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ACCELERATED PUBLICATION

The mitochondrial citrate carrier: a new player in inflammation

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The mitochondrial CIC (citrate carrier) catalyses the efflux of citrate from the mitochondrial matrix in exchange for cytosolic malate. In the present paper we show that CIC mRNA and protein markedly increase in lipopolysaccharide-activated immune cells. Moreover, CIC gene silencing and CIC activity inhibition significantly reduce production of NO, reactive oxygen species and prostaglandins. These results demonstrate for the first time that CIC has a critical role in inflammation.

Key words: citrate carrier (CIC), gene regulation, immune cell, nitric oxide (NO), prostaglandin E_2, reactive oxygen species (ROS).

INTRODUCTION

Inflammation is an immune response to infection, tissue injury and other noxious insults [1]. It is characterized by the production of inflammatory mediators such as cytokines, PGE_2 (prostaglandin E_2), NO and ROS (reactive oxygen species) by cells of the innate and adaptive immune system. The nuclear transcription factor NF-κB (nuclear factor κB) is a pivotal orchestrator of inflammation [2,3]. Exposure of cells to LPS (lipopolysaccharide) or other pro-inflammatory stimuli leads to the activation of NF-κB, which stimulates target gene expression [4]. Many studies have shown that fatty acids modulate NF-κB activation and macrophage functions [5,6]. However, until now there has been no publications on the role of the mitochondrial CIC (citrate carrier) in inflammation. CIC is an integral inner mitochondrial membrane protein that catalyses the export of citrate from the mitochondrial matrix in exchange for cytosolic malate [7,8]. This transporter is essential for fatty acid biosynthesis because citrate in the cytosol is cleaved to acetyl-CoA and oxaloacetate by citrate lyase. Acetyl-CoA is directly used for fatty acid synthesis, and oxaloacetate produces NADPH plus H^+ (also necessary for fatty acid production) via malate dehydrogenase and malic enzyme [9].

In the present study we show that CIC expression increases in LPS-activated immune cells and CIC silencing as well as CIC activity inhibition reduces the production of NO, ROS and PGE_2 in LPS-activated U937 cells. Moreover, evidence is provided that CIC gene expression is up-regulated by NF-κB. To our knowledge, this is the first study to state the key role of CIC in the production of inflammatory mediators.

EXPERIMENTAL

Cell culture, RNA interference and transient transfection

The PBMC (peripheral blood mononuclear cell) fraction was obtained as reported in the Supplementary Experimental section (at http://www.BiochemJ.org/bj/438/bj4380433add.htm). Human monocytic/macrophage cells from hystiocytoma, U937 cells (ICLC HTL 94002-Interlab Cell Line Collection), were cultured as described for PBMCs (see the Supplementary Experimental section). U937 cells were differentiated with 10 ng/ml PMA (Sigma) and allowed to adhere for 24 h. Where indicated, both macrophages and U937/PMA (differentiated U937) cells were treated with 2 μg/ml bacterial LPS (Sigma) for 24 h; U937/PMA cells were treated with 20 μM Tos-Phe-CH_2Cl ('TPCK'; tosylphenylalanylchloromethane; Sigma) for 1 h and then with LPS (2 μg/ml) for 24 h; and U937/PMA cells were treated with 2 mM BTA (1,2,3-benzentricarboxylic acid; Sigma) for 4 h and then with LPS (2 μg/ml) for 24 h (NO and ROS measurements) and for 48 h (PGE_2). In the latter case, to aid the entry of BTA into the cells, 25 μl of a 6:1 (v/v) mixture of Fugene HD (Roche) and RPMI 1640 medium with and without BTA at a final pH of 7.3, were added to 500 μl of U937/PMA cells. After 4 h, the medium was replaced with fresh medium and the BTA-treated U937/PMA cells were incubated with LPS (2 μg/ml).

