



Metformin protects against diclofenac-induced toxicity in primary rat hepatocytes by preserving mitochondrial integrity via a pathway involving EPAC

Fabio Alejandro Aguilar Mora^a, Nshunge Musheshe^e, Johanna C. Arroyave Ospina^a, Yana Geng^a, Juan M. Soto^b, José A. Rodrigo^b, Tatiana Alieva^b, Manon Buist-Homan^{a,f}, Frank Lezoualc'h^c, Xiaodong Cheng^d, Martina Schmidt^e, Han Moshage^{a,f,*}

^a Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

^b Department of Optics and Faculty of Physical Sciences, Complutense University of Madrid, Spain

^c Inserm UMR-1048, Institut des Maladies Métaboliques et Cardiovasculaires, Univ Toulouse Paul Sabatier, Toulouse, France

^d Department of Integrative Biology & Pharmacology, Texas Therapeutics Institute, University of Texas Health Science Center at Houston, Houston, TX, USA

^e Department Molecular Pharmacology, Groningen Research Institute of Pharmacy, Groningen Research Institute for Asthma and COPD, GRIAC, University Medical Center Groningen University of Groningen, Groningen, The Netherlands

^f Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

ARTICLE INFO

Keywords:

CAMP
Diclofenac
Hepatocyte
Apoptosis
EPAC
Metformin
Mitochondria

ABSTRACT

Background and purpose: It has been shown that the antidiabetic drug metformin protects hepatocytes against toxicity by various stressors. Chronic or excessive consumption of diclofenac (DF) - a pain-relieving drug, leads to drug-induced liver injury via a mechanism involving mitochondrial damage and ultimately apoptotic death of hepatocytes. However, whether metformin protects against DF-induced toxicity is unknown. Recently, it was also shown that cAMP elevation is protective against DF-induced apoptotic death in hepatocytes, a protective effect primarily involving the downstream cAMP effector EPAC and preservation of mitochondrial function. This study therefore aimed at investigating whether metformin protects against DF-induced toxicity via cAMP-EPACs.

Experimental approach: Primary rat hepatocytes were exposed to 400 $\mu\text{mol/L}$ DF. CE3F4 or ESI-O5 were used as EPAC-1 or 2 inhibitors respectively. Apoptosis was measured by caspase-3 activity and necrosis by Sytox green staining. Seahorse X96 assay was used to determine mitochondrial function. Mitochondrial reactive oxygen species (ROS) production was measured using MitoSox, mitochondrial MnSOD expression was determined by immunostaining and mitochondrial morphology (fusion and fission ratio) by 3D refractive index imaging.

Key results: Metformin (1 mmol/L) was protective against DF-induced apoptosis in hepatocytes. This protective effect was EPAC-dependent (mainly EPAC-2). Metformin restored mitochondrial morphology in an EPAC-independent manner. DF-induced mitochondrial dysfunction which was demonstrated by decreased oxygen

List of abbreviations and definitions in alphabetical order: AC, Adenyl cyclase; ADR, Adverse drug reaction; AMPK, AMP-activated protein kinase; ATP, Adenosine triphosphate; AUF, Arbitrary units of fluorescence; cAMP, Cyclic adenosine monophosphate; CNG, Cyclic nucleotide-gated; CTCF, Corrected total cell fluorescence; DF, Diclofenac; DILI, Drug-induced liver injury; DISC, Death-inducing signaling complex; ECAR, Extra-Cellular Acidification Rate; EPAC, Exchange Protein Directly Activated by cAMP/cAMP-regulated guanine nucleotide exchange factors; EM, Emission; EX, Excitation; GSH, Glutathione; GSSG, Glutathione disulfide; H₂O₂, Hydrogen peroxide; IntDen, Integrated density; JNK, NH₂-terminal kinase; PKA, Protein kinase A; MGV, Mean gray value; MMP, Mitochondrial membrane polarization; MPTP, Permeability transition pore; NSAID, Non-steroidal anti-inflammatory drug; OCR, Oxygen consumption rate; OCTs, Organic cation transporters; OXPHOS, Oxidative phosphorylation system; O₂•⁻, Mitochondrial superoxide anion generation; PC-ODT, Partially coherent optically diffraction tomography; PDE, Phosphodiesterases; PGE₂, Prostaglandin E₂; Popdc, Proteins Popeye domain; P13-K/Akt, Phosphoinositide-3-kinase; RI, Refractive index; ROS, Reactive oxygen species; sAC, Soluble adenyl cyclase; 4-OH, 4'-hydroxydiclofenac; 5-OH, 5'-hydroxydiclofenac.

* Corresponding author at: University Medical Centre Groningen, Department of Gastroenterology and Hepatology Groningen, NL 9713 GZ, Groningen, The Netherlands.

E-mail addresses: f.a.aguilar.mora@umcg.nl (F.A.A. Mora), n.musheshe@rug.nl (N. Musheshe), carroarroyave87@gmail.com (J.C. Arroyave Ospina), y.geng@umcg.nl (Y. Geng), juansoto@ucm.es (J.M. Soto), jarmar@ucm.es (J.A. Rodrigo), talieva@ucm.es (T. Alieva), m.buist-homan@umcg.nl (M. Buist-Homan), Frank.Lezoualc'h@inserm.fr (F. Lezoualc'h), Xiaodong.Cheng@uth.tmc.edu (X. Cheng), m.schmidt@rug.nl (M. Schmidt), a.j.moshage@umcg.nl (H. Moshage).

¹ Postal address: University Medical Centre Groningen, Dept. Gastroenterology and Hepatology Groningen, NL 9713 GZ; Groningen, The Netherlands.

<https://doi.org/10.1016/j.bioph.2021.112072>

Received 26 April 2021; Received in revised form 23 July 2021; Accepted 17 August 2021

Available online 28 August 2021

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consumption rate, an increased ROS production and a reduced MnSOD level, were all reversed by metformin in an EPAC-dependent manner.

Conclusion and implications: Metformin protects hepatocytes against DF-induced toxicity via cAMP-dependent EPAC-2.

1. Introduction

Metformin, a biguanide initially identified in the herb *Galega officinalis*, is often used as the first-line drug to treat type 2 diabetes symptoms due to its capacity to lower both basal glucose and postprandial glucose. The use of metformin is not limited to its glucose-lowering effects. Previously it was shown that metformin protects against various stressors, including bile acids [67], superoxide anions [9] and saturated fatty acids [18]. Moreover, it was also shown that this protective effect of metformin is (partially) due to preservation of mitochondrial integrity and function. The exact mechanism of the protective effect of metformin against various stressors remains to be elucidated.

Diclofenac (DF) is an over-the-counter, non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, antipyretic and analgesic properties. Although diclofenac consumption is well-tolerated, overconsumption and chronic consumption can be toxic to the liver and lead to severe hepatic adverse drug reactions (ADRs) [7,37].

The toxicity of DF is due to the accumulation of highly reactive metabolites, 4-OH and 5-OH. These metabolites subsequently react with glutathione (GSH) and NAD(P)H and are excreted via kidneys and urine. However, when GSH/NAD(P)H is depleted, these reactive metabolites react with macromolecules inside the cell, causing cell injury and eventually cell death [7].

Both 4-OH and 5-OH can induce mitochondrial damage by increasing the mitochondrial membrane permeability transition pore (MPTP). This leads to the depolarization of the mitochondrial membrane and the inhibition of adenosine triphosphate (ATP) synthesis [57,62], ultimately inducing apoptotic death of hepatocytes [16].

3',5'-cyclic adenosine monophosphate (cAMP) is a secondary messenger synthesized by the plasma membrane-bound enzyme adenylyl cyclase (AC) using ATP, and by soluble adenylyl cyclase (sAC) [65]. cAMP is degraded by phosphodiesterases (PDE) [25] thereby terminating cAMP downstream effects.

Via its effectors EPAC and PKA, cAMP is implicated in various processes, including but not limited to inhibition of apoptosis [3,23], increased cell proliferation in response to partial hepatectomy [53] and protection against several noxious stresses in the liver, e.g., bile acids [6,12,17,27].

