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Tumor Necrosis Factor-α Induces Apoptosis in Immortalized Hypothalamic Neurons: Involvement of Ceramide-Generating Pathways

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ABSTRACT

To investigate possible effects that may contribute, together with a direct action on neurohormone secretion, to the impairment of gonadal axis function during inflammation, we evaluated the effect of TNFα on the growth and viability of GT1–7 hypothalamic neurons and the intracellular transduction pathways involved in these effects. TNFα caused a reduction of cell number and an induction of apoptotic death. These effects were mimicked by cell-permeable analogs of ceramide and by neutral or acidic sphingomyelinase. Exposure to acidic sphingomyelinase induced a persistent (up to 48 h) reduction of cell growth and apoptosis, whereas the effect of neutral sphingomyelinase was time limited. The involvement of acidic sphingomyelinase in TNFα action was demonstrated by the partial prevention of ceramide generation, apoptosis, and reduced cell growth by the inhibitor of the acidic sphingomyelinase-generating pathway, D609, whereas the involvement of ceramide was proved by complete prevention of TNFα-induced effects by treatment with okadaic acid at concentrations inhibiting ceramide-dependent protein phosphatase. The present data indicate that TNFα, through activation of ceramide-generating pathways, is able to affect GT1–7 cell viability, suggesting an additional effect that may contribute to the global action of this cytokine on neuroendocrine activities. (*Endocrinology* **140:** 4841–4849, 1999)

TUMOR NECROSIS factor-α (TNFα) as well as other cytokines are known to affect neuroendocrine secretory activities (1, 2); this phenomenon may be relevant in the alterations of endocrine parameters, including disruption of reproductive function, that accompany infectious states. The hypothalamus-pituitary-gonadal axis, in fact, exhibits marked sensitivity to the effects of various cytokines (3, 4), and TNFα can affect the activity of this axis exerting its action at different levels. Thus, TNFα mediates lipopolysaccharide-induced suppression of the GnRH pulse generator activity (5) and reduces basal and stimulated LH release by acting either at central level (6) or at the pituitary (7). In addition, TNFα is able to stimulate apoptotic death in ovarian follicles, suggesting a causative role for this cytokine in the genesis of follicular atresia (8).

Besides a direct effect on neuroendocrine secretory activity, TNFα may also act by regulating neuronal viability at the hypothalamus. In fact, in other cellular systems, TNFα is known to act as a trophic, toxic, or differentiating agent (9). At the central nervous system, the responses to TNFα exhibit large variability (10), and neurotoxic (11–17) as well as neuroprotective (18–20) effects of this cytokine in neuronal cultures have been reported. This appears particularly intriguing, as definition of the action of TNFα on hypothalamic neuronal viability could provide a significant contribution to the understanding of the global action of this cytokine at the hypothalamus and of the possible role of TNFα at the intersection between the neuroendocrine and the immune systems. The relevance of these phenomena may be related not only to the pathological events that lead eventually to the impairment of neuroendocrine activity, but also to physiological conditions that control development and maturation of selected central nervous system areas, including the hypothalamus. In this respect, the dual action exerted by TNFα may be critical, as it may combine a general neurotropic effect with the induction of programmed cell death that takes place during the course of normal development. The complexity of the response to TNFα may be partly related to the activation of two distinct receptors that mediate TNFα signaling: TNFR1 (p55), whose activation is known to generate intracellular signals that are responsible also for cell death, and TNFR2 (p75), whose role has not been completely characterized, but which probably mediates proliferation and survival events (21). Activation of TNFR1 leads to the hydrolysis of sphingomyelin and the generation of ceramide, an intracellular second messenger involved in survival and death phenomena (22, 23). Focusing our attention on this particular TNFα receptor subtype, we have studied the effect of TNFα in a hypothalamic cell population. The availability of the GT1–7 cell line (24) allowed us to study the action of TNFα on cell viability directly in GnRH-secreting neurons. The choice of this homogeneous cell population was based on the double opportunity to use an experimental model that allowed the evaluation of cell viability (25, 26) and the investigation of intracellular mechanisms. Hence, in GT1–7 cells we have studied the action of TNFα on neuronal viability and

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proliferation, TNFα-activated transducing mechanisms, and, in parallel, the effects produced by intracellular mediators that may be responsible for the transduction of TNFα signaling in GnRH-producing neurons.

Materials and Methods

Cell culture

GT1–7 cells were maintained under sterile conditions in DMEM supplemented with 10% FCS and antibiotics in a temperature-controlled and humidified atmosphere of 5% CO_2. All cell culture materials and plasticware were obtained from Life Technologies, Inc. (Milan, Italy).

Cell counting

GT1–7 cells were plated into 24-well multiwell plates in FCS-containing DMEM for 24 h and then maintained in the presence of the tested drugs for 3–12 h (short term studies) or 24–96 h (long term studies). Cells were then harvested with a 0.01% trypsin solution and counted with the aid of a hemocytometer.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT cell proliferation assay is based on the conversion of a diphenyltetrazolium salt into blue formazan detectable in an enzyme-linked immunosorbent assay plate reader. After exposure to various treatments, GT1–7 cells were incubated with MTT (0.9 mg/ml, final concentration) for 2 h at 37 C and then solubilized with isopropanolol containing 0.1 N HCl. Formazan production was evaluated in a plate reader with a 560-nm test wavelength and a 690-nm reference wavelength.

[^{3}H]Thymidine incorporation

GT1–7 cells were plated into 24-well multiwell plates and exposed to different agents for various lengths of time. [^{3}H]Methylthymidine (Amersham Pharmacia Biotech, Milan, Italy; SA, 20 Ci/mmol; 1 μCi/ml) was added during the last 6 h of incubation. Cells were then extracted with 1 N $HClO_4$, and the incorporated radioactivity was determined by scintillation counting.

