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Multidrug resistance related protein (ABCC1) and its role on nitrite production by the murine macrophage cell line RAW 264.7

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ABSTRACT

Multidrug resistance related protein 1 (MRP1/ABCC1) is an ABC transporter protein related to the extrusion of reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH-conjugates, as well as leukotriene C_4 and cyclopentane prostaglandins. Inhibition of ABCC1 activity impairs lymphocyte activation. The present work studied ABCC1 expression and activity on a murine macrophage cell line, RAW 267.4 and the effects of ABCC1 classical inhibitors, as well as GSH metabolism modulators, on LPS induced activation. Approximately, 75% of resting cells were positive for ABCC1 and the classical ABCC1 reversors (indomethacin, 0.1–2 mM; probenecid, 0.1–10 mM and MK571, 0.01–1 mM) were able to enhance intracellular CFDA accumulation in a concentration-dependent manner, suggesting ABCC1 inhibition. After LPS (100 ng/ml) activation 50% of the population was positive for ABCC1, and this protein was still active. In LPS-activated cells, ABCC1 activity was also impaired by BSO (1 mM), an inhibitor of GSH synthesis. Conversely, GSH (5 mM) reversed the BSO effect. ABCC1 inhibition by indomethacin, probenecid or MK571 decreased LPS induced nitrite production in a concentration-dependent manner, the same result was observed with BSO and again GSH reversed its effect. The ABCC1 reversors were also able to inhibit iNOS expression. In conclusion, LPS modulated the expression and activity of ABCC1 transporters in RAW macrophages and inhibitors of these transporters were capable of inhibiting nitrite production suggesting a role for ABCC1 transporters in the inflammatory process.

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1. Introduction

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins capable of transporting a wide variety of substrates across biological membranes in an energy-dependent manner. So far, 48 human ABC genes have been identified and divided into 7 distinct subfamilies (ABCA–ABCG) on the basis of their sequence homology and domain

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organization [1]. ABC transporters such as ABCB1 (P-glycoprotein) and the ABCC1 (multidrug resistance associated protein 1, MRP1 also known as GS-X) were initially detected in tumour cells. Their overexpression is associated with increased efflux of chemotherapeutic drugs, leading to a phenomenon known as multidrug resistance [2]. However, it is currently known that ABC transporters, especially ABCC1 have an ubiquitous expression, being also expressed in normal immune cells [3–5].

It has been demonstrated that ABCC1 activity is involved in cellular detoxification [1], macrophage activation [6] and lymphocyte stimulation [3]. Furthermore, there is evidence that ABCC1 participates in the migration of mature T lymphocytes and dendritic cells to lymph nodes [4]. This action seems to be mediated by endogenous substrates of this pump.

ABCC1 recognizes neutral and anionic hydrophobic natural products and transports glucoronides, both forms of gluthatione, oxidized (GSSG) and reduced (GSH) gluthatione, and its conjugates [7,8] such as the inflammatory mediators leukotriene C_4 (LTC_4) [9], prostaglandin A (PGA) and the 15-deoxy-delta 12,14 prostaglandin J_2 (15-d-PGJ_2) [10–12].

During inflammation, oxidant-generating enzymes are expressed or activated in phagocytes such as macrophages. These cells overproduce reactive oxygen species (ROS) through the NADPH oxidase a key enzyme that catalyzes the generation of superoxide and hydrogen peroxide, in a process called respiratory burst [13]. Other ROS are generated non-enzymatically as side products of reactions that utilize electron transfer [13]. Macrophages are also able to overproduce nitric oxide (NO) through the inducible nitric oxide synthase (iNOS) which generates NO from L-arginine [14]. NO can react directly with target molecules or is capable of generating potent oxidants called reactive nitrogen species (RNS) [15]. These RNS and ROS radicals are important in host defence because they behave as signalling molecules that activate expression of proinflammatory cytokine genes [15,16] and, also, because of their direct bactericidal activity. While these radicals can kill invading pathogens, they can also kill phagocytes themselves. However, macrophages have antioxidant defence systems that can be up regulated by activation, protecting themselves from radicals toxicity and contributing to the homeostasis of the intracellular redox state [17].

Gluthatione is present in virtually all eukaryotic cells with concentrations ranging from 0.5 to 10 mM and probably represents the most important antioxidant inside the cells, protecting cellular components against oxidative stress, maintaining proteins sulfhydryl groups (SH) in a reduced form [18]. GSH form conjugates with endogenous and exogenous compounds, being involved on cellular detoxification and transport [19]. This thiol is also important to ABCC1 activity and its depletion can alter drug resistance caused by ABCC1 overexpression [20–22].

Although it is known that ABCC1 participates in the maintenance of the redox state, in the process of cellular detoxification and it is able to secrete inflammatory mediators, little information is known about the expression, activity and role of this protein in macrophages. In the present study we investigated the expression and activity of ABCC1 in a murine macrophage cell line, RAW 264.7 as well as its role on macrophage activation measured by nitrite (NO_2) secretion and iNOS expression.

