Activation of serine/threonine protein phosphatase-1 is required for ceramide-induced survival of sympathetic neurons

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In sympathetic neurons, C₆-ceramide, as well as endogenous ceramides, blocks apoptosis elicited by NGF (nerve growth factor) deprivation. The mechanism(s) involved in ceramide-induced neuronal survival are poorly understood. Few direct targets for the diverse cellular effects of ceramide have been identified. Amongst those proposed is PP-1c, the catalytic subunit of serine/threonine PP-1 (protein phosphatase-1). Here, we present the first evidence of PP-1c activation by ceramide in live cells, namely NGF-deprived sympathetic neurons. We first determined PP activity in cellular lysates from sympathetic neurons treated with exogenous ceramide and demonstrated a 2–3-fold increase in PP activity. PP activation was completely blocked by the addition of the specific type-1 PP inhibitor protein I-2 as well as by tautomycin, but unaffected by 2 nM okadaic acid, strongly indicating that the ceramide-activated phosphatase activity was PP-1c. Inhibition of PP activity by phosphatidic acid (which has been reported to be a

selective inhibitor of PP-1c) and tautomycin (a PP-1 and PP-2A inhibitor), but not by 10 nM okadaic acid, abolished the anti-apoptotic effect of ceramide in NGF-deprived neurons, suggesting that activation of PP-1c is required for ceramide-induced neuronal survival. Ceramide was able to prevent pRb (retinoblastoma gene product) hyperphosphorylation by a mechanism dependent on PP-1c activation, suggesting that two consequences of NGF deprivation in sympathetic neurons are inhibition of PP-1c and subsequent hyperphosphorylation of pRb protein. These findings suggest a novel mechanism for ceramide-induced survival, and implicate the involvement of PPs in apoptosis induced by NGF deprivation.

Key words: ceramide, neuronal survival, protein phosphatase, retinoblastoma gene product.

INTRODUCTION

Ceramide is an essential second messenger in a variety of processes downstream of stress stimuli (reviewed in [1,2]). The finding that neurotrophins induce ceramide accumulation in certain neuronal types by binding to the p75 neurotrophin receptor and activating a sphingomyelinase [3–5] underscores the relevance of ceramide as a second messenger in the nervous system [6,7]. Interestingly, ceramide can both induce and protect neurons from apoptosis [3,4,8–10]. Work from our laboratory indicated that exogenous and endogenous ceramides are able to abort the apoptotic programme in sympathetic neurons triggered by NGF (nerve growth factor) deprivation [11]. Our studies showed that C₆-Cer (C₆-ceramide) is converted into long-chain ceramides and that the anti-apoptotic effect of C₆-Cer is due to both the short-chain analogue and the long-chain ceramides. C₆-Cer causes activation of the neurotrophin receptor TrkA and signalling pathways downstream of phosphoinositide 3-kinase/Akt. Nevertheless, TrkA is not activated by endogenous ceramides; therefore additional mechanisms must exist to mediate ceramide-induced neuronal survival.

Although ceramide produces a variety of cellular effects, only a few direct targets for ceramide have been identified, namely ceramide-activated protein kinase (CAPK), CAPPs (ceramide-activated protein phosphatases), cathepsin D and protein kinase C ζ (reviewed in [12]). PPs (protein phosphatases) activated by ceramide belong to the PP2A (type-2A serine/threonine PP) [13,14] and PP-1 (type-1 serine/threonine PP) families [15,16]. Amongst the substrates of PP-1 and PP-2A phosphatases, pRb

(retinoblastoma gene product) plays a particularly important role in cell cycle regulation [17–19] and neuronal death [20,21]. Progression of a cell through the G1 and S phases requires inactivation of pRb by CDK (cyclin-dependent kinase)-mediated phosphorylation [22,23]. Conversely, dephosphorylation of pRb by PP-1 [24] favours cell cycle arrest [17,25,26]. Accordingly, ceramide causes pRb dephosphorylation and cell cycle arrest in leukaemia cells [27,28] and activates PP-1 *in vitro* [15,16]. Until now, however, regulation of PPs by ceramide has not been demonstrated in live cells.

Deregulation of the cell cycle has been linked to apoptosis in post-mitotic neurons. According to the ‘cell cycle theory’, neuronal apoptosis could result from an abortive attempt to re-enter the cell cycle [29]. Consistent with this, after NGF deprivation of sympathetic neurons and neuron-like cells, there is an increased expression of proteins involved in cell cycle regulation [30–32]. Moreover, cell cycle inhibitors protect PC12 cells and sympathetic neurons from apoptosis [33–35].

In the present study, we examined whether ceramide activates PP-1 or PP-2A, leading to dephosphorylation of pRb in sympathetic neurons, thus blocking any attempt to re-enter the cell cycle after NGF withdrawal. We demonstrate for the first time that ceramide activates PP-1 in cultured neurons and that activation of PP-1 is required for ceramide to inhibit apoptosis caused by NGF deprivation. We also present evidence that, on NGF withdrawal, there is a decrease in serine/threonine PP activity accompanied by hyperphosphorylation of pRb. Both phenomena are prevented by ceramide. Moreover, PA (phosphatidic acid) inhibits PP-1 in cultured neurons, abolishes the effect of ceramide on pRb and

Abbreviations used: C₆-Cer, C₆-ceramide; CDK, cyclin-dependent kinase; DAG, diacylglycerol; DHCer, dihydroceramide; I-2, inhibitor-2; NGF, nerve growth factor; OA, okadaic acid; PA, phosphatidic acid; LPA, lyso PA; PP, protein phosphatase; pRb, retinoblastoma gene product.

