

Original Paper

Ceramide Kinase Contributes to Proliferation but not to Prostaglandin E₂ Formation in Renal Mesangial Cells and Fibroblasts

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Uwe Zangemeister-Wittke^a Josef Pfeilschifter^b Andrea Huwiler^a^aInstitute of Pharmacology, University of Bern, Bern, Switzerland, ^bPharmazentrum Frankfurt/ZAFES, Johann Wolfgang Goethe Universität, Frankfurt am Main, Germany**Key Words**Ceramide kinase • NVP-231 • Mesangial cells • Fibroblasts • Proliferation • M phase arrest • Apoptosis • Prostaglandin E₂**Abstract**

Background/Aims: Ceramide kinase (CerK) catalyzes the generation of the sphingolipid ceramide-1-phosphate (C1P) which regulates various cellular functions including cell growth and death, and inflammation. Here, we used a novel catalytic inhibitor of CerK, NVP-231, and CerK knockout cells to investigate the contribution of CerK to proliferation and inflammation in renal mesangial cells and fibroblasts. **Methods:** Cells were treated with NVP-231 and [³H]-thymidine incorporation into DNA, [³H]-arachidonic acid release, prostaglandin E₂ (PGE₂) synthesis, cell cycle distribution, and apoptosis were determined. **Results:** Treatment of rat mesangial cells and mouse renal fibroblasts with NVP-231 decreased DNA synthesis, but not of agonist-stimulated arachidonic acid release or PGE₂ synthesis. Similarly, proliferation but not arachidonic acid release or PGE₂ synthesis was reduced in *CERK* knockout renal fibroblasts. The anti-proliferative effect of NVP-231 on mesangial cells was due to M phase arrest as determined using the mitosis markers phospho-histone H3, cdc2 and polo-like kinase-1, and induction of apoptosis. Moreover, loss of CerK sensitized cells towards stress-induced apoptosis. **Conclusions:** Our data demonstrate that CerK induces proliferation but not PGE₂ formation of renal mesangial cells and fibroblasts, and suggest that targeted CerK inhibition has potential for treating mesangioproliferative kidney diseases.

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Introduction

Mesangial cells are specialized cells localized in the renal glomerulus that exert important functions such as preserving the structural integrity of the glomerulus, and regulating the glomerular filtration rate by their smooth muscle cell-like contractile properties. In addition, mesangial cells also play a central role in various pathological processes of the renal glomerulus [1-3]. Mesangial cells are activated by classical pro-inflammatory cytokines including interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF α). Upon activation, they respond with a variety of reactions which are considered hallmarks of many forms of chronic inflammatory kidney diseases, including accelerated proliferation, increased pro-inflammatory mediators production, and enhanced extracellular matrix production [1, 3].

A key event commonly associated with inflammation is the production of prostaglandin E₂ (PGE₂) by sequential activation of phospholipases A₂ (PLA₂) and cyclooxygenases (COX). This has attracted attention due to the broad clinical impact of anti-inflammatory drugs. Best reported are COX inhibitors such as aspirin which have been used for many years. In addition, alternative approaches have been described such as inhibition of PLA₂, which represents an attractive target due to the fact that the activity of this class of enzymes is the rate-limiting step in PGE₂ formation. However, the development of PLA₂ inhibitors turned out to be difficult due to the large number of isoforms which are redundantly involved in inflammatory reactions [4-8].

Sphingolipids are crucial not only for providing structural support to cellular membranes, but also as signalling molecules in many biological systems, and previous studies demonstrated a regulatory function of sphingosine 1-phosphate (S1P) in various physiological and pathophysiological processes [9, 10].

Recently, another phosphorylated lipid, i.e. ceramide 1-phosphate (C1P), was proposed as a signalling molecule regulating cellular responses, including synaptic vesicle release, mast cell degranulation, phagocytosis, inflammation and proliferation [11]. The only direct target of C1P described so far is cytosolic cPLA₂, which was shown to directly bind C1P at its calcium-dependent lipid-binding (CaLB) domain and is activated upon C1P binding [12].

C1P is generated by the action of a ceramide kinase (CerK), which was cloned from various species including human and mouse [13]. The enzyme shows sequence homology to diacylglycerol kinase and sphingosine kinase, and contains a N-terminal myristoylation site and a pleckstrin homology domain, which both seem to be indispensable for membrane association. The enzyme is localized at various subcellular sites including Golgi, cytoplasm and nucleus [14, 15]. Since the N-terminal sequence of CerK contains a nuclear import signal and the C-terminal sequence contains a nuclear export signal [15], it was speculated that nucleocytoplasmic shuttling of CerK occurs and that CerK is active both inside and outside of the nucleus.

Here, we investigated the cellular function of CerK in renal mesangial cells and in renal fibroblasts. We demonstrate that in neither cell type, inhibition of CerK using the novel catalytic inhibitor NVP-231 attenuated agonist-stimulated arachidonic acid release or PGE₂ formation, thus, excluding its role in inflammation. However, we instead report that inhibition of CerK decreases cell proliferation by induction of M phase arrest and apoptosis, and facilitates apoptosis induced by various stress stimuli. Our data suggest that pharmacological inhibition of CerK has potential for treating proliferation-associated kidney diseases such as in mesangioproliferative glomerulonephritis and renal cancer.

Materials and Methods

Chemicals

[³H]-arachidonic acid (specific activity: 180-240 Ci/mmol) and [6-³H]-thymidine (specific activity: 10 Ci/mmol) were from Hartmann Analytic GmbH, Braunschweig, Germany; the PGE₂ ELISA was from Enzo Life Sciences, Lörrach, Germany; the Cell Death Detection ELISA^{PLUS} was from Roche Diagnostics, Mannheim,

Germany; secondary horseradish peroxidase-coupled IgGs, Hyperfilm MP and enhanced chemiluminescence reagents were from GE Health Care Systems, Glattbrugg, Switzerland. Human recombinant IL-1 β was from Cell Concept, Umkirch, Germany; TNF- α was a gift from Knoll AG, Ludwigshafen, Germany; PDGF-BB was kindly provided by Dr. Michael Pech, F.Hoffmann-La Roche Ltd., Basel, Switzerland; antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), total histone H3, cleaved caspase-3, PARP-1, phospho-Tyr¹⁵-cdc2, total cdc2, phospho-Ser¹³³-cyclin B1, total cyclin B1, phospho-Thr²¹⁰-PLK1, and total PLK1 were obtained from Cell Signaling, Schwalbach, Germany; COX2 and β -actin were from Santa Cruz Biotechnology Inc., Heidelberg, Germany; phospho-Ser¹⁰-histone H3 was from EMD Millipore Corporation; ATP, TPA, ionomycin, forskolin, and NVP-231 were obtained from Sigma Aldrich Fine Chemicals, Buchs, Switzerland; all cell culture additives were from Invitrogen AG, Basel, Switzerland.