Primary rat hepatocyte mitochondria express both EPAC1 and EPAC2 [3]. It was demonstrated in a previous study that elevating cAMP levels prevented DF-induced toxicity in hepatocytes, and the protective effect was mediated by EPACs partly by preservation of mitochondrial integrity [3].

Therefore, the present study aimed at investigating whether metformin protects against diclofenac-induced toxicity and, in the affirmative, whether the protective effect is via the cAMP-EPAC pathway.

2. Materials and methods

2.1. Animals and compliance with requirements for studies using animals

Specified pathogen-free male Wistar rats (220–250 g) aged 5–8 weeks were purchased from Charles River Laboratories Inc (Wilmington, MA, USA). Rats were housed in polypropylene cages at room temperature (25 ± 2 °C) with standard bedding, regular cycle (12 h light/12-hour dark), and free access to standard laboratory chow and water.

All experiments were performed according to the Dutch law on the welfare of laboratory animals (The Animal act 2011) and permission no

16778-01-002 of the local Committee for care and use of laboratory animals of the University of Groningen.

2.2. Rat hepatocyte isolation

Primary rat hepatocytes were isolated using the two-step collagenase perfusion method under anesthesia (isoflurane 5%, followed by 60 mg/kg ketamine (Alfasan, Netherlands BV) combined with 0.25 mg/kg medetomidine (Orion Pharma, Finland)) as described previously [41]. After isolation, hepatocyte viability was assessed using trypan blue, and only hepatocyte isolations with viability above 85% were used. Hepatocytes were plated and cultured in collagen-coated (PureCol® Advanced BioMatrix) plates in Williams E medium (Thermo Fisher Scientific, Waltham, MA, USA Cat N32551020) supplemented with 50 µg/ml gentamycin (Thermo Fisher Scientific), 50 nmol/L dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands), 5% fetal calf serum (Thermo Fisher Scientific) and penicillin, streptomycin and fungizone for 4 h at 37 °C in an atmosphere containing 5% CO₂ to allow cell attachment.

2.3. Experimental design

Experiments started after the attachment period of 4 h. As a model of DILI-induced apoptosis, we used diclofenac (2-[(2,6-dichlorophenyl) amino] benzene acetic acid sodium salt (Sigma-Aldrich Zwijndrecht, the Netherlands). This model has previously been described and induces predominantly apoptotic cell death in primary rat hepatocytes. The optimal concentration for the experiments was defined previously [3,16,48]. Apoptotic cell death determined by caspase-3 activity assay peaked at 400 µmol/L and 12 h exposure. These conditions did not lead to necrotic cell death assessed by Sytox green staining. The optimal concentration for metformin (Sigma-Aldrich Zwijndrecht, the Netherlands) was defined by performing a dose-response curve against diclofenac (400 µmol/L for 12 h). Metformin (0.5–4 mmol/L) was added 30 min before diclofenac addition. One mmol/L metformin was then chosen since it was the lowest concentration that significantly reduced caspase-3 activity in DF-treated hepatocytes and corresponded to the concentration used previously [18] (Fig. 1A,B). Experiments with metformin and EPAC inhibitors were performed in fresh Williams E medium (Thermo Fisher Scientific, Waltham, MA, USA Cat N32551020) supplemented with 50 µg/ml gentamycin (Thermo Fisher Scientific), 50 nmol/L dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands), penicillin, streptomycin and fungizone. CE3F4 (5,7-Dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2H)-quinoline carboxaldehyde) was used as EPAC-1 inhibitor as previously described [11] and ESI-O5 (1,3,5-Tri-methyl-2-[(4-methylphenyl) sulfonyl]-benzene, mesityl (4-methylphenyl) sulfone) was used as EPAC-2 inhibitor as previously described [59]. Samples were randomly labeled to avoid group or treatment identification until after the analysis was completed.

2.4. Caspase 3 enzyme activity assay

Caspase-3 enzyme activity was assayed as described previously [50]. Fluorescence was quantified using a spectrofluorometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The arbitrary units of fluorescence were normalized to the control condition and expressed as fold induction vs. control to avoid an unwanted source of variation. The results represent at least three independent hepatocyte isolations (n = 3) with duplicate conditions.

Mann-Whitney u test was used to determine statistical significance ($n = 6$). All samples were included in the analysis.

2.5. Sytox green nuclear staining

Rupture of the plasma membrane distinguishes necrotic from apoptotic cell death. To estimate necrotic cell death, hepatocytes were incubated for 15 min with Sytox Green (Thermo Fisher Scientific®) nucleic acid stain. Sytox Green only penetrates necrotic cells with compromised plasma membranes but does not cross the membrane of viable cells or apoptotic bodies. Hepatocytes exposed to 5 mmol/L hydrogen peroxide (H_2O_2) for 24 h were used as a positive control for necrosis [8]. Fluorescent nuclei were visualized in three randomly selected areas from three independent hepatocyte isolations ($n = 9$) for each condition. The images were obtained on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems, Amsterdam, The Netherlands) using a 10X objective. Quantification of the necrotic cells was performed by determining the % of cell viability with the ImageJ

software. Quantification was performed in three biological replicates with three or five technical replicates of each image and counting the viable cells (non-stained nuclei) vs. necrotic cells (green stained nuclei) [8] the raw percentage of cell viability of selected conditions was obtained.

2.6. Mitochondrial fusion and fission rates

Mitochondrial morphology was assessed using the 3D refractive index (RI) reconstruction based on partially coherent optically diffraction tomography (PC-ODT). RI works as an intrinsic imaging contrast for 3D marker-free cell imaging, widely used in biomedical science for its advantages over traditional imaging methods [10,20]. The RI tomogram can provide useful biological information such as shape, density, volume, dry mass concentration, and mitochondrial morphology without the requirement of using fluorescent labels [44,54,55]. Briefly, primary rat hepatocytes (4.5×10^5) were seeded on glass coverslips in 12-well plates. After 4 h of attachment and 12 h after incubation, the culture