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prevents ceramide-induced neuronal survival. Our results indicate that serine/threonine PPs play an active role in neuronal apoptosis.

EXPERIMENTAL

Materials

Leibovitz L15-CO₂ culture medium was purchased from Life Technologies (Burlington, ON, Canada). Rat serum was prepared from adult rat blood supplied by Health Science Lab Animal Services (University of Alberta). Rat-tail collagen was isolated as described previously [36]. Mouse NGF (2.5 S) was purchased from Alomone Laboratories (Jerusalem, Israel). Anti-NGF antibody (α -NGF) was purchased from Cedarlane Laboratories (Hornby, ON, Canada). C₆-Cer and C₆-DHCer (C₆-dihydroceramide) were obtained from Matreya (Pleasant Gap, PA, U.S.A.). OA (okadaic acid) and I-2 (inhibitor-2) were purified as described in [37,38]. LPA (lyso PA) was a gift from Dr D. Brindley (University of Alberta). MitoTracker[®] Orange (CM-H₂-TMRos) and MitoTracker[®] Green FM were purchased from Molecular Probes (Eugene, OR, U.S.A.). Polyclonal anti-Rb antibody and Molt-4 pRb control lysates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti- α / β -tubulin and PA 1,2-di (9-*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphate were purchased from Sigma-Aldrich (Oakville, ON, Canada). Monoclonal and polyclonal secondary antibodies were purchased from Pierce (Rockford, IL, U.S.A.). All other materials were purchased from Sigma-Aldrich or Fisher Scientific (Nepean, Ontario, Canada).

Culture of sympathetic neurons

Sympathetic neurons were isolated from superior cervical ganglia of newborn Harlan Sprague-Dawley rats (Health Science Lab Animal Services, University of Alberta) and cultured as described in [11]. Neurons were plated on collagen-coated 24-well plates at a density of 1 ganglion/well for immunoblot analysis and phosphatase activity assays or on 96-well plates at a density of 0.16 ganglion/well for survival experiments. Cultures were maintained in 50 ng/ml NGF for 7 days before the experiments.

NGF withdrawal experiments

Neurons were deprived of NGF and treated with C₆-Cer as described previously [11]. Medium containing no NGF but with 24 nM anti-NGF antibody (α -NGF) to eliminate residual NGF was used. Control cultures were given 50 ng/ml NGF throughout the experiment.

Detection of apoptosis

Apoptosis was evaluated by nuclear staining with Hoechst 33258 [11]. Alternatively, we examined loss of mitochondrial membrane potential using MitoTracker[®] Green FM and MitoTracker[®] Orange. Staining was performed according to manufacturer's instructions (Molecular Probes, Eugene, OR, U.S.A.). Neurons were visualized using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with Nikon digital camera DXM-1200 (Nikon Canada, Toronto, ON, Canada). Images were analysed using Northern Elite V6.0 image capturing and analysis software (Empix Imaging, Mississauga, ON, Canada).

Determination of PP activity

Neurons were treated with 20 μ M C₆-Cer or 20 μ M C₆-DHCer for 12 h. Some cultures were provided with PA (30 μ M) or OA (10 nM) during the incubation to test the effects of these PP inhibitors in live neurons. After the appropriate treatment, neuro-

nal cell extracts were prepared by rinsing and harvesting the neurons on ice-cold 50 mM Tris/HCl buffer (pH 7.4) containing leupeptin (1 μ g/ml), pepstatin (0.1 μ g/ml), PMSF (1 mM) and aprotinin (0.5 μ g/ml) followed by a centrifugation at 20080 *g* for 5 min at 4 °C. Pellets were washed three times with ice-cold PBS, resuspended in 40 μ l of 50 mM Tris/HCl buffer (pH 7.4) containing protease inhibitors and lysed by passage through a 100 μ l Hamilton syringe. Protein content was determined by the BCA assay (Pierce).

Serine/threonine phosphatase activity in neuronal cell lysates was assayed as reported previously [37]. Briefly, 10 μ M [³²P]-phosphorylase *a* was used as a substrate and the release of soluble phosphate was measured. The assay was standardized to obtain approx. 15 % release of total phosphate from the substrate under control conditions. PA was prepared immediately before the application to neurons as follows: 1 mg of PA dissolved in chloroform was dried under a constant low-pressure nitrogen stream for 30 min followed by rehydration in 1 ml of 50 mM Tris/HCl (pH 7.4), for 1 h at room temperature (23 °C) before sonication. Sonication was performed using a Vibra Cell 50 probe sonicator (Sonics & Materials, Newtown, CT, U.S.A.) at 15 % power in four 30 s pulses with 30 s cooling period between pulses.

To examine the effect of PP inhibitors *in vitro*, 200 nM I-2, 2 nM OA or up to 100 nM tautomycin were added to the neuronal cell extract just before the determination of phosphatase activity.