Immunocytochemistry

GT1–7 cells were stained for TNFR1 and TNFR2 using rabbit polyclonal antibodies specifically recognizing each subtype. Cells were fixed with 4% paraformaldehyde and exposed to the primary antibody (Sanbio, Uden, The Netherlands; 1 μg/ml) for 1 h at room temperature before exposure to antirabbit IgG for 1 h. After reaction with avidin-biotin-horseradish peroxidase (Elite ABC Vectastain, Vector Laboratories, Inc., Burlingame, CA), staining was developed by exposure to 0.05% diaminobenzidine-0.01% H_2O_2.

Flow cytometry

For specific detection of TNFR1/R2, growing GT1–7 cells were fixed with 4% paraformaldehyde for 30 min, repeatedly washed, and subsequently treated with anti-TNFR1/R2 (5 μg/ml·30 min) and fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (1:100 for 30 min). All incubations were carried out at 4 C. Controls included omission of the primary antibody and substitution with nonimmune serum. Samples were analyzed with an ELITE flow cytometer (Coulter Electronics, Hialeah, FL) with an excitation wavelength of 488 nm and monitoring of fluorescence at 525 nm. At least 10,000 forward and side scatter gated events/sample were evaluated.

Ceramide-1-phosphate measurement

GT1–7 cells were cultured in 35-mm dishes and exposed to TNFα for the time indicated. Lipids were extracted and subjected to mild alkaline hydrolysis, and ceramide levels were measured using a modified diacylglycerol kinase assay (27) with a commercially available kit (Amersham Pharmacia Biotech).

Evaluation of apoptotic death

Quantitative analysis of DNA fragmentation was performed with the cell death detection enzyme-linked immunosorbent assay based on the photometric sandwich immunoassay of cytoplasmic histone-associated DNA fragments (Roche Molecular Biochemicals, Mannheim, Germany).

For cytofluorometric analysis, after fixation with 70% ethanol overnight at −20 C, cells were incubated with ribonuclease (100 μg/ml) for 2 h at 37 C and stained with the nuclear dye propidium iodide (final concentration, 50 μg/ml). Analysis was carried out on a Coulter ELITE flow cytometer and was restricted to cells with diploid and hypodiploid DNA contents.

Laddered patterns of DNA fragmentation were resolved by conventional gel electrophoresis on 1.5% agarose gel impregnated with ethidium bromide and visualized by UV illumination.

Drugs

Unless otherwise specified, all chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). D-Erythro-sphingosine *N*-octanoyl (C8-ceramide; Calbiochem, La Jolla, CA) was dissolved in dimethylsulfoxide and stored at −80 C. Okadaic acid (Calbiochem, La Jolla, CA) solubilized in water was stored at −20 C. Neutral sphingomyelinase (N SMase) from *Staphylococcus aureus* (Sigma Chemical Co.) was provided in a solution containing 50% glycerol and 0.25 M phosphate buffer, pH 7.5, and stored at 4 C. Acidic sphingomyelinase from human placenta (Sigma Chemical Co.) was provided in 50% glycerol, 25 mM potassium phosphate, 0.1% Triton X-100, and 0.05 mM phenylmethylsulfonylfluoride, pH 4.5, and stored at −20 C. Human TNFα was obtained from PeproTech EC Ltd. (London, UK).

Results

The expression of the low and high affinity TNFα receptors, TNFR1 and TNFR2, in GT1–7 cells was analyzed by flow cytometry (Fig. 1), immunocytochemistry (Fig. 1), and Western blot analysis (data not shown) using antibodies specifically recognizing each receptor subtype. In immunohistochemical studies, staining was marked and diffuse within the cell body, but was also present in neuritic extensions. Interestingly, the expression was more pronounced for TNFR1 and was selective for a defined cell population, whereas a small percentage of cells did not show positive immunostaining.

Long term (24- to 96-h) exposure of GT1–7 cells to TNFα (20 ng/ml) produced a significant reduction of cell number that was already present after 24 h of incubation and persisted throughout all time points examined (Fig. 2A). Indeed, a marked reduction of cell number was already evident after short term (6- to 12-h) exposure to TNFα, as measured by the MTT proliferation assay (Fig. 2B). The inhibitory effect of TNFα was concentration dependent, significant at 1 ng/ml, and maximal between 10–100 ng/ml (Fig. 2, *inset*).

The reduction of GT1–7 cell number by TNFα is correlated to induction of apoptotic cell death. Characteristic features of apoptosis, such as DNA laddering, in fact appeared after only 6 h of exposure to TNFα and were particularly evident after 24–48 h (Fig. 3A). Induction of apoptosis was confirmed by cytofluorometric analysis; Fig. 3B reveals a pronounced predyploid cell population in TNFα-treated GT1–7 cells stained with the nucleic acid probe, propidium iodide.