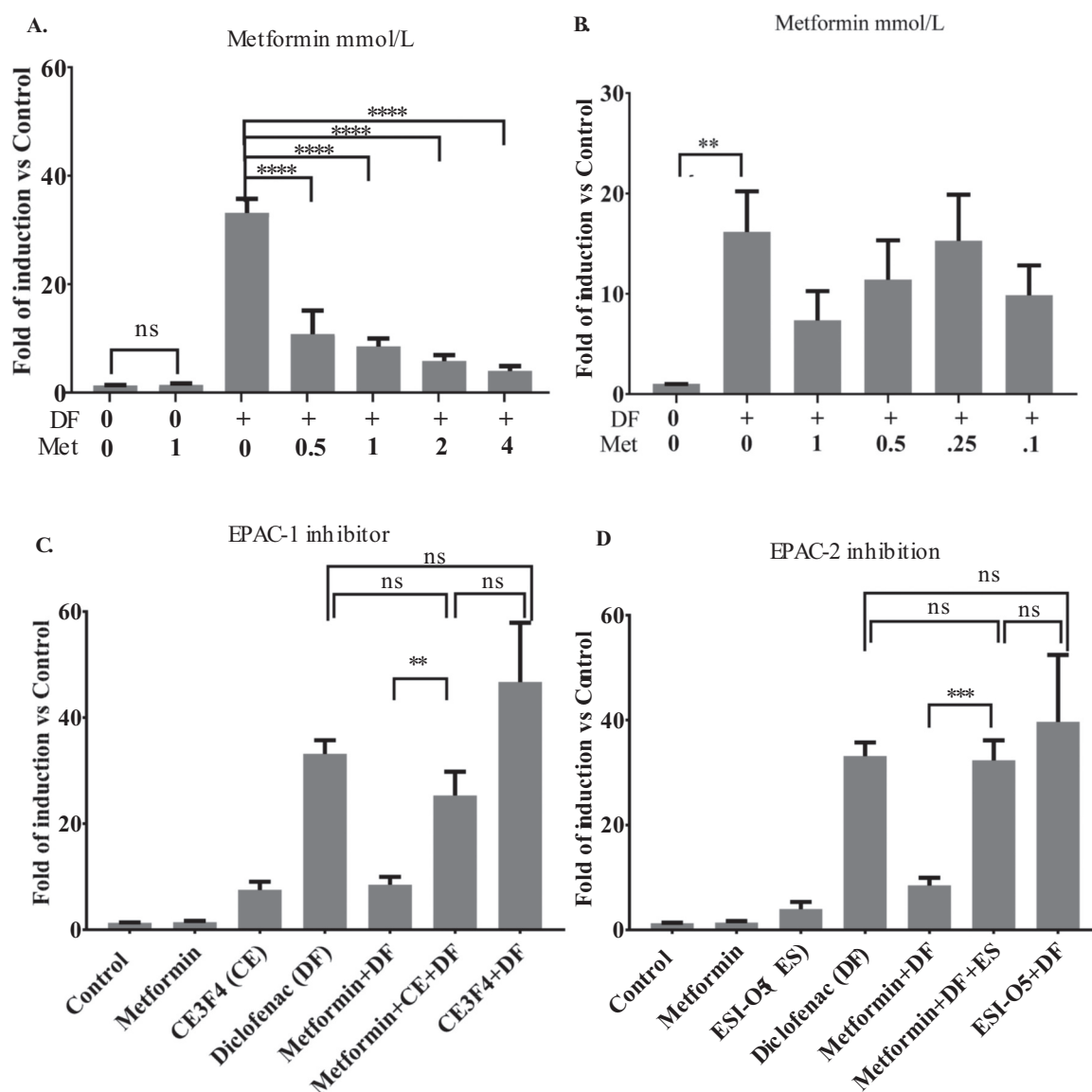


Fig. 1. Metformin prevents diclofenac (DF)-induced caspase-3 activation in primary rat hepatocytes via EPAC. A) Metformin alone protects against caspase-3 activation. B and C) Both EPAC-1 and EPAC-2 inhibition abolish metformin's protective effect against DF-induced caspase-3 activation in primary hepatocytes without causing a significant increase of caspase-3 activity. Cells were pretreated with ESI-05 (15 μ mol/L) or CEFA4 (10 μ mol/L) before metformin (1 mmol/L) and DF exposure (400 μ mol/L). A two-tailed Mann-Whitney u test was used to determine statistical significance ns: $P = 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. $n = 3$ biological replicates.

medium was removed and coverslips were washed three times with PBS (Thermo Fisher Scientific). Cells were fixated using 4% paraformaldehyde (Merck) solution (PFA) in PBS 1X for 10 min at room temperature (RT) and washed three times with PBS 1X at RT. Permeabilization was performed using 0.1% Triton X-100 in PBS at RT for 10 min. The setup includes a conventional Nikon Eclipse T-U inverted microscope equipped with a quasi-monochromatic broadband LED illumination source filtered with a bandpass filter (Nikon, Texas Red, central wavelength 560 nm, 60 nm FWHM), a high numerical aperture (NA) oil immersion objective (60x, 1.45 NA, Nikon Plan-Apo with immersion oil RI=1.515) and an air condenser lens (CSC2001, Thorlabs, NA = 0.78). An electronically tunable lens (ETL, Optotune EL-10-30-C) was attached to the microscope to provide an automatic axial scan of the sample with 95 planes (500 × 500 pixels). Then, the intensity stack registered by the microscope is collected by an sCMOS camera (Hamamatsu, Orca Flash 4.0, 16-bit gray-level) with an exposure time of 2 ms (2 ms × 95 planes per volume). To further improve the signal-to-noise (SNR) ratio, 25 stacks were averaged for each measurement, thus obtaining a 5-fold SNR enhancement. The spatial resolution is diffraction-limited and the theoretical limits provided by such configuration are 251.1 nm (lateral direction) and 520.4 nm (axial direction). To analyze the sample, we took the surrounding medium of the samples as the reference RI (Vectashield Antifade Mounting Medium with DAPI, Vector Laboratories). RI is $n_m = 1.450 \pm 0.001$ measured by the manufacturer with a calibrated ATAGO PAL-RI refractometer at 25 °C [10], and defined RI contrast as the difference (in absolute value) between the sample RI and that of the surrounding medium ($\Delta n = |n_{\text{sample}} - n_m|$). The resolution of Δn values provided by PC-ODT is 0.002. After the 3D RI tomogram was obtained, a segmentation step based on RI gradient information was performed to analyze certain regions of the sample [54]. Briefly, low gradient values are associated with more homogeneous areas of the sample (in this case, the immersion or background medium). In contrast, the cell exhibits more considerable variations (linked to higher gradient values). In this way, one can perform background removal and separate the cell from the surrounding medium. Moreover, some organelles show characteristic Δn values. In particular, mitochondria display higher Δn than neighboring organelles, so they can be segmented taking into account both RI and RI gradient. For 3D RI visualization and analysis Tomviz software (Kitware, Inc).

2.7. Cellular bioenergetics analysis

Mitochondrial activity and ATP production were assessed by XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara CA, USA). Primary rat hepatocytes (2×10^4) were seeded in Seahorse XF96 cell culture plates and treated as indicated for 12 h. Oxygen consumption rate (OCR) and Extra-Cellular Acidification Rate (ECAR) were assessed after the addition of glucose diluted in water as a substrate for the glycolysis pathway (10 mmol/L), oligomycin A (2.5 $\mu\text{mol/L}$) as a Complex V inhibitor, rotenone as a Complex I inhibitor (2 $\mu\text{mol/L}$), dinitrophenol as uncoupler for the proton gradients across bioenergetic membranes, antimycin A as a Complex III inhibitor (40 $\mu\text{mol/L}$) and bromopyruvate diluted in water as a pyruvate analog targeting GAPDH (100 $\mu\text{mol/L}$). All the molecules were purchased from (Sigma-Aldrich Zwijndrecht, the Netherlands). All samples were at a pH of 7.4 before its analysis to avoid unwanted sources of variation. All results represent at least three independent hepatocyte isolations with five or six technical duplicates. Kruskal-Wallis test was used to compare ranks of the complete test, while a two-tailed Mann-Whitney *U* test was used to determine differences between individual parameters.

2.8. Immunofluorescence microscopy

Primary rat hepatocytes (4.5×10^5) were seeded on glass coverslips in 12-well plates. After 4 h of attachment, cells were treated according to the protocol described above. Twelve hours after incubation, the culture

medium was removed, and coverslips were washed three times with phosphate-buffered saline 1X (PBS) (Thermo Fisher Scientific). Then, cells were fixated using 4% paraformaldehyde (Merck, Germany) solution (PFA) in PBS 1X for 10 min at room temperature (RT) and washed three times with PBS 1X at RT. Permeabilization was performed using 0.1% Triton X-100 (Sigma Aldrich) in PBS at RT for 10 min 2% Bovine serum albumin (BSA) (Sigma Aldrich) in PBS 1X (blocking buffer) was used to block non-specific binding of the antibodies for 30 min. Polyclonal antibody against manganese superoxide dismutase (MnSOD) (Cell Signaling Technology, Danvers, Massachusetts, USA) was used at a dilution of 1:200 in 0.5% BSA in PBS 1X at RT for 1 h. Samples were then washed three times with blocking buffer and incubated for 45 min at RT in the dark with goat-anti-rabbit green Alexa 488 (Thermo Fisher Scientific) and mounted on a coverslip using DAPI nucleic acid staining (Sigma-Aldrich Zwijndrecht, the Netherlands). Images were obtained on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems, Amsterdam, the Netherlands) at excitation (EX)/emission (EM) wavelengths of 460–500 and 512–542, respectively, with a 40X objective and at least three areas were randomly evaluated using the LAS X software (Leica). The quantification of the expression of MnSOD was measured using ImageJ. The quantification was performed by quantifying the integrated density (intDen) and mean gray value (MGV) of the green channel to obtain the corrected total cell fluorescence (CTCF) against the intDen and MGV value of the background of each image to avoid image bias. Also, over-saturated parts of the image were deleted at the time of quantification as they can be a source of discrimination in the interpretation. $\text{CTCF} = ((\text{intDen} - \text{Area of the cell}) * \text{MGV of the background})$. CTCF was then normalized to the average of CTCF of the control and expressed as Normalized CTCF ($\text{Normalized CTCF} = \frac{\text{CTCF}}{\text{Average CTCF of the control group}}$). At least three independent experiments using hepatocytes from different isolations were performed with triple technical duplicates.