To measure CIC gene expression activity, U937/PMA cells were transiently transfected as reported previously [10] using 0.5 μg of pGL3 basic-LUC vector, containing the $-1785/-20$ bp region of the CIC gene promoter [11] and 10 ng of pRL-CMV (Promega) to normalize the extent of transfection [12]. After 24 h, transfected U937/PMA cells were treated with LPS (2 μg/ml) and after a further 24 h were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). In RNA interference experiments, U937 cells were differentiated by adding PMA and simultaneously transfected with the specific pre-designed siRNA (small interfering RNA) targeting human SLC25A1 (solute carrier family 25 member 1; s13095, Ambion) using siPORT™ NeoFX™ Transfection Agent (Ambion). After 24 h, the medium was replaced with fresh medium and the siRNA-transfected U937/PMA cells were treated with LPS (2 μg/ml); CIC mRNA and protein, ROS and NO were measured 24 h after the addition of LPS, whereas PGE_2 was measured 48 h after the addition of LPS. In these experiments, siRNA

Abbreviations used: BTA, 1,2,3-benzentricarboxylic acid; CIC, citrate carrier; COX2, cyclo-oxygenase 2; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cell; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; siRNA, small interfering RNA; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane.

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(C6A-0126; Ambion) with no significant similarity to human, mouse or rat gene sequences was used as a negative control.

Real-time PCR and Western blotting

Total RNA was extracted from 1×10^6 U937 cells and reverse transcribed as described previously [13]. Real-time PCR was performed as described previously [14]. Assay-on-demand for human CIC (Hs00761590_sH) and human β-actin (4326315E) were purchased from Applied Biosystems. All transcript levels were normalized against the β-actin expression levels. For Western blot analysis, proteins were electroblotted on to nitrocellulose membranes (Bio-Rad) and treated with anti-CIC [15] or anti-β-actin (Santa Cruz Biotechnology) antibodies. The immunoreaction was detected by Immobilon Western Chemiluminescent HRP (horseradish peroxidase) Substrate (Millipore).

NO, ROS and PGE_2 detection

Nitrite, the oxidation product of NO, was measured using the Griess reaction [16]. For ROS analysis, the cells were incubated with 10 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate; Molecular Probes) for 30 min. The fluorescence was measured by a Victor^3 plate reader (PerkinElmer) at 485 nm excitation and 530 nm emission wavelengths [17]. PGE_2 was detected by the DetectX High Sensivity PGE_2 Enzyme Immunoassay Kit (K018-HX1) according to the manufacturer's instructions (Arbor Assays).

RESULTS

Expression of mitochondrial CIC in LPS-activated macrophages and U937 cells

If activated by an inflammatory stimulus, macrophages synthesize and release modulators of inflammation, such as the eicosanoid PGE_2 [18]. As eicosanoids originate from fatty acids, in particular from arachidonic acid, we investigated whether the upstream process of the fatty acid synthesis pathway, i.e. the exit of citrate from the mitochondrial matrix and the production of acetyl-CoA, is involved in inflammatory signalling. Acetyl-CoA is produced by citrate lyase, which cleaves the citrate transported from the mitochondria to the cytosol by CIC. Total RNA, extracted from primary blood monocytes, macrophages and LPS-stimulated macrophages, was retrotranscripted and used in real-time PCR experiments. Surprisingly, an increase of approximately 15-fold and 28-fold of CIC mRNA was found in monocyte-derived macrophages and LPS-stimulated macrophages respectively, compared with undifferentiated monocytes (Figure 1A). Consistently, a parallel increase of CIC protein content in macrophages after differentiation and after activation by LPS was found (Figure 1B).