Detection of pRb phosphorylation

The phosphorylation state of pRb was examined by immunoblot analysis. Neurons were washed with ice-cold PBS containing the protease inhibitors indicated above and harvested in a modified RIPA buffer (25 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.5 % sodium deoxycholate, 2 % Nonidet-40, 0.2 % SDS and protease and phosphatase inhibitors). Proteins were separated by SDS/PAGE (7.5 % gel) containing modified ratios of acrylamide/bis-acrylamide as described in [39]. Gels were transferred on to PVDF membranes overnight at 4 °C in 25 mM Tris, 192 mM glycine, 16 % methanol buffer (pH 8.3) containing 0.1 % SDS. Membranes were blocked for 1 h in Tris-buffered saline, containing 0.1 % Tween 20 and 5 % (w/v) skimmed milk. Immunoblotting for pRb was performed using a polyclonal anti-pRb c-15 antibody (1:250 dilution; Santa Cruz Biotechnology) overnight at 4 °C, followed by polyclonal rabbit anti-IgG (1:5000 dilution; Pierce) for 1 h at room temperature. Bands were visualized using Super-Signal West Femto Substrate (Pierce). In all experiments, Molt-4 cell lysates (Santa Cruz Biotechnology) were used as control (results not shown). Tubulin was used to control for equal loading by immunoblotting with a monoclonal anti- α / β tubulin antibody (1:1000; Sigma-Aldrich), followed by a secondary antibody (1:2000).

Measurement of the mass of ceramide

Lipids were extracted by the Bligh-Dyer method and mass amounts of ceramide in cellular extracts were measured by a modification of the DAG (diacylglycerol)-kinase enzymic method as before [11]. Results are expressed with reference to the mass of total phospholipids.

RESULTS

Ceramide prevents the loss of mitochondrial membrane potential in NGF-deprived sympathetic neurons

In the absence of NGF, cultured sympathetic neurons undergo apoptosis with nearly complete death observed in 2 days

Figure 1 Ceramide inhibits loss of mitochondrial membrane potential in NGF-deprived sympathetic neurons

Neurons were deprived of NGF for 36 h and given one of the following: a medium containing 50 ng/ml NGF (NGF); a medium without NGF but supplemented with 24 nM NGF neutralizing antibody (α -NGF); a medium containing the anti-NGF antibody and 20 μ M C_6 -Cer (α -NGF + C_6 -Cer); a medium containing the anti-NGF antibody and 20 μ M C_6 -dihydroceramide (α -NGF + C_6 -DHCer). After 36 h, the mitochondrial membrane potential was evaluated using MitoTracker[®] and nuclei were stained with Hoechst 33258 as described in the Experimental section. Fluorescent micrographs are shown. In the merged images, arrows indicate normal neurons and arrowheads indicate apoptotic neurons.

(reviewed in [40]). The neuronal death programme initiated by NGF deprivation is blocked by the short-chain ceramide homologue C_6 -Cer as well as by endogenous ceramides [11].

In the present study, we analyse whether ceramide is capable of blocking a critical event that occurs during apoptosis, namely the loss of mitochondrial membrane potential. To examine the mitochondrial membrane potential, we used a modification of the MitoTracker[®] Orange staining method [41]. The reduced form of MitoTracker[®] Orange labels active mitochondria since it only fluoresces when it is sequestered and oxidized in mitochondria of actively respiring cells. On the other hand, MitoTracker[®] Green FM becomes fluorescent only when it accumulates in the lipid environment of mitochondria independent of mitochondrial membrane potential and, therefore, all mitochondria will be green-labelled. By merging red and green fluorescence images two populations of neurons can be identified: green neurons that had lost mitochondrial membrane potential and orange neurons bearing intact mitochondria. Hence, mitochondrial membrane potential was analysed in sympathetic neurons deprived of NGF and treated with C_6 -Cer. To confirm that the effect was specific to ceramide, some cultures were given C_6 -DHCer, a structural isomer of ceramide lacking the 4–5 trans double bond required for biological activity [42]. After 36 h, neurons maintained in NGF displayed normal mitochondrial membrane potential, appearing orange in the merged images and contained nuclei evenly stained with Hoechst 33258 (Figure 1). Conversely, most neurons deprived of NGF (α -NGF) had lost mitochondrial membrane potential, appearing green and showing condensed/fragmented nuclei in the merged image (Figure 1). Addition of C_6 -Cer to NGF-deprived neurons caused a significant decrease in the number of neurons that had lost mitochondrial membrane potential as well as the number of condensed/fragmented nuclei (Figure 1). The

relatively inactive isomer C_6 -DHCer was unable to prevent the loss of mitochondrial membrane potential (Figure 1).

The percentage of neurons bearing condensed/fragmented nuclei and neurons that had lost mitochondrial membrane potential was evaluated (Figure 2A). The neuronal population undergoing nuclear fragmentation that was saved by ceramide was also protected from the loss of mitochondrial membrane potential, a late event that occurs subsequent to loss of cytochrome *c* in sympathetic neurons [41]. These experiments indicate that ceramide is a true anti-apoptotic agent for sympathetic neurons.