It is well established that stimulation of TNFR1 results in activation of the sphingomyelin cycle with ensuing produc-

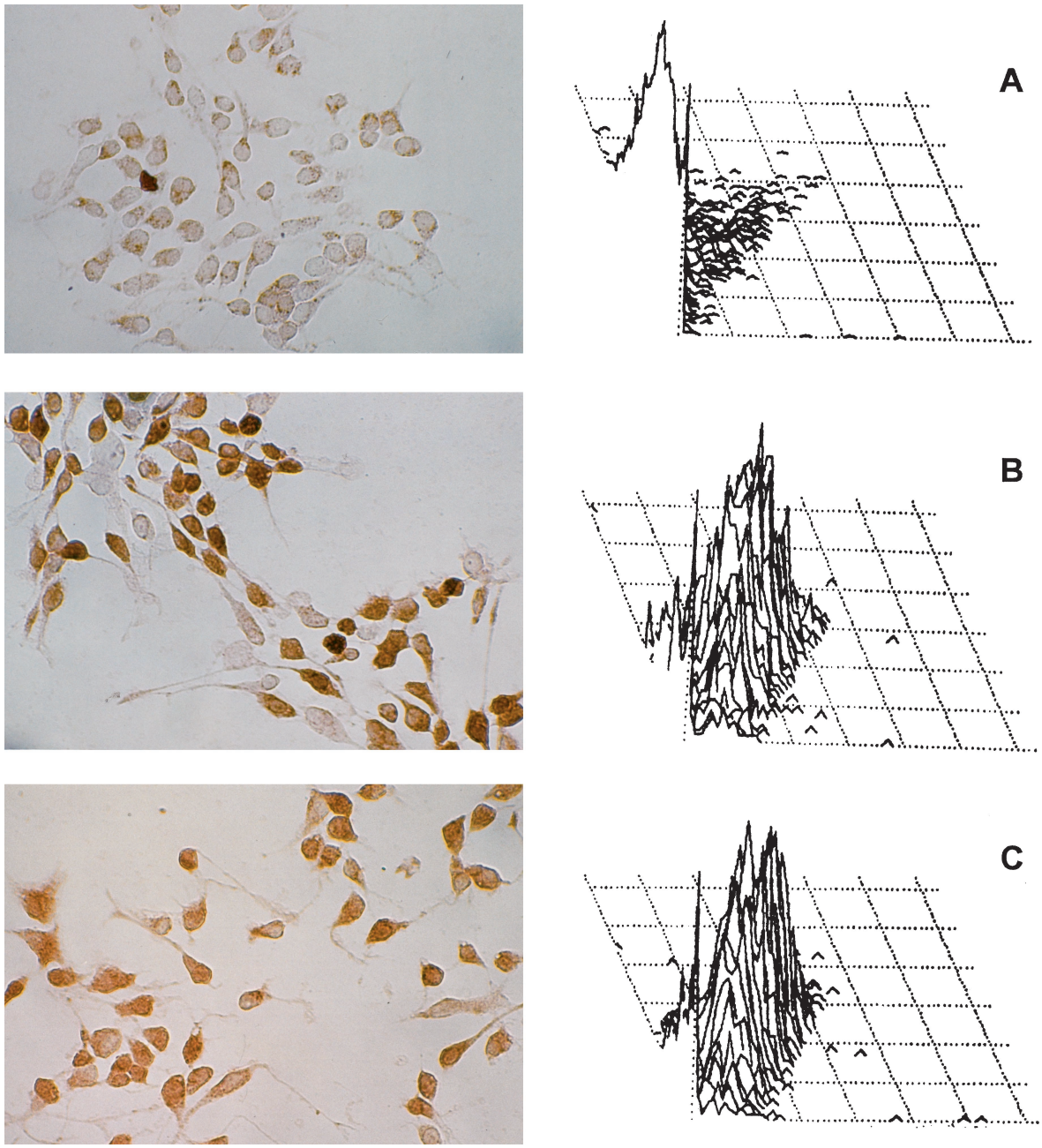

FIG. 1. GT1–7 cells are immunopositive for both TNFα receptors subtypes, TNFR1 (B) and TNFR2 (C). Cells were fixed in 4% paraformaldehyde, incubated with rabbit antimouse-TNFR1 and -TNFR2, and processed for immunocytochemistry (*left panels*) or flow cytometry (*right panels*) as described in *Materials and Methods*. Control rabbit IgG were used to determine nonspecific labeling (A).

tion of ceramide. Accordingly, treatment of GT1–7 cells with TNFα produced a significant stimulation of ceramide formation, as assessed by measurement of the metabolite ceramide-1-phosphate (Fig. 4); this effect exhibited time dependency, being present after 5 min, peaking at 15 min, and remaining elevated up to 60 min.

To establish a functional link between activation of the sphingomyelin cycle and proliferation and viability of hypothalamic neurons, GT1–7 cells were treated with N SMase (200 mU/ml) or the permeable analog of ceramide, C8-ceramide (25 μM). At the same concentration, another cell-permeable analog of ceramide, C2-ceramide, behaved in a very similar manner (not shown). Long term treatment with N SMase and C8-ceramide produced an inhibition of cell proliferation, measured as a reduction of cell number, that was time dependent, significant at 24 h, and more pronounced, particularly for C8-ceramide, after 48 h (Fig. 5A). However, for longer time points (72 and 96 h), the behavior of the two compounds was divergent, as C8-ceramide was still able to maintain a reduced cell number, whereas GT1–7 cells were no longer sensitive to the inhibitory action of N SMase, and their number was significantly increased (Fig. 5A). Accordingly, GT1–7 cells exposed to N SMase very rapidly escaped the inhibitory action of the enzyme and exhibited an in-