In subsequent experiments, we used differentiated U937 cells, which have a large repertoire of macrophage functions and can be stimulated with LPS to mimic the inflammatory response of activated macrophages [19]. Both real-time PCRs and Western blots revealed that CIC mRNA and protein levels were increased in U937/PMA cells and even more in LPS-activated U937 cells as compared with untreated U937 cells, although to a lesser extent than macrophages in the presence and absence of LPS as compared with monocytes (Figures 1C and 1D). These findings clearly show that CIC expression significantly increases in immune system cells, suggesting that CIC can play a role in inflammation.

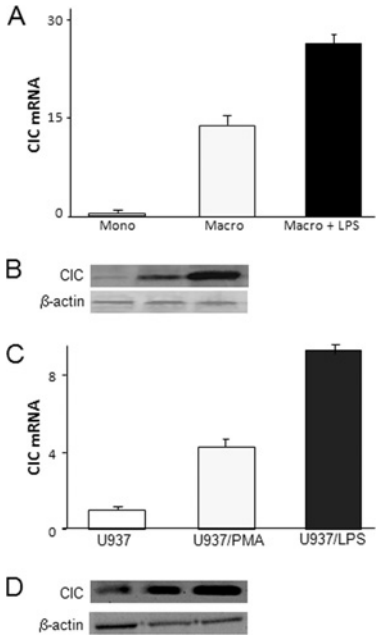

Figure 1 CIC expression in immune cells

(**A** and **C**) Total RNA from monocytes (Mono), macrophages (Macro), LPS-treated macrophages (Macro + LPS), U937 cells, U937/PMA cells and LPS-treated U937/PMA (U937/LPS) cells was used to quantify CIC mRNA. Means ± S.D. of three duplicate independent experiments are shown; differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA). (**B** and **D**) CIC and β-actin of the immune cells, shown in (**A**) and (**C**) respectively, were immunodetected with specific antibodies.

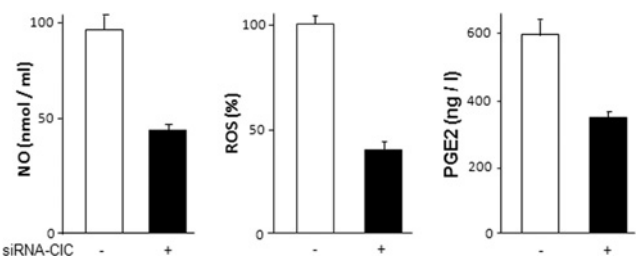

Figure 2 Effect of CIC silencing on the inflammatory response

U937 cells, differentiated and transfected with siRNA targeting human CIC (+) or control siRNA (−), were treated with LPS and then used to quantify NO, ROS and PGE_2. Means ± S.D. of six duplicate independent experiments are shown; differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA).

Effect of CIC silencing on the production of inflammatory molecules

To understand the role of CIC in the inflammatory pathway, we extended our investigation on a possible relationship between CIC expression and molecules involved in inflammation. U937 cells were transfected with siRNA targeting human CIC or control siRNA, differentiated by adding PMA, stimulated with LPS and assayed for CIC expression, and NO, ROS and PGE_2 levels. CIC silencing efficiency was confirmed by a significant decrease in CIC mRNA and protein levels (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380433add.htm). The amount of NO, ROS and PGE_2 was significantly decreased in silenced PMA-differentiated and LPS-stimulated U937 cells as compared with non-silenced cells (Figure 2). These results indicate a direct involvement of CIC in the inflammatory pathway.

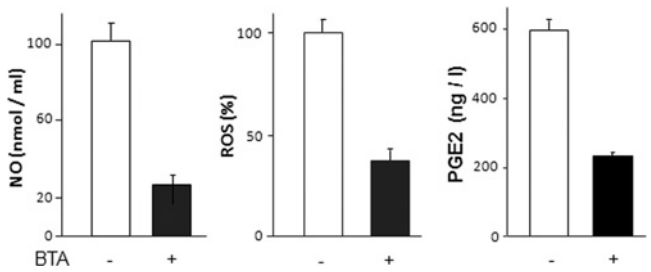

Figure 3 Effect of CIC activity inhibition on the inflammatory response

U937/PMA cells, treated with (+) or without (−) BTA, were activated with LPS and assayed for NO, ROS and PGE_2. Means ± S.D. of six duplicate independent experiments are shown; differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA).

