# Negative regulation of interleukin-1β-activated neutral sphingomyelinase by protein kinase C in rat mesangial cells

Marietta Kaszkin[a,*], Andrea Huwiler[a], Kirsten Scholz[a], Henk van den Bosch[b], Josef Pfeilschifter[a]

[a] *Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany*
[b] *Center for Biomembranes and Lipid Enzymology, University of Utrecht, NL-3584 CH Utrecht, The Netherlands*

Received 11 September 1998; received in revised form 21 October 1998

**Abstract Endogenous ceramide is produced by the action of acidic or neutral sphingomyelinases (SMase) in response to stimuli such as proinflammatory cytokines or other inducers of stress. Interleukin-1β (IL-1β) is known to stimulate ceramide formation in rat renal mesangial cells; however, the respective subtype of SMase and its regulation have not been investigated. We found that IL-1β induced an increase in endogenous ceramide levels via the action of a neutral SMase but not an acidic SMase in rat mesangial cells. Cytokine-induced activation of neutral SMase was inhibited by stimulation of protein kinase C (PKC) by the phorbol ester TPA which caused a reduction of ceramide back to control levels. This inhibitory effect of TPA was reversed by the specific PKC-inhibitor Ro-318220. Long-term incubation (24 h) of mesangial cells with TPA, which downregulates PKC-α, -δ, and -ε isoenzymes, resulted in a recovery of IL-1β-stimulated neutral SMase activity as well as ceramide formation. These data implicate an important modulatory function of PKC in ceramide production in IL-1β-activated mesangial cells.**



*Key words:* Ceramide; Mesangial cell; Phorbol ester; Protein kinase C; Sphingomyelinase

## 1. Introduction

Rat renal mesangial cells are a suitable model system to study cytokine-regulated mechanisms which reflect proinflammatory processes involved in the pathogenesis of glomerulonephritis [1]. Under pathological conditions, mesangial cells have the potential to produce a cascade of inflammatory mediators including eicosanoids, nitric oxide and other reactive oxygen species as well as growth factors [2]. Another more recently characterized class of lipid second messengers are ceramides which have emerged as potent mediators in the regulation of proliferation of different cell systems [3–6]. In mesangial cells ceramides have been shown to activate the c-Raf kinase via specific binding to this enzyme [7]. Endogenous ceramides can be formed by the stimulation of different sphingomyelinases (SMase) by inflammatory cytokines [8]. The action of a neutral cytosolic or membrane-bound form and an acidic form located in lysosomes determine the major route of sphingomyelin degradation resulting in ceramide and phosphocholine. The acidic form of SMase had already been cloned and is well characterized [9]. Recently, a neutral SMase was cloned in human embryonic kidney 293 cells [10]. In mesangial cells the activation of an SMase by IL-1β was described without further distinguishing the different isoenzymes [11]. The aim of this study was to investigate which type of SMase is responsible for IL-1β-stimulated ceramide formation. Furthermore, we looked for regulatory signaling pathways that might interfere with the hydrolysis of SM by the respective SMase. We found that IL-1β stimulated a neutral SMase, and that this enzyme as well as ceramide formation were inhibited by TPA treatment. This inhibition could be reversed completely by specific PKC inhibitors or by downregulation of TPA-sensitive PKC-isoenzymes indicating that PKC may act as an early negative modulator of IL-1β-induced signaling through the SM pathway.

## 2. Materials and methods

### 2.1. Materials

Recombinant IL-1β was generously supplied by Dr. C. Rordorf, Novartis Pharma, Basel, Switzerland. [1-$^{14}$C]Oleic acid, L-[3-$^{14}$C]serine and [N-*methyl*-$^{14}$C]sphingomyelin were from Amersham-Buchler, Frankfurt, Germany. TPA was purchased from Sigma (Munich, Germany). {3-[1-[3-(Amidinothio)propyl-1*H*-indoyl]-3-yl]-3-(1-methyl-1*H*-indoyl-3-yl)maleimide methane sulfonate}, Ro-318220, was a generous gift of F. Hoffmann-LaRoche (Basel, Switzerland). All cell culture media and nutrients were from Gibco-BRL (Eggenstein, Germany), and all other chemicals used were from either Merck (Darmstadt, Germany) or Fluka (Deisenhofen, Germany).

### 2.2. Cell culture

Rat renal mesangial cells were cultivated as described previously [12]. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin (0.66 units/ml). Twenty-four hours prior to stimulation and during the experiments cells were incubated in DMEM containing 0.1 mg/ml fatty acid-free bovine serum albumin (BSA).

