the extent of which was maximal at 18 h post-dose, as previously described (Ramirez-Alcantara et al., 2009). In contrast, vehicle-treated mice did not exhibit any apparent ulceration. The distribution of the ulcers among the four quartiles typically displayed no ulceration in the first quartile and very few ulcers or small erosions in the second quartile, whereas in the third and fourth quartiles, large and confluent ulcers were abundant (Figs. 3B and 3C). In contrast, in the animals that received both DCF and CsA, there was a significant decrease in both the number of ulcers and the degree of mucosal tissue damage (Figs. 3A–C; for definition of the scores see Materials and Methods section). The distribution of ulcers across the jejunum/ileum did not change in the combined DCF/CsA-treated mice, but the number of large ulcers (score ≥ 2) was greatly reduced, and the tissue lesions were predominantly small erosions. These findings were confirmed by quantitative determination of serum ALP activity, a clinical-chemical biomarker of small intestinal injury (Ramirez-Alcantara et al., 2005, 2009). Although DCF alone significantly attenuated ALP activity, as expected, we found that CsA fully prevented this decrease in ALP activity (Fig. 3D). Collectively, these data indicate that CsA afforded significant tissue protection against the ulcerogenic effects of DCF in the small intestine, suggesting that the mPT may be involved in mediating enterocyte cell death in vivo.

Debio 025, a Novel CypD Inhibitor, Affords Protection Against DCF-Induced Enteropathy without Involvement of the Calcineurin Pathway

Because CsA not only binds to mitochondrial CypD but also to the cytosolic CypA and other cyclophilins in the low nanomolar range (Bergsma et al., 1991), it is possible that CsA’s protective effects were mediated through a mitochondria-independent pathway. Specifically, the CsA-CypA complex can bind to and inhibit calcineurin, a Ca$^{2+}$/calmodulin-activated serine/threonine protein phosphatase that has been mechanistically implicated in the immunosuppressive effects of CsA (Liu et al., 1991). To circumvent potential calcineurin-dependent pathways, we used a novel cyclosporin analog and specific CypD inhibitor, Debio 025, whose complex with CypA does not inhibit calcineurin and which, therefore, is nonimmunosuppressive (Tiepolo et al., 2009). We found that Debio 025, similar to CsA, afforded protection against DCF-induced cell injury in cultured IEC-6 cells (data not shown). Next, mice were treated with DCF followed by an ip injection of Debio 025 (10 mg/kg, ip) 1 h post-dose. Gross pathologic and biochemical analysis of the small intestinal damage revealed that Debio 025 afforded protection to a similar degree as CsA (Fig. 4A). Specifically, DCF-exposed mice cotreated with Debio 025 exhibited a significantly smaller number of ulcers in quartiles three and four, and the number of large ulcers (score > 2) was greatly decreased as compared with DCF-alone–treated mice (Figs. 4B and 4C). Consistent with these data, serum ALP activity was restored to normal control values after combined DCF/Debio 025 treatment (Fig. 4D). Collectively, these data indicate that Debio 025 protected from DCF-induced enteropathy through calcineurin-independent pathways and suggests that CypD plays a critical role in drug-induced small intestinal ulceration.
CypD-Deficient Mice (Ppif<sup>−/−</sup>) are Resistant to DCF-Induced Enteropathy

To exclude other CypD-independent pathways through which Debio 025 might confer protection (e.g., toxicokinetic interactions) and to further confirm a key role of CypD in DCF enteropathy, we used a genetic approach for eliminating CypD. Recently, gene targeting of Ppif (coding for mitochondrial CypD) has produced viable mice featuring homozygous deficiency in mitochondrial CypD by a number of independent laboratories (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Therefore, we subjected littermates of B6.129 wild-type mice (Ppif<sup>+/+</sup>) and homozygous knockout mice (Ppif<sup>−/−</sup>) to the standard treatment using a single ulcerogenic dose of DCF (60 mg/kg, ip) or vehicle. After 18 h, gross pathological examination of the small intestinal mucosa revealed that the wild-type mice had developed enteropathy to a similar extent as the previously used genotype (C57BL/6J), suggesting a comparable sensitivity of the two genetic strains (Fig. 5A). In contrast, the knockout mice exhibited little mucosal injury across the entire length of the jejunum/ileum, with the lesions being small erosions, and no large ulcers in quartile four (Figs. 5A–C). Again, serum ALP activity correlated well with the pathology scoring; Ppif<sup>−/−</sup> mice treated with DCF exhibited normal values for serum ALP activity, whereas the wild-type Ppif<sup>+/+</sup> mice exposed to DCF had significantly reduced ALP activity (Fig. 5D). Taken together, these results demonstrate that genetic inactivation of the mitochondrial CypD greatly attenuated the ulcerogenic effects of DCF, strongly suggesting a role for the mPT in enterocyte demise in vivo.