Cell culture and stimulation

Rat glomerular mesangial cells were isolated, characterized and cultured as described [16]. CerK deficient mice were kindly provided by Dr. Bornancin (Novartis Pharma AG, Basel) [17]. Mouse renal fibroblasts were isolated from wildtype BalbC mice or CerK deficient mice as described [18] and plated on collagen-coated plastic dishes. Rat cells were cultivated in RPMI medium supplemented with 10 % fetal bovine serum (FBS), 10 mM HEPES pH 7.4, 6 μ g/ml bovine insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Mouse cells were cultivated in the same medium but additionally supplemented with 1% non-essential amino acids and 45 μ M β -mercaptoethanol. All cells were grown at 37°C in a humidified atmosphere containing 5 % CO₂.

Cell homogenization and Western blot analysis

After treatment cells were washed with phosphate-buffered saline (PBS), scraped and homogenized in lysis buffer [19] and centrifuged for 10 min at 13'000 x g. The supernatant was taken for protein determination and 30 μ g of protein was separated by SDS-PAGE, transferred to nitrocellulose membrane and immunostained using antibodies as indicated in the figure legends.

Determination of arachidonic acid release

Confluent cells in 24-well plates were labelled for 24 h with 1 μ Ci/ml of [³H]-arachidonic acid in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg/ml of fatty acid-free bovine serum albumin (BSA). Cells were then washed three times with DMEM containing 1 mg/ml of BSA to remove all unincorporated arachidonic acid. Thereafter, cells were stimulated as indicated and the supernatant was taken for determination of released radioactivity.

Determination of DNA synthesis

To measure DNA synthesis in cells, the incorporation of [³H]-thymidine into *de novo* synthesized DNA was detected as described [19].

Cell cycle analysis by flow cytometry.

Cells were harvested by trypsinization, washed and fixed in 70 % ethanol for at least 1 h at -20°C. 30 min prior to analysis, cells were resuspended in PBS containing 50 μ g/ml propidium iodide and 1 mg/ml RNase A in PBS. Cell cycle distribution was analyzed using a FACSCalibur flow cytometer and the Cell Quest software (both from Becton Dickinson Biosciences, Allschwil, Switzerland). At least 20'000 cells were counted for each sample.

Determination of apoptosis

Cells in 96-well plates were stimulated as indicated and directly taken for detection of DNA fragmentation using a Cell Death detection ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

C16-ceramide and sphingosine quantification by LC-MS/MS

Stimulated cells in 6-well plates were scraped into methanol containing internal C17-ceramide and C17-sphingosine standards and subjected to lipid extraction and LC/MS/MS analysis as described [20].

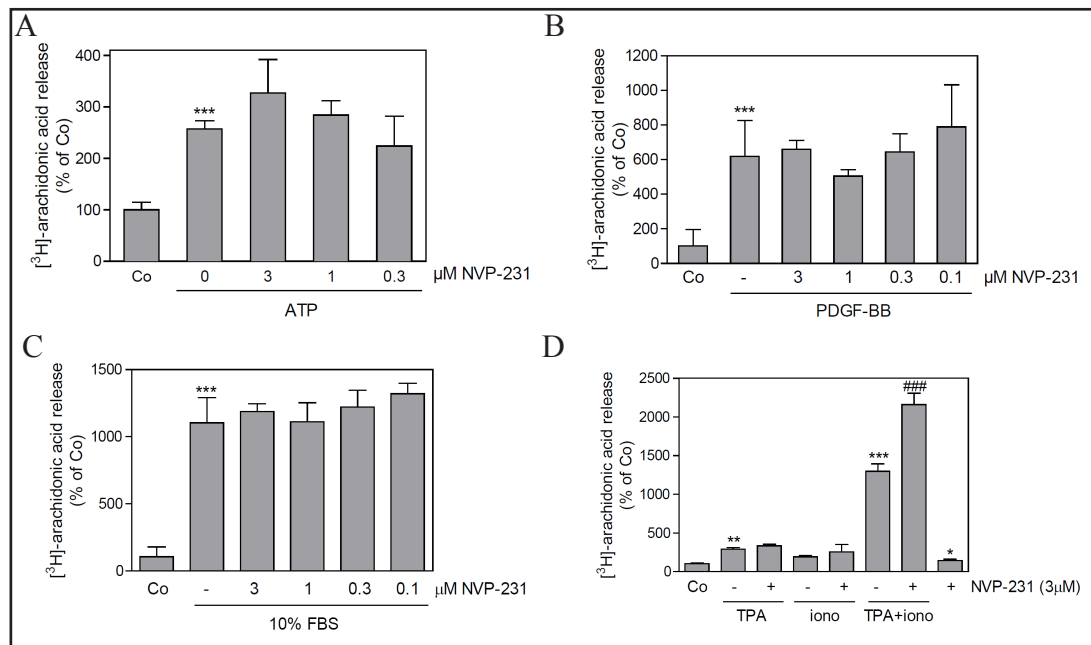


Fig. 1. Effect of NVP-231 on agonist-stimulated arachidonic acid release in rat renal mesangial cells. $[^3\text{H}]\text{-arachidonic acid}$ -labelled rat mesangial cells were stimulated for 1 h with either vehicle (Co), 100 μM of ATP (A), 20 ng/ml of PDGF-BB (B), 10 % FBS (C), TPA (100 nM) (D), ionomycin (1 μM) (D) or a combination of TPA plus ionomycin (D) in the absence or presence of the indicated concentrations of NVP-231. Released ^3H -arachidonic acid was determined as described in the Methods Section. Data are expressed as % of control and are means \pm S.D. (n=4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ considered statistically significant when compared to the control values; ### $p < 0.001$ when compared to the TPA plus ionomycin-stimulated value.

Statistical analysis

Statistical analysis of data was performed using one-way ANOVA following Bonferroni post-hoc test for multiple comparisons or unpaired t-tests when only two groups were compared.