### 2.3. Assay of neutral and acidic sphingomyelinase

The assays for detecting activity of a neutral and an acidic SMase were performed according to Robinson et al. [13] under optimal assay conditions for the respective enzyme using [N-*methyl*-$^{14}$C]sphingomyelin. Briefly, for determination of the activity of the neutral SMase aliquots of the cell lysates containing 10 μg of protein were incubated with [N-*methyl*-$^{14}$C]sphingomyelin (0.05 μCi) in 50 μl final volume of 20 mM HEPES, 1 mM $MgCl_2$ buffer, pH 7.4 at 37°C for 2 h. The amount of [$^{14}$C]phosphocholine liberated from the substrate was assessed by partitioning the reaction mixture with 800 μl chloroform/methanol (2:1 by vol.) and 250 μl water, and counting the radioactivity of the aqueous phase (200 μl) in a liquid scintillation counter. To measure acidic SMase activity, aliquots of cell lysates were incubated with [N-*methyl*-$^{14}$C]sphingomyelin in 50 μl final vol. of 250 mM sodium acetate, 1 mM EDTA buffer, pH 5.0 and the amount of [$^{14}$C]phosphocholine produced was determined as described for the neutral SMase assay.

### 2.4. Lipid extraction and analysis of endogenous ceramides

Cells were prelabeled for 24 h with L-[3-$^{14}$C]serine (0.4 μCi/ml) in DMEM plus 0.1 mg/ml fatty acid-free BSA, and after medium change

*Corresponding author. Fax: (49) (69) 6301-7942.
E-mail: kaszkin@em.uni-frankfurt.de


PII: S0014-5793(98)01445-8

they were treated with the compounds to be tested. The reaction was stopped by removing the supernatant and adding 2 ml ice-cold methanol to the cells. Cellular lipids were extracted with 2 ml 1 N HCl and 2 ml chloroform according to Bligh and Dyer [14]. The concentrated chloroform phases were applied to silica gel 60 TLC plates and developed in a first run for the first 10 cm in chloroform-methanol-25% ammonia (65:35:7.5 by vol.) and in a second run to the top of the plate in chloroform-methanol-acetic acid (90:10:10 by vol.). The detection and quantification of ceramides was performed by scanning the lanes using a Linear Analyzer (Berthold, Wildbad, Germany). For localization of ceramides on the TLC-plates $C_{16}$-ceramide as well as ceramides from bovine brain (Sigma) were used as reference substances and were visualized by iodine vapor.