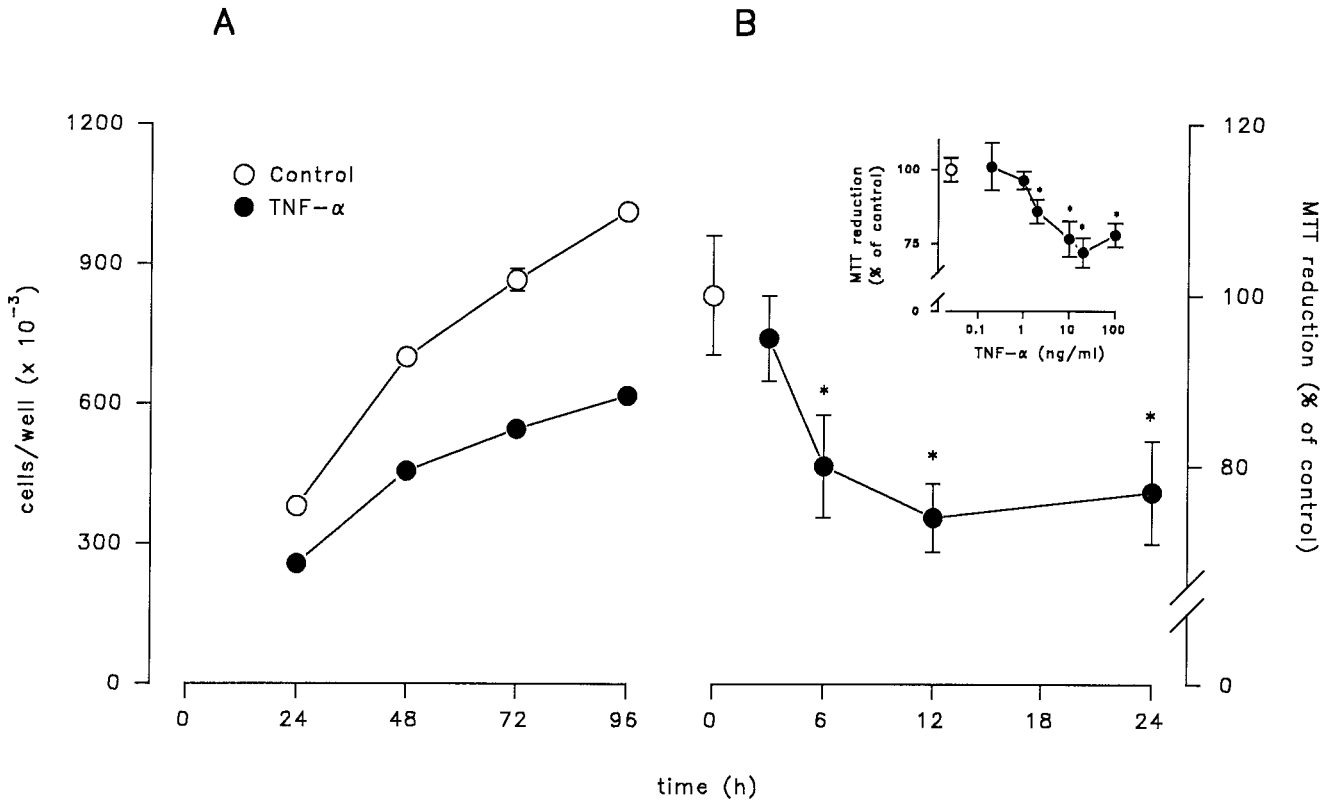

FIG. 2. Time and concentration dependency of the TNFα effect on GT1–7 cell proliferation. Cells plated at low density were exposed to TNFα (20 ng/ml) for the time indicated (single treatment) and either counted with the aid of a hemocytometer (A) or quantitated by the MTT proliferation assay (B). In the *inset*, the concentration-response curve of a 48-h treatment with TNFα is shown. Values are mean ± SE of at least three independent studies. A significant effect of treatment ($P < 0.05$) on GT1–7 cell number (A) was observed at all time points examined (by two-way ANOVA followed by Duncan's multiple range test). *, $P < 0.05$ *vs.* control (by one-way ANOVA followed by Newman-Keuls *t* test for significance).

creased proliferation rate, as shown by enhanced [^{3}H]thymidine incorporation, evident after 48–96 h of incubation (Fig. 5B). The behavior of C8-ceramide was again different, as a reduction of [^{3}H]thymidine incorporation could be observed at all time points examined (Fig. 5B). The lack of effect of long term treatment with N SMase on GT1–7 cell proliferation might be ascribed to rapid metabolism and/or inactivation of the enzyme; in support of this possibility, reexposure of GT1–7 cells to N SMase after 48 h (total exposure time, 96 h) produced a reduction of GT1–7 cell number, comparable to that induced by a single treatment for 48 h (Table 1). The inhibitory action of N SMase was very rapid; the MTT proliferation assay revealed a very early reduction of the number of cells that was already significant after 3 h of incubation and was maximal at 12 h (Fig. 6). This prompt effect is suggestive of induction of cell death; accordingly, short term (6–12 h) treatment with N SMase (200 mU/ml) determined the appearance of clear signs of apoptotic death. These were evaluated by measurement of oligonucleosome formation (Fig. 7A) and analysis of DNA laddering (Fig. 7B). The time-course studies indicated that induction of apoptosis by N SMase was very rapid (6–12 h) and was not present after 48 h (Fig. 7). Conversely, as shown for cell counting, C8-ceramide (25 μM) treatment produced a marked and sustained (up to 48 h) apoptotic effect (Fig. 7B).

TNFα is known to activate an acidic sphingomyelinase (Ac SMase) located in specific intracellular compartments (28). Thus, we analyzed the effect of this enzyme on the proliferation and viability of GT1–7 cells. Treatment with 200 mU/ml Ac SMase induced a time-dependent inhibition of cell number. A significant inhibition, as assessed by the MTT proliferation assay, was present after only 6 h of treatment and progressively increased, reaching a maximum after 48 h (Fig. 8A). This effect was accompanied by the appearance of DNA fragmentation, indicative of apoptotic death. Figure 8B reports a representative DNA laddering observed in GT1–7 cells exposed to Ac SMase for 48 h.