2.9. Determination of mitochondrial reactive oxygen species production

Mitochondrial superoxide anion generation ($\text{O}_2^{\bullet-}$) was detected using the fluorescent MitoSox probe (Invitrogen). Primary rat hepatocytes (4.5×10^5) were seeded on glass coverslips in 12-well plates. After four hours of attachment, cells were treated according to the protocol described above. 12 h after incubation, the cell culture medium was removed, and coverslips were washed three times with PBS. Then cells were incubated in PBS with 200 nmol/L MitoSox-Red for 10 min at 37 °C in a 5% CO_2 atmosphere protected from light. Next, cells were washed three times with a warm buffer. Images were obtained on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems, Amsterdam, the Netherlands) using excitation (EX)/emission, (EM) wavelengths of 460–500/510–580 nm respectively with a 40X objective and at least three areas were randomly imaged using the LAS X software (Leica). The quantification of superoxide anion generation was measured using ImageJ. The quantification was performed by quantifying the intDen and MGV of the red channel to obtain the corrected total cell fluorescence (CTCF), then divided against the background intDen and MGV of each image to avoid image bias. Over-saturated parts of the image were deleted at the time of quantification as they can be a source of artifacts in the interpretation. $\text{CTCF} = ((\text{intDen} - \text{Area of the cell}) * \text{MGV of the background})$. Then, CTCF was normalized to the average of CTCF of the control and expressed as Normalized CTCF ($\text{Normalized CTCF} = \frac{\text{CTCF}}{\text{Average CTCF of the control group}}$). At least three independent experiments using hepatocytes from different isolations were performed with triple technical duplicates.

2.10. Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental designs and analysis in pharmacology [13]. All results

represent at least three independent hepatocytes isolations ($n = 3$) with duplicates as technical replicates. Normality test was done using the Shapiro-Wilk test. Due to the samples' non-normal distribution, statistical significance was analyzed using a two-tailed Mann-Whitney U test to compare differences between two groups, and the Kruskal-Wallis test was used to compare ranks of a complete test in the case of the Seahorse analysis. Results are presented as mean \pm standard error (mean \pm SEM) unless specified, and the threshold for statistical significance was defined as ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $p \leq 0.001$, ****: $P \leq 0.0001$. Statistical analysis was performed using GraphPad Prism 7 version 7.04 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. The protective effect of metformin against diclofenac-induced caspase-3 activity in primary rat hepatocytes is mediated by EPACs

Caspase-3 activity was used as a readout for DF-induced apoptosis. Metformin prevented DF-induced caspase-3 activity in primary rat hepatocytes in a dose-dependent manner (Fig. 1A,B). To investigate whether the protective effect of metformin was mediated by EPACs, cells were treated with diclofenac and either CE3F4 (EPAC-1 inhibitor), or ESI-O5 (EPAC-2 inhibitor) in the presence or absence of metformin. Both inhibitors abolished the protective effect of metformin against DF-induced caspase-3 activation (Fig. 1C,D). ESI-O5 or CEFA4 alone did not increase caspase-3 activity as compared to untreated controls. Sytox green nuclear staining for necrosis demonstrated that metformin did not increase necrotic cell death indicating that metformin prevents DF-induced hepatotoxicity and does not switch cell death mode from apoptosis to necrosis (Table 1).

3.2. Metformin preserves mitochondrial morphology in diclofenac treated hepatocytes independently of EPAC

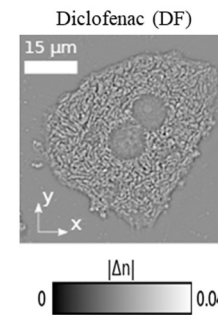
Next, the effects of metformin on DF-induced changes in mitochondrial morphology were investigated (Fig. 2 and Supplemental Fig. 1 & 2). By comparing the Control vs. Diclofenac conditions, it was observed that DF decreased the RI contrast by 0,0092 (indicating that diclofenac shifted the mitochondrial fusion and fission rate towards mitochondrial fission).

Metformin-pre-treated hepatocytes displayed both elongated-connected and rounded mitochondria with a median RI contrast of 0,0241 (0,0159- 0,0327). Compared to the control condition, metformin shifted the RI contrast by 0,0053, indicating that metformin shifted the mitochondrial fusion/fission rate towards a higher mitochondrial fission rate in comparison to the control condition. However, pre-treatment with metformin protected hepatocytes against the DF-induced increase in mitochondrial fission rate: a recovery of the RI contrast of 0,0038 was observed. The results indicate that pre-treatment with metformin prevents the mitochondrial fission caused by DF (Fig. 2B).

To investigate whether the protective effect of metformin was mediated via EPAC, cells were incubated with DF, EPAC-1 inhibitor or EPAC-2 inhibitor in the presence or absence of metformin. EPAC-1 or EPAC-2 inhibitors alone increased the fission rate of the mitochondria expressed as RI contrast 0007 and 0,0096, respectively, in comparison to the control (Control vs. CE3F4 and Control vs. ESI-O5). In the presence of diclofenac and metformin, EPAC-1 or EPAC-2 inhibitors did not abolish the protective effect of metformin, and the RI contrast remained higher 0,0031 and 0.0045 (Metformin+DF+CE vs. Diclofenac and Metformin+DF+ES vs. Diclofenac) (Fig. 2B). The results suggest that metformin's protective effect on mitochondrial morphology against diclofenac-induced mitochondrial fission rate is independent of the EPAC pathway.

A.

Representative segmentation of 3D RI tomogram.



B.

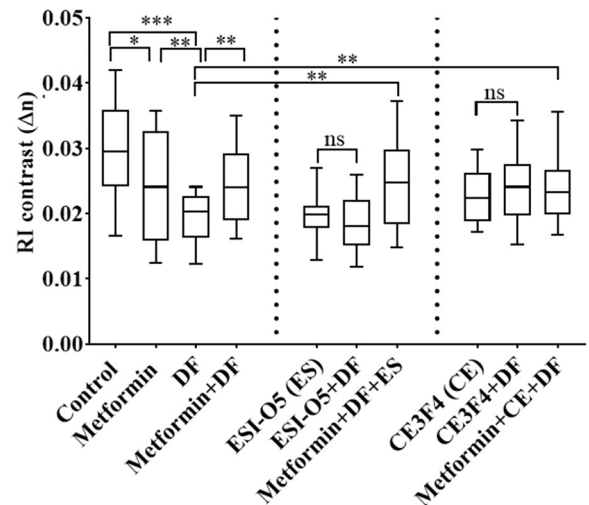


Fig. 2. Metformin is protective against mitochondrial fission induced by diclofenac (DF) in primary rat hepatocytes independently of EPAC. (A) Representative segmentation of a single cell 3D refractive index (RI) tomogram (cell is treated with diclofenac). Scale bar: 15 μ m. (B) The panels show the RI contrast (Δn) of rat hepatocytes pretreated with ESI-O5 (15 μ mol/L) or CEFA4 (10 μ mol/L) before metformin (1 mmol/L) and DF exposure (400 μ mol/L). ns: $P = 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $p \leq 0.001$, ****: $P \leq 0.0001$. A two-tailed Mann-Whitney u test was used to determine statistical significance $n = 3$ biological replicates.