Results

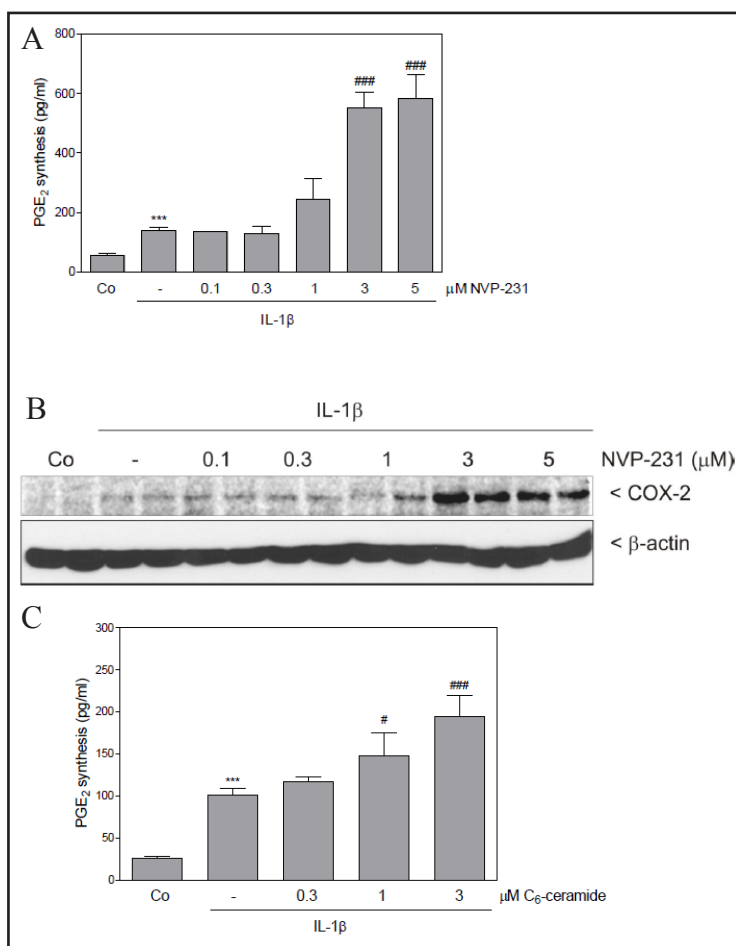
CerK inhibition does not abrogate agonist-induced arachidonic acid release or PGE_2 formation in renal mesangial cells

It was previously suggested that C1P is involved in cPLA_2 activation by directly binding and activating the enzyme [12]. To investigate this further, we measured the effect of CerK on cPLA_2 activation in rat renal mesangial cells using a novel potent catalytic inhibitor of CerK, NVP-231 [21]. This inhibitor demonstrated an in vitro IC_{50} value for CerK of 12 nM [21]. NVP-231 competitively binds to the ceramide binding site of the enzyme, is highly cell-permeable and active under intracellular conditions [21].

As a cellular activity assay of cPLA_2 , the release of $[^3\text{H}]\text{-arachidonic acid}$ upon agonist stimulation was detected. $[^3\text{H}]\text{-arachidonic acid}$ -labelled cells were stimulated with either extracellular ATP or platelet-derived growth factor-BB (PDGF-BB), two well-known cellular cPLA_2 activators acting via the P2Y2 purinoceptor or $\text{PDGFR}\beta$, respectively. Both agonists stimulated a pronounced arachidonic acid release after 30 min of stimulation (Fig. 1A and 1B). In the presence of NVP-231 no reduction of $[^3\text{H}]\text{-arachidonic acid}$ release was observed upto 3 μM as the highest concentration tested.

Additional stimuli were also tested including serum, 12-O-tetradecanoylphorbol 13-acetate (TPA), ionomycin and the combination of TPA plus ionomycin. Whereas

Fig. 2. Effect of NVP-231 on cytokine-stimulated PGE₂ formation in rat renal mesangial cells. Rat mesangial cells were stimulated for 24 h with either vehicle or IL-1 β (2 nM) in the absence or presence of the indicated concentrations of NVP-231 (A, B), or C₆-ceramide (C). Supernatants were taken for PGE₂ quantification using a PGE₂-ELISA (A and C). Protein in the remaining cell monolayer were extracted and taken for Western blot analysis (B) of COX2 (upper panel) or β -actin (lower panel). Data in A and C are expressed as pg/ml supernatant and are means \pm S.D. (n=4). ***p<0.001 considered statistically significant when compared to the control values; #p< 0.05, ###p< 0.001 when compared to the IL-1 β -stimulated values.



stimulation of cells with 10 % FBS induced strong [³H]-arachidonic acid release (Fig. 1C), TPA alone or ionomycin alone exerted only marginal effects and only the combination of TPA plus ionomycin was synergistically active (Fig. 1D). This confirms previous findings that phosphorylation of cPLA₂ by the protein kinase C/MAPK cascade alone, or increased intracellular Ca²⁺ alone is not sufficient to fully activate cPLA₂, and that only the combination of phosphorylation and increased Ca²⁺ fully activates cPLA₂ [22, 23]. Again, in the presence of NVP-231 no reduction of either serum, TPA or ionomycin-induced arachidonic acid release was found. The combined stimulation with TPA plus ionomycin was even enhanced in the presence of NVP-231. Our data thus demonstrate that cPLA₂ activity induced by the various stimuli described above is not abrogated by CerK inhibition, a finding which questions the current hypothesis that C1P is necessary for cPLA₂ activity.

We also stimulated rat mesangial cells for 24 h with the pro-inflammatory cytokine IL-1 β which is known to induce PGE₂ synthesis in these cells by combined induction and activation of cPLA₂ and group IIA sPLA₂ [24, 25]. Again, as shown in Fig. 2A, in the presence of low concentrations of NVP-231, PGE₂ formation was not reduced (Fig. 2A), whereas it was even enhanced at higher concentrations (1-5 μ M). Analogous to the increased PGE₂, COX2 protein expression was also upregulated (Fig. 2B). Increased PGE₂ synthesis (Fig. 2C) was also seen when cells were exposed to IL-1 β in the presence of increasing concentrations of C₆-ceramide suggesting that ceramide, which is expected to accumulate upon CerK inhibition, is responsible for the increased PGE₂ formation.