Effect of the CIC inhibitor BTA on LPS-induced inflammation

We also tested the effect of BTA, a specific inhibitor of CIC [20,21], on the production of the inflammatory mediators NO, ROS and PGE_2. In this case, U937/PMA cells were treated with BTA, activated by LPS and assayed for NO, ROS and PGE_2. As shown in Figure 3, a marked reduction in NO, ROS and PGE_2 production was found in BTA-treated U937 cells compared with untreated cells. The BTA delivery into the cells was also verified by staining intracellular lipids (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/438/bj4380433add.htm). These results show that CIC activity is essential for the production of the inflammatory mediators NO, ROS and PGE_2.

The CIC gene promoter contains NF-κB active responsive elements

Finally, to clarify molecular mechanisms responsible for CIC gene activation during inflammation, we performed *in silico* analysis of the human CIC gene promoter and we found two NF-κB-responsive elements. NF-κB binding site activity was tested by transfecting LPS-activated U937/PMA cells with the pGL3 basic-LUC vector containing the −1785/−20 bp region of the CIC gene in the presence or absence of Tos-Phe-CH_2Cl, a specific NF-κB inhibitor [22]. Figure 4(A) shows that the luciferase gene reporter activity was approximately 50 % lower in cells transfected with the CIC-pGL3 basic-LUC vector in the presence of Tos-Phe-CH_2Cl than with the vector in the absence of Tos-Phe-CH_2Cl. Moreover, when U937/PMA cells were treated with both LPS and Tos-Phe-CH_2Cl, a reduction of CIC mRNA and protein levels was observed as compared with cells treated with LPS alone (Figures 4B and 4C). It is worth mentioning that NO, ROS and PGE_2 contents measured in Tos-Phe-CH_2Cl-treated cells were also diminished as expected (results not shown). These results demonstrate that the increased CIC gene expression in LPS-activated U937 cells is mediated by NF-κB, and the CIC gene represents a newly identified target of the inflammatory cascade.

DISCUSSION

Until now, CIC function has been mainly related to liver and pancreas metabolism. For the first time, we show in the present study that CIC is involved in inflammation. Among the reported results, the following supporting evidence can be mentioned. Both CIC transcript and protein levels greatly increase when macrophages or differentiated U937 cells are activated by LPS. Moreover, CIC gene silencing and CIC activity inhibition drastically reduce the production of the inflammatory mediators NO, ROS and PGE_2.

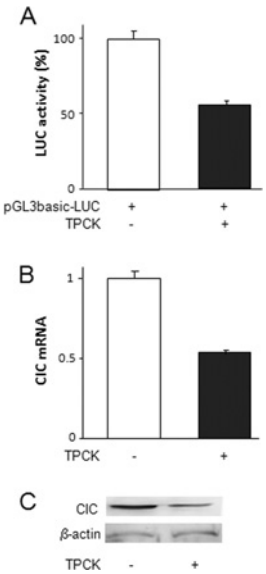

Figure 4 Effect of Tos-Phe-CH_2Cl on CIC gene expression

(**A**) U937/PMA cells, transfected with the pGL3 basic-LUC vector containing the −1785/−20 bp region of the CIC gene promoter and incubated with (+) or without (−) Tos-Phe-CH_2Cl (TPCK) were treated with LPS and assayed for luciferase activity. (**B**) U937/PMA cells, incubated with (+) or without (−) Tos-Phe-CH_2Cl, were treated with LPS and used to quantify CIC mRNA. In (**A**) and (**B**), means ± S.D. of five duplicate independent experiments are shown; differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA). (**C**) CIC and β-actin of U937/PMA cells treated as in (**B**) were immunodetected with specific antibodies.

