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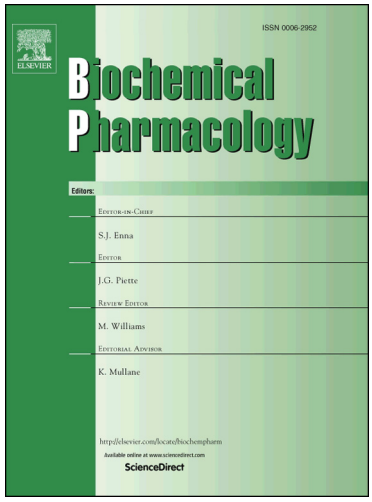

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Abbreviations: Abcc2: multidrug resistance-associated protein 2, TNFα: tumor necrosis factor alpha, ERK: extracellular signal–regulated kinases, ROS: reactive oxygen species, NOX: NADPH oxidase, CMFDA: 5-chloromethylfluorescein diacetate, GS-MF: glutathione methylfluorescein, DMSO: dimethyl sulfoxide, RHC: rat hepatocyte couplets, cVA: canalicular vacuolar accumulation.

Abstract

TNFα is a cytokine whose levels are increased in inflammatory pathologies that are associated with cholestasis. Endocytic internalization of Abcc2 (multidrug resistance-associated protein 2), a canalicular transporter of organic anions that is implicated in the clearance of clinically important drugs, is a phenomenon that occurs in inflammatory liver diseases, and it has been established that cytokines act as mediators. However, the intracellular mechanism involved in this effect remains unknown. The aim of the present work was to characterize the internalization of Abcc2 induced by TNFα and to study the role of ERK1/2 and reactive oxygen species as signaling mediators of transporter internalization. Using rat hepatocyte couplets, we found that TNFα (6.25 pg/ml) induced a decrease in Abcc2 activity estimated by the accumulation of the Abcc2 substrate glutathione methylfluorescein in the canalicular vacuole that was accompanied by internalization of Abcc2 from the canalicular membrane. Inhibition of MEK1/2 (upstream of ERK1/2) partially prevented TNFα effects on Abcc2 internalization and activity impairment. Reactive oxygen species (ROS) scavengers such as vitamin C and mannitol partially prevented both TNFα-induced decrease in Abcc2 activity and ERK1/2 phosphorylation. Apocynin, a NADPH oxidase inhibitor, prevented the increase in ROS and the phosphorylation of ERK1/2 produced by TNFα. Taken together, these results indicate that TNFα activates a pathway involving NADPH oxidase, ROS and MEK1/2-ERK1/2 that is partially responsible for the internalization of Abcc2. This internalization leads to an altered transport activity of Abcc2 that could impair drug disposal, enhancing drug toxicity in patients suffering from inflammatory liver diseases.

Keywords: Mrp2, ROS, NOX, cholestasis, ABC transporters

1. Introduction

The canalicular transporter Abcc2 (Mrp2, multidrug resistance protein 2) is a transporter that mediates the biliary excretion of many organic anions including glucuronides (*e.g.*, bilirubin, bile salt and estrogen glucuronides), glutathione *S*-conjugates (*e.g.*, of leukotriene C4 or bromosulfophthalein), oxidized glutathione (GSSG), and GSH, the latter with lower affinity than GSSG [1], [2]. Both GSSG and GSH are major determinants of the so called canalicular bile salt-independent fraction of canalicular bile flow [3]. Abcc2 is also implicated in the clearance of numerous clinically important drugs, including antibiotics, anti-inflammatory and anticancer agents; as well as dietary and environmental toxins [4].

The amount of Abcc2 present in the canalicular membrane is regulated by transcriptional and post-transcriptional mechanisms. Among the latter, there is a balance between Abcc2 in the canalicular membrane and in a subapical vesicular compartment. This balance is regulated by signaling proteins [5]. Cholestatic agents such as estradiol 17ß-D-glucuronide [6], or taurolithocholate [7] activate signaling proteins that favor the internalization of transporters and their traffic from the canalicular membrane to the subapical space, whereas, dibutyl-cAMP [8] or taurocholate [9] activate signaling proteins that favor the reinsertion of Abcc2 from the subapical space to the canalicular membrane.

TNFα is a cytokine whose production is increased in inflammatory diseases that directly or indirectly affect the liver such as primary sclerosing cholangitis (PSC) [10], primary biliary cholangitis (PBC) [11], [12], autoimmune hepatitis [13], sepsis [14] and obstructive cholestasis [15]. In some of these inflammatory diseases, there are evidences of endocytic retrieval of Abcc2 [16], [17]. Experimental data that support the role of cytokines as key mediators in the endocytic internalization of transporters arise from an animal model of sepsis using LPS [17]. However, the mechanisms by which TNFα internalizes canalicular transporters are still unknown. In several physiological and pathological processes, different intracellular signaling pathways activated by the cytokine have been reported. Among these signaling proteins, TNFα is known to induce MEK/ERK kinase activation [18] which has been already implied in Abcc2 internalization induced by other cholestatic agents [19], [20]. Hence, our aim was to evaluate the role of this kinase in TNFα-induced internalization of Abcc2.

2. Materials and Methods

2.1 Materials

Collagenase type A (from *Clostridium histolyticum*), Leibovitz-15 (L-15) culture medium, dimethyl sulfoxide (DMSO), tumor necrosis factor-alpha from rat (TNFα), vitamin C (VitC), D-mannitol (Man), apocynin (APO), bovine serum albumin (BSA), Triton X-100, protease and phosphatase inhibitor cocktail, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) were acquired from Sigma Chemical Co (St. Louis, MO, USA). PD980589 (PD) was from Calbiochem (San Diego, CA, USA). 5-Chloromethylfluorescein diacetate (CMFDA), Alexa Fluor 568 phalloidin and 4,6-diamidino-2-phenylindole were from Molecular Probes (Eugene, OR, USA). Primary Abcc2 antibody (M2 III-6 clone) was purchased from Alexis Biochemicals (San Diego, CA, USA) and primary Rab5 antibody was from Abcam (Cambridge, MA, USA). Secondary Cy2-conjugated anti-mouse IgG antibody and Cy3-conjugated anti–rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (Philadelphia, USA). Rabbit anti-phospho-ERK1/2 and rabbit anti-ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM) culture medium, secondary horseradish peroxidase (HRP)-conjugated antirabbit IgG and the chemiluminescence reagent were acquired from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2 Animals

Adult female Wistar rats weighing 250-300g, bred in our animal house as described [21], were used in all studies under ketamine/xylazine anesthesia (100 mg/3 mg/kg of b.w., i.p.). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" Eighth Edition (National Academy of Sciences, 2011). Experimental procedures were carried out according to the local Guideline for the Use of Laboratory Animals (Resolution Nº 6109/012, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina). The use of animals for the project was approved by the Ethical Committee for the Use of Laboratory Animals of the Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina. (Res Nº 1074/2014 and 348/2016).

2.3 Rat hepatocytes couplets and treatments

An enriched preparation of rat hepatocytes couplets (RHC) were obtained by liver perfusion according to two-step collagenase procedure and centrifugal elutriation [22]. The final preparation, containing 70-80% of RHC with viability of > 95%, were seeded onto 24-well plate at a density of 5 x 10^4 couplets/ml (20.000 couplets per well) in L-15 culture medium, and incubated at 37ºC for 5 hours to restore couplet polarity. Then, RHC were exposed to the vehicle

(DMSO, control group) or increasing concentrations of TNFα (0-100 pg/ml) for 20 minutes. To evaluate the protective effects of glucagon and salbutamol, RHCs were incubated for 15 min with Glu (0.1 μM) or Sal (1 μM) and then exposed to DMSO (control group) or TNFα (6.25 pg/ml) for 20 min in the presence of Glu or Sal. To assess the involvement of MEK/ERK in TNFα effects, RHCs were preincubated 15 minutes with MEK inhibitor, PD 5 μM, followed by the addition of TNFα for another 20-minutes period. To study the effects of antioxidant agents, vitamin C (1 mM) [23] and mannitol (60 mM) [24] were added to the culture medium for 20 minutes prior to exposure to TNFα and kept further during the incubation of TNFα for another 20 minutes period. The possible activation of NADPH oxidase, as a major source of intracellular ROS production, was evaluated by pretreating RHCs with the inhibitor apocynin (APO, 300 μM, 30 minutes) [25] followed by TNFα incubation (6.25 pg/ml, 20 minutes).

