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THE MITOCHONDRIAL CITRATE CARRIER: A NEW PLAYER IN INFLAMMATION

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Synopsis

The citrate carrier (CIC) catalyzes the efflux of citrate from the mitochondrial matrix in exchange for cytosolic malate. Herein we show that CIC mRNA and protein markedly increase in LPS-activated immune cells. Moreover, CIC gene silencing and CIC activity inhibition significantly reduce production of nitric oxide, reactive oxygen species and prostaglandins. These results demonstrate for the first time that CIC has a critical role in inflammation.

Keywords

Citrate carrier, immune cells, gene regulation, nitric oxide, reactive oxygen species, prostaglandin E2

Abbreviations

BTA, 1,2,3-Benzenetricarboxylate; CIC, Citrate carrier; COX2, cyclooxygenase 2; $DCFH_2$-DA, 2´,7´-dichlorofluorescein diacetate; ELISA, Enzyme-Linked ImmunoSorbent Assay; IkB, inhibitor of kB; iNOS, the inducible nitric oxide synthase; LPS, lipopolysaccharide; LUC, luciferase; NADPH OX, NADPH oxidase; NF-kB, Nuclear Factor-kappa B; NO, nitric oxide; PMA, Phorbol-12-myristate-13-acetate; PGE2, prostaglandin E2; ROS, reactive oxygen species; siRNA, small interfering RNA; TPCK, N-p-Tosyl-L-phenylalanine-chloromethyl-ketone.

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, prostaglandin E2 (PGE2), nitric oxide (NO) and reactive oxygen species (ROS) by cells of the innate and adaptive immune system. The nuclear transcription factor, Nuclear Factor-kappa B (NF-κB), is a pivotal orchestrator of inflammation [2, 3]. Exposure of cells to lipopolysaccharide (LPS) 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-kB activation and macrophage functions [5, 6]. However, until now there is no report on the role of the mitochondrial citrate carrier (CIC) 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 + H^+ (also necessary for fatty acids production) via malate dehydrogenase and malic enzyme [9].

In this study we show that CIC expression increase in LPS-activated immune cells and CIC silencing as well as CIC activity inhibition reduces the production of NO, ROS and PGE2 in LPS-activated U937 cells. Moreover, evidence is provided that CIC gene expression is upregulated by NF-kB. To our knowledge, this is the first study to state the CIC key role in the production of inflammation mediators.

EXPERIMENTAL

Cell culture, RNA interference, and transient transfection

The peripheral blood mononuclear cell (PBMC) fraction was obtained as reported in Supplementary experimental. Human monocytic/macrophage cells from hystiocytoma, U937 cells (ICLC HTL 94002-Interlab Cell Line Collection), were cultured as described for PBMC (see Supplementary experimental). U937 cells were differentiated with 10 ng/ml of phorbol-12-myristate-13-acetate (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 of bacterial lipopolysaccharide (LPS, Sigma) for 24 h; U937/PMA cells were treated with 20 μM of N-p-Tosyl-L-phenylalanine-chloromethyl-ketone (TPCK, Sigma) for 1 h and then with LPS (2 μg/ml) for 24 h; and U937/PMA cells were treated with 2 mM of 1,2,3-benzentricarboxylic acid (BTA, Sigma) for 4h and then with LPS (2 μg/ml) for 24 h (NO and ROS measurements) and for 48 h (PGE2). In the latter case to favor 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 final pH 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 previously reported [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 further 24 h were assayed for LUC 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 small interfering RNA (siRNA) targeting human SLC25A1 (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, while PGE2 was measured 48 h after the addition of LPS. In these experiments, siRNA (Cat. No. C6A-0126, Ambion) with no significant similarity to human, mouse, or rat gene sequences was used as negative control.

Real-time PCR and western blotting

Total RNA was extracted from 1 x 10^6 U937 cells and reverse transcripted as reported [13]. Real-time PCR was performed as previously described [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 onto 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 Substrate (Millipore).

NO, ROS and PGE2 detection

Nitrite, the oxidation product of NO, was measured by the Griess reaction [16]. For ROS analysis the cells were incubated with 10 μM $DCFH_2$-DA (Molecular Probes) for 30 min. The fluorescence was revealed by Victor^3 plate reader (Perkin-Elmer) at 485 nm excitation and 530 nm emission wavelengths [17]. PGE2 was detected by the DetectX High Sensivity PGE2 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 PGE2 [18]. Because 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 the inflammatory signaling. Acetyl-CoA is produced by citrate lyase, which cleaves the citrate transported from the mitochodria 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. Surprensingly, an increase of about 15-fold and 28-fold of CIC mRNA was found in monocyte-derived macrophages and LPS-stimulated macrophages, respectively, compared to 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 to untreated U937 cells, although to a lesser extent than macrophages in the presence and absence of LPS as compared to monocytes (Figures 1C and 1D).