The outcome of our experiments can be explained on the basis of the important role played by CIC in intermediary metabolism. Indeed, CIC supplies acetyl-CoA necessary for PGE_2 synthesis and NADPH plus H^+ for NO and ROS production (Figure 5). At the same time, CIC gene expression is activated by NF-κB, the hallmark of inflammation, which activates virtually all of the genes whose expression is crucial in driving the inflammatory response [23]. In fact, Tos-Phe-CH_2Cl, a specific NF-κB inhibitor, strongly reduces CIC transcription in the inflammatory response.

Thus it seems reasonable to conclude that the PGE_2, ROS and NO increase in inflammation is due to a direct effect of NF-κB not only on the *COX2* (cyclo-oxygenase 2), *iNOS* (inducible NO synthase) and NADPH oxidase genes but also on the CIC gene, which causes an increased availability of cytosolic acetyl-CoA and NADPH plus H^+, needed for the synthesis of all of the above-mentioned mediators. Furthermore, macrophage activation involves profound transcriptional and translational modifications, leading, among other effects, to changes in cellular metabolism [24]. Thus other mitochondrial carriers, which catalyse the translocation of solutes across the inner mitochondrial membrane, may display altered expression levels and play a role in activated macrophages.

AUTHOR CONTRIBUTION

Vittoria Infantino conceived and designed the experiments. Vittoria Infantino, Paolo Convertini, Liana Cucci, Maria Antonietta Panaro, Maria Antonietta Di Noia and Rosa Calvello performed the experiments. Vittoria Infantino and Paolo Convertini analysed the data. Ferdinando Palmieri and Vito Iacobazzi contributed reagents, materials and analysis tools. Vittoria Infantino, Paolo Convertini, Vito Iacobazzi and Ferdinando Palmieri wrote the paper.

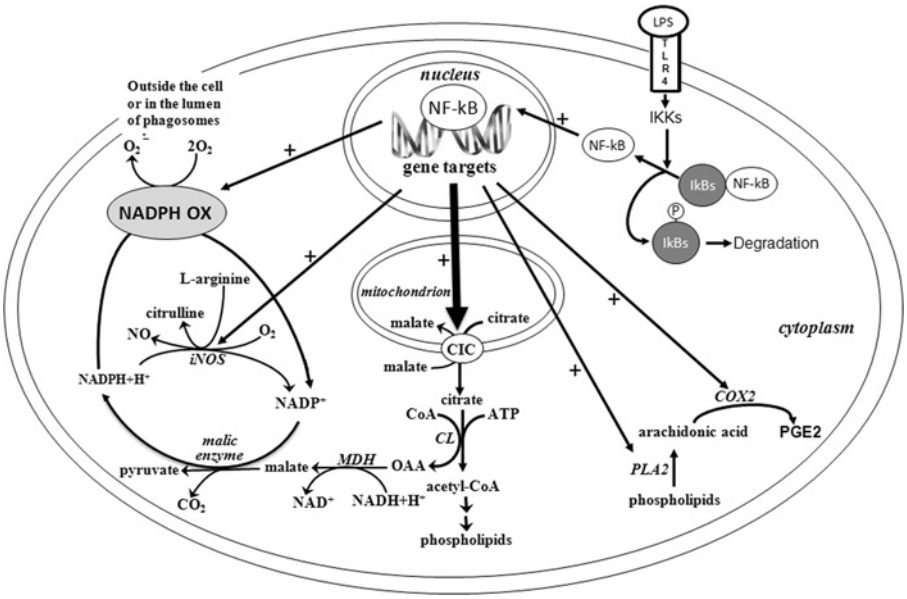

Figure 5 Role for CIC in inflammation

Following LPS stimulation, activated IκB (inhibitory κB) kinases (IKKs) induce the phosphorylation of IκBs and the release of NF-κB. In the nuclei, NF-κB binds to NF-κB-responsive elements and activate target genes, such as CIC, iNOS, NADPH oxidase (NADPH OX), COX2 and phospholipase A_2 (PLA2). Arrows marked with + indicate activation. CL, ATP-citrate lyase; MDH, malate dehydrogenase; OAA, oxaloacetate; TLR4, toll-like receptor 4.