We next investigated what effect the complete genetic knockout of CerK has on arachidonic acid release and PGE₂ formation in mouse renal fibroblasts isolated from CerK (-/-) mice and control littermates (Wt). As shown in Fig. 3A, CerK knockout had no effect

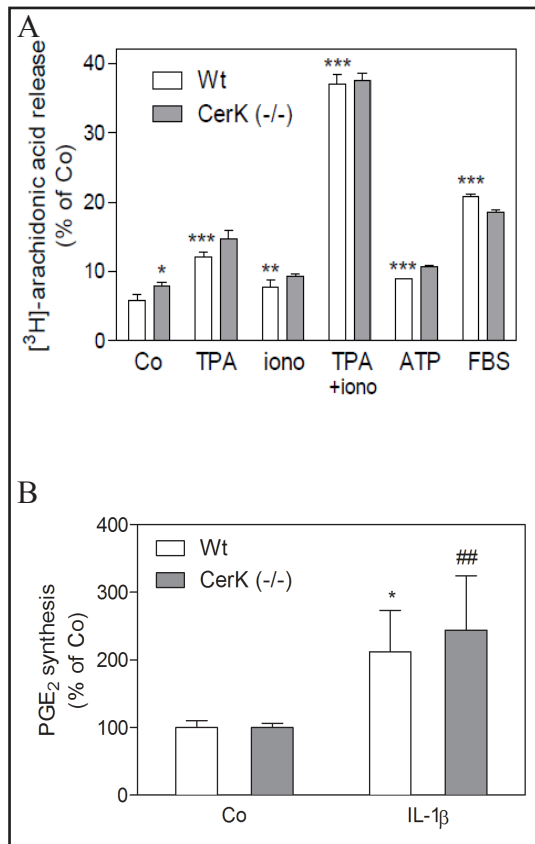


Fig. 3. Effect of CerK knockout on agonist-stimulated arachidonic acid release in mouse renal fibroblasts. (A) Mouse renal fibroblasts isolated from either wildtype BalbC mice (Wt, open columns) or CerK deficient mice (CerK(-/-), closed columns) were labelled for 24 h with $[^3\text{H}]$ -arachidonic acid and thereafter stimulated for 1 h with either vehicle (Co), TPA (100 nM), ionomycin (iono, 1 μM), TPA (100 nM) plus ionomycin (1 μM), ATP (100 μM), or fetal bovine serum (FBS, 10 %). Samples were processed as described in the Methods Section. (B) Mouse renal fibroblasts isolated from either wildtype BalbC mice (Wt, open columns) or CerK deficient mice (CerK (-/-), closed columns) were stimulated for 24 h with either vehicle (Co) or IL-1 β (1 nM). Supernatants were taken for a PGE₂ ELISA. Data are expressed as % of control and are means \pm S.D. (n=4). *p<0.05, **p<0.01, ***p<0.001 considered statistically significant when compared to the control values; ##p<0.01 when compared to the CerK (-/-) values.

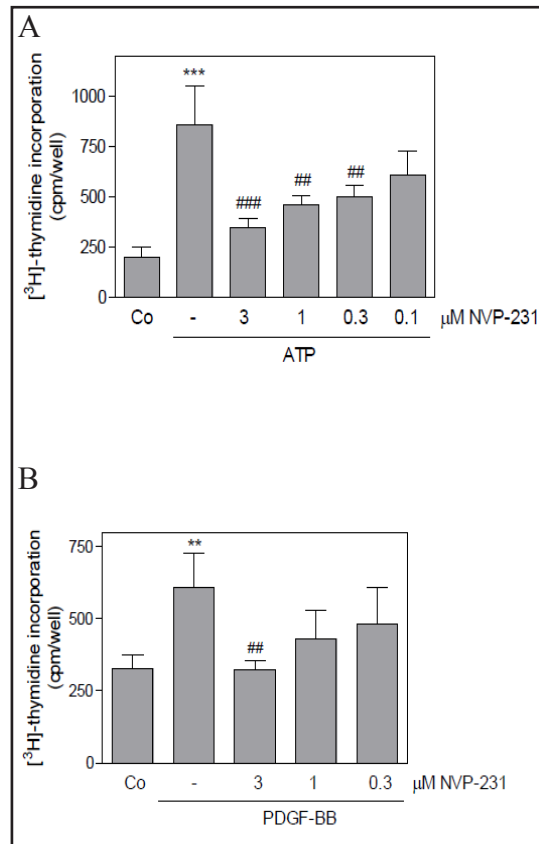


Fig. 4. Effect of NVP-231 on ATP- and serum-stimulated mesangial cell proliferation. Quiescent rat mesangial cells were stimulated for 24 h with ATP (100 μM , A) or 48 h with PDGF-BB (20 ng/ml, B) in the absence or presence of the indicated concentrations of NVP-231 (in μM) and in the presence of $[^3\text{H}]$ -thymidine. Samples were processed as described in the Methods Section. Data are expressed as % of control and are means \pm S.D. (n=4). **p<0.01, ***p<0.001 considered statistically significant when compared to the control values. ##p<0.01, ###p<0.001 when compared to the agonist-stimulated value.

on the various agonists-triggered arachidonic acid release including TPA, ionomycin, the combination of TPA plus ionomycin, ATP and serum. Also, no difference in IL-1 β -stimulated PGE₂ synthesis was observed in CerK (-/-) cells (Fig. 3B).

Altogether, our data do not confirm previous findings that CerK is positively involved in cPLA₂ activation and subsequent PGE₂ formation, but rather suggest the ability of the substrate ceramide to stimulate arachidonic acid release and promote COX-2 induction and subsequent PGE₂ formation.