To support the involvement of Ac SMase in the effect of TNFα on GT1–7 cell viability, we used the xanthate D609, an inhibitor of phosphatidylcholine-specific phospholipase C (29), a key enzyme in the activation of Ac SMase (but not N SMase) by TNFα (30). Pretreatment of GT1–7 cells with D609 (5 μg/ml) reduced by about 50% the accumulation of ceramide-1-phosphate induced by a 15-min exposure to 20 ng/ml TNFα (Table 2). Similarly, preincubation of GT1–7 cells with D609 for 1 h before the addition of TNFα for 48 h significantly reduced the inhibitory effect of the cytokine on cell number and partially prevented TNFα-induced apoptosis (Table 2). The latter evaluation was carried out by flow cytometry, which allowed a quantitative analysis of the protective effect.

Recently, a specific cytosolic protein phosphatase has been

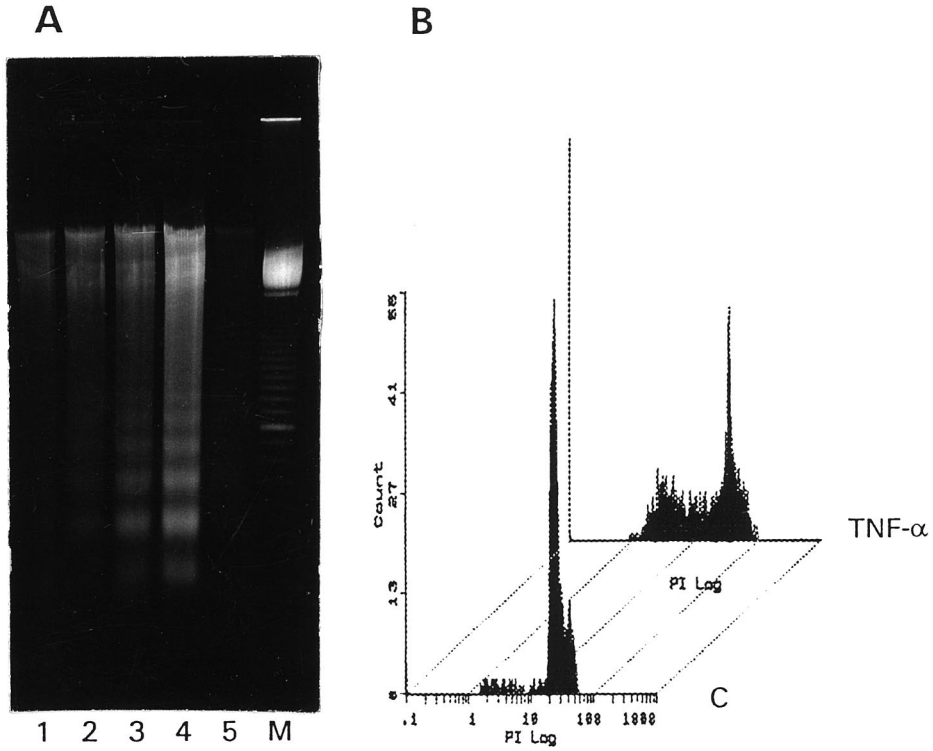

FIG. 3. TNFα induces apoptosis in GT1–7 cells, as assessed by DNA laddering (A) and cytofluorometry (B). In A, DNA was extracted from cells exposed to TNFα for different lengths of time: 1, 3 h; 2, 6 h; 3, 24 h; 4, 48 h; and 5, untreated control at 48 h. M, 100-bp markers. In B, the appearance of a predyploid population after exposure to TNFα (20 ng/ml·48 h) is detected by cytofluorometric analysis.

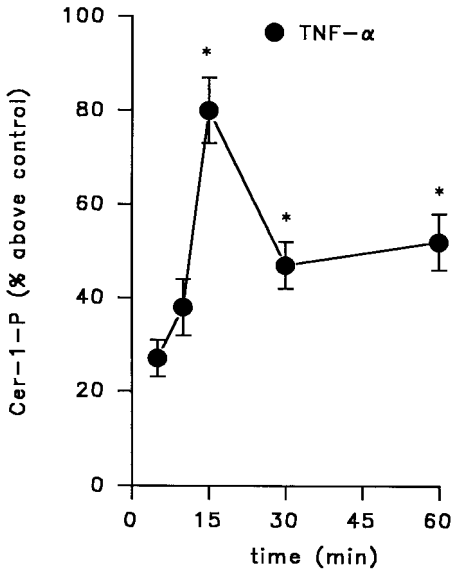

FIG. 4. TNFα stimulates the accumulation of ceramide, as assessed by measurement of ceramide-1-phosphate (Cer-1-P). After exposure to TNFα (20 ng/ml) for the time indicated, lipids were extracted, and ceramide levels were measured using a modified diacylglycerol kinase assay. Data are expressed as the percentage above the control value of untreated samples incubated for the same length of time. Values obtained in untreated cells were consistent with time; variations were less than 10%. Cer-1-P accumulation in control cultures at 5 min was 4478 ± 223 cpm/10^6 cells. Values reported are the mean ± SE of three independent determinations. *, $P < 0.05$ *vs.* untreated control, by Student's *t* test.

suggested as a mediator of ceramide effects (31, 32). To assess the involvement of this pathway in the action of TNFα, GT1–7 cells were preincubated with okadaic acid. This compound is a potent inhibitor of various protein phosphatases (33); however, at low concentrations, it appears to act as a relatively specific inhibitor of ceramide-activated protein phosphatase (31). Okadaic acid, in a range of concentrations shown to inhibit both protein phosphatases 1 and 2 (>5 nM) caused a concentration-dependent reduction of cell number (~32% reduction at 10 nM and >70% reduction at 100 nM). In contrast, smaller concentrations (0.5–1 nM) did not affect cell number or viability, but completely prevented the inhibitory effect of TNFα on cell number and the induction of apoptosis (Table 3), as assessed by cell counting and cytofluorometric analysis, respectively. In addition, to exclude further the involvement of protein phosphatase 1 in okadaic acid action, GT1–7 cells were pretreated with 1 nM tautomycin (a compound that, at this concentration, is known to specifically affect protein phosphatase 1) (34). Under these conditions, the inhibitory effect of TNFα on GT1–7 cell proliferation was not affected (data not shown).