We next determined the lowest concentration of exogenous C_6 -Cer needed to protect NGF-deprived neurons from apoptosis. In the absence of NGF, 10 μ M C_6 -Cer did not inhibit apoptosis; however, 20 and 30 μ M C_6 -Cer significantly prevented apoptosis (Figure 2B). In contrast, C_6 -DHCer did not inhibit apoptosis at any concentration tested (Figure 2B). On the basis of these results, for further studies we decided to use the lowest concentration of C_6 -Cer (20 μ M) that could inhibit apoptosis.

Ceramide activates PP-1 in live neurons

PP-1 and PP-2A are recognized targets of ceramide *in vitro* [14,16,43]; however, activation of these phosphatases in live cells has not been directly demonstrated. Since direct substrates of serine/threonine PPs such as pRb and Bcl-2 play an important role in the balance between survival and apoptosis, we considered the possibility that ceramide activates PPs to block apoptosis in sympathetic neurons. We examined phosphatase activity in cell extracts of neurons treated with C_6 -Cer or C_6 -DHCer. We found that C_6 -Cer caused an increase in phosphatase activity of 2.5 ± 0.3 -fold (Figure 3A). PP activation was specific to ceramide since neurons treated with the inactive isomer C_6 -DHCer retained

Figure 2 Anti-apoptotic effect of ceramide is dose-dependent

(A) Neurons were treated as indicated in Figure 1. Nuclear fragmentation was evaluated by Hoechst 33258 staining and compared with MitoTracker staining. Results are means $\pm$ S.D. for three experiments. Each experiment consisted of 4 wells per treatment. More than 500 neurons/treatment were counted. (B) Neurons were deprived of NGF and treated with α -NGF and increasing concentrations of C₆-Cer or C₆-DHCer. After 36 h, apoptosis was evaluated by Hoechst 33258. Results are means $\pm$ S.D. for three experiments. Each experiment consisted of 4 wells/treatment. More than 500 neurons were counted per treatment. Statistically significant differences from cultures treated with α -NGF ($P < 0.001$) are indicated by * and were evaluated by the Kruskal–Wallis test with a post hoc comparison test.

unaltered phosphatase activity (Figure 3A). This experiment demonstrates for the first time that C₆-Cer activates PPs in live sympathetic neurons.

To determine whether the ceramide-activated phosphatase belonged to the PP-2A or PP-1 class, we tested the effect of phosphatase inhibitors added to neuronal lysates *in vitro*. We used OA, tautomycin and I-2. At 2 nM concentration OA added *in vitro* selectively inhibits PP-2A; however, at this concentration, OA was unable to block the increase in phosphatase activity induced by ceramide and caused a slight decrease in phosphatase activity in lysates prepared from untreated neurons (Figure 3B). Tautomycin, an equally potent PP-1 and PP-2A inhibitor [45], on the other hand, significantly decreased phosphatase activity even at concentrations lower than 1 nM. I-2, an endogenous and very specific inhibitor of PP-1 [44,45], completely abolished ceramide-induced phosphatase activation and inhibited approx. 75% of PP activity in lysates from both untreated and ceramide-treated

Figure 3 C₆-Cer increases PP-1 activity in sympathetic neurons

(A) Neurons were treated with 20 μ M C₆-Cer or 20 μ M C₆-DHCer for 12 h. Phosphatase activity in cell lysates was determined using the phosphorylase a phosphatase assay. Results represent means $\pm$ S.D. from a representative experiment for samples processed in quadruplicate. The experiment was repeated three times with similar results. Statistically significant differences from untreated cultures are indicated by ** ($P < 0.001$) and were evaluated by the Kruskal–Wallis test with a post hoc comparison test. (B) Effect of PP inhibitors *in vitro*. Neurons (7-day old) were treated with 20 μ M C₆-Cer for 12 h. Cellular extracts were prepared and the following phosphatase inhibitors were added *in vitro* for 30 min immediately before the determination of enzymic activity: no inhibitors (black bars), 2 nM OA (grey bars), 20 nM tautomycin (hatched bars), 200 nM I-2 (white bars) and 2 nM OA + 200 nM I-2 (hatched bars). Results are the means $\pm$ S.D. from three independent experiments for samples processed in quadruplicate. *, Statistically significant differences versus respective groups without inhibitors ($P < 0.001$) as evaluated by the Kruskal–Wallis test with a post hoc comparison test. (C) Effect of PP inhibitors in live neurons. Neurons were incubated for 12 h with or without 20 μ M C₆-Cer in the presence of 10 nM OA (grey bars), 50 nM tautomycin (hatched bars) or 30 μ M PA (white bars). Phosphatase activity determined as in (A) is presented as a percentage of the activity in lysates from neurons incubated in the absence of ceramide and inhibitors. Results are the means $\pm$ S.D. from three independent experiments for samples processed in triplicate. Statistically significant differences from cultures not treated with inhibitor are represented by * ($P < 0.05$) and ** ($P < 0.001$); ***, statistically significant differences from cultures given ceramide alone ($P < 0.001$), evaluated by the Kruskal–Wallis test with a post hoc comparison. Inset (top left): different concentrations (0–100 nM) of tautomycin were given to neurons in the absence and presence of ceramide. Phosphatase activity was determined after a 12 h treatment. Results refer to untreated neurons in the absence of ceramide. Inset (top right): different concentrations (0–50 μ M) of PA were tested for 12 h in neurons in the absence and presence of ceramide. Phosphatase activity was determined as for tautomycin.