2. Material and methods

2.1. Materials and reagents

Cell culture plates and flasks were obtained from Corning Costar (Bodenheim, Germany). Calf serum (CS), Dulbecco's modified Eagle medium (DMEM), penicillin and streptomicyn were obtained from GIBCO BRL Life technologies (Rockville, MD, USA). L-Glutamine, hepes, bovine serum albumin (BSA), trypsin, LPS (serotype O111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 1,4-dithio-D,L-threitol (DTT), Tween-80, phenylmethanesulfonyl fluoride (PMSF), beta-mercaptoethanol, probenecid, indomethacin, L-buthionine-[S,R]-sulfoxide (BSO), reduced gluthatione (GSH), *N*-acetylcysteine (NAC), rabbit polyclonal antibody against iNOS and the goat anti-rat secondary (fluorescein conjugated) antibody were purchased from Sigma Co. (St. Louis, MO, USA). KCl was obtained from VETEC (Brazil). Primary rat monoclonal antibody anti-MRP1 (ABCC1) was purchased from Alexis Biochemicals (Lausen, Switzerland). Peroxydase-conjugated anti-rabbit IgG and peroxydase-conjugated anti-goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MK571 was gently donated by Merk (Kirkland, Que., Canada). The Griess reagents sulphanilamide and naphthyletilenediamine were obtained from Merk Research Laboratories (West Point, PA, USA). Ethylenediaminetetraacetic acid (EDTA) and glycerol were purchased from Invitrogen (Paisley, Scotland, UK). Sodium dodecyl sufate (SDS), polyacrylamide, and Tris were obtained from USB Corporation (Cleveland, OH, USA). The polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bredford, MA, USA). Reagents for SDS-eletrophoresis and enhanced chemiluminescence assay system were purchased by Amersham Biosciences (Piscataway, NJ, USA). (5-and-6)-Carboxyfluorescein diacetate (CFDA) was purchased from Molecular Probes (Eugene, OR, USA). The iNOS inhibitor 1400 W was obtained from Tocris Bioscience (MO, USA). Trizol, RNAse-free DNAse I Amplification Grade, RNAseOUT, Accuprime *Pfx* SuperMix, DNA Ladder were all purchased from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase, oligo dT 15, dNTP mix, DTT Molecular Grade were from Promega (Madison, WI, USA) and GoldView (SBS, Beijing, China).

The ABCC1 inhibitors were dissolved as follows: probenecid was dissolved in alkaline water and then the pH was adjusted to 7.8 in a stock solution of 140 mM. Indomethacin was first dissolved in Tris–HCl (0.15 M, pH 7.6) in a stock solution of 50 mM. MK571 was dissolved in saline. All the subsequent dilutions were made in culture medium.

2.2. Cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% heat inactivated calf serum, 10 U/ml penicillin 10 mg/ml streptomycin and maintained at 37 °C in a humidified

incubator with 5% CO_2. RAW 264.7 cells, 1.25×10^5; 5×10^5 or 2×10^6, were seeded in 96-, 24- or 6-well plates, respectively, and incubated at 37 °C for 18 h in a DMEM 10% supplemented with heat inactivated calf serum to reach confluence. The medium was removed and the plates washed to remove non-adherent cells. After this procedure different experiments were conducted. In experiments were NO_2 production was analyzed, cells were exposed to different concentrations of ABCC1 inhibitors: indomethacin (0.05–0.3 mM); probenecid (0.1–1 mM) or MK571 (0.01–0.1 mM) 5 min before and left throughout LPS (100 μg/ml) stimulation which extended for 24 h. In another set of experiments, cells were treated for 12 h before and during LPS stimulation with 1 mM of L-buthionine-[S,R]-sulfoxide (BSO) or 5 mM of *N*-acetylcysteine (NAC). In some experiments untreated cells as well as cells pre or co-treated with BSO were supplemented with 5 mM of GSH throughout LPS stimulation.

2.3. Nitrite assays and cell viability

For NO_2 quantification, macrophages were seeded in 24- or 96-well plates and treated as described above. Following the 24 h of activation, 100 μl of supernatant were harvested for NO_2 determination. Macrophage supernatant were mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid (w/v) and 0.1% naphtylethylenediamine dihydrochloride in water; 1:1, v/v) and incubated for 10 min at room temperature. The NO_2 concentration was determined by measuring mixture absorbance at 550 nm in an enzyme-linked immunosorbent (ELISA) assay plate reader (EL808, Bio-Tek Instruments Inc.). Nitrite concentration was calculated on the basis of standard solutions of sodium nitrite ($NaNO_2$).

Cell viability was determined by mitochondrial-dependent reduction of MTT to formazan as described by Mosmann [23] with minor modifications. Briefly, 1.25×10^5 cells were incubated in a 96-well plate with 10 μl of 5 mg/ml MTT in a total volume of 100 μl for 1 h. The supernatant was removed and the pellet was suspended in DMSO. The optical density (OD) was measured in an ELISA reader, using the wavelength of 550 nm.

2.4. Measurement of iNOS enzyme activity

Cells were activated with LPS (100 ng/ml) and after a 12 h period, the medium was discarded and the cells were treated with ABCC1 inhibitors: MK571 (0.1 mM), probenecid (1 mM), indomethacin (0.5 mM) or with the iNOS inhibitor, 1400 W (30 μM), without LPS. Afterwards cells were incubated for another 12 h, supernatants were removed and levels of nitrite and cell viability were determined using the Griess reagent and MTT assay, respectively, as described in Section 2.3.

2.5. CFDA accumulation by flow cytometry

To measure ABCC1 activity, macrophage cells were seeded in 24-well plates and allowed to reach confluence in DMEM 10% supplemented with calf serum. Twenty-four hours after LPS activation the supernatant was removed and cells were incubated in DMEM 10% calf serum at 37 °C for 30 min with 2.5 μM of CFDA in the presence or absence of reversors (probenecid, indomethacin and MK571) in different concentrations. Cells were then washed with PBS and left in DMEM 10% at 37 °C for a further 30 min with or without reversors, washed re-suspended with trypsin (0.5% trypsin and 0.03% EDTA), washed again, suspended in cold PBS and kept on ice.