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SUPPLEMENTARY ONLINE DATA
The mitochondrial citrate carrier: a new player in inflammation

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EXPERIMENTAL

Cell culture

Research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the University of Bari Ethics Committee. Blood collection was performed after each patient was informed of the purpose, nature and risk of all procedures used. All patients gave their consent to extract and use their samples. Heparinized blood from healthy adult volunteers was mixed with 5 % dextran in PBS, pH 7.3, at a ratio of 4:1 (v/v) and allowed to settle at 37 °C for 30 min. The PBMC (peripheral blood mononuclear cell) fraction was separated from blood by Ficoll-Paque (Sigma) density centrifugation at 400 ***g*** for 40 min at room temperature (25 °C). Cells were adjusted to 5×10^6 cells/ml and cultured in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin and 100 μg/ml streptomycin at 37 °C in 5 % CO_2. After 24 h of incubation, adherent mononuclear cells (1×10^6/ml) were washed three times with PBS and then incubated in fresh complete medium for 8 days to permit the differentiation of monocytes into macrophages. During this period, the medium was replaced every 48–72 h. Cell viability, determined by Trypan Blue exclusion, was greater than 98 %. Morphological features, such as increased size, the presence of short, blunt pseudopodia and non-specific esterase staining, showed that more than 96 % of the cells had differentiated into macrophages.

Oil-Red-O stain

For visualization of intracytoplasmic lipid accumulation, the cells were washed twice with PBS, fixed in 10 % formaldehyde for 1 h and washed with 60 % propan-2-ol. Immediately after this, Oil-Red-O solution (Sigma) was added for 10 min. The stained culture dishes were observed by optical microscopy and photomicrographs were acquired.

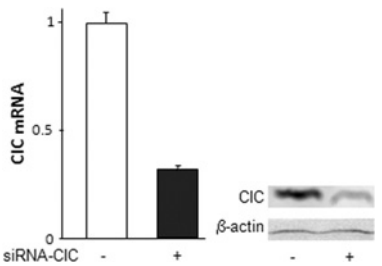

Figure S1 Effect of siRNA on CIC gene expression

To test the silencing efficiency of siRNA directed against CIC, U937 cells, differentiated and transfected with siRNA targeting human CIC (+) or control siRNA (−), were treated with LPS and then used to quantify CIC transcript and protein levels. Transfection of CIC-specific siRNA strongly reduced both CIC mRNA and protein levels. Means ± S.D. of six duplicate independent silencing experiments are shown; differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA). In Western blot experiments, β-actin was used as a control.

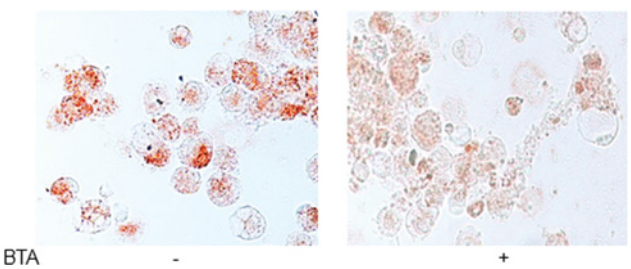

Figure S2 Effect of CIC activity inhibition on lipid content

The entry of BTA into the cells was tested by staining intracellular lipids. U937/PMA cells treated with (+) or without (−) BTA for 72 h were assayed for lipid content. The strong decrease of lipid accumulation was proof of BTA delivery into the cells. The magnification of the photomicrographs is ×100. Photographs typical of those taken in more than three separate experiments are shown.

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