3.3. Metformin preserves mitochondrial function via EPAC2

The oxygen consumption rate (OCR) was used to study the effect of metformin on DF-induced mitochondria dysfunction (Fig. 3, Supplementary Table 1). In addition, total extra-cellular acidification rate (ECAR) an indicator of the glycolytic conversion of glucose into pyruvate, was used (Fig. 4, Supplementary Table 2).

DF intoxication significantly reduced the total OCR compared to the control condition (Fig. 3). Further analysis revealed that DF reduced the basal respiration, maximal respiration rate, spare respiration, ATP-linked respiration, proton leak and non-mitochondrial-respiration (Fig. 3B, Supplementary Table 1).

Pre-treatment with metformin did not restore DF-induced reduction of total OCR to the control level but increased the basal respiration rate, maximal respiration rate, proton leak, non-mitochondrial respiration and spare respiration rate, the latter being an indicator of the cell's capability to respond to an energetic demand (Fig. 3B, Supplementary Table 1).

EPAC-2 inhibition, but not EPAC-1 inhibition, attenuated the metformin-mediated recovery of proton leak and spare respiration capacity to levels comparable to those of diclofenac alone (Fig. 3B). These results suggest that metformin's protective effect against diclofenac toxicity is involved in preserving total OCR and preventing proton leak

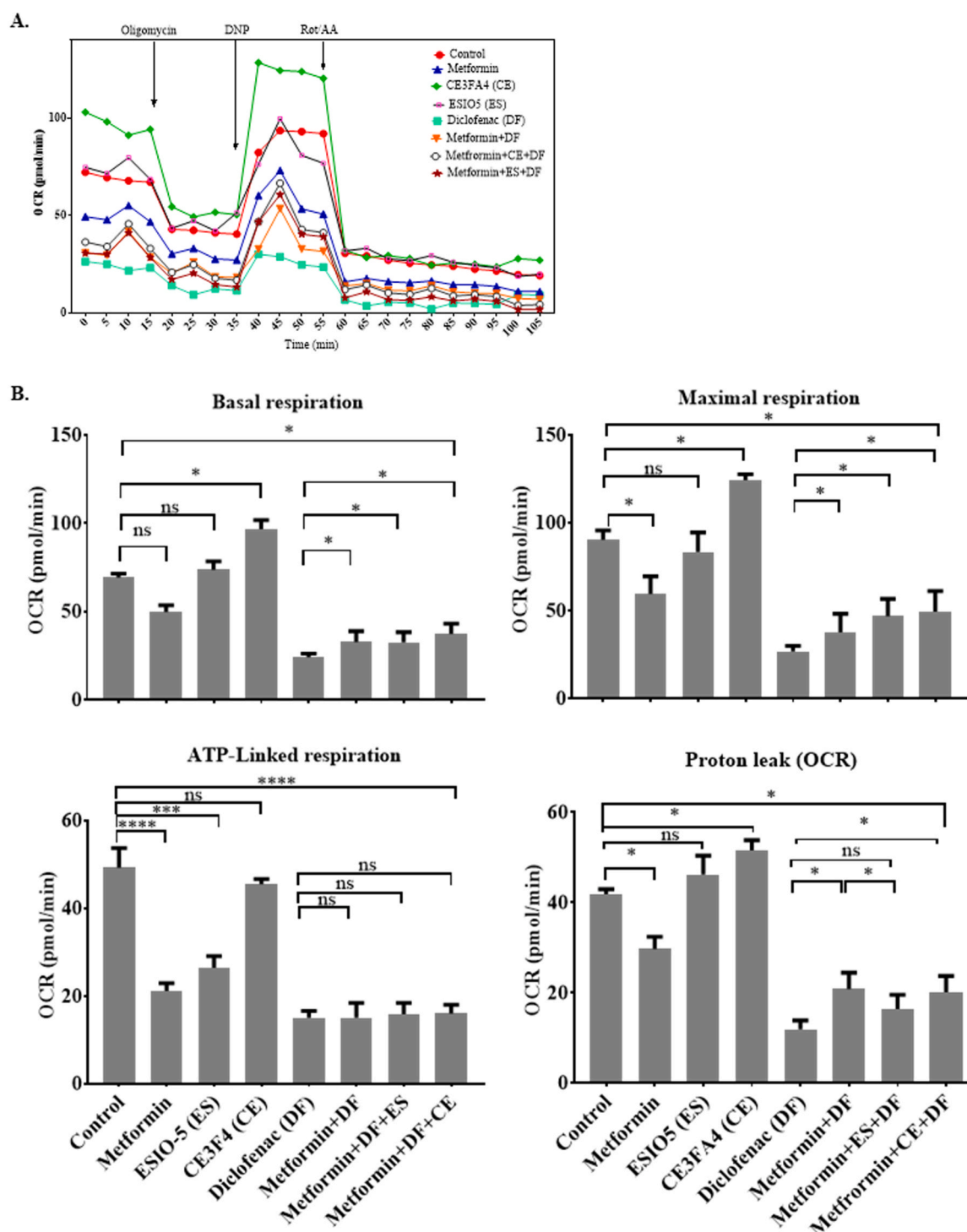


Fig. 3. Metformin preserves mitochondrial function via EPAC2. A) Oxygen consumption (OCR) measurements in rat hepatocytes treated with either metformin, diclofenac, ESI-05 (ESI), CE3FA4 (CE), Metformin + CE + DF or Metformin + ESI + DF, measured over time (min) using an extracellular flux analyzer (Seahorse Bioscience). B) The mitochondrial stress test was used to obtain bioenergetics parameters, by adding substrates. Maximal OCR was stimulated by addition of DNP (50 $\mu\text{mol/L}$). Graphs show the OCR in pmoles per min. Rat hepatocytes were pretreated with ESI-05 (15 $\mu\text{mol/L}$) or CEFA4 (10 $\mu\text{mol/L}$) before metformin (1 mmol/L) and DF exposure (400 $\mu\text{mol/L}$ n = 3 biological replicates. Data is shown as \pm SEM. ns: $P = 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $p \leq 0.001$, ****: $P \leq 0.0001$.

in an EPAC-2 dependent manner.

In addition, DF decreased glycolysis, the glycolytic capacity, the glycolytic reserve and the non-glycolytic acidification as compared to the control condition (Fig. 4, Supplemental Table 2). Pre-treatment with metformin did not restore the DF-induced reduction of total ECAR to the control level. However, a recovery of glycolysis, glycolytic capacity and non-glycolytic acidification after pre-treatment with metformin was

observed (Fig. 4B). EPAC-2 inhibition, but not EPAC-1 inhibition, abolished the metformin-mediated recovery of DF-induced reduction of glycolytic capacity and non-glycolytic acidification (Fig. 4B). These results suggest that EPAC-2 mediates the protective effect of metformin on mitochondrial respiration and glycolysis.