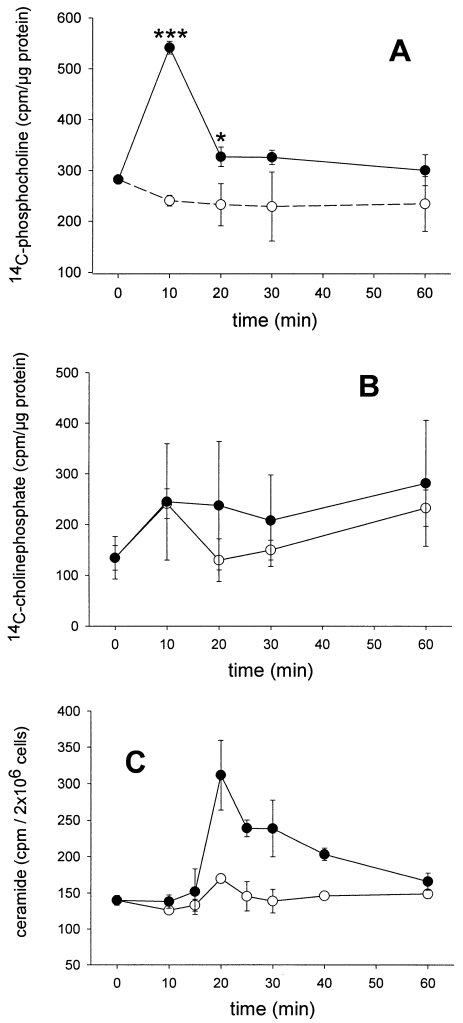


Fig. 1. Time course of IL-1β-stimulated activity of neutral (A) and acidic SMase (B) and ceramide formation (C) in rat mesangial cells. For measuring neutral (A) and acidic SMase activity (B) cells were incubated with IL-1β (2 nM; ●) or vehicle (○) for the time points indicated. Cell lysates were assayed for neutral and acidic SMase activity with [N-*methyl*-$^{14}$C]sphingomyelin and analyzed for released [$^{14}$C]phosphocholine as described in Section 2. For detection of endogenous ceramide (C) cells were prelabeled for 24 h with [$^{14}$C]serine and then treated with IL-1β (●) or vehicle (○) for the time points indicated. After lipid extraction ceramides were analyzed by thin layer chromatography as described in Section 2. Each value represents the means ± S.D. of 3 experiments, $n = 3$. Statistical analysis in A: $^{***}P < 0.001$, $^{*}P < 0.05$ (Student's *t*-test). None of the data points in B was statistically significant from its corresponding control value (Student's *t*-test).

## 3. Results

First we investigated whether an acidic or a neutral SMase is activated in mesangial cells after IL-1β treatment. To this end a time course of [$^{14}$C]phosphocholine release from radioactively labeled sphingomyelin was performed for up to 3 h under the specific conditions required for optimal activity of acidic SMase and neutral SMase, respectively. As shown in Fig. 1A we found a rapid and transient increase in neutral SMase activity with a maximum after 10 min of IL-1β treatment. During this time period no activation of an acidic SMase could be observed (Fig. 1B). In parallel we measured the ceramide formation in [$^{14}$C]serine-prelabeled cells after IL-1β treatment. In Fig. 1C it is shown that ceramide formation followed the neutral SMase activity with a rapid and transient increase 20 min after IL-1β treatment.

When cells were preincubated with TPA, a direct activator of PKC, for 10 min and then treated with IL-1β, the neutral SMase activity (Fig. 2A) as well as ceramide formation (Fig. 2B) were completely inhibited. During this short-term incubation period TPA alone did not stimulate an increase in ceramide levels. These data indicate that phorbol ester-activated PKC isoenzymes negatively regulate IL-1β-stimulated neutral SMase activation and ceramide formation.

In earlier studies we have shown that a 24-h treatment of mesangial cells with 500 nM TPA is necessary to downregulate the TPA-sensitive PKC-isoforms -α, -δ and -ε and to remove cellular functions mediated by these isoenzymes [15–17]. Performing different preincubation periods with TPA the effects on neutral SMase activity and ceramide formation were investigated. The data in Fig. 2 show that after a 4–8-h treatment with TPA the IL-1β-stimulated neutral SMase activity (Fig. 2A) and ceramide formation (Fig. 2B) were still completely inhibited indicating that under these conditions PKC-isoenzymes were still active. However, after a 24-h treatment with TPA the activity of neutral SMase as well as ceramide formation were fully reconstituted thus indicating that one or more PKC-isoenzymes seem to be responsible for the inhibitory effect of TPA on SM hydrolysis.

In order to confirm the role of PKC as a negative regulator of IL-1β-induced neutral SMase activation we used Ro-318220 as a specific inhibitor of PKC [18]. The data in Fig. 3 show that Ro-318220 inhibited TPA action and resulted in a complete recovery of IL-1β-stimulated neutral SMase activity. Under this condition we also detected a nearly complete reconstitution of ceramide formation with Ro-318220 reaching 86 ± 4% ($n = 3$).

## 4. Discussion

In this study we could show that (i) the activation of a neutral SMase and formation of its product ceramide are early events in the signaling pathway induced by IL-1β and (ii) that this effect can be inhibited by PKC in mesangial cells. Activation of PKC is already known to inhibit hormone-stimulated phosphoinositide signaling in mesangial cells [19–21]. Now with the inhibitory effect on neutral SMase activation we report a new negative regulatory function of PKC which may contribute to the coordination of signal flow in different signalling pathways in mesangial cells.

A membrane-associated neutral SMase activity has been known for many years, and several studies have suggested