In another set of experiments, cells were treated for 12 h before and during LPS stimulation with 1 mM of BSO. In some experiments untreated cells, as well as cells pre and co-treated with BSO, were supplemented with GSH (5 mM) throughout LPS stimulation. Twenty-four hours after LPS activation cells were incubated with CFDA as described above.

For those experiments data were acquired in a mode of 10,000 events, and a gate based on forward scatter (FSC) and side scatter (SSC) parameters was made to analyze only viable cells. Analyses were performed by flow cytometry using a FACScan (Beckton and Dickinson, USA). The data were analyzed by WinMID software. The autofluorescence value of the cells was subtracted from the mean intensity fluorescence of cells labelled with CFDA. Results are presented by mean fluorescence intensity or as percentage of CFDA intensity.

2.6. Western blotting analysis

RAW 264.7 cells were cultured in 6-well plates until confluence, exposed to ABCC1 inhibitors; MK571 (100 μM), probenecid (1 mM) and indomethacin (0.5 mM) and stimulated with LPS (100 ng/ml) for 24 h. The cells were washed, scrapped in PBS, and centrifuged ($1000 \times g$) for 10 min at 4 °C. Pellets were re-suspended in lysis buffer (10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Hepes, pH 7.6) and allowed to rest for 20 min on ice. Samples were sonicated for 5 min and centrifuged ($12{,}000 \times g$) for 5 min at 4 °C. Supernatants (cytosolic extracts) were used for Western blot assay. Protein concentrations were determined using the Bradford method [24]. Lysates were boiled in equal volumes of loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% beta-mercaptoethanol) and aliquots corresponding to 10 μg protein were run in a 10% polyacrylamide gel, transferred on to PVDF membrane at 4 °C 300 mA, which was blocked overnight at 4 °C with phosphate-buffered saline containing 0.1% Tween-20 (v/v) and 5% (w/v) defatted milk. Following washing, the membrane was probed with rabbit polyclonal anti-iNOS (1:10,000, v/v), or goat polyclonal anti-actin (1:500, v/v) for 2 h, and detected with a 1:2000 diluted peroxydase-conjugated anti-rabbit IgG and peroxydase-conjugated anti-goat IgG (1:5000, v/v), respectively. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amershan Biociences).

2.7. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 (1.5×10^6) cells were cultured in flasks (25 cm^2) for 2 days, the medium (5 ml) was changed and the cells were exposed to ABCC1 inhibitors; MK571 (100 μM), probenecid (1 mM) or indomethacin (0.5 mM) and stimulated with LPS (100 ng/ml) for 8 h. Total RNA was extracted from treated cells by using the Trizol reagent protocol. To eliminate the

passenger DNA, the samples were treated with RNAse-free DNAse I Amplification Grade using the kit protocol. The reverse transcription assay was performed as described in the M-MLV Reverse Transcriptase protocol. Briefly, the reverse transcription reaction was performed by using 0.5 μg of total RNA, 1 μl of oligo dT 15, 1 μl of dNTP mix (10 mM) and ultra pure water up to 12 μl. The mixture was heated for 5 min at 65 °C and quick chilled in ice. To the mixture were added 4 μl of first strand buffer, 2 μl of 0.1 M DTT Molecular Grade and 1 μl of RNAseOUT. The mixture was incubated at 37 °C for 2 min and 1 μl (200 U) of M-MLV reverse transcriptase was added. The samples were then incubated for 50 min at 37 °C and the reaction was inactivated at 75 °C for 15 min. The c-DNAs were stores at 4 °C until the PCR reaction was done. The amplification reaction was realized as described in Accuprime™ *Pfx* SuperMix protocol. Briefly 0.75 μl of the c-DNA was added to 11.25 μl of the Accuprime™ *Pfx* SuperMix. To this mixture 0.25 μl of the Antisense prime for iNOS (CTTCTGGTCGATGTCATGA) or β-actin (CGTCTCCGGAGTCCATCACA), 0.25 μl of the Sense prime for iNOS (CAGAAGCAGAATGTGACCATC) or β-actin (TCCTTCGTTGCCGGTCCACA) and 0.5 μl of $MgCl_2$ (25 mM) (Qbiogene, Irvine, CA, USA) were added. The conditions for PCR were 5 min at 95 °C followed by different cycle numbers of 15 s at 95 °C, 30 s at 50 °C and 1 min at 68 °C. The PCR step was performed for 20, 25, 30 and 35 cycles for iNOS and β-actin in an DNA thermal cycler (Eppendorf Mastercycler personal, Hamburg, Germany). The PCR products were size fractionated in 1.8% agarose gels stained with 1 μl of GoldView™. The DNA band sizes were confirmed by using the 50 pb DNA Ladder.

