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Transcriptional repression of ceramide kinase in LPS-challenged macrophages

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ABSTRACT

Ceramide kinase (CERK) produces the bioactive lipid ceramide 1-phosphate (C1P). Both CERK and C1P have been identified as mediators of cell growth and survival. Recent evidence showed that CERK is down-regulated during M1-type macrophage activation, which is known to promote cell growth arrest. However, the mechanism has not been investigated yet and, in particular, whether growth arrest might be the signal for down-regulation of CERK is currently unknown. Here, we found that LPS-mediated TLR-4 engagement reduces *Cerk* mRNA levels in mouse primary macrophages. Reporter gene assays in RAW264.7 macrophages showed that LPS inhibits the transcriptional activity of the *Cerk* proximal promoter. The G1-cell cycle blocker mimosine did not inhibit *Cerk* transcription, suggesting that transcriptional repression of *Cerk* by LPS is not a primary consequence of LPS-induced cell cycle blockade.

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1. Introduction

Ceramide kinase (CERK) belongs to the diacylglycerol kinase family of lipid kinases and is the only enzyme known to produce ceramide 1-phosphate (C1P) [1]. However, studies with CERK deficient (*Cerk*$^{-/-}$) mice have shown that another route for production of C1P must exist, at least in mammals [2,3]. The best described signaling properties reported for C1P include, on the one hand, a positive effect on cell proliferation and cell survival [4,5] and, on the other hand, the control of cytosolic phospholipase A2 (cPLA$_2$) activity [6–9]. Of note, however, neither knocking down the *Cerk* gene [3] nor using a CERK inhibitor [10] could recapitulate these findings, which may suggest compensation by other C1P pools that do not depend on CERK for their synthesis.

The physiological role of CERK and its relevance to disease is only starting to be addressed. Using a gene knockdown strategy Igarashi and coworkers have shown preliminary evidence for a role of CERK in emotional behavior [11]. Based on ex- vivo work with *Cerk*-deficient endothelial cells together with use of the CERK inhibitor NVP-231 [10] our laboratory has recently proposed a role for CERK in the regulation of angiogenesis [12]. CERK may also be relevant to immune cell biology. In fact, neutrophils represent one of the first cell types where CERK/C1P were described [13–15] and subsequently shown to promote phagolysosome formation [16]. That CERK is important for neutrophil function was further evidenced by the neutropenia observed in *Cerk*-deficient animals together with their impaired capacity to fight an *S. pneumoniae* infection [3]. Macrophages represent another innate immune cell type where high levels of CERK activity were detected [2,3]. Thus, it was unexpected that *Cerk*-deficient macrophages did not reveal impaired immune responses [3]. However, during these investigations we noted that lipopolysaccharide (LPS) could potently down regulate CERK activity in macrophages. In fact, treatment with LPS led to "ceramide anabolic switching" because, in contrast to CERK, both glucosylceramide synthase (GCS) and sphingomyelin synthase (SMS) activities were up-regulated [17]. This prompted us to investigate the mechanism for down-regulation of CERK by LPS.

2. Materials and methods

2.1. Chemicals

Ultrapure LPS and the mouse TLR Agonist kit were from InvivoGen. NBD-C6 ceramide was from Molecular Probes. All other chemicals were from Sigma unless otherwise stated.

2.2. Mice

Balb/c mice were kept under standard housing conditions. Macrophages were obtained from 6–12 week old mice. The

Abbreviations: Cer, ceramide; CERK, ceramide kinase; GCS, glucosylceramide synthase; IFN-γ, interferon gamma; M-CSF, macrophage colony stimulation factor; MΦ, macrophage (s); LPS, lipopolysaccharide; NBD, *N*-[7-(4-nitrobenzo-2-oxa-l,3-diazole)]; NF-κB, nuclear factor kappa B; SMS, sphingomyelin synthase; RT-PCR, real-time polymerase chain reaction; TLC, thin-layer chromatography; TLR, Toll-like receptor; TSS, transcription start site.