2.4 Assessment of Abcc2 secretory function on RHC

Transport function of Abcc2 was analyzed by the measurement of canalicular vacuolar accumulation (cVA) of the substrate glutathione-S-methylfluorescein (GS-MF), a fluorescent glutathione-conjugated metabolite of chloromethylfluorescein diacetate (CMFDA). The last-mentioned probe is a lipophilic compound that diffuses passively the basolateral membrane, and is intracellularly converted into GS-MF by esterases and glutathione-S-transferases. For transport studies, RHCs were washed with L-15 and exposed to 2,5 μM CMFDA [26]. After 15 minutes of incubation, RHC were washed twice with L-15 and images were captured under an inverted microscope (Axiovert 25, Zeiss, Göttingen, Germany) coupled to a digital camera (Q-Color5, Olympus America, Center Valley, PA). The cVA of GS-MF was determined as the percentage of RHCs in the images displaying visible green fluorescence in their canalicular vacuoles from a total analysis of at least 200 couplets per preparation.

2.5 Confocal immunofluorescence of Abcc2 on RHC

Intracellular distribution of Abcc2 was evaluated by confocal immunofluorescent microscopy (LSM880, Carl Zeiss LLC, NY, USA). After treatments, RHCs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with PBS–Triton X-100 0.2% v/v–bovine serum albumin 3% w/v. For Abcc2 labeling, RHC were incubated with mouse anti-human Abbc2 (1:100, 1 h), followed by incubation with a Cy2-conjugated donkey anti-mouse IgG (1:200, 2 h) and Alexa Flour 568 phalloidin (1:100, 2 h) for F-actin staining. In colocalization studies of Abcc2 with Rab5, after fixation, transporters were stained using the primary and secondary antibodies just described. Rab5 was detected by using a rabbit monoclonal antibody (1:100, 1 h), followed by incubation with a Cy3-conjugated goat anti–

rabbit IgG (1:200, 2 h). Finally, cellular nuclei were stained by incubating during 5 min with 1,5 µM 4,6-diamidino-2-phenylindole. Densitometric analysis of confocal microscopy images was performed along a line perpendicular to the canalicular vacuole with ImageJ 1.52f, as described [27].

2.6 Western Blot analysis of ERK phosphorylation

Isolated rat hepatocytes were obtained by collagenase perfusion and mechanical disruption [28] and cultured in 3-cm Petri dishes with DMEM supplemented with 10% of fetal calf serum and antibiotics (penicillin and streptomycin) at a density of 1,5 x 10^6 cells/ml. Cells were treated with DMSO (control) or TNFα 6.25 pg/ml for 10 to 30 minutes. For additional studies, hepatocytes were pretreated with PD (5 µM, 15 min), VitC (1 mM, 20 min) or APO (300 µM, 30 min). After treatments, cells were washed with sucrose 0.3 M and lysed using sucrose 0.3 M supplemented with protease and phosphatase inhibitor cocktail, followed by sonication (Amplitude 30%, 1s, 5 pulses). Total protein concentration was quantified according to Sedmak and Grossberg method [29]. Preparations with equal amount of protein content were loaded onto 12% SDS–polyacrylamide gel and subjected to electrophoresis. After electrotransfer onto Immobilon-P membranes, the blots were probed overnight at 4 ºC with rabbit anti-phospho-ERK1/2 (1:1000). Stripped membranes were reprobed with rabbit anti-ERK1/2 (1:1000). Immune complexes were detected by incubation with donkey anti-rabbit IgG secondary antibody (1:5000, 1 h) followed by chemiluminescence detection. Immunoreactive bands were quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

2.7 Reactive oxygen species accumulation in rat hepatocytes

ROS production was measured by using the fluorescent probe 2′,7′-dichlorofluorescin-diacetate (DCFH-DA). This probe is cell-permeable and is hydrolyzed intracellularly by esterases to 2′,7′-dichlorofluorescin (DCF), a fluorescent product which is retained in the cell. DCF is then able to react with intracellular ROS resulting in a detectable fluorescent signal that is proportional to ROS level within the cell [30]. Isolated rat hepatocytes were cultured in 6-wells plates at a density of 7,5 x 10^5 cells/ml. Once hepatocytes had attached, cells were exposed to TNFα 6.25 pg/ml at different times (1, 3, 5, 10, 15 and 20 min) for time-response analysis. For further studies, hepatocytes were pretreated with VitC (1 mM, 20 min) or APO (300 µM, 30 min) followed by the addition of TNFα (6.25 pg/ml) for another 10-min period. After treatments, the cells were washed with PBS and loaded with 5 µM DCFH-DA for 30 min at 37 ºC with 5% CO_2. Then, hepatocytes were washed and scraped off the plates to monitored DCF formation at

an excitation wavelength of 488 nm and an emission wavelength of 525 nm in a DTX 880 multimode detector plate reader (Beckman Coulter, Brea, Calif.).

2.8 Statistical analysis

Results are expressed as mean ± standard error of the media (SEM). One-way ANOVA, followed by Newman-Keuls' test, was used for multiple comparisons. The variances of the densitometric profiles of Abcc2 localization were compared with the Mann–Whitney U test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 TNFα decreases Abcc2 transport activity in RHCs

In concentration-response studies, TNFα caused an impairment of Abcc2 transport function. The percentage of couplets able to accumulate the fluorescent Abcc2 substrate glutathione-S-methylfluorescein (GS-MF) is shown in Fig. 1. TNFα treatment induced a significantly decrease in the percentage of couplets accumulating GS-MF in their canalicular vacuoles. This effect was maximum at the concentration of 6.25 pg/ml [65 ± 3 %] and remained in similar values up to the concentration of 50 pg/ml. Based on these results, we had chosen the minimal concentration of the cytokine that produced the maximal effect, i.e. 6.25 pg/ml.

3.2 TNFα effect on Abcc2 transport activity is not reverted by salbutamol

Pretreatment of RHC with salbutamol or glucagon, agents that increase intracellular cAMP, produced different effects on TNFα-induced impairment of Abcc2 transport activity. Fig. 2 shows that glucagon partially prevented the effect of TNFα (TNF [64 ± 1%] vs. TNF+Glu [80 ± 4%]), whereas salbutamol pretreatment did not affect TNFα actions on Abcc2 transport (TNF [64 ± 1%] vs. TNF+Sal [61 ± 5%]).

3.3 MEK-ERK participate in TNFα-induced impairment of canalicular Abcc2 function

As shown in Fig. 3A, RHC pretreated with the MEK 1/2 inhibitor PD98059 [86 ± 2 %] partially prevented TNFα-induced impairment [67 ± 2 %] in cVA of the Abcc2 substrate. PD alone did not modify cVA of GS-MF respect to control [102 ± 5 %]. These results suggest that MEK/ERK pathway may have a role mediating TNFα effects.

For further elucidation of MEK/ERK involvement, western blot studies were performed. The result shown a time-dependent increase in the phosphorylation of ERK 1/2 after exposure of cultured rat hepatocytes to TNFα (Fig. 3B). Treatment with TNFα (6.25 pg/ml) induced a

significant transient increase of ERK 1/2 phosphorylation levels with a peak at 20 minutes [224 ± 17 %], which was prevented by pretreatment with PD [63 ± 11 %], an inhibitor that targets MEK 1/2, a kinase immediately upstream of ERK 1/2.