2.8. Statistical analysis

All results are presented as the mean ± S.E.M. Statistical comparison of the data was performed using ANOVA one-way analysis of variance, followed by Neuman–Keuls multiple comparison test. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Expression and activity of ABCC1 in resting RAW 264.7 macrophages

The ABCC1 protein has been described already in murine macrophages by functional assays [6]; however, to our knowledge, it has not been demonstrated in this cell line. To analyze the expression of ABCC1 on RAW macrophages, cells were stained with anti-ABCC1 antibodies and studied by flow cytometry (Fig. 1A). Approximately, 75% of the population was positive for ABCC1. Thus, the activity of this protein was evaluated using the fluorescent substrate CFDA. It has been shown that efflux of this fluorescent dye from pre-labeled cells

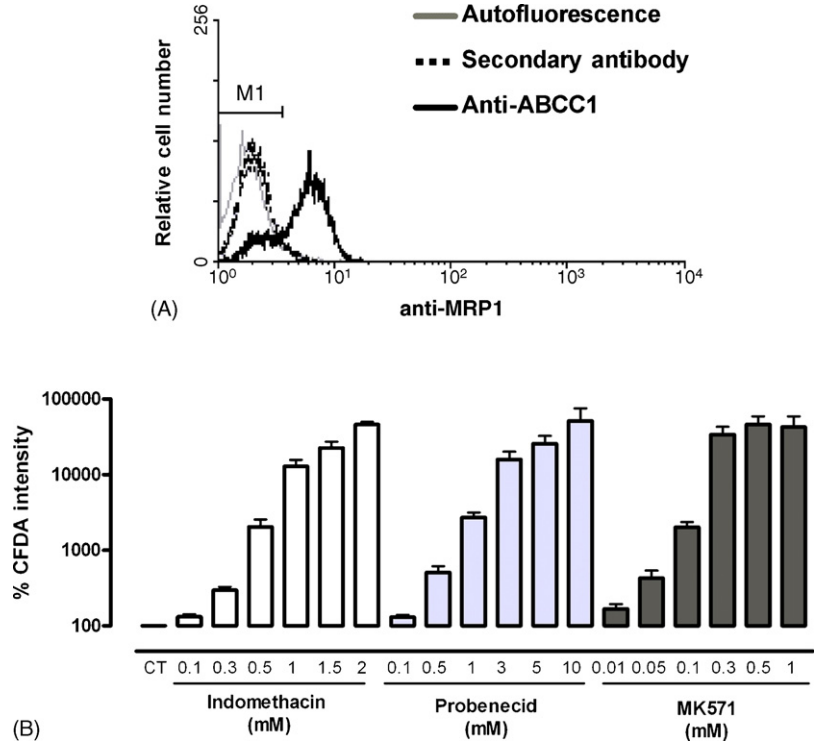

Fig. 1 – ABCC1 expression and related activity of resting RAW macrophages. (A) Expression of ABCC1 in resting cells. Cells were permeabilized, labelled with mAb anti-ABCC1 and incubated with the secondary antibody. Control was labelled only with the secondary antibody. (B) Inhibition of CFDA efflux by different reversors. Resting RAW macrophages were incubated with 2.5 μM CFDA in the presence or absence of indomethacin (0.1–2 mM), probenecid (0.1–10 mM) or MK571 (0.01–1 mM). The columns represent CFDA mean intensity fluorescence as percentage relative to control cells (cells incubated only with CFDA). Results represent mean ± S.E.M. of at least four independent experiments.

correlates with ABCC1 activity [25]. To test the sensitivity of ABCC1 activity to different modulators/reversors, RAW macrophages were treated with indomethacin (0.1–2 mM), probenecid (0.1–10 mM) and MK571 (0.01–1 mM). These classical ABCC1 reversors were able to enhance intracellular CFDA accumulation in a concentration-dependent manner, suggesting ABCC1 inhibition (Fig. 1B).

3.2. *Expression and activity of ABCC1 in LPS-activated RAW 264.7 macrophages*

There are evidences that inflammation regulates ABC transporters expression [26], it has also been shown that murine macrophages stimulated by Bacille Calmette Guerin (BCG) show an enhanced GS-X activity, which is known to correspond to ABCC1 [6]. The expression of ABCC1 on LPS-activated RAW macrophages was, therefore, analyzed. Cells were activated by LPS (100 ng/ml) for 24 h and ABCC1 expression was measured (Fig. 2A). Approximately, 50% of the population was positive for ABCC1, demonstrating that LPS activation negatively modulates ABCC1 expression. To verify if ABCC activity was altered in LPS-activated RAW 264.7 macrophages compared to resting cells, activated macrophages were concomitantly incubated with CFDA and ABCC1 reversors. Indomethacin, probenecid and MK571 were able to raise CFDA accumulation in a concentration-dependent manner (Fig. 2B), showing that this protein is active in LPS-activated RAW macrophages.

3.3. *Effect of ABCC1 modulators on nitrite production*

It has been demonstrated that ABCC1 activity inhibition blocks the activation of T CD4 Th1 lymphocytes [3]. To verify if ABCC1 function is important for macrophage activation, cells were incubated with ABCC1 reversors 5 min prior to LPS activation. As NO_2 production is a feature of activated macrophages, the amount of NO_2 was analyzed in the supernatant 24 h after LPS activation. Fig. 3A shows that ABCC1 reversors inhibited NO_2 production in a concentration-dependent manner, suggesting that ABCC1 activity is important to NO_2 production. Alternatively, to verify if ABCC1 reversors were able to inhibit iNOS activity, cells were firstly activated with LPS for a 12 h period, when iNOS expression was already detected by Western blot (data not shown). Afterward, the medium was removed and cells were incubated for another 12 h period without LPS in the presence or absence of the ABCC1 reversors MK571 (100 μM), probenecid (1 mM), indomethacin (0.5 mM) or with the iNOS inhibitor, 1400 W. Subsequently the ability of ABCC1 reversors to diminish nitrite production was analyzed. As shown in Fig. 3B the ABCC1 reversors were not able to reduce NO production after LPS activation, suggesting that the reversors dot not act by inhibiting the iNOS enzyme activity per se.