Discussion

TNFα is recognized as one of the key molecules mediating neuroimmune interactions; at the hypothalamus this results in modulation of hormonal secretion (1–3, 5, 6), but a role for this cytokine in hypothalamic neuronal viability can also be hypothesized. The action of TNFα at the central nervous system has been mainly related to its ability to modulate neuronal survival, but very little is known about the intracellular events that mediate cytokine action at this level. In this respect, the immortalized hypothalamic GT1–7 cells represent a useful neuronal *in vitro* model, as they express receptors for TNFα, TNFR1, and TNFR2. TNFα recognizes, in fact, two distinct receptors (21), one of which, the TNFR1 or p55, belongs to the nerve growth factor/TNF receptor family characterized by the presence of a specific intracellular se-

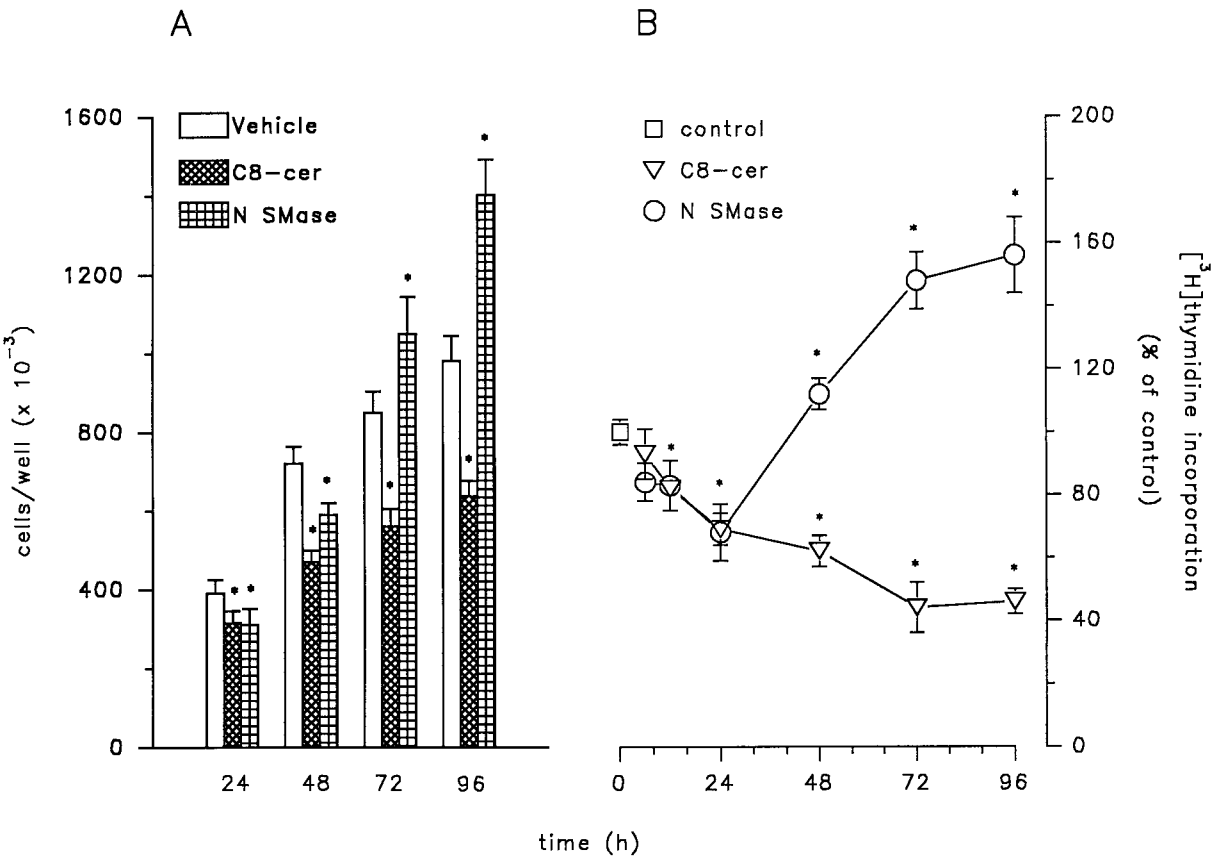

FIG. 5. N SMase and C8-ceramide (C8-cer) differently affect growth and proliferation rates of GT1–7 cells. Cells were exposed to N SMase (200 mU/ml) or C8-cer (25 μM) for the time indicated (single administration) and either counted with a hemocytometer (A) or assessed for [^{3}H]thymidine incorporation (B). *, $P < 0.05$ *vs.* control, by one-way ANOVA followed by Newman-Keuls *t* test for significance.

TABLE 1. Effect of single or repeated long term treatment with N SMase (200 mU/ml) and C8-cer (25 μM) on GT1-7 cell growth

Treatment	No. of cells (% of control)
Control	100 ± 1.8
N SMase (48 h)	81 ± 5.2[a]
N SMase (96 h)	143 ± 4.3[a]
N SMase (48 + 48 h)	78 ± 4.8[a]
C8-cer (48 h)	65 ± 7.1[a]
C8-cer (96 h)	66 ± 4.0[a]
C8-cer (48 + 48 h)	71 ± 3.6[a]

Values are expressed as a percentage of the value in untreated cultures and represent the mean ± SE of three or four independent studies performed in triplicate.