3.4 TNFα induces internalization of the canalicular transporter Abcc2

TNFα-induced impairment of Abcc2 runs parallel with an alteration on transporter localization. Fig. 4A revealed that in TNFα-treated RHCs, there was a visible redistribution of Abcc2 from the canalicular membrane into intracellular vesicles that localized near the canalicular membrane and the nuclei (see insets in Fig. 4A). In control images, transporter-associated fluorescence was confined to the canalicular space delineated by F-actin staining. The pretreatment of RHCs with PD markedly prevented the internalization of Abcc2 induced by TNFα. All these visual analyses were confirmed by densitometric analysis which demonstrated a TNFα-induced redistribution of Abcc2 over a greater distance from the canalicular vacuoles that was fully prevented by ERK1/2 blockage (see Fig. 4B).

3.5 Intracellular distribution of Abcc2 after TNFα-induced internalization is similar to Rab5 distribution.

As shown in Fig. 5, Rab5-associated fluorescence in control RHCs was localized intracellularly with increased densities near the canalicular vacuola and the nuclei, whereas Abcc2-associated fluorescence was delimited to the canalicular area. After TNFα treatment, there was no overt difference in Rab5-associated fluorescence but part of Abcc2-associated fluorescence became intracellular, localized in pericanalicular and perinuclear areas rich in Rab5-associated fluorescence (insets in TNFα-treated couple image). There was not a clear colocalization of Rab5 and Abcc2 fluorescence. RHCs treated with TNFα+PD showed a similar distribution to control group, indicating that PD did not modify Rab5 distribution and confirming that MEK1/2 inhibition prevented the internalization of Abcc2 produced by TNFα.

3.6 ROS generated by TNFα partially mediates cytokine effects on Abcc2 activity

A time-dependent ROS production curve was observed after TNFα (6.25 pg/ml) treatment in hepatocytes (Fig. 6A): the cytokine significantly increased ROS level over time showing a peak of fluorescence intensity of DCF at 5 min [118 ± 3 %], which hold until 15 min [127± 2 %] and finally decreased to control (DMSO-treated) levels at 20 min [96 ± 9 %]. As shown in Fig. 6B, the stimulated ROS production by TNFα 10 min [117 ± 2 %] was prevented by pretreatment with antioxidant vit C [71 ± 2 %] as well as with NOX inhibitor APO [90 ± 8 %].

Then, to evaluate TNFα-induced ROS involvement in our Abcc2 functional studies, we analyzed cVA of Abcc2 substrate (GS-MF) in RHCs in the presence of antioxidants vitamin C or mannitol. As shown in Fig. 6C, cVA of GS-MF was not significantly affected by either of the antioxidants used, vit C [99 ± 3 %] or Man [93 ± 2 %], when administered alone; whereas, TNFα-induced impairment [70 ± 2 %] in cVA of the Abcc2 substrate was partially prevented by pretreatment RHCs with both antioxidants (TNF + VitC [89 ± 2 %]; TNF + Man [80 ± 4 %]).

3.7 The activation of MEK-ERK depends on the generation of ROS by TNFα

Since we have demonstrated that Abcc2 impairment caused by TNFα is partially prevented by the inhibition of MEK 1/2, a well-known ROS effector kinase, we assessed whether MEK/ERK activation is downstream of TNFα-induced ROS production, modulating Abcc2 functional activity. As depicted in Fig. 7A, TNFα-induced impairment of GS-MF cVA accumulation [65 ± 2 %] was reverted by the pretreatment of RHCs with vit C [82 ± 1 %] and no additive protective effects were observed when RHCs were exposed to vit C in presence of PD [85 ± 1 %] suggesting that TNFα-induced ROS production and MEK/ERK activation are in the same pathway.

In addition, western blot analysis shown that TNFα-dependent increase in phospho-ERK 1/2 levels in hepatocytes [150 ± 23 %] was partially prevented in presence of the antioxidant vitamin C [123 ± 11 %] (Fig. 7B) confirming that TNFα-induced ROS production precedes MEK/ERK activation. Apocynin also prevented the phosphorylation of ERK 1/2 induced by TNFα [TNF (168 ± 3 %) vs TNF+APO (105 ± 28 %)] pointing out NOX as the source of ROS.

4. Discussion

Liver can suffer many different diseases that are associated directly or indirectly with inflammation and increase in serum cytokines. There are reports of high serum levels of TNFα in: PSC [10], PBC [11], [12], sepsis [14] and obstructive cholestasis [15]. Abcc2 is affected in some of these pathologies via transcriptional and post-transcriptional mechanisms. Abcc2 expression is decreased in patients with PSC [31] and PBC [32] and in animal models of sepsis [33] and bile obstruction [34]. Transcriptional regulation is involved in PSC [31] and in sepsis [33] where hepatic ABCC2/Abcc2 mRNA levels are decreased, whereas in bile duct-ligated rats, transcriptional regulation is absent since Abcc2 mRNA levels are not modified [35]. Information about Abcc2 transcriptional regulation in PBC is lacking. Endocytic retrieval of hepatocyte

ABCC2/Abcc2, a post-transcriptional mechanism of transporter activity regulation, was reported in patients with PSC, obstructive cholestasis [16], PBC [36] and in endotoxemic rats [17].

TNFα may be, at least partially, responsible of the transcriptional and post-transcriptional regulation of Abcc2 in inflammatory diseases. There are direct evidences of transcriptional regulation [37] but direct evidence of post-transcriptional regulation is still lacking. Indirect evidences of the mediatory role of cytokines arise from experiments employing strategies known to decrease synthesis or release of inflammatory cytokines such as dexamethasone administration or heat stress, that counteract the effect of LPS on canalicular transporter internalization [17], [38]. Here, we present a direct evidence that indicates that TNFα also regulates post-transcriptionally the canalicular transporter Abcc2. TNFα acutely decreases Abcc2 transport activity and this effect is mediated by transporter internalization as shown in confocal images.

The effect of increasing concentrations of TNFα in Abcc2 activity differs from other cholestatic agents such as estradiol 17ß-D-glucuronide. TNFα effect was maximal from a concentration of 6.5 pg/ml onwards, associated with a partial decrease in transport activity of about 40%. Estradiol 17ß-D-glucuronide, on the other hand, produces a typical logistic concentration-effect curve with a maximal effect of 100% (no transport activity) [8]. Typically, internalization leads transporters to early endosomes, then transporters pass to recycling endosomes, returning to canalicular membrane through a microtubule-dependent mechanism [8]. The particular behavior of TNFα in comparison to estradiol 17ß-D-glucuronide could be due to differences in the internalization pathways used: the cytokine could retrieve transporters to an immediately reversible compartment whereas estrogen could internalize them to a less reversible compartment. Such dissimilar capacity to activate certain endosomal traffics by TNFα and estradiol 17ß-glucuronide is not reflected in the intracellular distribution of Abcc2. Confocal images show that TNFα internalized Abcc2 as deep as the perinuclear area with a distribution that resembles that of Rab5, an early endosomal marker, and similar results were obtained after estrogen-induced internalization, costaining Abcc2 with Rab5 [39] or Rab11, a recycling endosome marker [8]. However, our results show that TNFα-induced impairment in Abcc2 activity is reverted by glucagon, a hormone that increases cAMP and induces a PKA dependent and microtubule independent recycling of transporters from early endosomes [8] whereas, salbutamol, an agent that induces transporter reinsertion from recycling endosomes [8] fails to restore Abcc2 activity. These findings suggest that TNFα may be unable to induce the traffic of Abcc2 from early to recycling endosomes, allowing a rapid retrieval from early endosomes to the canalicular membrane. Further experiments with specific endosomal markers will be needed to

clarify the Abbc2 endosomal trafficking in this experimental model. A second hypothesis to explain the atypical concentration-effect curve of TNFα it that the receptor TNFR1, which is expressed in low quantities in normal hepatocytes [40], could be a limiting factor so that TNFα would saturate it and increasing concentrations would produce the same partial internalizing effect.