Table 1 Effects of PA and its metabolites on phosphatase activity

Sympathetic neurons were incubated for 12 h in the presence or absence of 20 μ M C₆-Cer and without or with PA, PLD (phospholipase D), lysoPA or C₆-dioctanoylglycerol at the indicated concentrations. Results are the means $\pm$ S.D. for two independent experiments for samples processed in triplicate. Statistically significant differences versus respective groups without inhibitors (** $P < 0.001$) as evaluated by the *t* test.

Treatment	Phosphatase activity [$\times 10^6$ units $\cdot$ (mg of protein) ⁻¹]	
	No ceramide	+20 μ M ceramide
None	2.40 $\pm$ 0.30	4.72 $\pm$ 0.44
30 mM PA	0.55 $\pm$ 0.12**	0.69 $\pm$ 0.26**
1 unit/ml PLD	1.93 $\pm$ 0.45	4.16 $\pm$ 0.34
10 units/ml PLD	0.80 $\pm$ 0.42**	0.81 $\pm$ 0.50**
30 mM LPA	2.29 $\pm$ 0.28	4.81 $\pm$ 0.35
30 mM diC ₈ -glycerol	2.17 $\pm$ 0.16	4.22 $\pm$ 0.18

neurons. This experiment indicates that the phosphatase activity activated by ceramide is quite probably PP-1. The combination of I-2 and OA *in vitro* produced a complete inhibition of neuronal PP activity (Figure 3B), suggesting the existence of a small pool of PP-2A difficult to detect by OA inhibition alone.

We next tested the effect of phosphatase inhibitors given to live neurons so as to confirm the identity of the PPs activated by ceramide. We used OA at concentrations ranging from 10 to 50 nM in an effort to inhibit both PP-1 and PP-2A [16,45]. Tautomycin was used at concentrations from 10 to 100 nM. Since I-2 is not cell-permeant, we chose PA that has been suggested to be a selective inhibitor of PP-1 [16]. As for the *in vitro* experiment, 10 nM OA failed to inhibit the increase in phosphatase activity caused by exogenous C₆-Cer and had a small effect on basal phosphatase activity in untreated neurons (Figure 3C). Unfortunately, we could not achieve inhibition of both forms of phosphatases using OA since it was toxic to neurons at concentrations above 15 nM. Tautomycin, on the other hand, was well tolerated by neurons at concentrations up to 100 nM. At the lowest concentration (50 nM) effective *in vivo* (see inset of Figure 3C) tautomycin eliminated almost all phosphatase activity. PA, on the other hand, was effective at concentrations above 30 μ M (inset to Figure 3C), causing a reduction in C₆-Cer-induced phosphatase activity to approx. 30% of the activity without ceramide and without inhibitor (Figure 3C). We excluded the possibility that the effect of PA was due to interference with C₆-Cer uptake by measuring the mass of ceramide in neurons given C₆-Cer in the absence and presence of PA. PA did not significantly change the mass of C₆-Cer (245 $\pm$ 35 pmol/nmol phospholipid in the absence of PA versus 230 $\pm$ 28 nmol/nmol phospholipid in the presence of PA), neither did it change the mass of long-chain ceramides generated endogenously (180 $\pm$ 25 pmol/nmol phospholipid in the absence of PA versus 198 $\pm$ 31 nmol/nmol phospholipid in the presence of PA). In addition, the basal phosphatase activity detected in untreated neurons was decreased by PA, indicating that the effect of PA was independent of C₆-Cer and directed to the PP. As an alternative to adding exogenous PA to the neurons, we treated the neurons with phospholipase D to generate PA within the plasma membrane. This procedure inhibited phosphatase activity to an extent similar to that obtained with PA (Table 1). In addition, the PA metabolites LPA (up to 30 μ M) and C₆-dioctanoylglycerol (30 μ M), a cell-permeable homologue of DAG, did not inhibit phosphatase activity (Table 1). Thus our findings confirm that PA is an active inhibitor of serine/threonine phosphatase in live sympathetic neurons.

Considering the high specificity of I-2 and the close correlation of the results using PA for PP inhibition in live neurons, these

Figure 4 Activation of PP-1 is required for the anti-apoptotic effect of C₆-Cer

Neurons were deprived of NGF in the absence or presence of 20 μ M C₆-Cer. Some neurons received 10 nM OA (grey bars), 50 nM tautomycin (hatched bars) or 30 μ M PA (white bars). Control cultures received 50 ng/ml NGF without or with the corresponding inhibitor. After 36 h, apoptosis was evaluated using MitoTracker[®] as indicated in the Experimental section. Results are means $\pm$ S.D. for three experiments. Each experiment consisted of 4–5 wells per group and > 1000 neurons/treatment were counted. Statistically significant differences within each group of treatments are indicated by **($P < 0.001$) and were evaluated by the Kruskal–Wallis test with a post hoc comparison.

experiments strongly suggest that ceramide activates PP-1 in sympathetic neurons.