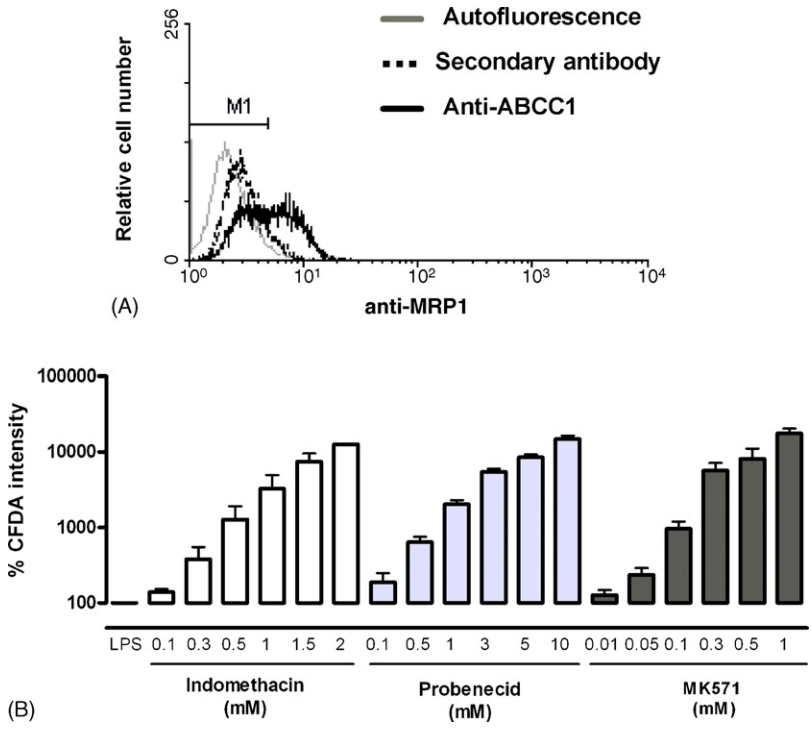

Fig. 2 – ABCC1 expression and related activity of LPS-activated RAW macrophages. (A) Expression of ABCC1 in LPS-activated cells. Twenty-four hours following LPS activation cells were permeabilized, labelled with mAb anti-ABCC1 and incubated with the secondary antibody. Control was labeled only with the secondary antibody. (B) Inhibition of CFDA efflux by different reversors. LPS-activated RAW macrophages were incubated with 2.5 μM CFDA in the presence or absence of indomethacin (0.1–2 mM), probenecid (0.1–10 mM) or MK571 (0.01–1 mM). The columns represent CFDA mean intensity fluorescence as percentage relative to control cells (cells incubated only with CFDA). Results represent mean ± S.E.M. of at least four independent experiments.

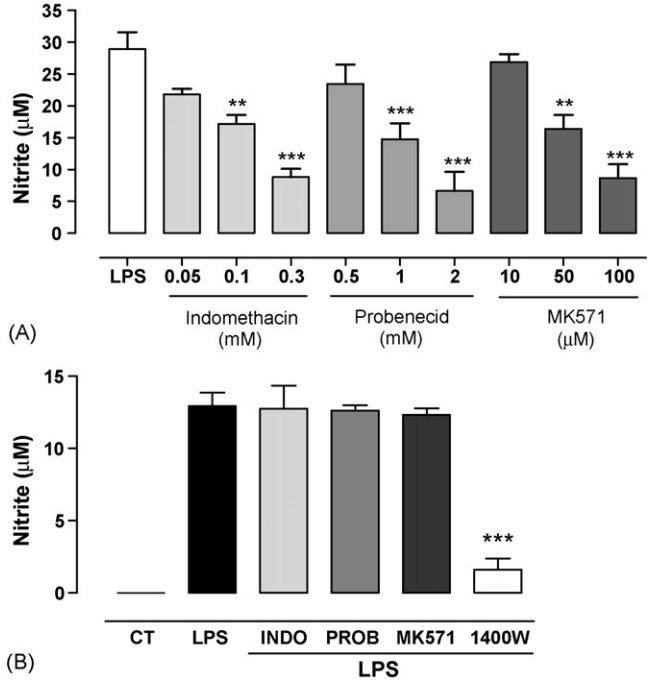

Fig. 3 – Effect of ABCC1 reversors on RAW macrophage NO_2 production. (A) ABCC1 reversors reduced NO_2 production when incubated before iNOS induction. RAW macrophages were stimulated with LPS 100 ng/ml in the presence or absence of different concentrations of indomethacin (50–300 μM), probenecid (0.5–2 mM) or MK571 (10–100 μM) and the supernatants were harvested 24 h later for determination of NO_2 production. (B) ABCC1 reversors effect on NO_2 production after iNOS induced expression. Cells were firstly activated with LPS for a 12 h period, the medium was removed and cells were incubated for another 12 h period without LPS in the presence or absence of the ABCC1 reversors MK571 (100 μM), probenecid (PROB, 1 mM), indomethacin (INDO, 0.5 mM) or with the iNOS inhibitor, 1400 W (30 μM). Subsequently the ability of ABCC1 reversors to diminish nitrite production was analyzed. Results represent mean ± S.E.M. of at least four independent experiments. $^{**}P < 0.01$ $^{***}P < 0.001$ relative to LPS treated cells.