[a] $P < 0.05$, by Student's *t* test.

quence responsible for the transduction of a death signal to the cell (35). Treatment of GT1–7 neurons with TNFα caused rapid and prolonged reduction of cell number, an effect accompanied by induction of apoptotic death. This confirms previous reports showing that TNFα induces apoptosis in neurons (13–15) and is involved in neurodegeneration processes (11, 16, 17, 36), but is in contrast with a large body of evidence that indicates a neuroprotective activity for this cytokine (18–20).

Activation of TNFR1 is linked to stimulation of a neutral and an acidic sphingomyelinase that differ on the basis of their location in distinct cellular compartments, but that are

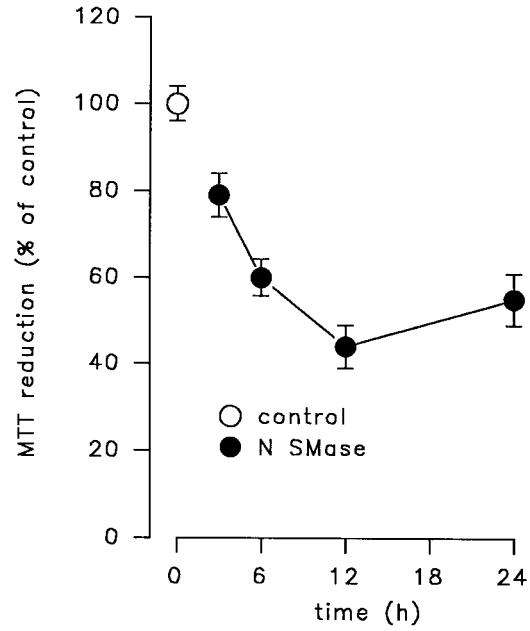

FIG. 6. N SMase very rapidly modifies the proliferation of GT1–7 cells. Cells were exposed to the enzyme (200 mU/ml), and MTT was added during the last 2 h of incubation. Cells were solubilized with acidified isopropanol, and their number was evaluated in a plate reader. A significant ($P < 0.05$) inhibitory effect of N SMase was observed at all time points examined.

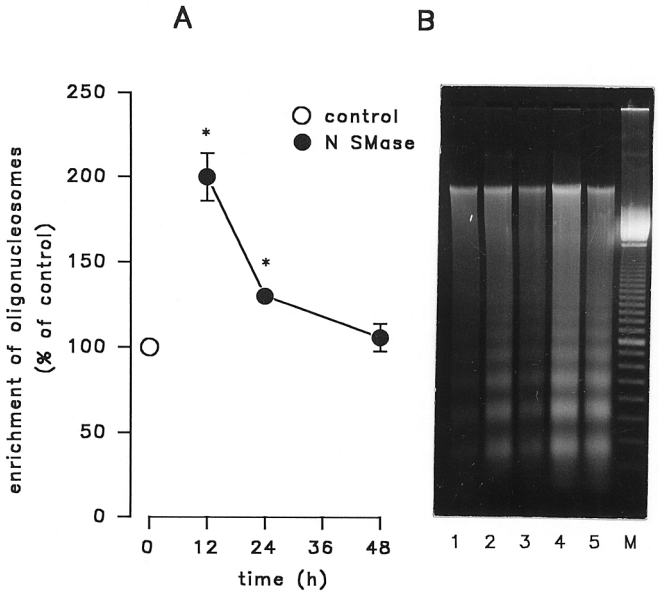

FIG. 7. Different time-course pattern of apoptosis induction by N SMase and C8-cer in GT1–7 cells. The appearance of oligonucleosomes after treatment with N SMase (200 mU/ml) was very rapid, disappearing after 48 h of exposure (A). Accordingly, DNA laddering was very pronounced after 12 h of exposure and was still present after 24 h. In contrast, the induction of DNA fragmentation by C8-cer (25 μM) was equally effective after short and long term treatment (B). 1, Control; 2, N SMase 12 h; 3, N SMase 24 h; 4, C8-cer 12 h; 5, C8-cer 48 h; M, 100-bp marker. *, $P < 0.05$ *vs.* control values.

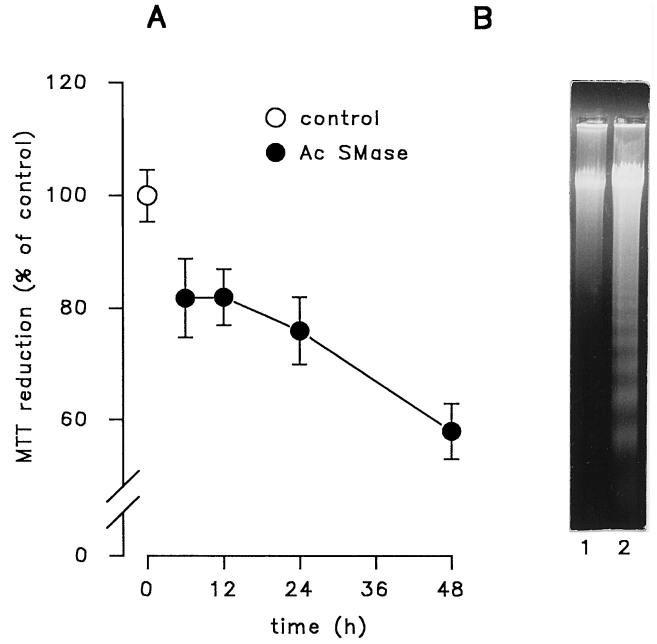

FIG. 8. Treatment of GT1–7 cells with Ac SMase affects proliferation and induces apoptotic death. Cells were exposed to the enzyme (200 mU/ml) for the time indicated, and the MTT solution was added during the last 2 h of incubation. MTT reduction, indicative of viable cell number, was then evaluated in an ELISA plate reader (A). A significant ($P < 0.05$) inhibitory effect of Ac SMase was present at all time points examined. B, DNA ladder of GT1–7 cells exposed to Ac SMase for 48 h. 1, Control; 2, Ac SMase 200 mU/ml.