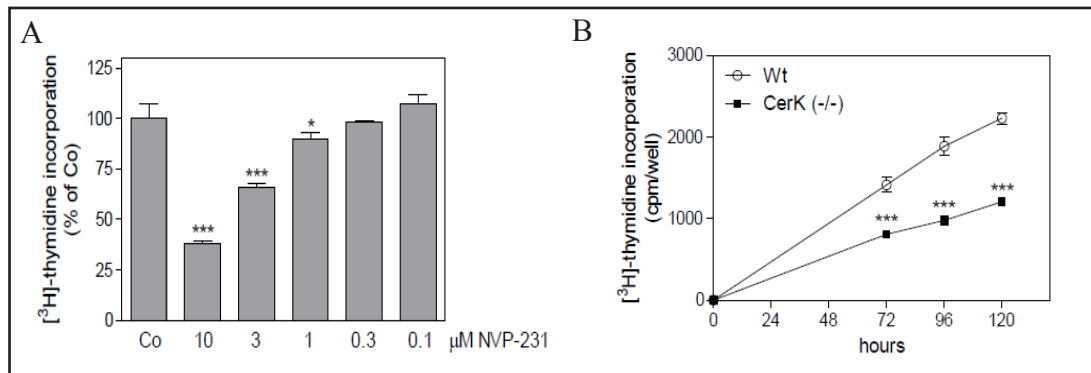


Fig. 5. Effect of NVP-231 and of CerK gene knockout on proliferation of mouse renal fibroblasts. (A) Renal fibroblasts isolated from BalbC mice were treated for 24 h with either vehicle (Co) or the indicated concentrations of NVP-231 in the presence of [³H]-thymidine. (B) 10⁴ cells of renal fibroblasts from wildtype BalbC mice (Wt, open circles) and from CerK knockout mice (CerK (-/-), closed squares) were seeded in the presence of [³H]-thymidine and DNA synthesis was measured after 72 h, 96 h, and 120 h. Samples were processed as described in the Methods Section. Data are expressed as % of control (A) or as cpm/well (B) and are means \pm S.D. (n=4). *p<0.05, ***p<0.001 considered statistically significant when compared to the respective control values.

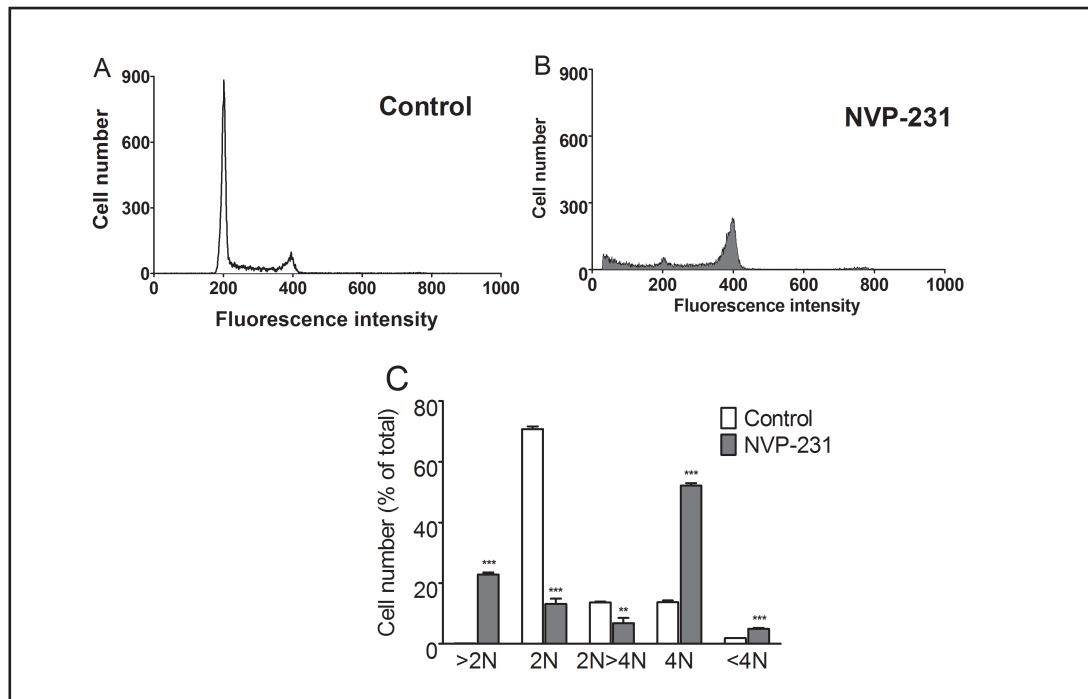


Fig. 6. Effect of NVP-231 on cell cycle distribution of rat renal mesangial cells. Rat mesangial cells were incubated for 24 h in growth medium containing either vehicle (control) (A) or 10 μ M of NVP-231 (B). Cells were stained with propidium iodide and DNA content was measured by flow cytometry using a FACSCalibur flow cytometer and the Cell Quest software for data processing. The graphs in A and B are representative of three independent experiments. (C) shows the % of total cells in the different cell cycle phases of control cells (open columns) and NVP-231-treated cells (closed columns) and are means \pm S.D. (n=3). **p<0.01, ***p<0.001 considered statistically significant when compared to the corresponding Wt values.

Inhibition of CerK decreases proliferation of renal mesangial cells and fibroblasts

A major biological function attributed to CerK is stimulation of cell proliferation [11]. Since mesangial cell proliferation is an important hallmark of many forms of chronic