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experimental procedures met all regulations and standards as approved by the Austrian government.

2.3. Macrophages

In this study, we used either thioglycollate-elicited peritoneal macrophages (MΦ) or bone-marrow derived MΦ, obtained and grown exactly as described in [17]. MΦ proliferation was measured using a BrdU assay, as previously described [3].

2.4. mRNA measurements

RNA was extracted directly from the cell lysates using the Absolute RNA Miniprep Kit, following the provider's instructions (Stratagene). 1 μg of RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied BioSystems). Real-time PCR, to quantify transcriptional levels of sphingolipid anabolism enzymes relatively to house keeping genes, was performed on a ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as previously described [3,18,19].

2.5. Cloning of human and murine promoter fragments

Cerk promoter fragments were amplified from human blood cells derived genomic DNA (Promega), and from murine Genomic DNA (Balb/c) (Promega). Fragments were cloned into the BglII and Mlu sites of the pGL3 Basic vector (Promega) which harbors a firefly luciferase gene driven by the inserted promoter fragment. Human promoter fragments were cloned into the XhoI and HindIII sites of pGL3. All promoter fragments were sequenced (VBC Genomics, Vienna).

2.6. Rapid amplification of cDNA ends

Total RNA, from a confluent layer of murine MΦ in a 10 cm-dish, was isolated and taken as input for the GeneRacer kit (Invitrogen). Mature mRNA molecules were selected by discriminating for a functional CAP site and a polyA tail. The primers, used for primary and nested PCR, were 5′cctacgcacacgatgccatcatagct3′, and 5′cgtagtgatggaagccaaggtaaac3′, respectively.

2.7. Ceramide anabolic activity assay using NBD-ceramide

This assay has been set up and validated previously [2]. In brief, to evaluate CERK, GCS and SMS activities in parallel, cells were incubated for 2 h in the presence of 5 μM NBD-Cer which will readily accumulate at the Golgi complex to serve as substrate for these ceramide anabolic enzymes. Cells were rinsed with HBSS buffer supplemented with 10 mM EDTA. Then, lipids were extracted with 200 μl methanol, 200 μl chloroform and 150 μl HBSS/EDTA. Samples were vortexed and after centrifugation the organic phase was taken and dried using a Thermo SPD Speed Vac. Dried samples were dissolved in 10 μl methanol:chloroform 1:1 and analyzed on Silica Gel 60 HPTLC plates (Merck) using butanol:acetic acid:water, 3:1:1 as the mobile phase. TLC plates were dried and imaged using Fuji film LAS3000 intelligent dark box in SYBR Green fluorescent light.

2.8. Transfection and luciferase reporter assay

RAW 264.7 cells were seeded into 24-well plates at a density of 1×10^5 cells per well. On the next day cells were transfected with 50 ng of the phRL-TK vector, which codes for the renilla luciferase serving as expression control, and 250 ng of the promoter fragment in pGL3 Basic. The GeneJuice reagent (Novagen) was used for transfection. OptiMEMI medium was brought to 37 °C and, per transfection, 30 μl of medium were mixed together with 1 μl of the GeneJuice reagent. After 5 min incubation at ambient temperature the promoter fragment-containing pGL3 and the pRL-TK control vector were added to the mixture which was incubated for another 20 min. The transfection mix was applied directly to the cells and afterwards samples were incubated at 37 °C, 5% CO_2 in a humidified atmosphere. In case of further treatments the transfection medium was taken off after 7 h and treatment was applied. On the next day cells were harvested using the passive lysis buffer from the dual luciferase assay kit (Promega). Cells were vigorously shaken at ambient temperature for 20 min. Twenty micro litres were analyzed for both renilla and firefly luciferase activities; the firefly enzyme signal was normalized against that obtained with the renilla enzyme, thus yielding relative luciferase units (RLU).