Activation of PP-1 is required for the anti-apoptotic effect of C₆-Cer

To determine if PP-1 activation was required for ceramide to act as an anti-apoptotic agent in sympathetic neurons, we inhibited PP-1 with PA and examined the effect on ceramide-induced survival of neurons deprived of NGF. C₆-Cer was unable to protect the neurons from apoptosis when given together with PA (Figure 4). Similarly, complete inhibition of phosphatase activity by tautomycin reversed the anti-apoptotic effect of C₆-Cer. Conversely, OA (10 nM) did not affect ceramide survival. Importantly, PA and tautomycin did not cause significant apoptosis of neurons maintained with NGF, eliminating the possibility that the increased apoptosis observed in the group treated with ceramide and either of the inhibitors was due to an effect of PA (or its metabolites) or tautomycin independent of inhibition of phosphatases. Hence, our results indicate that activation of PPs, quite probably PP-1, is necessary for the anti-apoptotic effect of C₆-Cer and suggest an involvement of PPs in neuronal survival.

PP activity is decreased upon NGF deprivation

NGF deprivation causes significant changes within sympathetic neurons. Amongst these changes, activation of protein kinases and protein phosphorylation has been well characterized [35,46]. The contribution of a change in PP activity has been poorly investigated. Since our results demonstrated that increased phosphatase activity leads to inhibition of apoptosis, we considered the possibility that, in the absence of NGF (NGF deprivation), phosphatases would be inhibited. We examined the activity of PPs in sympathetic neurons after NGF withdrawal. Neurons were deprived of NGF for various times in the absence or presence of C₆-Cer and phosphatase activity was measured as above. NGF deprivation in the absence of ceramide caused a rapid and progressive decrease in phosphatase activity (Figure 5). In the presence of ceramide, no significant decrease in phosphatase activity

Figure 5 PP activity decreases during NGF deprivation

Neurons were deprived of NGF for various times in the absence (○) or presence (●) of C₆-Cer. At the end of the treatment, neurons were harvested and phosphatase activity was determined in neuronal extracts using the phosphorylase *a* assay. The Figure depicts means $\pm$ S.D. for three experiments with samples processed in quadruplicate. Results are expressed as a percentage of phosphatase activity from neurons maintained in NGF. Statistically significant values for neurons maintained in NGF (* $P < 0.05$ and ** $P < 0.001$) were evaluated by the Kruskal–Wallis test with a post hoc comparison test.

was observed and, instead, a transient increase in phosphatase activity was evident. In neurons deprived of NGF but treated with ceramide, two opposing processes take place. On one side, ceramide activates PPs and, on the other, NGF deprivation leads to inhibition of PPs. The phosphatase activity measured in the presence of ceramide represents a balance between these two opposing effects. Therefore the increase in activity found at early times might indicate that the activation by ceramide occurs faster than the inhibition caused by lack of growth factor support, which is significant only at 2 h after NGF deprivation. We did not extend our experiment past 18 h since protein degradation becomes important at that time, making the results more difficult to interpret. The residual phosphatase activity detected in neurons deprived of NGF in the absence of ceramide was inhibited *in vitro* by I-2 (> 85%) and completely blocked by a combination of I-2 and OA (results not shown), suggesting that NGF deprivation equally affected PP-1 and the small pool of PP-2A. This experiment indicates that NGF deprivation causes a reduction in PP activity in sympathetic neurons.

pRb is hyperphosphorylated during NGF deprivation

In view of our findings on PP-1 inhibition on NGF deprivation and since pRb is a direct target of PP-1 [24], we decided to examine whether pRb phosphorylation was similarly affected by NGF withdrawal. It is known that members of the family of CDKs, the kinases responsible for pRb phosphorylation *in vivo*, are altered during NGF deprivation [31,34,35,47]. It has also been suggested that unregulated hyperphosphorylation of pRb is detrimental to cell survival in neurons and non-neuronal cells [48]. Nevertheless, the phosphorylation status of pRb has not been determined in the paradigm of apoptosis induced by NGF deprivation in sympathetic neurons. To study the phosphorylation of pRb, sympathetic neurons were deprived of NGF for various times and pRb phosphorylation was examined by immunoblot analysis. We utilized an anti-pRb antibody generated against the spacer region of the pRb protein, since this antibody recognizes pRb independent of phosphorylation status. To confirm the identity of detected bands, lysate Molt-4 cells overexpressing pRb were used as a pRb positive control (results not shown). We found that NGF deprivation

Figure 6 Hyperphosphorylation of pRb is blocked by ceramide

Neurons were deprived of NGF for different times up to 18 h. Cells were harvested and pRb was examined by SDS/PAGE and immunoblot analysis. Control cultures received 50 ng/ml NGF throughout the experiment. Tubulin immunoblotting was used to control protein loading. (A) Neurons were deprived of NGF and given α -NGF. (B) Neurons were given 20 μ M C₆-Cer together with α -NGF. (C) Neurons were given α -NGF, 20 μ M C₆-Cer and 30 μ M PA. (D) Neurons were given α -NGF, 20 μ M C₆-Cer and 10 nM OA. The Figure is a representative blot. The experiment was repeated three times with similar results.

causes hyperphosphorylation of pRb demonstrated by the appearance of a new protein band at approx. 115 kDa (Figure 6A). Only in neurons deprived of NGF was the hyperphosphorylated protein band (ppRb) present. Therefore this experiment clearly demonstrates that NGF deprivation leads to pRb hyperphosphorylation.