Intracellular signaling cascades are fundamental for the development of cholestatic phenomena. TNFα is capable of activating different signaling proteins. Among them, MEK-ERK [18], PI3K-AKT [41] and isoforms of PKC (α, δ) [42], [43] have been already implied in other cholestasis models [44], [45], [20], [46]. We proved the role of MEK-ERK pathway in TNFα-induced Abcc2 retrieval. As previously reported, TNFα increases the phosphorylation of ERK using a concentration as low as 6.25 pg/ml, far below the concentration previously informed [47], [18]. ERK phosphorylation was transient, as was previously observed in estradiol 17ß-glucuronide-induced canalicular transporters internalization [19]. Inhibition of MEK partially prevented the decrease in Abcc2 activity induced by TNFα and fully prevented Abcc2 internalization induced by the cytokine. Partial protection in Abcc2 impaired activity indicates that other pathways activated by TNFα would participate in Abcc2 retrieval. PI3K-AKT and PKC are suitable candidates for the other/s pathways and further studies will be conducted to investigate them.

MEK-ERK activation could be produced by ROS. In particular, TNFα activation of MEK-ERK has been proposed to be mediated by ROS derived from TNF-induced NOX activation [48]. We have here demonstrated that TNFα 6.25 pg/ml produces a pulse of ROS, as measured by DCF fluorescence, that attains a maximum in 5 to 15 min and that decreases to control values in 20 min. Besides, the antioxidants vitamin C and mannitol partially prevented TNFα-induced decrease in Abcc2 activity indicating the participation of ROS as signaling molecules in this effect. Further experiments showed that ERK and ROS share the same pathway activated by TNFα: vitamin C and PD, MEK inhibitor, both produced a similar partial protection against TNFα actions, and their simultaneous presence did not increase the protection that either alone produced. The sequence of events was confirmed with western blot studies that showed that vitamin C prevented TNFα-induced phosphorylation of ERK. Finally, apocynin, a NOX inhibitor, prevented the rise of ROS produced by TNFα and prevented TNFα-induced activation of ERK1/2, indicating that activation of NOX is the origin of the ROS produced by TNFα.

The capacity of ROS, even when produced at very low levels, to induce biliary secretory failure and cholestasis is already known [49] as well as the mediatory action of ERK in ROS-

induced Abcc2 internalization [20]. The low and short-term increase of intracellular ROS produced by TNFα is consistent with the role of oxidative stress previously described in LPS-induced cholestasis. The antioxidant dimerumic acid prevents LPS-induced cholestasis and Abcc2 internalization after a short period of LPS exposure [50], [51] and oxidative stress levels induced by LPS appears to be low, since F-actin localization remained unaltered [52].

The results shown in the present work give evidence of the participation of NOX, ROS and MEK-ERK in TNFα-induced Abcc2 internalization. Which member of NOX family is activated and how TNFα activates it cannot be explained from our results, but a plausible mechanism can be proposed from the studies of Lee et al [48] and Woo et al [47] made in different cellular models. Woo et al. demonstrated that in Hela cells, ERK activation produced by TNFα is mediated by internalization of TNFR1 and activation of NOX1, whereas Lee et al. demonstrated that in tracheal smooth muscle cell, ERK is phosphorylated by TNFα activation of NOX2. Hence, in hepatocytes, TNFα probably binds to TNFR1 and after internalization there is an activation of either NOX1 or NOX2 generating ROS that finally activates MEK-ERK.

Downstream of ERK1/2, several effectors may be responsible for the internalization of Abcc2. Abcc2 is maintained in the canalicular membrane by an association with a PDZ protein that tethers Abcc2 to the actin cytoskeleton by interacting with the actin-binding protein radixin [53]. Internalization of Abcc2 implies disinsertion of the transporter from the actin cytoskeleton and a vesicular transport towards early endosomes [39]. Then from early endosomes, Abcc2 can recycle to the canalicular membrane via pathways dependent and independent of microtubules [8], though from our results using salbutamol, recycling in TNFα seems to use only the direct microtubule independent pathway. Hypothetically, ERK1/2 could disrupt the complex Abcc2-PDZ protein-radixin-actin, favoring Abcc2 delocalization. A high phosphorylation of radixin could decrease the specificity of the interaction of radixin with apical pools of F-actin to a more promiscuous interaction with both apical and basolateral pools of F-actin; leading to relocation of Abcc2 [54]. In the case of oxidative stress-induced internalization of Abcc2, Sekine et al [53] demonstrated that low rather than high phosphorylation of radixin was associated with the phenomenon. No matter the case, ERK1/2 could participate in Abcc2 internalization by changing the phosphorylation status of radixin. In what refers to vesicular transport, ERK1/2 could also modify the recycling of Abcc2-containing endosomes. ERK is present in endosomes where it can either favor endosomes internalization or restrain spontaneous reinsertion. The latter effect has been proved in estradiol 17ß-D-glucuronide-induced alteration of Abcc2 [19], though, in this effect, recycling endosomes were involved.

The effect of TNFα on Abcc2 may be relevant in inflammatory liver diseases. TNFα impairs Abcc2 activity both by transporter internalization, as reported here, and by decreasing the transporter expression affecting Abcc2 mRNA levels [37]. These alterations would decrease the biliary excretion and increase the intrahepatic accumulation of Abcc2 substrates which would lead to a major toxicity of drugs excreted via this transporter. In line with this, in vitro studies using the polarized hepatocyte cell line HepaRG demonstrate that cytokines [55] and TNFα in particular [56] enhance cytotoxicity of chlorpromazine and diclofenac, highlighting the role of cytokines in drug-induced idiosyncratic hepatotoxicity.

In conclusion, our results indicate that TNFα produces Abcc2 internalization and the consequent decrease in Abcc2 transport activity, confirming its role as one of the mediators in cholestasis of inflammatory origin such as that produced by LPS. TNFα provokes Abcc2 retrieval by the activation of at least two pathways; one of these pathways is constituted by NOX, ROS and MEK-ERK. This phenomenon could impair drug disposal enhancing drug toxicity in patients suffering from inflammatory liver diseases.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Legend to figures

Fig. 1 Concentration–response curve of TNFα-induced impairment of canalicular secretory function of Abcc2. After 5-hours polarization period, rat hepatocytes couplets (RHC) were exposed to increasing concentrations of TNFα (0-100 pg/ml) for 20 min. Finally, CMFDA (5 µM), a metabolic precursor of GS-MF, was added to the culture medium for an additional 15-min period. Canalicular accumulation of GS-MF (Abcc2 substrate) was determined as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation. Canalicular vacuolar accumulation (cVA) values are presented as percentage of control values (TNF 0 pg/ml). Data are expressed as mean ± SEM (n = 3). * significantly different from control ($p < 0.05$)

Fig. 2 Preventive effect of agents that regulate intracellular cAMP on TNFα-induced impairment of Abcc2 transport activity. RHCs were preincubated with glucagon (Glu 0.1 µM) or salbutamol (Sal 1 µM) for 15 min and then exposed to TNFα (6.25 pg/ml) for an additional 20 min. cVA of GS-MF was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to control cVA values. Data are expressed as mean ± SEM (n = 3). [a] significantly different from control ($p < 0.05$). [b] significantly different from TNF treatment ($p < 0.05$)

Fig. 3 Role of MEK/ERK pathway in TNFα-induced Abcc2 canalicular activity impairment. (A) RHC were preincubated with MEK 1/2 inhibitor PD980589 (PD, 5 µM) for 15 minutes, and then exposed to TNFα (6.25 pg/ml) (or DMSO in controls) for additional 20-min period. cVA was determined as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to control cVA values. (B) Activation of ERK 1/2 by TNFα: whole lysates of primary-cultured rat hepatocytes were incubated with TNFα (6.25 pg/ml) for 10 to 30 min, or TNFα (6.25 pg/ml) for 20 min pretreated with MEK inhibitor (PD 5 µM, 15 min). Finally, ERK 1/2 activity was determined by immunoblots using antibodies against phosphorylated ERK and total ERK. Phosphorylation status was calculated as the ratio of p-ERK/total ERK band density for each experimental condition. Data are expressed as mean ± SEM (n = 3). [a] significantly different from control ($p < 0.05$). [b] significantly different from TNF treatment ($p < 0.05$). [c] significantly different from TNF + PD ($p < 0.05$)

Fig. 4 Protective effect of MEK inhibition in Abcc2 localization in RHCs. Panel A: Representative confocal images show cellular distribution of Abcc2 and F-actin in RHCs under