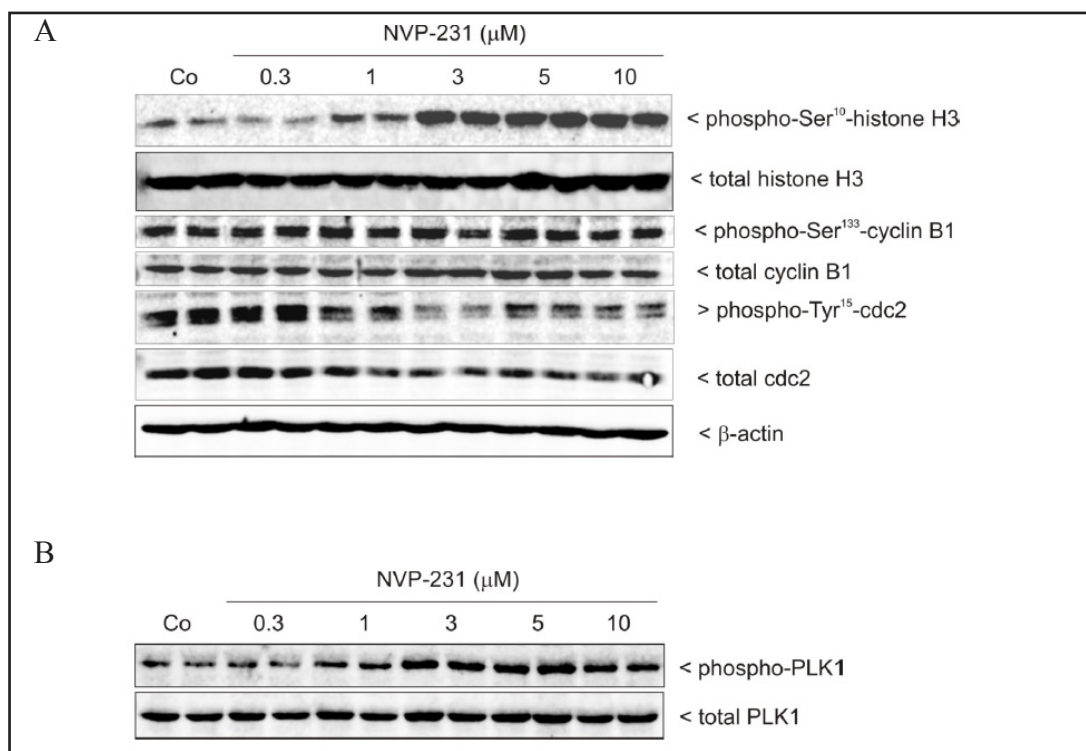


Fig. 7. Effect of NVP-231 on phospho-histone H3, cdc2, cyclin B1 and PLK1 expression in rat mesangial cells. Rat mesangial cells were incubated for 24 h in growth medium in the absence or presence of the indicated concentrations (in μM) of NVP-231. Thereafter, cell lysates were prepared and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and subjected to Western blot analysis using antibodies against phospho-histone H3 (A), total histone H3 (A), phospho-Tyr¹⁵-cdc2 (A), total cdc2 (A), phospho-cyclin B1 (A), total cyclin B1 (A), β -actin (A), phospho-PLK1 (B), and total PLK1 (B). A representative blot from two independent experiments performed in duplicates is shown.

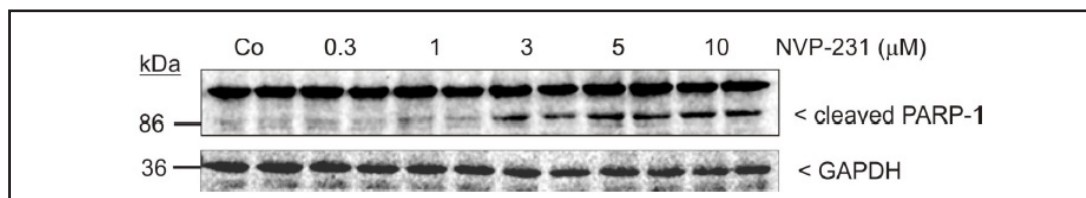


Fig. 8. Effect of NVP-231 on PARP-1 cleavage in rat mesangial cells. Rat mesangial cells were treated for 24 h with either vehicle (Co) or the indicated concentrations of NVP-231. Protein lysates were prepared and samples were separated by SDS-PAGE, transfer to nitrocellulose and subjected to Western blot analysis using antibodies against PARP-1 (upper panel) or GAPDH (lower panel). A representative blot from two independent experiments performed in duplicates is shown.

inflammatory kidney diseases [1-3], we investigated whether inhibition of CerK using NVP-231 affects mesangial cell growth determined by [³H]-thymidine incorporation. To this end, quiescent rat renal mesangial cells were stimulated with two well-known mitogens, i.e. extracellular ATP and PDGF-BB. As shown in Fig. 4A, ATP stimulated DNA synthesis already after 24 h. In the presence of NVP-231, this effect was dose-dependently decreased and resulted in almost complete inhibition at a dose of 3 μM NVP-231. PDGF-BB was a weaker mitogen for which 48 h of stimulation was required to increase DNA synthesis (Fig. 4B). Again, this effect was dose-dependently decreased by NVP-231 (Fig. 4B).

Similar to rat renal mesangial cells, a dose-dependent anti-proliferative effect of NVP-231 was also observed in mouse renal fibroblasts (Fig. 5A). Furthermore, we found that the