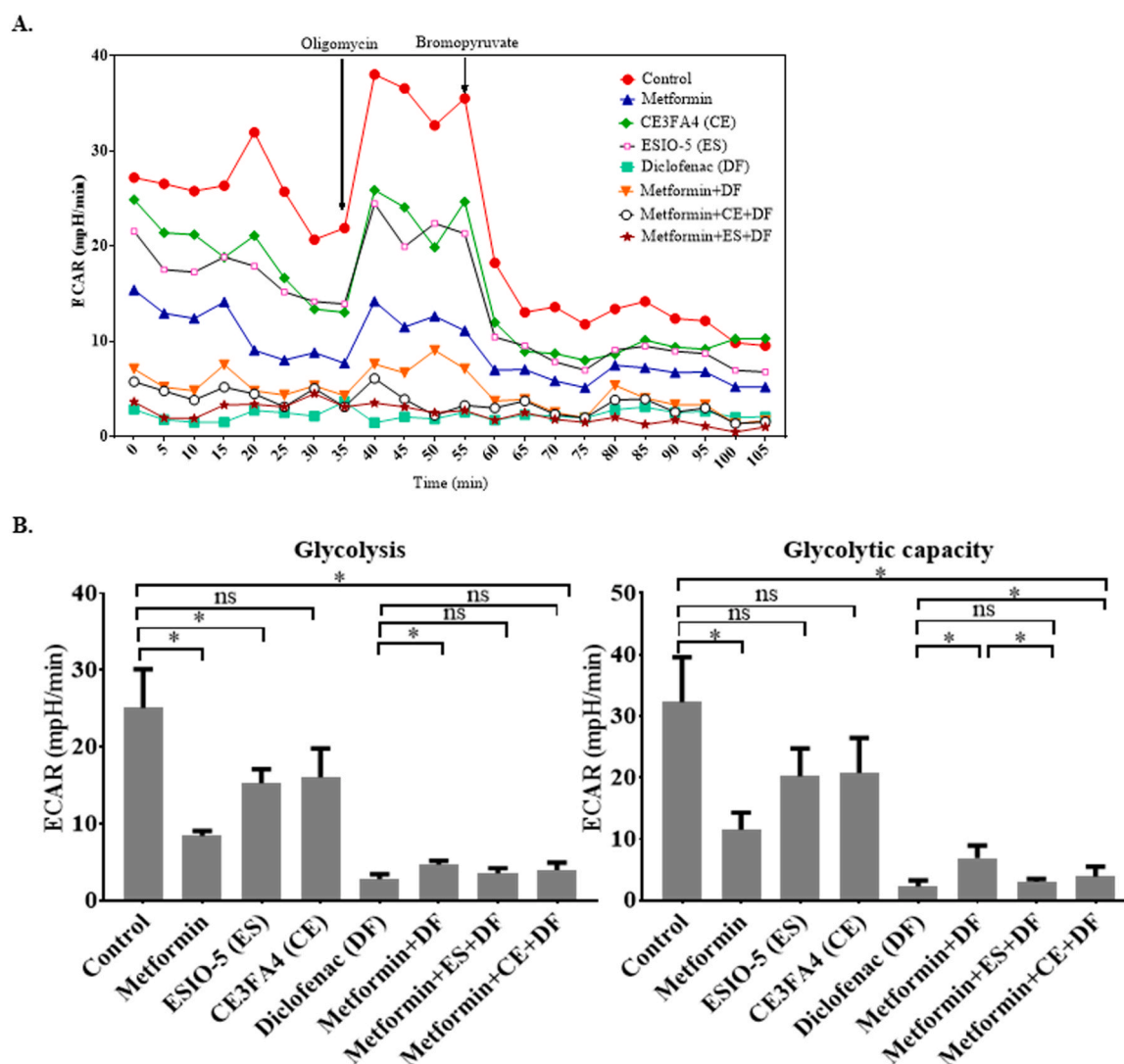


Fig. 4. Metformin restores diclofenac-induced impairment of glycolytic respiration via EPAC2. A) Extracellular acidification rate (ECAR) measurements in rat hepatocytes treated with either metformin, diclofenac, ESI-05 (ESI), CE3FA4 (CE), Metformin + CE + DF or Metformin + ESI + DF, measured over time (min) using an extracellular flux analyzer (Seahorse Bioscience). B) The mitochondrial stress test was used to obtain bioenergetics parameters, by adding substrates. Graphs show mpH/min. $n = 3$ biological replicates. Data is shown as \pm SEM. ns: $P = 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$.

3.4. Metformin restores the diclofenac-induced decrease of MnSOD in primary rat hepatocytes via EPAC2

Manganese superoxide dismutase (MnSOD, SOD2) is the mitochondrial enzyme that converts superoxide anions into hydrogen peroxide. Diclofenac significantly reduced the expression of MnSOD in comparison to the control condition. Pre-treatment with metformin preserved the expression of MnSOD (Fig. 5).

It was previously shown that both EPACs are expressed in rat hepatocyte mitochondria [3] and since MnSOD is exclusively located in the mitochondria and an early elevation of cAMP levels correlated with mitochondrial protection [1,31], the role of metformin in preservation of MnSOD expression in an EPAC-dependent manner was investigated. Hepatocytes were either incubated with diclofenac, with or without ESI-05 or CE3FA4, or in the presence or absence of metformin.

As shown in Fig. 5, inhibition of EPAC-1 did not abolish metformin's protective effect against the DF-induced reduction of MnSOD expression. In contrast, the inhibition of EPAC-2 abolished the protective effect of metformin against the DF-induced reduction of MnSOD expression (Fig. 5 A and B). The use of ESI-05 or CEFA4 alone did not affect the expression of MnSOD (ESI-05 vs. Control) and (CEFA4 vs. Control).

These results demonstrate an EPAC-2 dependent protective role of metformin against DF-induced reduction of MnSOD.

3.5. Metformin reduces diclofenac-induced generation of superoxide anions via EPAC2

As shown in Fig. 6, diclofenac increased superoxide anion production compared to the control condition. Pre-treatment with metformin alleviated augmentation of superoxide anion generation.

EPAC-1 inhibition did not abolish the effect of metformin against DF-induced superoxide anion production. In contrast, the inhibition of EPAC-2 abolished the effect of metformin against DF-induced superoxide anion production (Fig. 6 A & B). ESI-05 or CEFA4 alone did not result in a higher generation of ROS as compared to control.

These results suggest that metformin's protective effect involves reducing superoxide anion production and that this effect is mediated by EPAC-2.