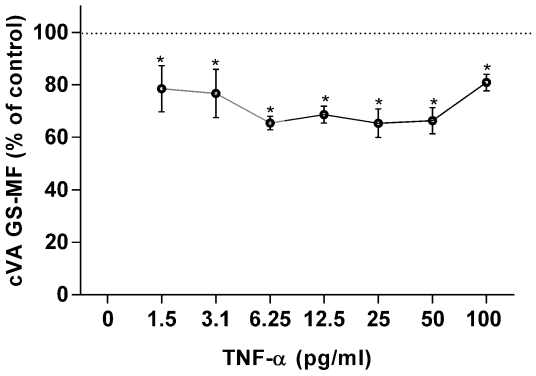
different treatments. Part of the images were amplified to show the canalicular and pericanalicular region of each image. Under control conditions, Abcc2 associated fluorescence (green) is mainly localized at the canalicular membrane in the area delimited by the pericanalicular F-actin ring (red). TNFα (6.25 pg/ml) induced a clear internalization of Abcc2 containing vesicles beyond the limits of the pericanalicular F-actin ring reaching the perinuclear area (see amplified image of perinuclear area in TNF image), a phenomenon significantly prevented by treatment of RHC with the MEK 1/2 inhibitor PD98059 (PD 5 μM). Panel B shows the densitometric analysis of the distribution of Abcc2 fluorescence intensity along an 8-mm line perpendicular to the canalicular vacuole (4 mm to each side of the vacuole center), using the ImageJ 1.52f software (NIH, USA). Each line profile measurement was normalized to the sum of all intensities of the respective measurement. The distribution of Abcc2 fluorescence, expressed as a percentage of the total, was then calculated for each canaliculus and compared statistically, using the Mann-Whitney test. Statistical analysis of the profiles revealed a significant internalization of Abcc2 under TNFα treatment ($p < 0.05$ vs control), which was completely abolished by PD ($p < 0.05$). Note that none of the treatments affected the normal distribution of F-actin, which showed similar profiles. For technical information see "Materials and methods". Results are expressed as mean ± SEM. n = 6–8 canalicular vacuoles per preparation, from three independent preparations

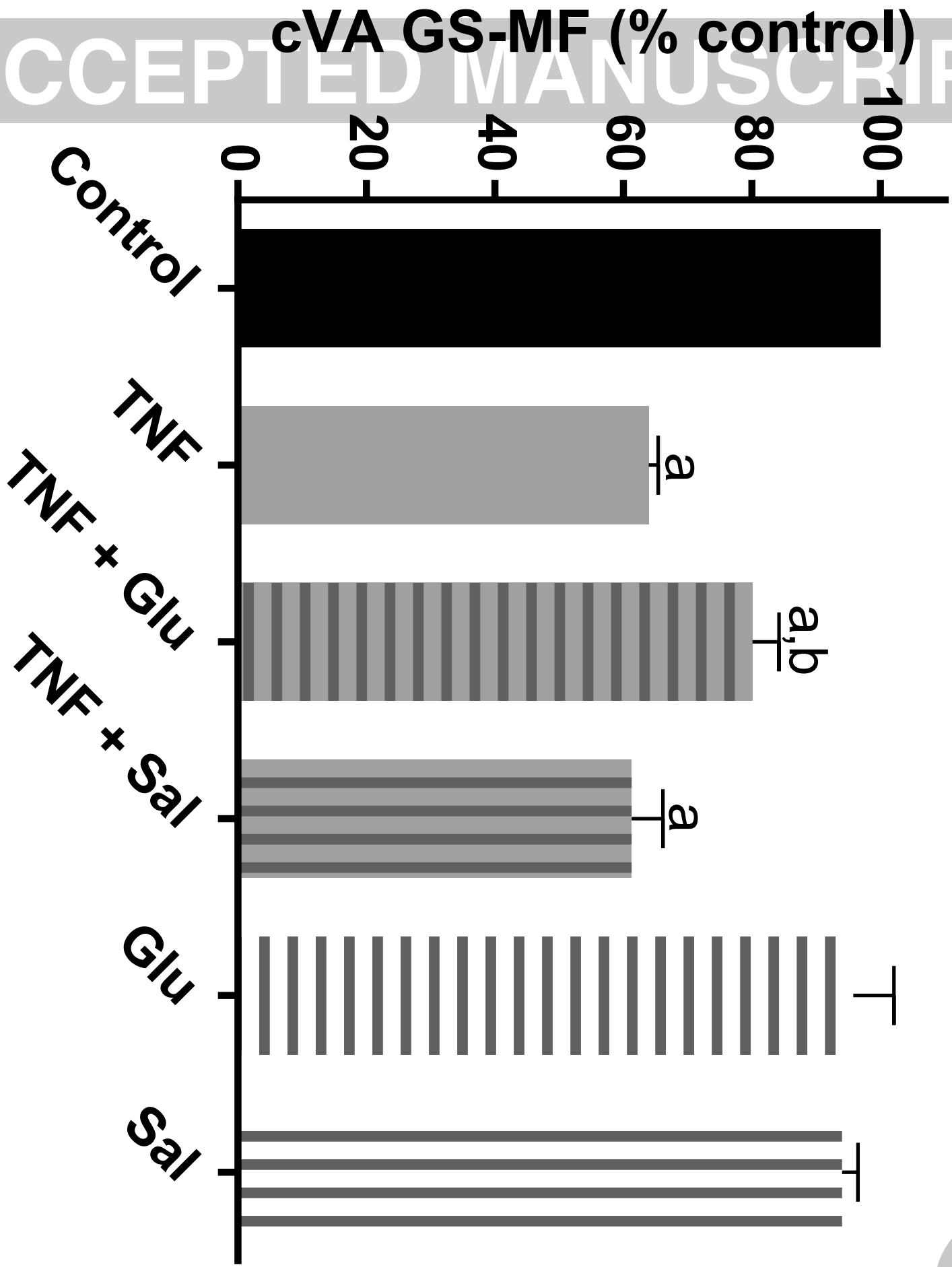
Fig. 5 Endocytic internalization of Abcc2 by TNFα is partly related to intracellular localization of endosomal Rab5 marker. In control RHCs, Abcc2 associated fluorescence (green) is delimited to the canalicular membrane and Rab5 (red) is mostly localized in perinuclear and pericanalicular areas. After TNFα treatment, Abcc2 is internalized from canalicular membrane to intracellular vesicles located in both perinuclear and pericanalicular regions that are surrounding by Rab5-associated fluorescence. TNFα by itself did not alter Rab5 cellular distribution. MEK1/2 inhibition by PD exhibited Abcc2 and Rab5 immunolocalization similar to control group.

Fig. 6 TNFα modulates Abcc2 activity by NOX-derived ROS production. (A) Time-response analysis of intracellular ROS levels: hepatocytes, cultured in 6-wells plates, were treated with TNFα (6.25 pg/ml) at different times, and then loaded for 30 min with 5 μM DCFH-DA. Fluorescence intensity of DCF formation was evaluated as reported in Material and Methods. Results are presented as percent of control values (dotted line at the value of 100%). (B) Effect of pretreatment with VitC (1 mM, 20 min) and the NOX inhibitor APO (300 μM, 30 min) in

DCF-fluorescence intensities in hepatocytes treated with TNFα (6.25 pg/ml, 10 min). (C) Effect of antioxidants in TNFα-induced impairment of GS-MF cVA. RHC were preincubated 20 minutes with VitC (1 mM) or mannitol (Man, 60 mM), and then exposed to TNFα (6.25 pg/ml) for a further 20-min period. The canalicular accumulation was expressed as the percentage of the total number of RHCs present in the field showing visible apical GS-MF-associated fluorescence, referred to control values (treated with DMSO). At least 200 RHCs were analyzed in each group. Data are shown as mean ± SEM (n = 3). [a] significantly different from control ($p < 0.05$). [b] significantly different from TNF ($p < 0.05$). [c] significantly different from TNF + Man ($p < 0.05$). [d] significantly different from TNF + VitC ($p < 0.05$)