DCF Induces an ER Stress Response in Murine Enterocytes

Although we provide evidence that DCF induced CypD-dependent mPT in enterocytes, the more upstream mechanisms that lead to the induction of the mPT are less clear. It has been previously demonstrated that DCF induces an ER stress response in gastric mucosal cells that is directly related to cell death (Tanaka et al., 2005). One of the hallmarks of this pathway that links ER stress and mitochondria-mediated cell death is the upregulation of the transcription factor Chop (Gadd153) (Tanaka et al., 2005; Tsutsumi et al., 2004). To explore whether DCF causes Chop induction in murine small intestinal epithelial cells, we exposed IEC-6 cells to DCF (100μM) for up to 24 h and determined the increases in Chop protein levels by Western immunoblotting (Fig. 6). We found that Chop levels were increased as early as 3 h, gradually being...
more abundant over time. These data confirm that DCF causes an ER stress response that may be a mechanistic link to the induction of the mPT (Wu et al., 2010).

Genetic Deletion of Chop Protects from DCF-Induced Enteropathy

To ascertain whether the DCF-induced increase in Chop expression was causally involved in the pathogenesis of enterocyte injury, we used a genetic approach with Chop-null mice. Male Chop−/− mice and their wild-type controls received a single ulcerogenic dose of DCF (60 mg/kg, ip) or vehicle, and the extent of small intestinal injury was evaluated at 18 h post-dose. We found that both the number and size of ulcers were significantly reduced and that serum ALP activity was at normal levels in Chop-null mice (Fig. 7), whereas their wild-type controls exhibited typical enteropathy. These data indicate that genetic ablation of Chop largely protects against DCF-induced small intestinal injury, suggesting that ER stress-mediated signaling through the Chop pathway is a critical upstream event in mitochondria-mediated enteropathy.

DISCUSSION

The aim of this study was to explore the mechanistic role of the mitochondrial matrix protein CypD in DCF-induced small intestinal ulceration and to assess the effects of inhibition of this regulator of the mPT as a potential cytoprotective strategy. We found that selective inhibition of mitochondrial CypD significantly protected from an ulcerogenic dose of DCF in mice and that these protective effects were mediated through the mPT rather than through a calcineurin-mediated pathway.

These conclusions were derived from a number of experimental findings. First, Debio 025, similar to CsA, afforded protection against DCF injury. Debio 025 is a novel cyclophilin inhibitor that, in contrast to CsA, does not inhibit calcineurin activity (determinant for the immunosuppressive activity of CsA). Second, DCF treatment did not induce small intestinal ulceration in Ppif-null mice, which are genetically deficient in CypD but which have an intact calcineurin pathway. Taken together, these data strongly suggest that mitochondrial CypD-regulated mPT is involved in DCF enteropathy and that inhibition of CypD can be a potentially new target to protect against NSAID-induced enteropathy.

CypD is the only mitochondrial form of a total of approximately 20 members of the cyclophilin protein family of PPIases in humans. Other cyclophilin forms include CypA, which is abundant in the cytosol and which is the major target and mediator for immunosuppressant activity of CsA. The CsA-CypA complex binds to and inhibits calcineurin,
a Ca\(^{2+}\)/calmodulin-activated serine/threonine protein phosphatase (Liu et al., 1991). Other forms (CypB and CypC) are abundant in the ER where they play a role in protecting from ER stress (Kim et al., 2008). One important function of the mitochondrially located CypD is to participate in the regulation of apoptotic and necrotic cell death mediated through the mPT (Halestrap et al., 2002; Leung and Halestrap, 2008; Zorov et al., 2009). In line with this concept are the findings that Debio 025, a high-affinity ligand for CypD, inhibited the mPT in brain mitochondria (Hansson et al., 2004). On the other hand, Debio 025 is approximately 3500-fold less potent than CsA in inhibiting calcineurin-mediated IL-2 production in Jurkat cells (Gallay, 2009), indicating minimal immunosuppressant effects at therapeutically relevant doses. Therefore, it is unlikely that the protective effects of Debio 025 on DCF-induced enteropathy were because of calcineurin-mediated effects on immune cells; instead, it is likely that Debio 025 prevented DCF-induced mitochondrial permeabilization. This was confirmed by our findings that CsA protected from DCF-induced mPT in cultured murine enterocytes.