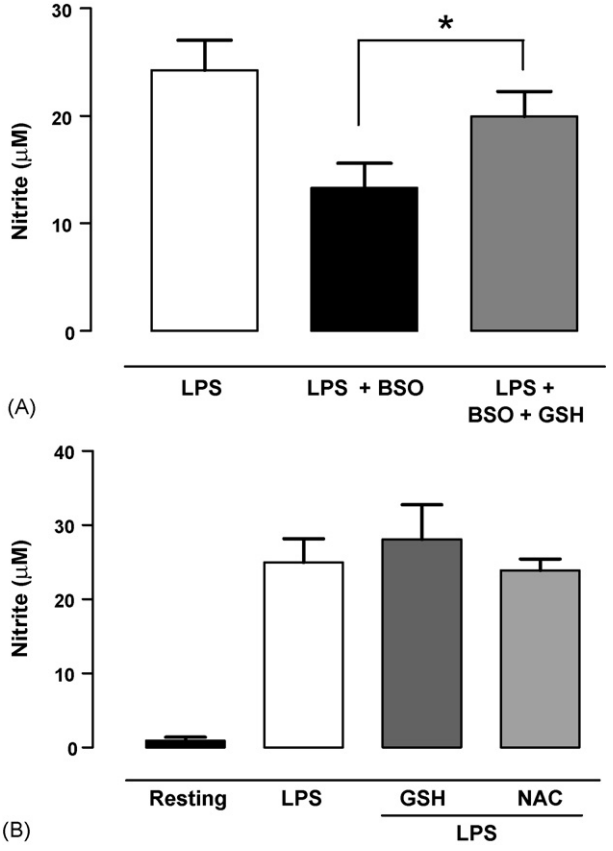

Fig. 4 – Effect of BSO, GSH and NAC on RAW macrophage NO_2 production. (A) RAW macrophages were treated or not with 1 mM of L-buthionine-[S,R]-sulfoxide (BSO) for 12 h before, and during LPS (100 ng/ml) stimulation. Cells pre-treated with BSO were stimulated with LPS in the presence or absence of gluthatione (GSH 5 mM). (B) Black column, resting cells. Cells were activated with LPS for 24 h in the absence (white column) or presence of GSH (dark grey column). Some cells were treated with N-acetylcystein (NAC, 5 mM) for 12 h before, and during 24 h of LPS (bright grey column). Twenty-four hours after LPS activation, supernatants were harvested for the determination of NO_2 production. Results represent mean ± S.E.M. of at least four independent experiments. $^{*}P < 0.05$ relative to LPS treated cells.

3.4. *Effect of BSO and GSH on nitrite production*

It is presently known that depletion of GSH by BSO reverses multidrug resistance reducing the transport of ABCC1 substrates [20–22]. Other authors suggested that glutathione status can alter mouse bone marrow monocyte-derived macrophage differentiation [27] and some characteristics of activation such as phagocytic activity [27] and NO_2 production [28,29]. To verify if BSO could modulate NO_2 production by RAW macrophages, cells were pre-treated or not with this drug for 12 h before LPS activation. After this period the untreated cells received LPS alone, while LPS plus BSO or LPS plus BSO and GSH were added to BSO pre-treated cells for a further 24 h. As shown in Fig. 4A BSO decreased NO_2 production in 46% while GSH partially restored it to 82% of control values. The modulation of NO_2 production did not represent a loss nor an increase in cellular viability, since none of the compounds tested altered it (data not shown). However, neither GSH nor NAC could modify NO_2 production per se (Fig. 4B).

3.5. *Effect of BSO and GSH on ABCC1 activity*

To verify if this diminished NO_2 production was a reflection of reduced ABCC1 activity, cells were treated as described above and incubated with CFDA. As shown in Fig. 5, BSO increased CFDA accumulation, suggesting ABCC1 inhibition, while GSH restores it. This result reinforced a possible role of ABCC1 activity on NO_2 production.

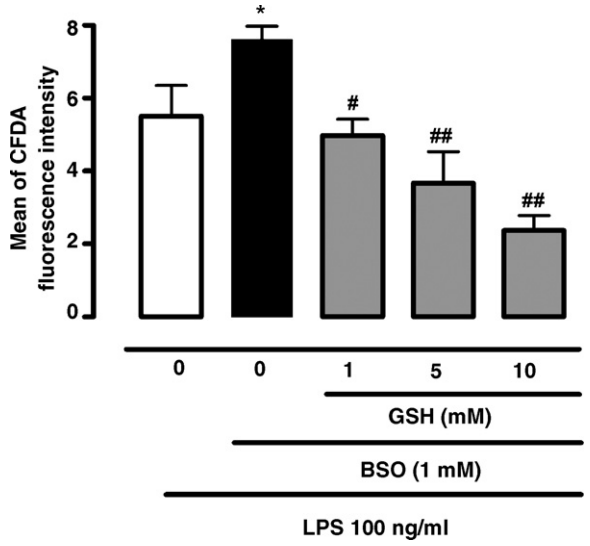

Fig. 5 – Effect of BSO and GSH on CFDA accumulation. Cells pre-treated with BSO (1 mM) for 12 h were stimulated with LPS (100 ng/ml) in the presence of BSO and in the presence or absence of GSH (1–10 mM). Twenty-four hours later CFDA accumulation was analyzed. White column, LPS-activated cells; black column, BSO treatment before and during LPS stimulation; grey column, cells pre-treated with BSO and stimulated with LPS in the presence of BSO (1 mM) and GSH (1–10 mM). The columns represent CFDA mean intensity fluorescence (arbitrary values). Results represent mean ± S.E.M. of at least three independent experiments. $^{*}P < 0.05$ different from LPS treated cells (CT). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ different from cells pre-treated with BSO and stimulated by LPS.