Fig. 7 ROS produced by NOX mediates TNFα-induced Abcc2 impairment through MEK/ERK pathway. (A) Effect of co-incubation with the antioxidant VitC and MEK inhibitor PD on TNFα-induced impairment of cVA of GS-MF. Couplets pretreated for 15 min with PD (5 μM) or 20 min with VitC (1 mM), either alone or together, were subsequently exposed to TNFα (6.25 pg/ml) for a further 20-min period. Canalicular vacuolar accumulation of GS-MF was analyzed as described before. (B) Effect of vitamin C and apocynin on TNFα-induced phosphorylation of ERK. Hepatocytes were pretreated with VitC (1 mM, 20 min) or APO (300 μM, 30 min) and then exposed to TNFα (6.25 pg/ml) for additional 20-min period. ERK 1/2 activation was determined by immunoblots using antibodies against phosphorylated ERK and total ERK. Phosphorylation status was calculated as described before. Data are expressed as percentages of the control group and are shown as mean ± SEM (n = 3). [a] significantly different from control ($p < 0.05$). [b] significantly different from TNF ($p < 0.05$)





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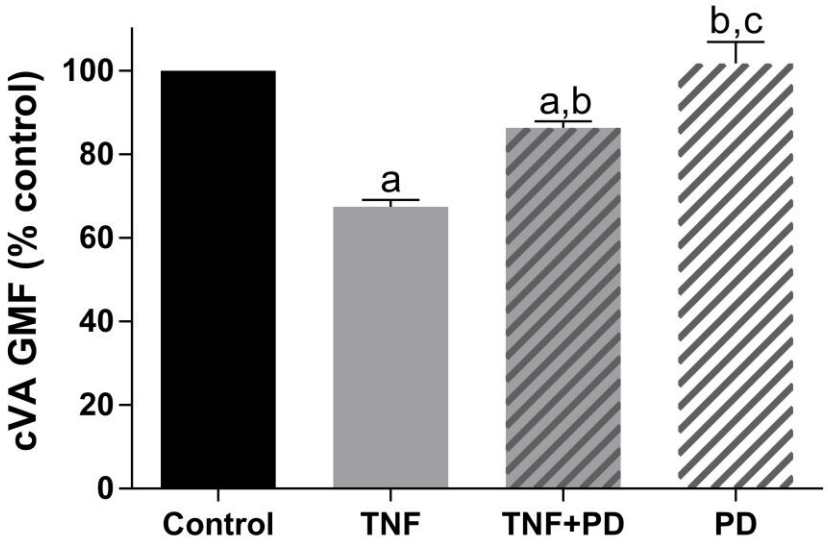
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100
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a
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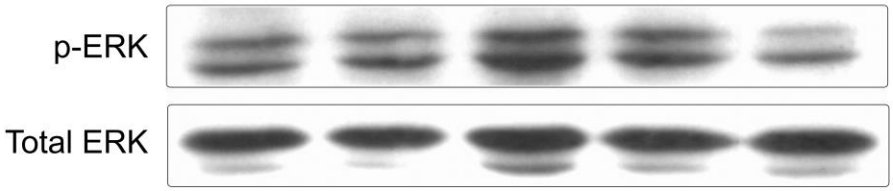
Control
TNF
TNF+PD
PD

B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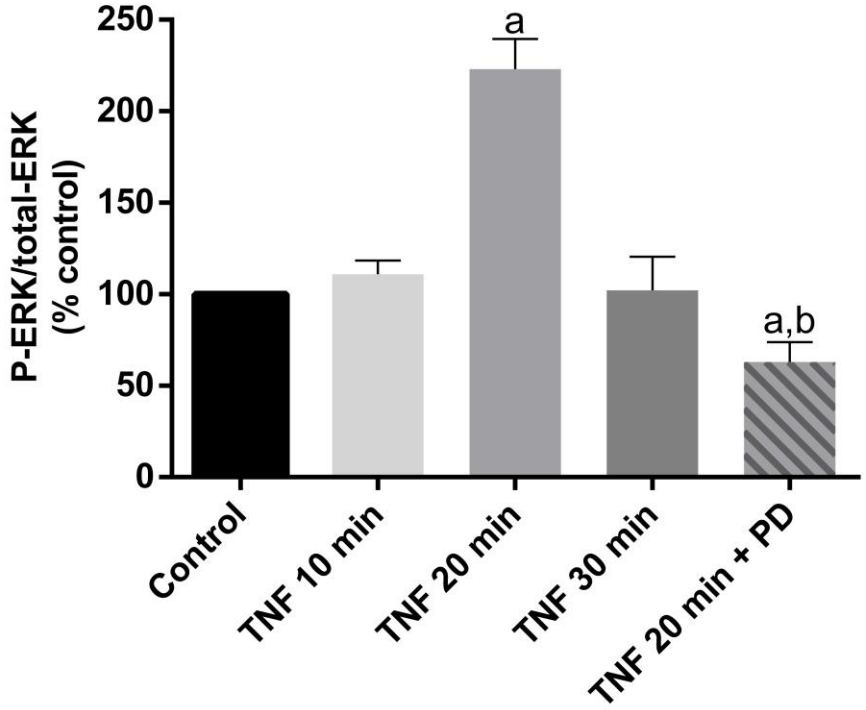
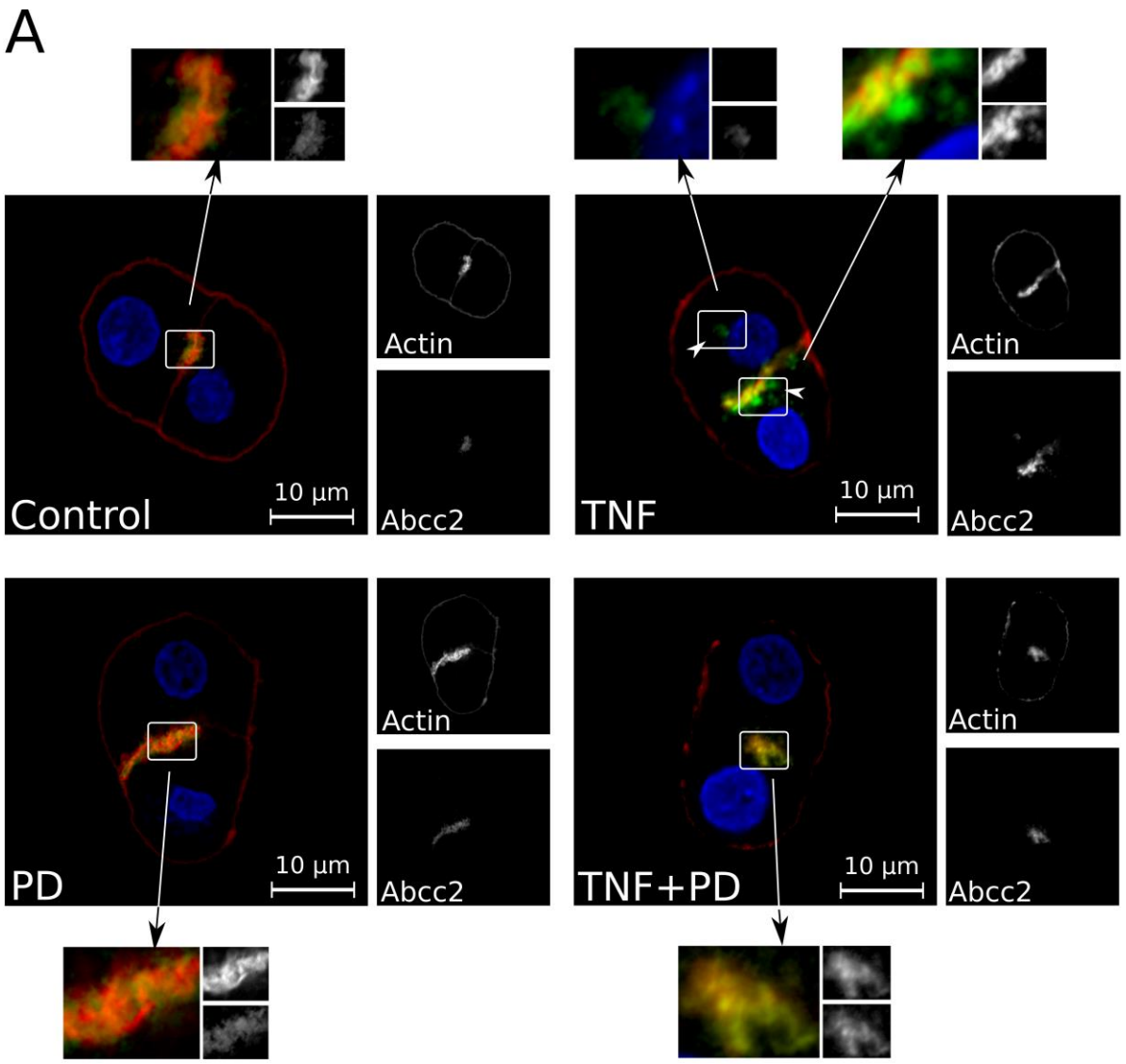