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These findings clearly show that CIC expression significantly increases in immune system cells suggesting that CIC can play a role in inflammation.

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, NO, ROS and PGE2 levels. CIC silencing efficiency was confirmed by significantly decrease of CIC mRNA and protein levels (Figure 1S). The amount of NO, ROS and PGE2 was significantly decreased in silenced PMA-differentiated, and LPS-stimulated U937 cells as compared to non-silenced cells (Figure 2).

These results indicate a direct involvement of CIC in the inflammatory pathway.

Effect of the CIC-inhibitor BTA on the 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 PGE2. In this case U937/PMA cells were treated with BTA, activated by LPS and assayed for NO, ROS, PGE2. As shown in Figure 3 a marked reduction in NO, ROS and PGE2 production was found in BTA-treated U937 cells compared to untreated cells. The BTA delivery into the cells was also verified by staining intracellular lipids (Figure 2S).

These results show that CIC activity is essential for the production of the inflammatory mediators NO, ROS and PGE2.

The CIC gene promoter contains NF-kB 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-kB responsive elements. NF-kB 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 TPCK, a specific NF-kB inhibitor [22]. Figure 4A shows that the luciferase gene reporter activity was about 50% lower in cells transfected with the CIC-pGL3 basic-LUC vector in the presence of TPCK (black bar) than with the vector in the absence of TPCK (white bar). Moreover, when U937/PMA cells were treated with both LPS and TPCK a reduction of CIC mRNA and protein levels was observed as compared to cells treated with LPS alone (Figures 4B and 4C). It is worth mentioning that NO, ROS and PGE2 contents measured in TPCK-treated cells were also diminished as expected (data not shown).

These results show that the increased CIC gene expression in LPS-activated U937 cells is mediated by NF-kB and 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 here 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 PGE2.

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 PGE2 synthesis and NADPH + H^+ for NO and ROS production (Figure 5). At the same time, CIC gene expression is activated by NF-kB, the hallmark of inflammation which activates virtually all the genes whose expression is crucial in driving the inflammatory response [23]. In fact, TPCK, a specific NF-kB inhibitor, strongly reduces CIC transcription in the inflammatory response.

Thus, it seems reasonable to conclude that PEG2, ROS and NO increase in inflammation by NF-kB direct effect not only on COX2, iNOS and NADPH oxidase genes but also on CIC gene which causes an increased availability of cytosolic acetyl-CoA and NADPH + H^+ needed for synthesis of all above mentioned mediators. Furthermore, macrophages activation involves profound transcriptional and translational modifications leading among others to changes in cellular metabolism [24]. So 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.

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FIGURE LEGENDS

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, LPS-treated U937/PMA (U937/LPS) cells was used to quantify CIC mRNA. Means ± SD 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 immunodecorated with specific antibodies.

Figure 2. Effect of CIC silencing on inflammatory response. U937 cells, differentiated and transfected with the siRNA targeting human CIC (+) or control siRNA (-), were treated with LPS and then used to quantify NO, ROS and PGE2. Means ± SD of six duplicate independent experiments are shown; differences between samples and relative controls were significant ($P << 0.05$, one-way ANOVA).

Figure 3. Effect of CIC activity inhibition on inflammatory response. U937/PMA cells, treated with (+) or without (-) BTA, activated with LPS and assayed for NO, ROS and PGE2. Means ± SD of six duplicate independent experiments are shown; differences between samples and relative controls were significant ($P << 0.05$, one-way ANOVA).

Figure 4. Effect of TPCK 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 (-) TPCK, were treated with LPS and assayed for LUC activity. **(B)** U937/PMA cells, incubated with (+) or without (-) TPCK, were treated with LPS and used to quantify CIC mRNA. In A and B means ± SD 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 immunodecorated with specific antibodies.

Figure 5. Role of CIC in inflammation. Following LPS stimulation, activated IKKs induce the phosphorylation of IkBs and the release of NF-kB. In the nuclei, NF-κB bind to NF-kB responsive elements and activate target genes, such as CIC, the inducible nitric oxide synthase (iNOS), NADPH oxidase (NADPH OX), cyclooxygenase (COX2) and phospholipase A2 (PLA2). Arrows marked with + indicate activation. Other abbreviations: TLR4, toll-like receptor 4; IKKs, IkB kinase; IkB, inhibitor of kB; CL, ATP-citrate lyase; OAA, oxaloacetate; MDH, malate dehydrogenase.