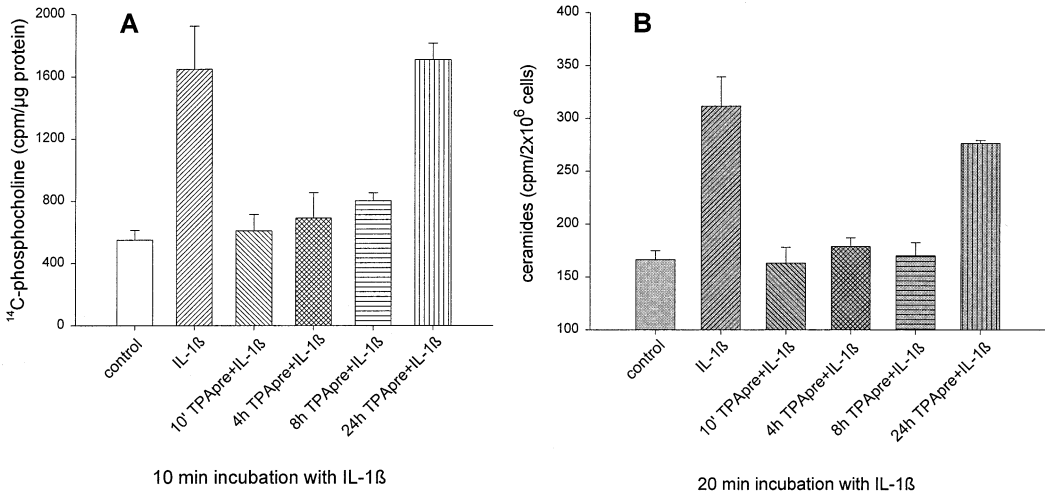


Fig. 2. Effect of different preincubation times with TPA on IL-1β-stimulated neutral SMase activity (A) and ceramide formation (B). Mesangial cells were preincubated for 10 min, 4 h, 8 h or 24 h with TPA (500 nM). Then without medium change cells were treated with IL-1β (2 nM) for 10 min to measure neutral SMase activity (A) or for 20 min to detect ceramide production (B) as described in Section 2. Each value represents the means ± S.D. of 3 experiments, $n = 3$. TPApre = preincubation with TPA.

an activation of this enzyme in response to cytokines and oxidative stress (for review see [22]). However, the mechanisms involved in regulating neutral SMase remained poorly defined. The recent cloning of the enzyme [10] may help to elucidate the regulation of neutral SMase signalling.

Earlier studies of our group have shown that phorbol ester-activated PKC isotypes interfere with IL-1β-stimulated signaling by inhibiting the expression of proinflammatory genes such as inducible nitric oxide synthase [23] and group IIA phospholipase $A_2$ (unpublished observations). As possible targets of PKC isoenzymes three possibilities were discussed: (i) phosphorylation and activation of transcription factors by PKC [24]; (ii) phosphorylation of the IL-1 receptor itself [25]; and (iii) phosphorylation of IL-1-receptor-associated kinases (IRAKs; [26]).

In our study we focussed on activation of the neutral SMase which is an early event in IL-1β signalling. Coincubation of mesangial cells with cytokine and phorbol ester negatively modulated the IL-1β-stimulated activation of neutral SMase thus leading to a complete inhibition of ceramide formation. This inhibition was reversed by downregulation of all TPA-sensitive PKC isoenzymes. In our earlier studies we observed that PKC-ε seems to be important for the negative modulation of cytokine-induced gene expression of inducible nitric oxide synthase and group IIA phospholipase $A_2$. Whether this is true also for neutral SMase regulation remains to be elucidated.

Further studies have to be performed to characterize the exact regulation of the neutral SMase by IL-1β-receptor activation and the target for PKC that allows for negative regulation of neutral SMase activity. In this context it is worth noting that the protein sequence of the neutral SMase shows five putative PKC-motifs ([S-T]-X-X-D-E) which suggests possible direct regulation by PKC-mediated phosphorylation. Moreover, we have recently shown that ceramide selectively binds to PKC-α and -δ isoenzymes in mesangial cells [27]. It will be of high priority to evaluate whether endogenous ceramide formed in mesangial cells after IL-1β stimulation activates one or both of these PKC isoforms, thereby inactivating neutral SMase and switching off ceramide formation in a negative feedback loop.