P-ERK/total-ERK (% control)
250
200
150
100
50
0
a
a,b
Control
TNF 10 min
TNF 20 min
TNF 30 min
TNF 20 min + PD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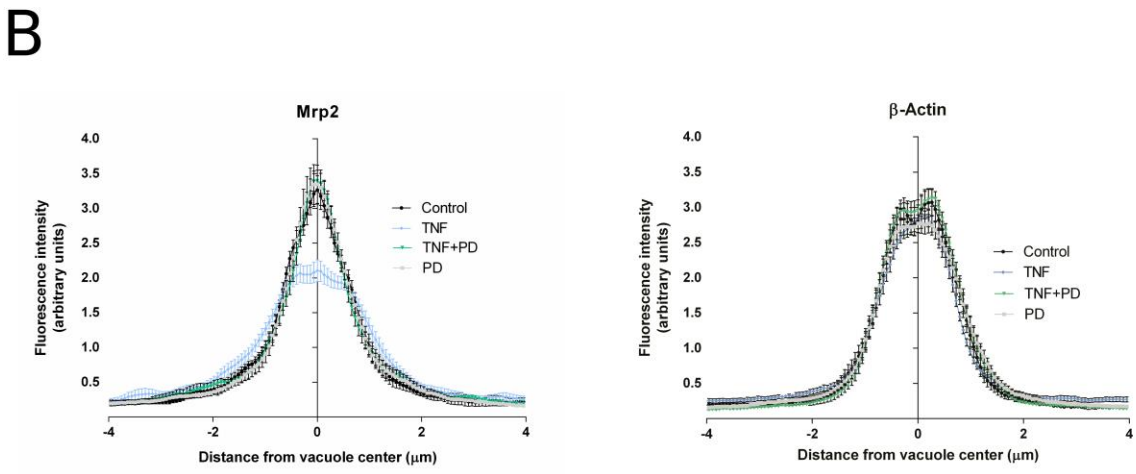
Actin
Abcc2


B
Mrp2
Fluorescence intensity (arbitrary units)
Distance from vacuole center (µm)
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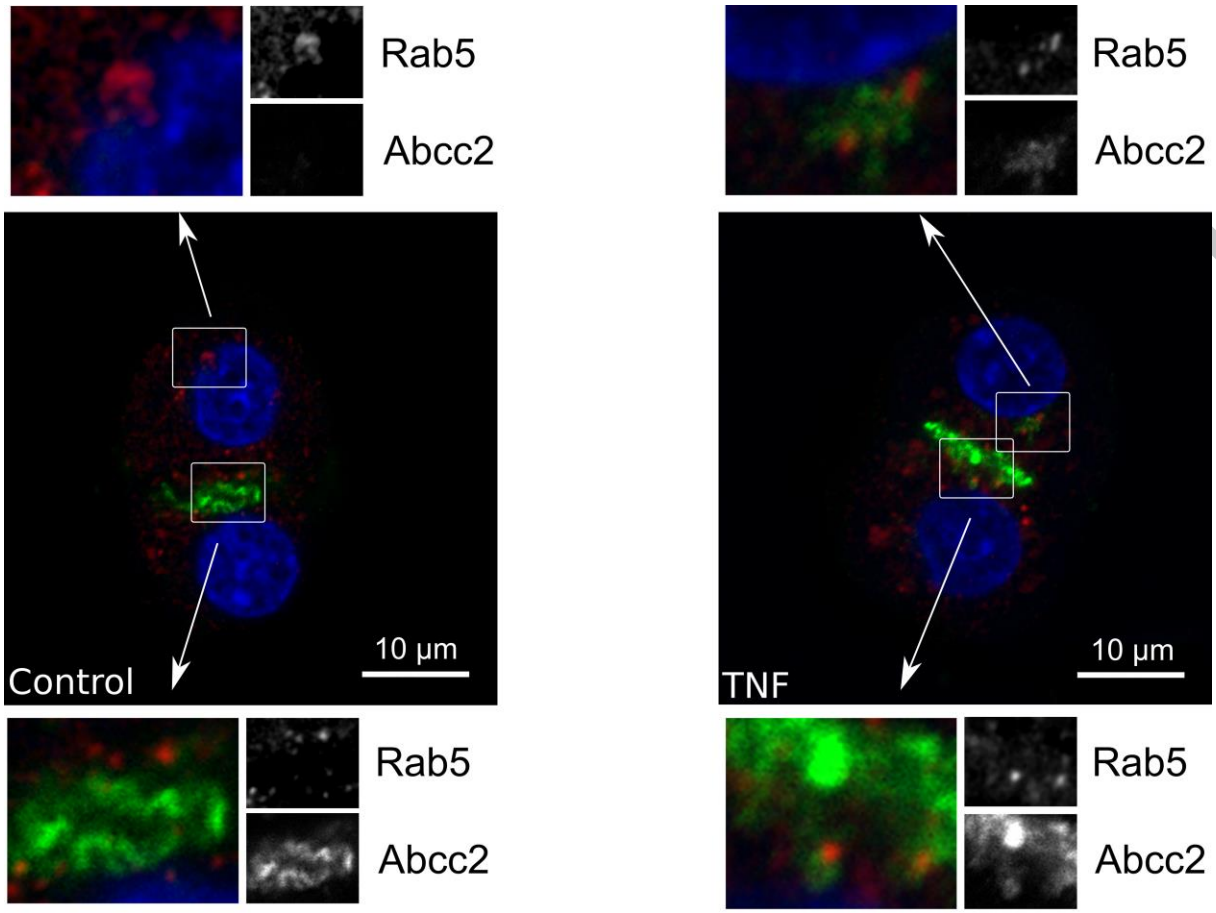
TNF+PD
PD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TNF
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Rab5
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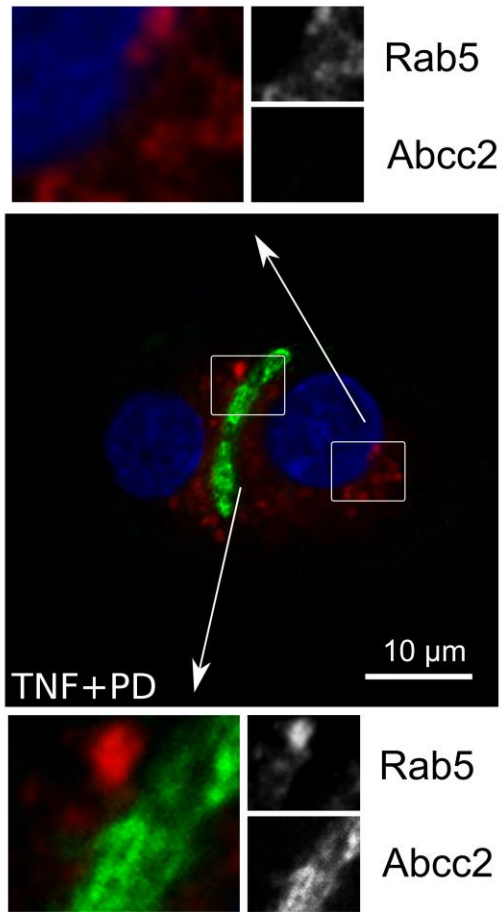
Abcc2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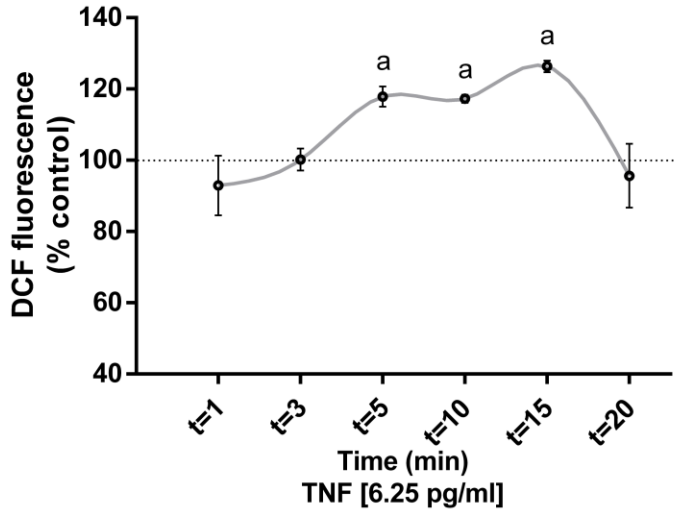
Abcc2
TNF+PD
10 μm
Rab5
Abcc2