3.6. *Effect of ABCC1 inhibitors on iNOS expression*

It has been demonstrated that BSO [28] as well as indomethacin [30] can inhibit iNOS expression on macrophages. Probenecid and MK571 reverse multidrug resistance as well as BSO and indomethacin, and we have shown that all these drugs are capable of diminishing NO_2 production. Thus, the effect of ABCC1 classical reversors on iNOS expression was analyzed. Fig. 6A and B shows that MK571 (100 μM), probenecid (PROB, 1 mM) and indomethacin (INDO, 0.5 mM) decrease the levels of iNOS induced by LPS. However, to more precisely define the relationship between ABCC1 and NO synthesis we evaluated whether the ABCC1 reversors were capable of modulating iNOS synthesis at transcriptional level. To better quantify the iNOS/β-actin mRNA expression ratio, PCR products resulted from different numbers of PCR reaction cycles were analyzed (Fig. 6C and D). Fig. 6E shows the iNOS/β-actin mRNA expression ratio quantification obtained with 30 cycles. The results shown in Fig. 6 demonstrate that MK571, probenecid (PROB) and indomethacin (INDO) were not able to inhibit the iNOS mRNA expression, limiting their effect to the amount of protein present.

4. Discussion

The present work describes the expression and activity of ABCC1 in resting and LPS-activated RAW 267.4 macrophages. It also indicates that ABCC1 activity may play a role in the regulation of NO_2 production by reducing the amount of iNOS protein expression but not iNOS mRNA expression, nor its activity, in these cells.

In cells stimulated for 24 h with LPS a decreased ABCC1 expression was observed. This result is in agreement with other studies showing that LPS is capable of decreasing the expression of some ABC transporters, such as mdr1a and abcc2 in human liver, rat jejunum and in mouse placenta [26,31,32]. Nevertheless, LPS was shown in another study to increase rat hepatic transporters such as abcc1, abcc3 and mdr1b [33] while, in mouse liver, it decreases the expression of abcc2 and abcc3 but did not alter abcc1 expression [34]. Collectively, these results indicate that the regulation of ABC transporters is complex and may vary between species and cell type. Despite the decreased expression observed by us, our results suggest that this pump is more active after LPS activation, as resting and activated cells present a similar degree of extrusion of the ABCC1 substrate CFDA. These results are in accordance with those of de Bittencourt Jr. et al. [6] that demonstrated that activated rat peritoneal macrophages display an enhanced ABCC1/GS-X activity. The possibility, however, that other ABCC transporters are involved in CFDA extrusion, cannot be discarded.

Although the present work was carried out using a murine macrophage cell line, human macrophages derived from peripheral blood monocytes [35], rat peritoneal macrophages [6] and mouse peritoneal macrophages (unpublished observation), all displayed ABCC1 expression or activity, suggesting that this molecule plays a role in these cells and is not just a feature of a transformed cell line.

In LPS-activated cells ABCC1 transport activity could be altered by modulators of GSH metabolism, such as BSO. This result is in agreement with other studies showing that BSO is capable of reversing multidrug resistance related to ABCC1 activity [21,22]. In our hands, BSO inhibited both CFDA and NO_2 secretion by LPS-activated RAW macrophages and the addition of GSH partially reversed this effect. These effects seem to be related to ABCC1 activity as ABCC1 reversors such as: indomethacin, probenecid and MK571, were capable of inhibiting both NO_2 secretion and iNOS protein expression, in addition to their well known function of inhibiting the transport of CFDA.

On the cross-talk between NOS and COX pathway contradictory results have been reported. It has been shown that when prostanoid synthesis was abrogated by several COX inhibitors, such as acetyl-salicyclic acid (ASA), 6-methoxy naphthalene acetic acid (6-MNA), as well as indomethacin a decrease in NO production and iNOS protein expression induced by LPS was observed [30]. Therefore, the inhibition produced by indomethacin could be explained by COX inhibition. However, in macrophages activated with zymosan the use of another COX inhibitor, NS398, had no effect on iNOS protein expression nor NO_2 production [36]. The present work presents an alternative explanation, indomethacin could be inhibiting LPS induced NO via ABCC1 inhibition as probenecid