Ceramide inhibits pRb hyperphosphorylation

Our finding that a consequence of NGF deprivation is the hyperphosphorylation of pRb provides further evidence that, after NGF withdrawal, sympathetic neurons attempt to re-enter the cell cycle, in turn leading to apoptosis. In non-neuronal cells, ceramide causes cell cycle arrest in G₀/G₁ through a pRb-dependent mechanism [28] and PP-1 has been recognized as the ceramide-activated PP involved in pRb dephosphorylation [16]. Therefore we considered the possibility that in sympathetic neurons ceramide could block pRb hyperphosphorylation to inhibit apoptosis. We first investigated if ceramide was able to decrease pRb phosphorylation during NGF deprivation. We examined the phosphorylation status of pRb on treatment of NGF-deprived sympathetic neurons with ceramide and found that C₆-Cer prevented hyperphosphorylation of pRb (Figure 6B). The effect of ceramide is rapid since ceramide was also able to prevent pRb hyperphosphorylation when given to neurons after 12 h of NGF deprivation (results not shown). This is in agreement with our previous observations that, to prevent apoptosis in the majority of neurons, ceramide could be provided to these neurons up to 12–15 h after NGF deprivation [11]. Since we have shown that ceramide activates PP-1, this experiment suggests that PPs activated by ceramides might also be involved in pRb dephosphorylation.

To demonstrate that the effect of ceramides on pRb was due to PP-1 activation, we used PA, which we showed was an effective inhibitor of PP-1 activation in cultured sympathetic neurons. As negative control we also treated some neurons with OA. In the presence of PA, C₆-Cer was unable to block the hyperphosphorylation of pRb that occurs on NGF deprivation (Figure 6C). Conversely, OA did not reverse the effect of ceramide (Figure 6D). This result implies that PP-1 plays an active role in the phosphorylation status of pRb in sympathetic neurons and that the prevention of

pRb hyperphosphorylation by C₆-Cer is probably linked to PP-1 activation. Taken together, these experiments suggest that C₆-Cer is able to induce pRb dephosphorylation by PP-1 activation and could block the neurons that attempt to re-enter the cell cycle.

DISCUSSION

The aim of the present study was to examine the mechanism(s) involved in the anti-apoptotic effect of ceramide in NGF-deprived sympathetic neurons. We focused on one family of enzymes that are modulated by ceramide, the serine/threonine directed PPs. Very elegant work by Hannun's group demonstrated that ceramide activates both PP-1 and PP2A *in vitro* [13–16]. We report here for the first time that ceramide activates PP-1 in live cells, underscoring the significance of PPs as physiological targets of ceramide. Our results indicate that, in the activation of a PP by sympathetic neurons, quite probably PP-1 is required for the anti-apoptotic effect of ceramide. In addition, we present evidence for two novel consequences of NGF deprivation in sympathetic neurons: the inhibition of PP-1 and the hyperphosphorylation of pRb. Our studies have important implications for the involvement of PPs in neuronal apoptosis.

Ceramide is a true anti-apoptotic agent in sympathetic neurons

Ceramide blocks apoptosis caused by NGF deprivation [49–51] but the mechanism(s) involved are not completely identified. Recent observations from our laboratory indicated that ceramide blocks caspase-3 activation and nuclear changes that accompany NGF deprivation [11]. Here we report that ceramide also inhibits the loss of mitochondrial membrane potential, a key process that occurs subsequent to cytochrome *c* release and which defines a second commitment point in sympathetic neurons [41]. Interestingly, sympathetic neurons can be rescued from death after they have lost cytochrome *c* but not after they have lost mitochondrial membrane potential [41]. The finding that ceramide blocks the loss of mitochondrial membrane potential, together with the inhibition of nuclear fragmentation and prevention of caspase activation further establishes an anti-apoptotic role for ceramide in NGF-deprived sympathetic neurons. However, our results are not sufficient to determine whether the inhibition of loss of mitochondrial membrane potential is due to a direct effect of ceramide at the level of the mitochondrial membrane or whether ceramide blocks upstream apoptotic signals.

Ceramide activates a PP-1 family PP

To date there is only a short list of direct targets of ceramide that includes, among others, ceramide-activated PPs PP-1 and PP-2A [12]. The catalytic subunit of PP-1 (PP-1c) is regulated by lipid mediators such as ceramide and PA *in vitro* [15,16]. Here, we demonstrate that short-chain ceramide activates PPs in live sympathetic neurons. The degree of activation we found is consistent with a similar activation of PP catalytic subunits reported *in vitro* [16].