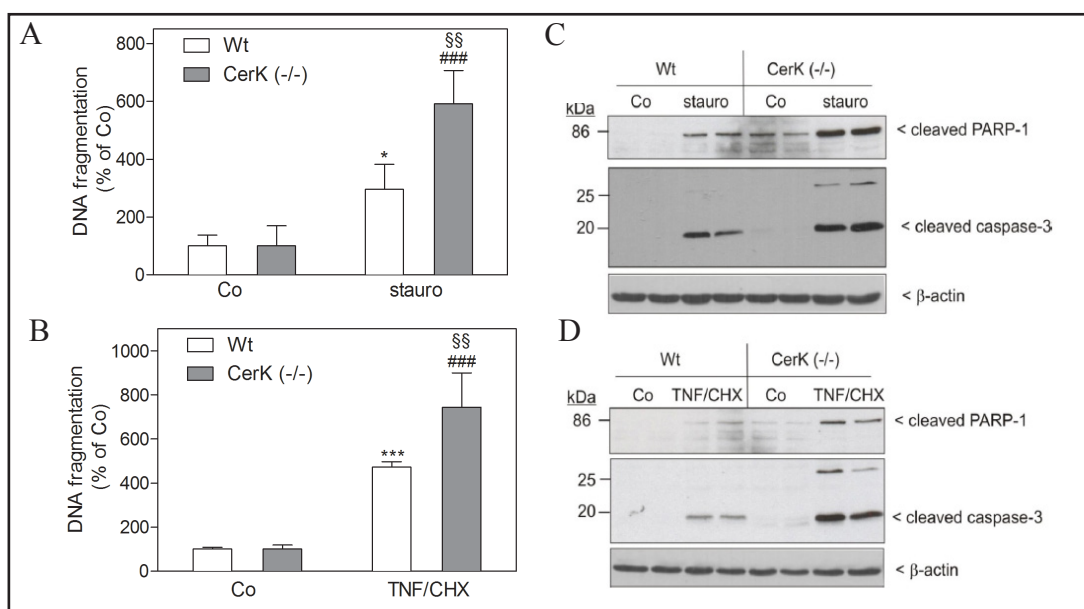


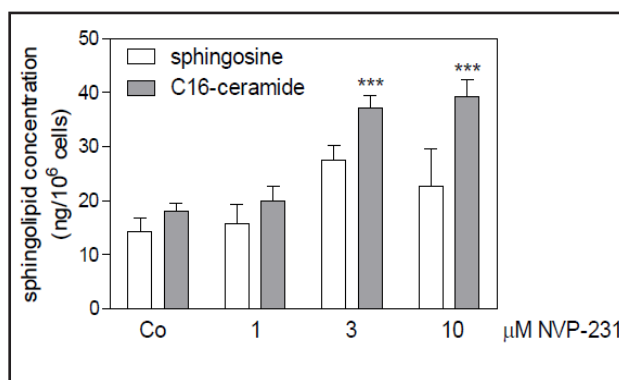
Fig. 9. Effect of CerK knockout on staurosporine- and TNF α /cycloheximide-induced apoptosis in renal fibroblasts. Mouse renal fibroblasts isolated from wildtype BalbC mice (Wt, open columns) or CerK-deficient mice (CerK (-/-), closed columns) were treated for 2 h with either vehicle (Co) and staurosporine (30 nM) (A and C), or for 24 h with vehicle (Co) and TNF α (1 nM) plus cycloheximide (10 μ M) (B and D). Samples were taken for a DNA fragmentation ELISA (A and B). Protein lysates were taken for SDS-PAGE, transfer to nitrocellulose and Western blot analysis (C and D) using antibodies against PARP-1 (upper panels), cleaved caspase-3 (middle panels) and β -actin (lower panels). Data in A and B are expressed as % of control values and are means \pm S.D. (n=3). *p<0.05, ***p<0.001 considered statistically significant when compared to the wildtype control values. ###p<0.001 when compared to the CerK (-/-) control values, §§p<0.01 when compared to the Wt stauro- (in A) or Wt TNF α /CHX (in B)-stimulated values respectively.

proliferation rate of CerK (-/-) fibroblasts detected over 6 days in culture was significantly lower compared to wildtype fibroblasts (Fig. 5B).

We next investigated whether CerK inhibition and decreased proliferation was associated with altered cell cycle distribution. To this end, rat mesangial cells were treated for 24 h with NVP-231 and the DNA content was determined. Strikingly, as shown in Fig. 6, upon NVP-231 treatment, the number of cells in G1 (2N DNA content) and S phase (2N>4N) decreased and cells accumulated in G2/M (4N DNA content). At the same time, also the number of cells with fragmented DNA (sub G1 peak) and of polyploid cells with a 8N DNA content increased (Fig. 6C).

To more specifically investigate whether cells accumulated at the G2/M boundary or were arrested in the M phase, lysates of NVP-231-treated cells were subjected to Western blot analysis and stained for cell cycle markers relevant for M phase regulation. As shown in Fig. 7A, the level of the mitosis marker phospho-histone H3 increased dose-dependently upon NVP-231 treatment indicating that cells were mainly arrested in M phase. The amount of total histone H3 remained unchanged (Fig. 7A). Furthermore, we determined the level of cdc2 (CDK1) as a major regulator of G2-M transition and execution of mitosis [26, 27]. Cdc2 interacts with cyclin B1 in a complex which is kept in an inactive state by Tyr¹⁵ phosphorylation of cdc2. At the G2/M boundary, cdc2 is dephosphorylated and thereby activated to promote mitotic entry. Here, we found that cdc2 Tyr¹⁵ phosphorylation was decreased upon NVP-231 treatment which further suggests that cells entered mitosis (Fig. 7A). No change of cyclin B1 was seen neither on the level of phosphorylation nor total protein expression (Fig. 7A). However, we found that treatment with NVP-231 also increased the phosphorylation and thus activation of polo-like kinase 1 (PLK1) (Fig. 7B), a kinase regulating mitosis entry and

Fig. 10. Effect of NVP-231 on C₁₆-ceramide and sphingosine levels in rat mesangial cells. Rat mesangial cells were treated for 24 h with either vehicle (Co) or the indicated concentrations of NVP-231. Lipids were extracted as described in the Methods Section and quantified by LC-MS/MS. Sphingosine (open columns) and C₁₆-ceramide (closed columns) are expressed as ng/10⁶ cells and are means ± S.D. (n=3). ***p<0.001 considered statistically significant when compared to the wildtype control values.



bipolar spindle assembly [28]. Cells remaining in the sub G1 phase upon NVP-231 treatment underwent classical apoptosis as further confirmed by increased poly-(ADP-ribose)-polymerase-1 (PARP-1) cleavage [29] shown in Fig. 8.

To investigate whether knockout of CerK in renal fibroblasts also facilitated apoptosis, DNA fragmentation detected by ELISA was measured in the presence of either staurosporine or TNF α /cycloheximide, two well-known inducers of apoptosis. As expected, both treatments induced DNA fragmentation (Fig. 9A and B) and this effect was significantly increased in CerK (-/-) fibroblasts (Fig. 9A and B). Concomitantly, cleavage of caspase-3 and PARP-1 was increased compared to wildtype cells (Fig. 9C and D).

Finally, to verify that in rat mesangial cells, CerK inhibition by NVP-231 results in the accumulation of cellular ceramide, the main ceramide subspecies C₁₆-ceramide, and sphingosine for comparison, were quantified by mass spectrometry. As shown in Fig. 10, C₁₆-ceramide indeed dose-dependently increased from basal 18 ± 1.5 ng/10⁶ cells to 39 ± 3.1 ng/10⁶ cells upon treatment with 10 μ M NVP-231, whereas sphingosine levels were not significantly changed.

Discussion

To date, the cellular function of CerK is still a matter of debate. Various studies demonstrated that CerK is an essential enzyme to produce C1P which, in turn, directly binds to and activates cPLA₂ resulting in the synthesis of pro-inflammatory PGE₂. These data unveil a key role for CerK in inflammation and suggest CerK targeting as a treatment modality for inflammatory diseases [30, 31]. However, data from CerK gene deficient mice do not support this hypothesis, since the loss of CerK did not ameliorate disease symptoms in inflammation-related disease models such as arthritis, peritonitis, thrombocytopenic purpura, and active or passive cutaneous anaphylaxis models [17]. Moreover, in a mouse model of fulminant pneumonia, CerK deficient mice showed even more severe symptoms and increased mortality. The lack of effect of CerK gene knockout in most of these experimental disease models may be due to systemic compensatory mechanisms to restore C1P homeostasis. Conflicting data were also reported from cells isolated from CerK (-/-) mice. On the one hand, Graf et al. [17] found no change of agonist-induced arachidonic acid release and PGE₂ formation in CerK (-/-) peritoneal macrophages compared to wildtype macrophages which fits well to our data. On the other hand, using mouse embryonic fibroblasts and bone marrow-derived macrophages from the same CerK (-/-) mouse strain, Mietla et al. [32] reported considerably decreased formation of arachidonic acid and various prostaglandin subspecies measured by mass spectrometry. The reason for this discrepancy is unclear. Similarly, as shown in RAG1-deficient mice, the additional depletion of CerK resulted in lower basal PGE₂ levels in bronchoalveolar lavage fluid [33]. However, upon ovalbumin stimulation as a model of experimentally-induced asthma, the induction of PGE₂ synthesis was manifold higher in

CerK (-/-) compared to CerK (+/+) mice, whereas the total amount of PGE₂ reached by ovalbumine stimulation was still identical in the two mouse strains as was the severity of disease symptoms [33].