2.9. Statistical determinations

Statistics were performed using the Student's *t*-test. $^{*}(p < 0.05)$; $^{**}(p < 0.01)$; $^{***}(p < 0.001)$.

3. Results

3.1. LPS down-regulates Cerk mRNA levels in mouse primary macrophages

MΦ, when treated with LPS, undergo "ceramide anabolic switching", which is characterized by down-regulation of CERK and up-regulation of GCS and SMS [17]. To characterize the LPS effect, we first evaluated the amplitude of the ceramide anabolic switch as a function of LPS concentration. This showed a clear LPS concentration dependency, with an IC_{50} in the low ng/ml range (Fig. 1A).

To understand the mechanism underlying ceramide anabolic switching, we measured mRNA levels of the responsible enzymes, i.e. CERK, GCS, and SMS. LPS treatment increased the levels of GCS, SMS-1 and SMS-2 mRNAs by 3- to 4-fold after 3 h of treatment (Fig. 1B). During the next 3 h of treatment both SMS-1 and -2 mRNAs returned to basal levels whereas GCS mRNA remained significantly elevated (Fig. 1B). In sharp contrast, CERK mRNAs time-dependently decreased during LPS treatment, reaching a minimum

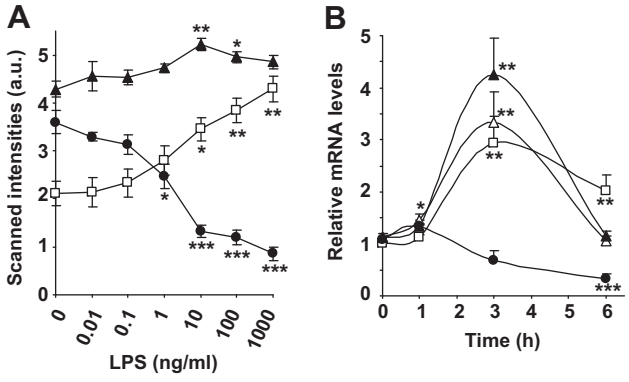

Fig. 1. Activity, and transcriptional regulation, of ceramide anabolic enzymes, in LPS-treated MΦ. (A) MΦ were seeded at 2×10^5 cells/well of a 24-well plate and treated for 18 h in the presence of a concentration range of LPS. To assay ceramide anabolic activities, 5 μM NBD-Cer was added to the medium during the last 2 h of incubation. Lipids were subsequently extracted and separated by TLC followed by densitometric analysis; NBD-GC (empty square), NBD-C1P (filled circle), NBD-SM (filled triangle); Mean of triplicate determinations + SD. (B) MΦ were treated with 100 ng/ml LPS for the indicated times. Total RNA was harvested, reversed transcribed and analyzed by real-time PCR: *gcs* (empty square), *cerk* (closed circle), *sms1* (empty triangle), *sms2* (filled triangle); average of six determinations + SD.

(3-fold decrease) after 6 h (Fig. 1B). These data indicated that the ceramide anabolic switch occurring during LPS-induced MΦ activation is regulated, at least in part, through transcriptional mechanisms.

3.2. LPS inhibits the Cerk proximal promoter

To study *Cerk* transcriptional regulation in more detail we cloned and characterized the promoter region of the *Cerk* gene. Consistent with published data on human *CERK*, we found the transcription start site in the mouse *Cerk* gene to be within 100-bp of the translation initiation site (Supplementary Fig. 1). Then, to probe the minimal promoter sequence, we made deletions starting from the 5′ end of a construct containing 1 kb of *Cerk* sequence downstream of the transcription start site, fused to a luciferase reporter construct. A 200 bp-long promoter fragment proved to be almost as effective as the full 1 kb *Cerk* construct (Fig. 2A), thus suggesting that critical functional elements of the *Cerk* promoter lay proximal to the transcription start site. Interestingly, this 200-bp region showed significant conservation between human and mouse promoter sequences; In fact, both the human and the mouse 200-bp region recapitulated transcriptional activity when fused to luciferase (Supplementary Fig. 1). Therefore, subsequent experiments focused on this proximal region.