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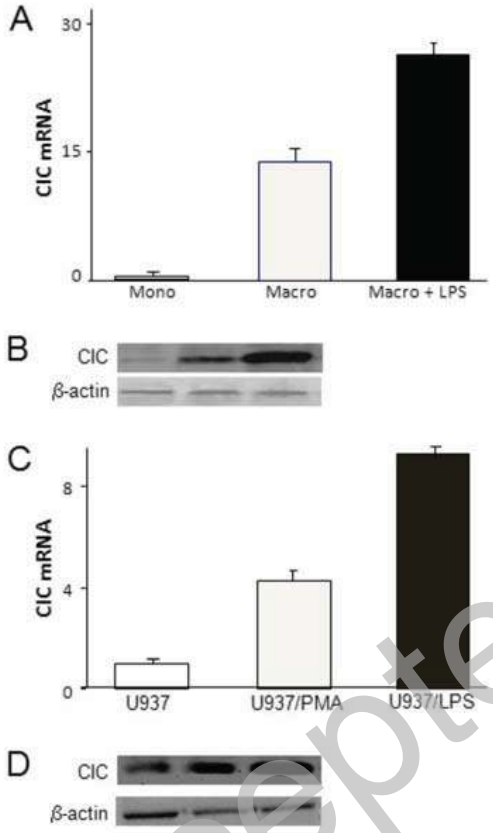

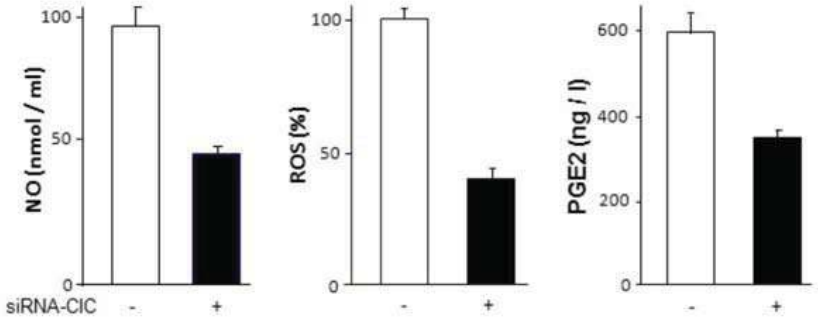

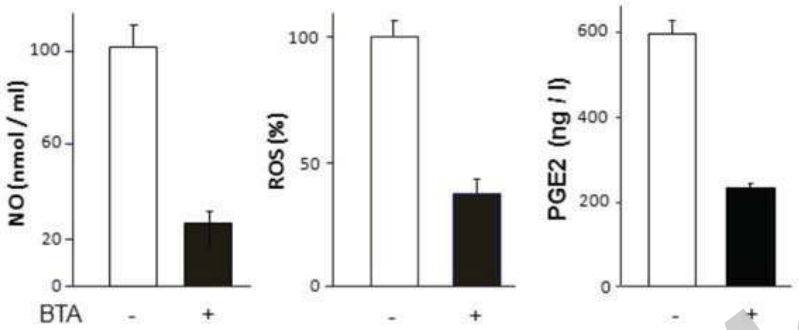

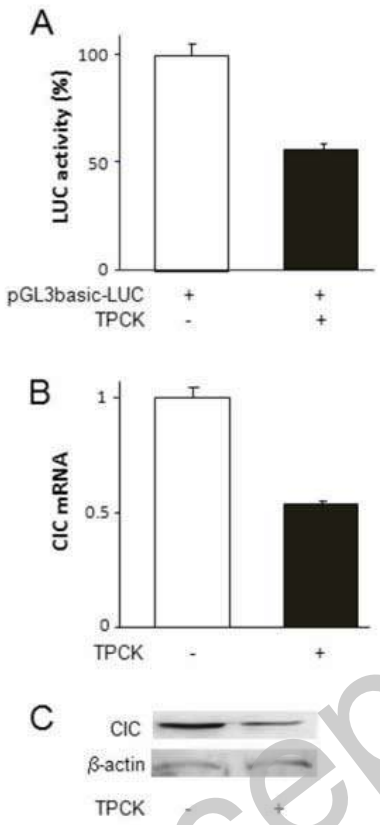

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