An alternative approach to overcome potential compensatory mechanisms of CerK gene knockout is the use of catalytic CerK inhibitors. Such an inhibitor, NVP-231, has been developed recently which demonstrated an in vitro IC₅₀ value for CerK of 12 nM [21]. The inhibitor proved to be highly cell-permeable and active under intracellular conditions [21]. Using NVP-231 in renal mesangial cells, we could clearly demonstrate that inhibition of CerK activity does not decrease either arachidonic acid release or PGE₂ formation in renal mesangial cells. Rather, we found an increase of PGE₂ (Fig. 2A) as a consequence of upregulated COX2 protein (Fig. 2B). This finding may be mechanistically explained by an increase of intracellular ceramide upon CerK inhibition (Fig. 10).

In fact, ceramide has been discussed as a pro-inflammatory lipid for many years. Mechanistically, ceramide was on the one hand shown to activate cPLA₂ [34-36] and also to enhance the expression of sPLA₂ [37] and COX2 [38-40] in various cell types, including mesangial cells and fibroblasts. Furthermore, in several inflammation-related disease models, ceramide was shown to contribute to an inflammatory phenotype such as in cystic fibrosis [41], pulmonary emphysema [42], and other acute and chronic lung injuries [43]. Whatever the pro-inflammatory mechanism of ceramide is, our data strongly suggest that CerK is not a suitable target for the treatment of inflammation and that its inhibition may result in the opposite effect i.e. acceleration of PGE₂ formation, which worsens symptoms even further.

Here we found that inhibition of CerK in renal mesangial cells using NVP-231 reduced proliferation and sensitized cells to stress-induced apoptosis.

A role for CerK in the regulation of apoptosis was first proposed by Liang et al. [44] who showed that in *Arabidopsis*, mutation of the plant homolog of CerK, also denoted "accelerated cell death 5" (*acd5*), not only led to an accumulation of ceramides, but also increased calcium mobilization and cell death upon pathogen infection [44, 45]. Similarly, downregulation of CerK using RNA interference was shown to inhibit proliferation and enhance apoptosis also in human A549 lung adenocarcinoma cells [46]. Apart from these preclinical findings, evidence for a key role of CerK in tumor progression was reported by Ruckhäberle et al. [47], who demonstrated that high CerK expression in tumor tissues correlated with a worse prognosis in patients with estrogen receptor-negative breast cancer. Moreover, recent data also suggest that the CerK protein is upregulated as a hormetic response of hepatoma cells exposed to UV irradiation to evade apoptosis, which could be counteracted by CerK silencing [48]. Based on this observation, pharmacological inhibition of CerK was suggested as a new concept in hepatoma therapy. The anti-proliferative and pro-apoptotic potential of CerK targeting is also corroborated by our data from CerK (-/-) fibroblasts demonstrating that the loss of CerK facilitates apoptosis induced by stress factors such as staurosporine or TNF α combined with cycloheximide (Fig. 9).

Further evidence that CerK targeting to decrease cell proliferation and survival may have therapeutic potential also derived from studies where CerK activity was affected indirectly. For instance, the anti-proliferative effect of vitamin D₃ on neuroblastoma cells was shown to involve the downregulation of CerK and C1P by vitamin D₃ [49], and CerK downregulation was also involved in all-trans retinoic acid (ATRA)-induced differentiation of neuroblastoma cells [50]. In mouse keratinocytes, it was correspondingly shown that the peroxisome proliferator-activated receptor β (PPAR β) promoted cell survival, which was dependent on PPAR β -mediated upregulation of CerK [51]. Consequently, in CerK deficient mice, PPAR β -mediated keratinocyte survival was strongly decreased [51]. These findings together with our data strongly support a key role for CerK in cell proliferation and survival.

Here, we report that in mesangial cells inhibition of CerK using NVP-231 induced M phase arrest and subsequent apoptosis. M phase arrest was confirmed by various parameters, including reduced Tyr¹⁵ phosphorylation of cdc2, reflecting enhanced cdc2 activity and mitotic entry, and enhanced phospho-histone H3 as a marker of mitosis. Furthermore,

treatment with NVP-231 increased the phosphorylation and thus activity of PLK1, a kinase regulating mitosis progression and proper bipolar spindle assembly [28].

This finding raises the intriguing question how CerK inhibition converges to the cell cycle machinery and whether the observed M phase arrest was due to ceramide accumulation or lack of C1P. That ceramide production is increased in CerK (-/-) mice and isolated cells was previously reported [17, 32]. Here, we confirm the accumulation of ceramide also in cells treated with NVP-231 (Fig. 10). In fact, ceramide is a well-characterized lipid with anti-proliferative and pro-apoptotic activity [9, 41]. Although the target of ceramide within the cell cycle machinery is still a matter of debate, it was shown to stimulate G1 arrest by inducing dephosphorylation of the retinoblastoma protein (Rb) [52, 53] and upregulation of the CDK inhibitory factor p21^{Cip1} [53, 54]. On the other hand, there is evidence that ceramide accumulation correlates with G2/M arrest [55-57]. This ambivalent effect may be due to cell type-specific differences in the equipment with ceramide-converting enzymes, such as sphingomyelin synthase, glucosylceramide synthase, ceramidases, and sphingosine kinases, all of which are essential determinants of ceramide action [58-61].

Alternatively, the here reported cell cycle effect may be attributed to the loss of C1P, which raises the question where in the cell C1P is generated. Based on the presence of nuclear import and export signals in the CerK sequence, both nuclear and cytoplasmic localizations were suggested [15]. Moreover, in COS-1 cells transfected with green fluorescence protein (GFP)-tagged CerK the enzyme was shown to localize in the cytoplasm, the nucleus and the nucleolus [15]. Further investigations are therefore warranted to reconcile the subcellular localization of CerK with the putative site of C1P activity and its link to cell cycle regulation.

In summary, we demonstrated here that CerK induces proliferation, but not PGE₂ formation, in renal mesangial cells and fibroblasts, and that its pharmacological inhibition has potential for the treatment of proliferative kidney diseases, but not for renal diseases associated with inflammation.

Disclosure Statement

None.

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