A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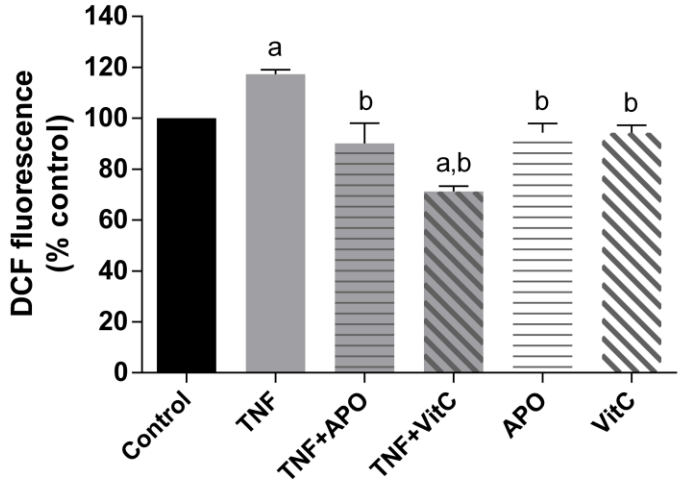
Time (min)
TNF [6.25 pg/ml]

B


DCF fluorescence (% control)
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APO
VitC

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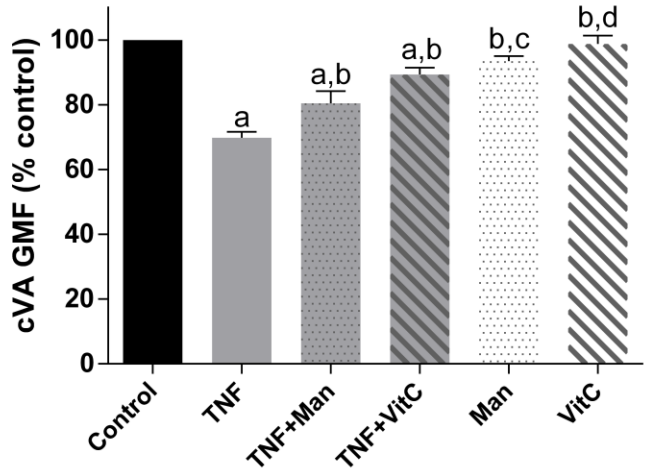
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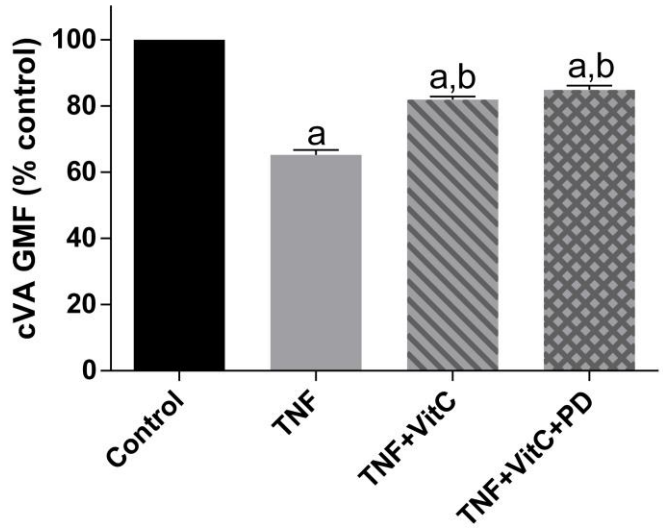
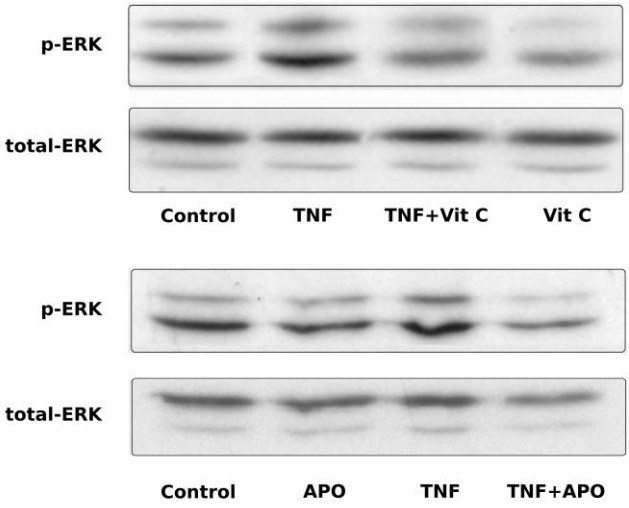
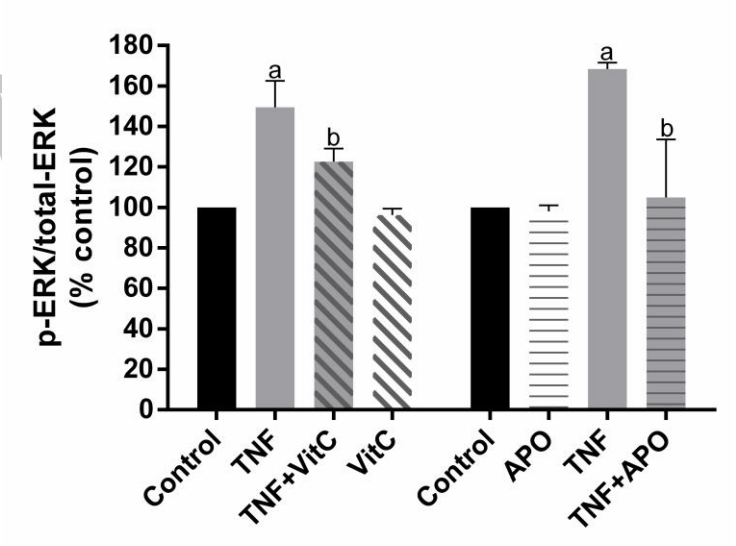
b,c
b,d
Control
TNF
TNF+Man
TNF+VitC
Man
VitC

A


cVA GMF (% control)
100
80
60
40
20
0
a
a,b
a,b
Control
TNF
TNF+VitC
TNF+VitC+PD

B


p-ERK
total-ERK
Control
TNF
TNF+Vit C
Vit C
p-ERK
total-ERK
Control
APO
TNF
TNF+APO


p-ERK/total-ERK (% control)
180
160
140
120
100
80
60
40
20
0
a
b
a
b
Control
TNF
TNF+VitC
VitC
Control
APO
TNF
TNF+APO

Schematic representation of TNFα-induced Abcc2 (Mrp2) internalization mediated by NOX-MEK/ERK pathway in rat hepatocytes