To address the LPS-dependency of the transcriptional activity of the *Cerk* proximal promoter, we used the macrophage-like RAW264.7 tumor cell line. In contrast to primary MΦ, RAW264.7 cells display very high levels of GCS activity but low levels of CERK activity (Fig. 2B), a finding consistent with the amounts of GC and C1P recently measured in this cell line [20]. Although less pronounced compared to primary cells, we could observe LPS-dependent down-regulation of CERK activity (Fig. 2B) and mRNA levels (Fig. 2C) in RAW264.7 cells. Cells were then transfected with the 200 bp *Cerk* promoter construct fused to luciferase and transcriptional activity was measured following a concentration range of LPS. This showed that the proximal promoter of CERK is negatively regulated by LPS (Fig. 2D).

3.3. CERK inhibition by LPS is not a consequence of cell cycle blockade

LPS is known to induce MΦ growth arrest by blocking the cell cycle in early G1 [21]. In fact, growth-arresting concentrations of LPS were efficient at down-regulation of CERK (Fig. 3A). Since CERK and C1P play a role in cell growth and survival [22] we wondered if down-regulation of CERK by LPS might be a direct consequence of cell cycle blockade in G1. To test this hypothesis, we applied mimosine, which blocks cell cycle in late G1 [23]. Mimosine impacted neither *Cerk* mRNA levels nor the activity of its proximal promoter (Fig. 3B). Therefore, down-regulation of CERK by LPS does not appear to be a consequence of cell cycle blockade. It may rather result from a specific transcriptional regulation mechanism triggered by LPS.

4. Discussion

In this work, we have reported that down-regulation of CERK by LPS involves transcriptional repression of the *Cerk* proximal promoter which does not appear to be the consequence of cell cycle blockade.

The LPS effects on CERK are likely to result from signaling through Toll-like receptor 4 (TLR-4) because paclitaxel, a molecule with no structural similarity to LPS but which can bind TLR-4, could also induce ceramide anabolic switching with decreased CERK activity (Supplementary Fig. 2). Furthermore, similar observations were made using alternative TLR agonists (Supplementary Fig. 2). Because Toll-like receptor 4 engagement leads to activation of the transcription factor NF-κB, we screened the *Cerk* promoter for putative response elements. However, we could not find any NF-κB consensus response elements within 5 kb downstream of the transcription start site. Further analysis of the *Cerk* promoter indicated that it is devoid of TATA-box but displays several CgG islands (Supplementary Fig. 3). Such regions were previously shown to be targeted by the specificity protein (Sp) family of transcription factors [24]. These transcription factors are known to regulate genes whose products play a role in cell survival. Because this property is shared by CERK, it remains to be evaluated if Sp transcription factors might play a role for regulating *Cerk* in LPS-challenged MΦ.