How exactly DCF induces the mPT in enterocytes is not clear. However, data from in vitro models suggest that uncoupling or ER stress may be involved (Somasundaram et al., 1997; Tsutsumi et al., 2004), which would result in oxidant stress and increases in [Ca\(^{2+}\)]\(_i\) (Tanaka et al., 2005). A key mediator that links ER stress–induced increases in [Ca\(^{2+}\)]\(_i\) and mitochondrial injury is the transcription factor Chop (Tsutsumi et al., 2004). Indeed, both cultured guinea pig gastric mucosal cells transfected with a dominant-negative form of Chop and peritoneal macrophages isolated from Chop-null mice were protected from NSAID-induced apoptosis. Furthermore, a recent study with human immunodeficiency virus protease inhibitor–induced enteropathy in mice provided compelling evidence for a causal role of Chop in drug enteropathy (Wu et al., 2010). Here, the demonstration that, in cultured murine enterocytes, the ER stress response marker Chop was upregulated by DCF at early time points and, more importantly, that genetic deletion of Chop protected from DCF toxicity in vivo is in line with the original reports on the consequences of genetic deletion of the Ppif gene in mice. These studies had revealed that hepatocytes from the homozygously knockout mice were protected from Ca\(^{2+}\)- or oxidant stress–induced necrotic cell death but that they were not protected from Bcl-2 protein–regulated cell death (Baines

FIG. 5. Protection from DCF-induced small intestinal ulceration in CypD-knockout mice (Ppif\(^{-/-}\)). Both wild-type (Ppif\(^{+/+}\)) and Ppif\(^{-/-}\) mice were administered a single ulcerogenic dose of DCF (60 mg/kg, ip) and sacrificed 18 h later. (A) Macroscopic evaluation of the mucosa in the third quartile of the small intestine in both genotypes. Note the complete absence of large ulcers and the presence of small erosions in the knockout mice. (B) Quantitative analysis and distribution of ulcers across the four quartiles. Each data point represents mean ± SEM for five animals per group. *p < 0.05 versus wild-type mice. (C) Distribution of large ulcers (score > 2). Each data point represents mean ± SEM for five to six animals per group. *p < 0.05 versus wild-type mice. (D) Serum ALP activity. Each data point represents mean ± SEM for five to six animals per group. *p < 0.05 versus wild-type mice.
et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Other, more recent reports have confirmed that CypD inhibitors or deficiency of CypD do not completely block the mPT but rather desensitize the mitochondria to mPT-inducing stimuli and/or unmask an inhibitory site for Pi (Basso et al., 2008).

These data provide new evidence to further support the concept that mitochondrial injury in enterocytes is an upstream event in the pathogenesis of NSAID-induced enteropathy. Thus, the initial “topical” phase of DCF-induced intestinal injury involves ER stress and mitochondrial stress (Somasundaram et al., 1997, 2000; Tsutsumi et al., 2004), resulting in mPT-mediated enterocyte demise. This is likely a consequence of the high luminal concentrations of DCF to which the mucosa is exposed (Seitz and Boelsterli, 1998). In fact, the acyl glucuronide, which is the major transport form for the hepatobiliary excretion of DCF, is cleaved in the distal parts of the jejunum by bacterial β-glucuronidase and, to a lesser extent, nonspecific carboxylesterases, and the released aglycone is taken up by the enterocytes (Boelsterli and Ramirez-Alcantara, 2010; Dickinson and King, 2001). This first hit (mitochondria-mediated enterocyte injury) is followed by an increase in epithelial permeability, which leads to an inflammatory response and further aggravation of mucosal damage (Watanabe et al., 2008) and which can be considered a second hit. Blocking the proximal steps can fully prevent the development of the downstream events. The critical involvement of initial mitochondrial damage has also been demonstrated recently in another murine model of NSAID enteropathy, where COX-2 inhibitors were administered to cytosolic phospholipase A2–knockout mice (Montrose et al., 2010). In this model, global expression profiling revealed that enteropathy was preceded by mitochondrial dysfunction. Based on those data, the putative role of COX inhibition in the pathogenesis of enteropathy has to be revisited because it was reported that small intestinal injury was not due to the absence of prostaglandins per se but rather to disturbance of mitochondrial function. For example, aspirin, which decreased enterocyte prostaglandin levels to a similar extent as the COX inhibitors, did not cause any small intestinal ulceration (Montrose et al., 2010).

In conclusion, this study demonstrates that chemical inhibition or genetic deletion of mitochondrial CypD protects against DCF-induced small intestinal ulceration in mice. In view of recent advances in the development of novel cyclophilin inhibitors (Gallay, 2009), the present data suggest novel potential therapeutic applications against drug enteropathy. Debio 025, the first oral non-immunosuppressive cyclophilin inhibitor that entered clinical trials is currently evaluated as a potential drug against hepatitis C virus infection. It remains to be seen whether these new CypD inhibitors that do not target

![FIG. 6. Early induction by DCF of Chop in cultured murine enterocytes. IEC-6 cells were exposed to DCF (100µM) or solvent (0.1% DMSO), and cell lysate proteins were resolved by Western blotting. (A) Time course. Membranes were probed with anti-Chop (1:1000, overnight at 4°C) followed by incubation with second antibody (1:8000, 2 h at room temperature). (B) Densitometric analysis of protein. β-Actin was used as loading control.](https://academic.oup.com/toxsci/article-abstract/118/1/276/1661278/fig6?highres=1)
calcineurin and, hence, are devoid of immunosuppressive effects will potentially find clinical applications in potential therapies against NSAID enteropathy.

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