4. Discussion

The present study shows that metformin prevents diclofenac-induced

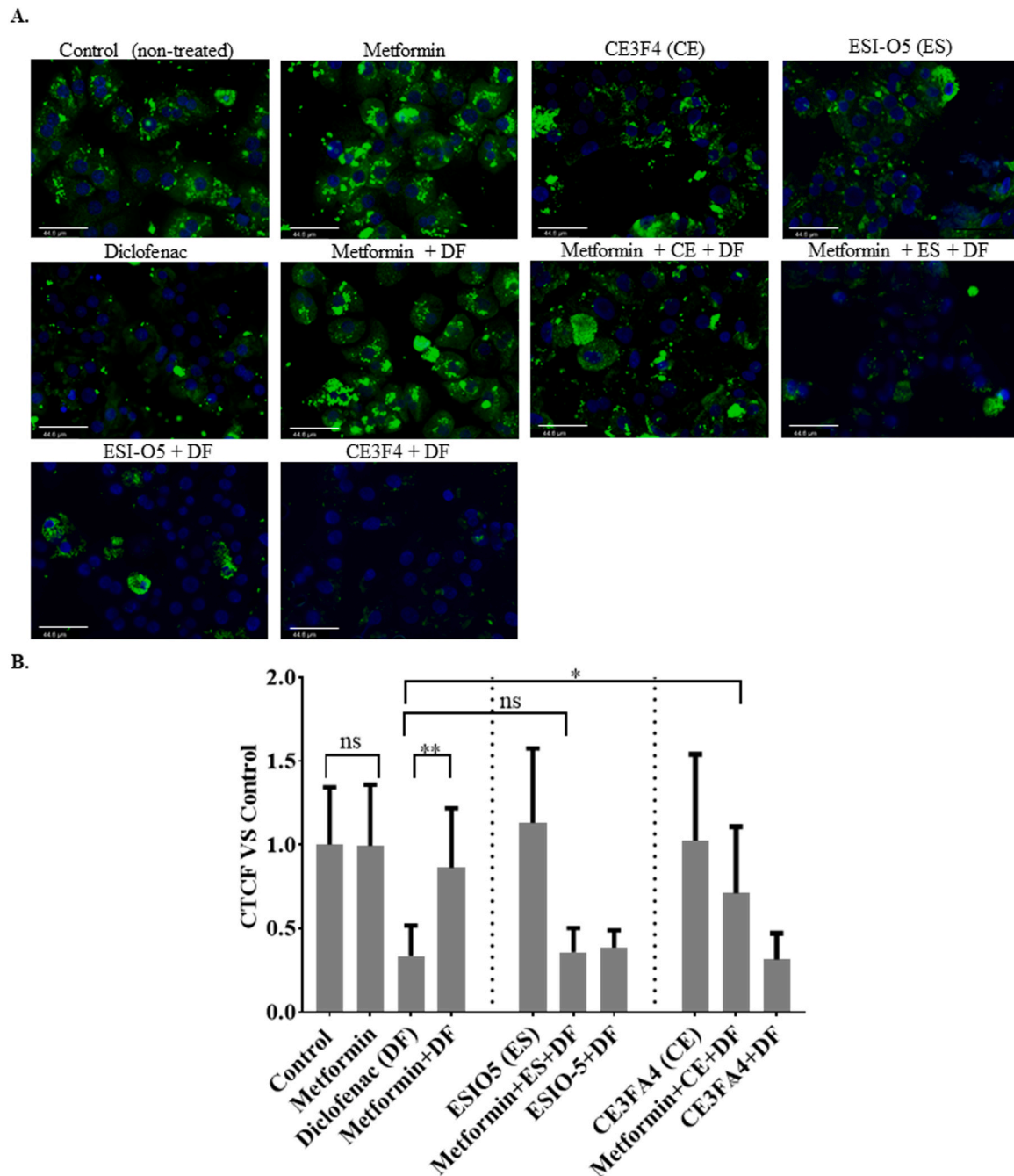


Fig. 5. Metformin prevents diclofenac-induced depletion of manganese superoxide dismutase (MnSOD) in primary rat hepatocytes via EPAC2. (A) Immunofluorescence analysis of MnSOD in primary rat hepatocytes treated with either metformin, diclofenac, ESI-05 (ESI), CE3F4 (CE), Metformin + CE + DF or Metformin + ESI + DF. (B) Quantitative analysis of MnSOD production in rat hepatocytes. Cells were pretreated with ESI-05 (15 $\mu\text{mol/L}$) or CEFA4 (10 $\mu\text{mol/L}$) before metformin (1 mmol/L) and DF exposure (400 $\mu\text{mol/L}$). Scale bar: 44.6 μm . A two-tailed Mann-Whitney u test was used to determine statistical significance. ns: $P = 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $p \leq 0.001$, ****: $P \leq 0.0001$. $n = 3$ biological replicates.

apoptosis in primary rat hepatocytes via EPAC-2, affecting both the antioxidant pathway and OXPHOS pathway, albeit not the mitochondrial morphology. These results help define the role of EPAC-2 in the treatment of DILI and liver toxicity, focusing on the mitochondria as a target, thereby providing avenues for targeted therapy.

In our study we used a metformin concentration of 1 mmol/L based on our previous studies and data from the literature. Therapeutic concentrations of metformin in experimental animals can reach levels as high as 1–4 mg/L [47,63]. Because of the lower bioavailability of metformin in rats compared with humans, 100–300 mg/kg/day metformin is needed to attain similar effects in rats [43,47] resulting in plasma

concentrations in rats within the range of the in vitro concentrations used in our study. In our previous in vivo study using a model of ischemic-reperfusion injury, we attained metformin plasma levels of 5 mg/L, without causing significant lactic acidosis when used prophylactically [64]. Finally, a systematic review that investigated therapeutic concentrations of metformin reported therapeutic metformin plasma concentrations ranging from 0.129 to 90 mg/ml [28]. Therefore, we believe that the concentrations used in our in vitro studies are relevant and within the range that can be attained in vivo.

Likewise, it is difficult to compare diclofenac plasma concentration in humans with the diclofenac concentrations used in in vitro studies.

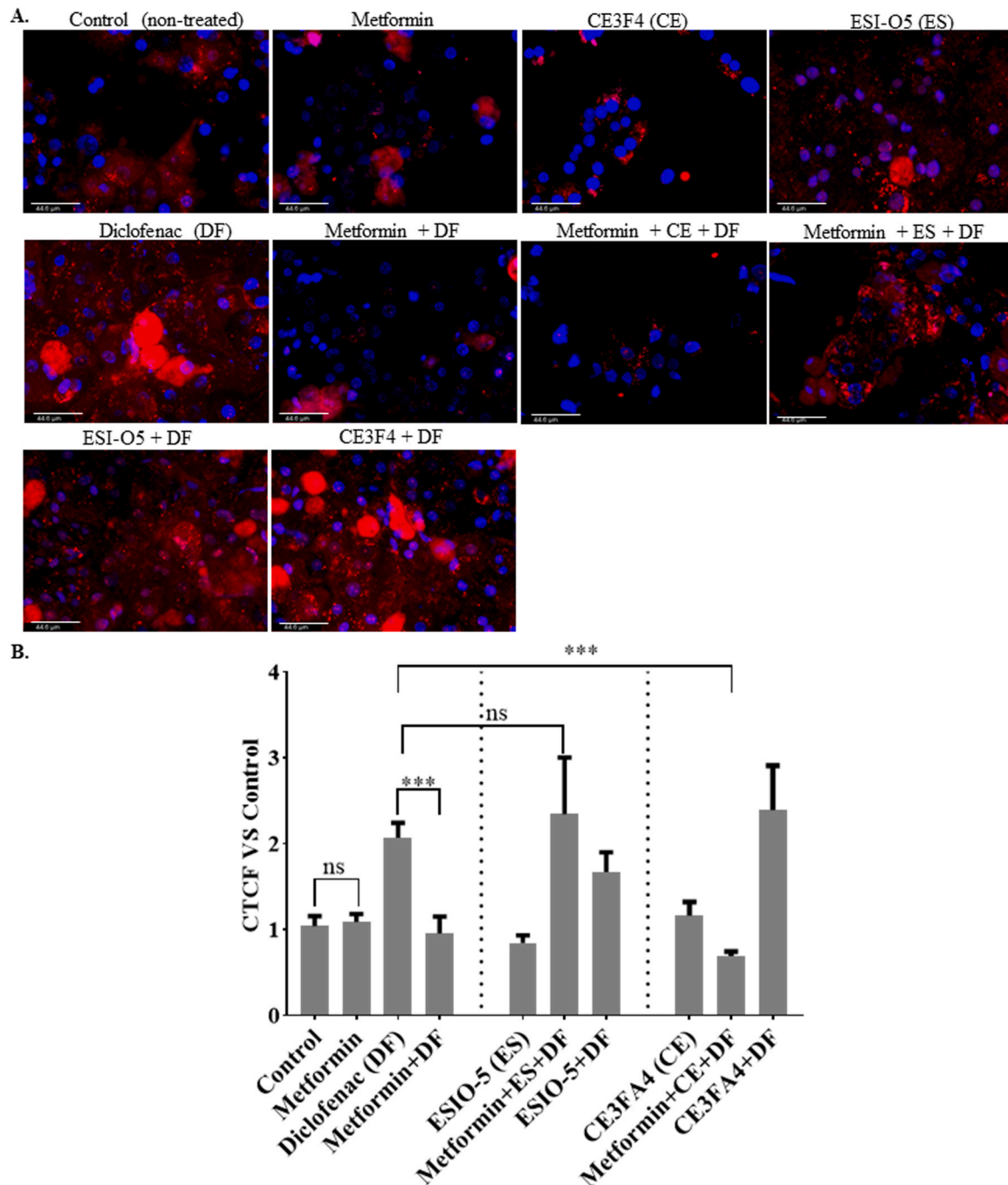


Fig. 6. Metformin reverses diclofenac-induced increase of mitochondrial superoxide anion production in primary rat hepatocytes via EPAC2. A) Immunofluorescence analysis to detect superoxide anion levels in primary rat hepatocytes treated with either metformin, diclofenac, ESI-O5 (ESI), CE3F4 (CE), Metformin + CE + DF or Metformin + ESI + DF. Scale bar is 44.6 μ m. B) Quantitative analysis of superoxide production in rat hepatocytes. Quantification of signals was performed as described in Materials and Methods. Cells were pretreated with ESI-O5 (15 μ mol/L) or CEFA4 (10 μ mol/L) before metformin (1 mmol/L) and DF exposure (400 μ mol/L). Scale bar: 44.6 μ m. A two-tailed Mann-Whitney u test was used to determine statistical significance (n = 6). ns: P = 0.05, *: P \leq 0.05, **: P \leq 0.01, ***: P \leq 0.001, ****: P \leq 0.0001. n = 3 biological replicates.