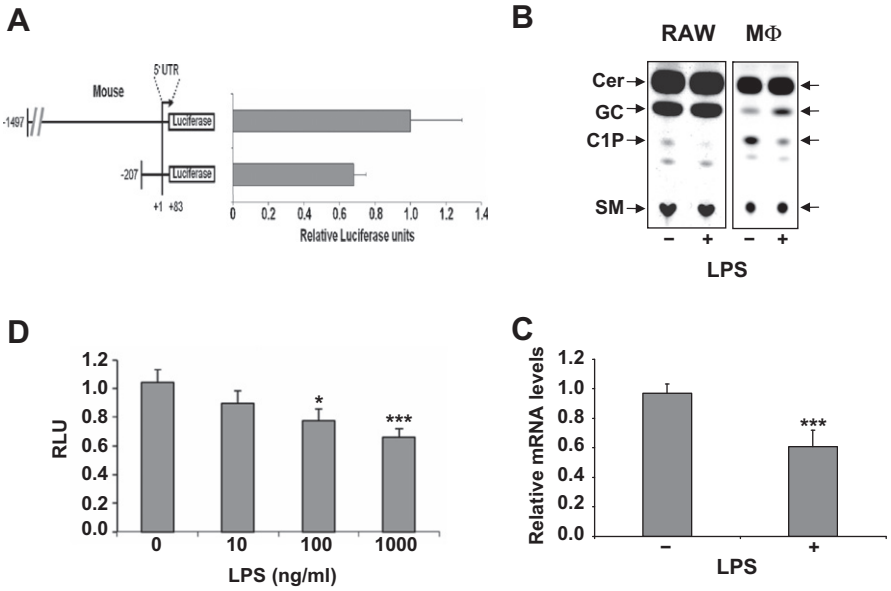

Fig. 2. Modulation of the *Cerk* proximal promoter by LPS in RAW264.7 cells. (A) Luciferase reporter assay in RAW264.7 cells transfected with a −1497 or a −207 *Cerk* promoter construct; (B) Effect of LPS on ceramide anabolic activities and (C) on *Cerk* mRNA levels, in RAW264.7 and MΦ, measured as described in the legend to Fig. 1. (D) Dependency on LPS concentration of the activity of the -207 *Cerk* promoter construct.

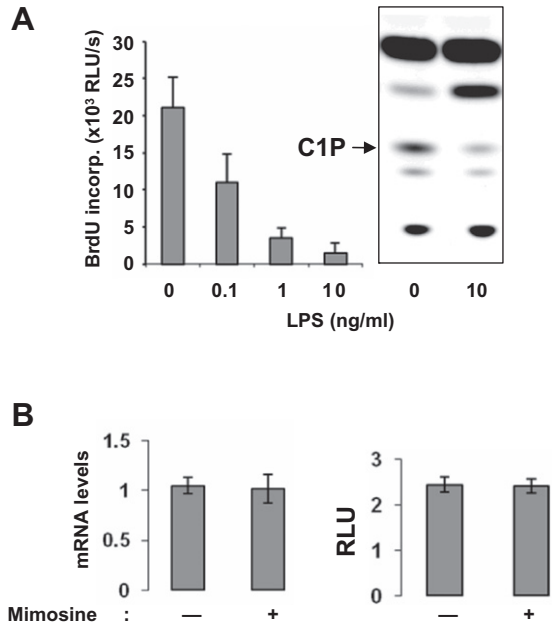

Fig. 3. Growth arrest in G1, *per se*, is not responsible for *Cerk* transcriptional inhibition. (A) Measurement of BrdU incorporation in MΦ, following a 18 h-treatment with a concentration range of LPS; A control TLC analysis showing ceramide anabolic activities is shown on the right; mean of quadruplicate determinations + SD (B) Relative *Cerk* mRNA levels (left) and relative activity of the *Cerk* proximal promoter (right) measured in HeLa cells, following 16 h treatment with or without 500 μM Mimosine. Similar results were obtained with human *CERK* promoter constructs (data not shown).

Down-regulation of *Cerk* mRNA levels also occurred when MΦ were treated with IFN-γ (Supplementary Fig. 4). Furthermore, IFN-γ like LPS, increased GCS mRNA levels. Therefore, transcriptional regulation of ceramide anabolic switching (decreased CERK/increased GCS, [17]) appears like a feature of the M1-MΦ differentiation and activation process. The regulation of SMS appears to be more contrasted because IFN-γ reduced (Supplementary Fig. 4), whereas LPS stimulated (Fig. 1), SMS-1 and SMS-2 mRNA levels. Further studies will be needed to unravel the consequences on macrophage function of the coordinated regulation of these ceramide metabolism enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.041.

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