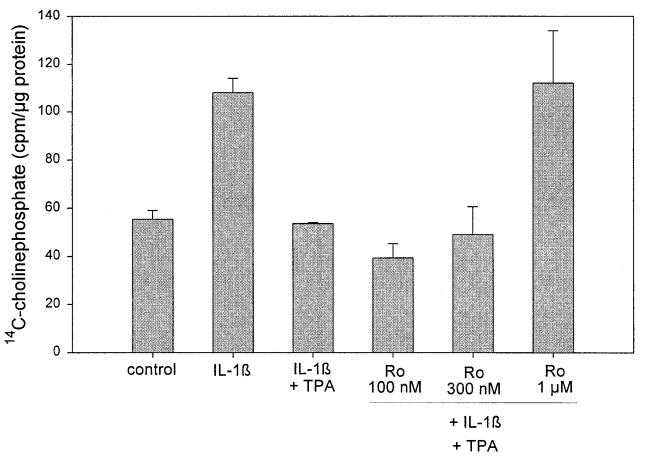


Fig. 3. Influence of the PKC inhibitor Ro-318220 on TPA-induced inhibition of IL-1β-stimulated neutral SMase activity in mesangial cells. Cells were pretreated for 30 min with different concentrations of Ro-318220 and then incubated for 10 min with TPA (500 nM) and finally treated with IL-1β (2 nM) for 10 min. Neutral SMase activity was measured as described in Section 2. Each value represents the means ± S.D. of 3 experiments, $n = 3$.

*Acknowledgements:* This work was supported by a grant of the Wilhelm Sander Stiftung to Kirsten Scholz and Josef Pfeilschifter. We thank Silke Spitzer for excellent technical assistance and Christiane Rordorf for the generous gift of IL-1β.

## References

[1] Pfeilschifter, J. (1994) News Physiol. Sci. 9, 271–276.
[2] Pfeilschifter, J. (1995) in: Molecular Approaches to Pathophysiology (Glaser, K.B. and Vadas, P., Eds.) pp. 25–51, CRC Press, New York, NY.
[3] Kolesnick, R. and Golde, D.W. (1994) Cell 77, 325–328.
[4] Hannun, Y.A. (1996) Science 274, 1855–1859.
[5] Pyne, S., Chapman, J., Stelle, L. and Pyne, N.J. (1996) Eur. J. Biochem. 237, 819–826.

[6] Spiegel, S. and Merrill, A.H. (1996) FASEB J. 10, 1388–1397.
[7] Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., van den Bosch, H. and Pfeilschifter, J. (1996) Proc. Natl. Acad. Sci. USA 93, 6959–6963.
[8] Kuno, K. and Matsushima, K. (1994) J. Leukocyte Biol. 56, 542–546.
[9] Quintern, L.E., Schuchman, E.H., Levran, O., Suchi, M., Ferlinz, K., Reinke, H., Sandhoff, K. and Desnick, R.J. (1989) EMBO J. 8, 2469–2473.
[10] Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M. and Stoffel, W. (1998) Proc. Natl. Acad. Sci. USA 95, 3638–3643.
[11] Coroneos, E., Martinez, M., McKenna, S. and Kester, M. (1995) J. Biol. Chem. 270, 23305–23309.
[12] Pfeilschifter, J., Kurtz, A. and Bauer, C. (1984) Biochem. J. 223, 855–859.
[13] Robinson, B.S., Hii, C.S., Poulos, A. and Ferrante, A. (1997) Immunology 91, 274–280.
[14] Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[15] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. J. 279, 441–445.
[16] Huwiler, A., Fabbro, D., Stabel, S. and Pfeilschifter, J. (1992) FEBS Lett. 300, 259–262.
[17] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1994) Biochem. Pharmacol. 48, 689–700.
[18] Davis, P.D., Hill, C.H., Keech, E., Lawton, G., Nixon, J.S., Sedgwick, A.D., Wadsworth, J., Westmacott, D. and Wilkinson, S.E. (1989) FEBS Lett. 259, 61–63.
[19] Pfeilschifter, J. (1986) FEBS Lett. 203, 262–266.
[20] Pfeilschifter, J. (1988) Biochim. Biophys. Acta 969, 263–270.
[21] Pfeilschifter, J., Fandrey, J., Ochsner, M., Whitebread, S. and de Gasparo, M. (1990) FEBS Lett. 261, 307–311.
[22] Hannun, Y.A. and Obeid, L.M. (1997) Biochem. Soc. Trans. 25, 1171–1175.
[23] Mühl, H. and Pfeilschifter, J. (1994) Biochem. J. 303, 607–612.
[24] Papavassiliou, A.G., Treier, M. and Bohmann, D. (1995) EMBO J. 14, 2014–2019.
[25] Bird, T.A., Woodward, A., Jackson, J.L., Dower, S.K. and Sims, J.E. (1991) Biochem. Biophys. Res. Commun. 177, 61–67.
[26] Yamin, T.T. and Miller, D.K. (1997) J. Biol. Chem. 272, 21540–21547.
[27] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1998) Biochemistry 37, 14556–14562.