Many factors determine diclofenac plasma and tissue concentrations in humans or in in vivo models [4,51,66]. Peak plasma concentrations of diclofenac of 1700 ng/ml to 2000–3000 ng/ml have been observed in healthy volunteers [33,35]. Moreover, within the context of clinical studies, plasma concentrations of diclofenac between 600 ng/ml (after a single infusion) up to 13000 ng/ml have been described [52,56]. These studies indicate that high (micromolar range) plasma concentrations of diclofenac can be attained when used chronically and/or excessively, as is the topic of our study).

The actions of metformin are believed to be related to the inhibition of Complex 1 in the OXPHOS system, although the exact mechanism is still open to debate [5,19]. It has been documented that metformin prevents DILI-mediated injury by activating AMP-activated protein kinase (AMPK) and triggering fusion of the mitochondria [30]. This study shows that metformin prevents DF hepatotoxicity and that this protection is related to cAMP-EPAC-2 signaling. This statement is supported by previous research [3]. Metformin has been demonstrated to decrease cAMP generation, a process linked to AMPK and EPAC1/2 signaling [19,

36,40,45]. Our observation that metformin protects via a cAMP-EPAC dependent pathway appears to be contradictory to the observation that metformin decreases cAMP generation. However, the modulation of the cellular cAMP content most likely involves a sophisticated interplay of different cAMP “pools”. Measurements of total global cAMP pool may not reflect the cAMP pool modulated primarily by EPAC2 [38,49]. To gain further insights into such distinct cAMP “pools” – for example the mitochondrial associated cAMP “pool” – would require the usage of sophisticated FRET measurements using targeted sensors. However, such studies are far beyond the scope of our current manuscript.

Importantly we show, using distinct inhibitors for EPAC1 and EPAC2, that both cAMP effectors differentially affect the protective effect of metformin against DF-induced toxicity in primary hepatocytes. Our data match the current literature implicating that metformin modifies the cellular level of cAMP explaining the distinct mode of action of the EPAC inhibitors used in our current study.

Fusion and fission of the mitochondria is a dynamic response towards changes in mitochondrial homeostasis, allowing the mitochondrial network to maintain functionality during stressful events [61]. Hepatocyte mitochondria are characterized by spheres or ovoid of 1–10 μm in length and a diameter of ~ 700 nm [68]. Using the PC-ODT technique, we observed and quantified the 3D distribution of mitochondria rather than the 2D view that is typically obtained [20,69]. We showed that diclofenac toxicity is characterized by fragmentation of mitochondria, probably related to the mitochondrial damage caused by ROS accumulation [24]. On the other hand, metformin reverses fragmentation and increases mitochondria fusion, an effect previously shown to be PKA dependent [26]. We have shown previously that EPAC is essential for mitochondrial function [3]. EPAC-1 has been linked to the transport of Ca^{2+} between the mitochondria and endoplasmic reticulum, and impaired Ca^{2+} homeostasis induces mitochondrial fission [15,34]. Our results demonstrate that the inhibition of both EPACs does not abolish metformin's effect on mitochondrial fusion. These results suggest that the cAMP-EPAC protective pathway against DF does not involve metformin-induced mitochondrial fusion. Instead, the cAMP-EPAC protective pathway could be involved solely in the mitochondrial function but not morphology.

The primary function of the mitochondria is ATP synthesis via the OXPHOS system. Diclofenac cytotoxicity is characterized by the inhibition of ATP synthesis in both the liver and the kidney [14,57]. Our results are consistent with these reports, showing a decrease in the spare respiration capacity related to the reduction in ATP synthesis compared to the control. We provided more information about the events that follow diclofenac intoxication, including the decrease in complex V activity, the decline in proton transport across the cell membrane, reduction of glycolysis and the inhibition of conversion of glucose into glucose-6-phosphate. Our data support previous studies by showing a reduction in Complex I and III's function, a decrease in protons' transport across the cell membrane, and a reduction of the glycolytic pathway on treatment with metformin. Noteworthy is that metformin showed a small protective effect against diclofenac-induced impairment of several mitochondrial protein complexes i.e. complex I, complex III and complex V transfers protons from the intermembrane space back to the matrix, converting ADP and inorganic phosphate into ATP [39]. Finally, metformin decreases proton leakage, promoting proton transport across the cell membrane and preventing the inhibition of glycolytic capacity. These results suggest that the protective effect of metformin involve OXPHOS.

The OXPHOS system and ROS production have been linked to cAMP generation by sAC in the mitochondrial external membrane. There is supporting evidence that cAMP generated from soluble adenylyl cyclase (sAC) in the mitochondrial external membrane can serve as a metabolic modulator of the OXPHOS system and ROS production [1,2,21]. Our results suggest that EPAC-2 inhibition alone does not affect OXPHOS by itself but abolishes the protective effect of metformin on complex III and V activity and glycolytic capacity. These results suggest that EPAC-2 is

involved in regulating complex III and V function in primary rat hepatocytes. It has been shown that EPAC is involved in intracellular Ca^{2+} flow between the endoplasmic reticulum and the mitochondria and ATP production [22,29,42,62]. However, whether EPAC-2 can regulate Ca^{2+} flow in hepatocytes remains to be determined.

Our study adds to previous studies indicative of EPACs physiological functions previously attributed to PKA [32,46,60]. In our present study, we showed that EPAC-2 maintains the expression of MnSOD in primary rat hepatocytes. Indeed, previous research indicated that EPAC-2 but not EPAC-1 is involved in antioxidant pathways [58]. Our results are the first to demonstrate the antioxidant effect of EPAC-2 in the mitochondria of primary rat hepatocytes, thereby suggesting for the first time a potential therapeutic role of EPAC2 in protection against DILI following metformin intake.

5. Conclusion

In this study, we demonstrated EPAC2's essential role in metformin's protective effect against diclofenac toxicity. We showed that metformin protects against diclofenac-induced hepatocyte toxicity by reducing oxidative stress and preserving mitochondria integrity. Most importantly, we demonstrate that even though the protective effects of metformin on mitochondrial morphology are independent of EPAC2, the protective effects of metformin on mitochondrial function are dependent on EPAC2, opening avenues for further understanding of this cAMP microdomain in DILI. This research therefore points to mitochondrial EPAC2 signaling as a potential target in the treatment of DILI.

Financial support

Fabio Aguilar Mora was supported by a personal grant from Conacyt (CVU614256); Deutsche Forschungsgemeinschaft (IRTG1874 DIAMICOM-SP2) and Novartis unrestricted grant 50199468 to M.S.

CRediT authorship contribution statement

Fabio Alejandro Aguilar Mora and Nshunge Musheshe: The authors contributed equally to the conception, design, analysis, execution, writing, project administration, and interpretation of the reported study.

Johanna Carolina Arroyave Ospina: The author contributed with the execution, analysis and reviewing of the reported study.

Manon Buist-Homan: The author contributed to the instruction for the execution and reviewing of the reported study.

Frank Lezoualc'h: The author contributed by providing analysis tools for the execution of the reported study.

Xiaodong Cheng: The author contributed by providing analysis tools for the execution of the reported study.

Han Moshage and Martina Schmidt: The authors contributed equally to the Conceptualization, design, execution, writing, Funding acquisition, Validation, Writing – review & editing, Supervision and interpretation of the reported study.

Chemical compounds

Diclofenac sodium (PubChem CID;5018304); Metformin (PubChem CID; 4091); CE3F4 (PubChem CID; 21781066); ESI-O5 (PubChem CID; 272513).

Conflict of interest statement

The authors declare that they have no conflict of interest with other people or organizations during the preparation of this work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.biopha.2021.112072.

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