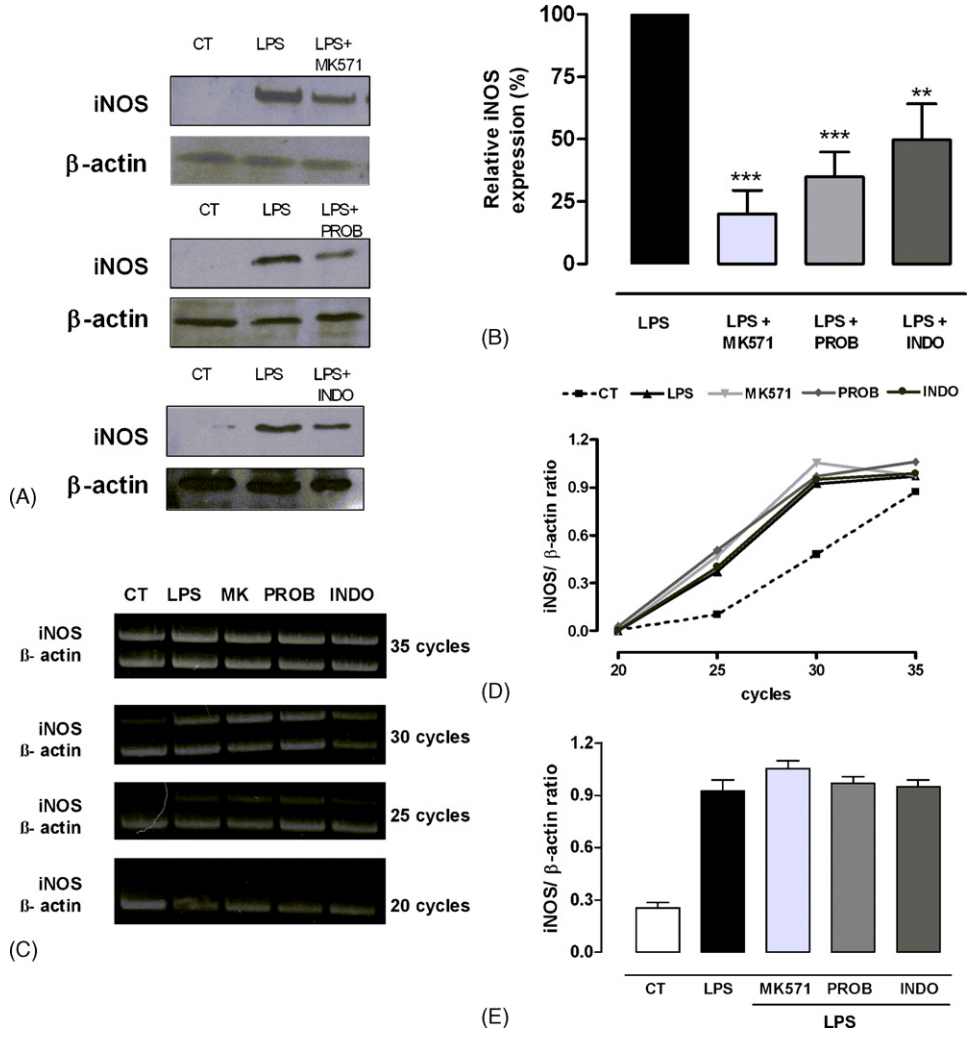

Fig. 6 – Effect of ABCC1 reversors on iNOS expression induced by LPS. Cells were activated by LPS in the presence or absence of MK571 (100 μM), probenecid (PROB, 1 mM) and indomethacin (INDO, 0.5 mM). Twenty-four hours later cells were lysed and the cytosolic extract was used for Western blot analysis. Alternatively, 8 h after activation, RNA was extracted and the semi-quantitative RT-PCR realized. The expression of the mRNA of iNOS and β-actin were analyzed by agarose-gel electrophoresis (1.8% gel) and staining with GoldView. (A) Representative Western blot gel. (B) The quantification as percentage of the ratio between iNOS and β-actin protein bands relative to LPS treated cells. (C) Representative RT-PCR products from different numbers of PCR reaction cycles. (D) The quantification of the ratio between iNOS and β-actin expression along the PCR reaction cycles. (E) The quantification of the ratio between iNOS and β-actin expression obtained with 30 cycles of PCR reaction. Results represent mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

and MK571, two other ABCC1 reversors, presented the same effect.

It has been suggested that cisteinyl leukotrienes may be involved in the NO production induced by LPS [37], being known that the relaxant effect of LTD4 over the endothelium is via the release of NO [38]. This leukotriene was shown to be formed in vivo through the conversion of exogenously administered LTC_4 [39]. Leukotrienes are also produced by peritoneal macrophages [40,41] as well as by RAW cells [42,43] under different stimuli. In fact, MK571 is a $CysLT_1$ receptor antagonist and was able to reduce NO production. Moreover, ABCC1 is an LTC_4 transporter, and its inhibition by indomethacin, probenecid or MK571 could inhibit LTC_4 release and, as a consequence, its conversion to LTD4 and its function as an autocrine signal to produce NO. It has been reported that iNOS protein expression and NO production in RAW cells is decreased by the 5-lipoxygenase inhibitor ZM 230.487 [36]. Glutathione also participates in the synthesis of LTC_4, what could explain the inhibitory effect of BSO on NO production as well as its partial reversion by GSH.

Recently, Watts et al. demonstrated that NO-mediated Fe and GSH efflux was significantly decreased by BSO and other ABCC1 inhibitors such as MK571 and probenecid [44] suggesting an interaction between these two systems.

Alternatively, all the observed effects could be the result of an alteration in the cell redox status, important to allow translocation of NF-κB to the nucleus where it will regulate iNOS expression [16]. BSO, which inhibits the new synthesis of

reduced glutathione, can inhibit this translocation [16], and inhibition of ABCC1 transport by indomethacin, MK571 or probenecid may change the ratio GSH/GSSG as this transporter has a higher affinity to oxidized glutathione than to reduced glutathione [1]. However, neither indomethacin, probenecid nor MK571 were capable of decreasing iNOS mRNA expression, suggesting that the regulation was not at the transcriptional level. In this regard, other reports have shown post-transcriptional regulation of iNOS expression such as iNOS mRNA translation and protein stability [45]. Moreover, it has been demonstrated in RAW 264.7 cells that iNOS is subjected to ubiquitination and protein degradation by the proteasome pathway [46] which could contribute to the observed effects of ABCC1 reversors upon iNOS expression. Although speculative at this moment, this hypothesis warrants further investigation.

In conclusion, LPS modulates the expression and activity of ABCC1 transporters in RAW macrophages and inhibitors of these transporters are capable of inhibiting NO_2 production suggesting a role for ABCC1 transporters in the inflammatory process.

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