Low concentrations of OA failed to block ceramide-activated phosphatase in live neurons as well as in neuronal lysates *in vitro*. We initially used OA to inhibit PP-1 and PP-2A [52]; however, this compound proved unusually toxic to sympathetic neurons. Fortunately, tautomycin did not exhibit the same toxicity and could be used to inhibit both phosphatases *in vitro* and in live neurons. The PP-1 inhibitor I-2 completely blocked the increase in phosphatase activity induced by ceramide in lysates prepared from neurons treated with a short-chain ceramide. Moreover, I-2 blocked more than approx. 70 % of the phosphatase activity in untreated neu-

rons. Similarly, PA induced a significant reduction in basal and C₆-Cer-activated phosphatase activity in live neurons. Although PA is a bioactive lipid, in our system, PA and its metabolites LPA and DAG did not have any significant effect on neuronal survival, which made it possible to use PA as a PP-1c inhibitor for our studies. It is possible, however, that in other cell systems PA might have cellular effects unrelated to PP-1c inhibition. Taken together, our findings indicate that most of the basal phosphatase activity detected in sympathetic neurons belongs to the PP-1 class of phosphatases and that ceramide activates a PP-1-like phosphatase. On the basis of data using toxin inhibitors, we cannot completely rule out a role for PP-2A; however, it is highly unlikely since phosphatase activation induced by ceramide was completely abolished by I-2. To our knowledge, our work represents the first direct evidence for PP-1 activation by ceramide in live cells.

We examined the importance of PP-1 activation for the anti-apoptotic effect of ceramide. Ceramide was unable to prevent neuronal apoptosis when provided to neurons with the phosphatase inhibitors PA or tautomycin. Therefore the anti-apoptotic action of C₆-Cer depends on its ability to activate a PP, probably PP-1c.

pRb as a substrate of PP-1 in sympathetic neuron apoptosis

pRb is a good candidate to explain the inhibition of apoptosis according to the 'cell cycle' theory [29]. Cell cycle progression requires hyperphosphorylation of pRb [53]. However, in post-mitotic cells such as neurons, the presence of hyperphosphorylated pRb could lead to an inappropriate attempt to enter the cell cycle, thus activating apoptotic mechanisms [29]. Evidence for such an attempt to re-enter the cell cycle includes the stimulation of CDKs during neuronal apoptosis [32] and the increased expression of cyclin D1, a binding partner for the pRb-directed CDKs [30]. Importantly, the phosphorylation state of pRb results from the balance between phosphorylation by CDKs and dephosphorylation by PP-1 [24,53]. Accordingly, we found that after NGF withdrawal there is a significant decrease in serine/threonine PP activity. Previous work in neuronally differentiated PC12 cells indicated that, during NGF deprivation-induced apoptosis, there is a significant decrease in PP-2A and PP-2B activities [54]. Interestingly, changes in PP-1 activity were not observed in PC12 cells. The reasons for the differences between sympathetic neurons and PC12 cells are unknown. Most of the serine/threonine PP activity that we detected in sympathetic neurons was of type-1 and a smaller pool of PP-2A activity was found, suggesting that some of the cellular functions performed by PP-2A in certain cell types are performed by PP-1 in sympathetic neurons.

Our experiments demonstrated that, in sympathetic neurons maintained with NGF, pRb is in a hypophosphorylated state, which corresponds to growth arrest/terminal differentiation [53] and that NGF withdrawal leads to hyperphosphorylation of pRb. Previous studies on NGF deprivation in sympathetic neurons focused on CDK activation after NGF withdrawal; however, to our knowledge, our work is the first to assess directly the phosphorylation status of pRb in this neuronal class.

We considered that pRb hyperphosphorylation could be a pro-apoptotic signal. Consequently, C₆-Cer prevented hyperphosphorylation of pRb in NGF-deprived sympathetic neurons by a mechanism that probably involves PP-1 activation.

Dephosphorylation of pRb by ceramide-dependent mechanisms has been indirectly demonstrated in non-neuronal Jurkat cells [55]. In addition, previous findings by Hannun and co-workers [16] in Molt-4 cells indicated that dephosphorylation of pRb in response to C₆-Cer treatment can be blocked with inhibitors of PP-1. Our studies, however, provide the first direct evidence of PP

modulation (activation by ceramide, inhibition by PA) in live cells. This is particularly important, since no other activator of PP-1 has been identified. Site-directed mutagenesis and molecular modelling studies with C₆ ceramides and derivatives thereof indicate that ceramides interact directly with the catalytic subunit of PP-1 (PP-1c) (K. Perreault, H. A. Luu, E. I. Posse de Chaves and C. F. B. Holmes, unpublished work). The site of interaction has been localized to the vicinity of a hydrophobic groove that also binds lipophilic toxins such as the tumour promoter OA [38].

Although we have demonstrated that pRb is hyperphosphorylated on NGF deprivation and that ceramide treatment reverses this effect, we do not have direct evidence that supports pRb dephosphorylation as an anti-apoptotic event.

In conclusion, our results not only suggest a mechanism for inhibition of neuronal apoptosis by ceramides through PP-1 activation, but also suggest that PPs are pivotal players in neuronal apoptosis and that their role deserves additional investigation.

We appreciate the excellent technical assistance of Mrs B. Zielnik-Drabick and Mrs V. Mancinelli. These studies were supported by a grant from the Canadian Institutes of Health Research and an establishment grant from the Alberta Heritage Foundation for Medical Research (AHFMR) to E.I.P.deC. K.R.P. is supported by a studentship from the AHFMR. E.I.P.deC. is a Medical Scholar and C.F.B.H. is a Medical Scientist of the AHFMR.

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Received 2 June 2004/3 September 2004; accepted 10 September 2004

Published as BJ Immediate Publication 10 September 2004, DOI 10.1042/BJ20040929