both responsible for generation of the intracellular messenger, ceramide (22, 23, 28, 37). Exposure of GT1–7 cells to TNFα caused the accumulation of ceramide, as assessed by the formation of ceramide-1-phosphate. Ceramide is known to induce apoptosis in a series of different cellular systems, but its role at the central nervous system is still controversial. Ceramide has, in fact, been shown to induce apoptosis in cultured mesencephalic neurons (38), but it also exerts neuroprotective activity in cultured neurons deprived of trophic support (39) or exposed to excitotoxic or oxidative insult (40). In addition, ceramide induces neuronal differentiation (41) and regulates the balance between neuritic formation and apoptosis in hippocampal cultures (42). Treatment of GT1–7 cells with cell-permeable ceramide analogs induced a marked reduction of cell number and the appearance of distinct features of apoptotic death at all time points examined (either short or long term treatments). To evaluate the relative contribution of the ceramide-generating pathways in the induction of apoptotic death in GT1–7 cells, cultures were exposed to N or Ac SMase. Treatment with both enzymes produced a reduction of cell number and induction of apoptotic cell death. However, the effects observed were temporally divergent, as the action of N SMase was rapid in its onset but restricted to a short period of time, whereas Ac SMase induced a reduction of cell number and apoptotic death that were sustained with time (up to 48 h). This different time-related behavior may be ascribed to specific, prompt metabolism of the neutral enzyme whose action is rapidly achieved and completed or, alternatively, it may be due to the activation of intracellular pathways able to counterbalance the effect of N SMase on neuronal viability. One such example is represented by activation of protein kinase C, whose action on cell survival in our system (data not shown) as well as in other cellular systems (43) is that of counteracting the effect of N SMase. However, in our conditions, the involvement of protein kinase C is partially ruled out by the fact that GT1–7 cells are still responsive to the action of N SMase once the initial effect has ended, as demonstrated by the reduction of viable cells observed after repeated (twice, every 48 h) treatment with the drug. Interestingly, GT1–7 cells exhibited a very prompt capacity to recover after completion of N SMase action, and they responded with an increased proliferation rate starting at 48 h, when cell number was still decreased in N SMase-treated cultures, and a significant enhancement at 72 h. Hence, the time-limited action of N SMase on GT1–7 cell growth and viability revealed the rapid reversibility of the effect observed. Indeed, the current knowledge of TNFR1 signaling suggests that activation of Ac SMase is responsible for the transduction of the death signal, whereas the neutral enzyme would, instead, mediate proliferation and survival events (44). Based on these results, an alternative interpretation of the effect observed could be made. The nature of the early N SMase response could be due to the enormous amount of ceramide generated within the cell by exogenous addition of the enzyme (data not shown); under these conditions, the true response of increased cell growth would be completely masked and appear late.

In our hands, the activation of Ac SMase seems to be only partially involved in the action of TNFα on GT1–7 cell number and viability as demonstrated by the partial reduction of

TABLE 2. Treatment with D609 modifies the response of GT1-7 cells to TNFα on cell proliferation, induction of apoptosis, and ceramide formation

Treatment	Cell no. (% of control)	Apoptotic population (% of total cells)	Cer-1-P (% of control)
Control	100 ± 5.6	8.4 ± 0.4	100 ± 3.6
TNFα	79.5 ± 4.1[a]	32.2 ± 2.1[a]	183 ± 8.9[a]
D609	97.1 ± 3.9	7.8 ± 0.3	108 ± 7.6
TNFα + D609	89.5 ± 2.6[a,b]	22.1 ± 1.3[a,b]	141 ± 5.5[a,b]

Cells were pretreated with D609 (5 μg/ml) for 1 h before the addition of TNFα (20 ng/ml) for 48 h. GT1-7 cell number was evaluated by the MTT proliferation assay. The apoptotic population was assessed by flow cytometry after staining cells with propidium iodide. Ceramide-1-phosphate (Cer-1-P) generation was evaluated using a diacylglycerol kinase assay kit in GT1-7 cells exposed to D609 (5 μg/ml) for 1 h before treatment with TNFα (20 ng/ml) for 15 min. Values are the mean ± SE of three to five independent determinations (cell counting and apoptotic assessment) and of one study (run in triplicate) representative of two in the case of ceramide-1-phosphate measurement.

[a] $P < 0.05$ *vs.* untreated control (by one-way ANOVA and Neuman-Keuls *t* test).

[b] $P < 0.05$ *vs.* TNFα-treated group (by one-way ANOVA and Neuman-Keuls *t* test).

TABLE 3. Treatment with okadaic acid modifies the response of GT1-7 cells to TNFα on cell proliferation and induction of apoptosis

Treatment	Cell no. (% of control)	Apoptotic population (% of total cells)
Control	100 ± 3.1	11.1 ± 0.9
TNFα	80.1 ± 6.0[a]	37.6 ± 4.0[a]
OKA	95.7 ± 2.6	9.8 ± 0.7
TNFα + OKA	102.5 ± 6.6	9.6 ± 1.1

Cells were pretreated with okadaic acid (0.5 nM) for 1 h before the addition of TNFα (20 ng/ml) for 48 h. GT1-7 cell number was evaluated by the MTT proliferation assay. The apoptotic population was assessed by flow cytometry after staining cells with propidium iodide. Values are the mean ± SE of three separate studies.

[a] $P < 0.05$ *vs.* control (by Student's *t* test).

TNFα-induced effects by treatment with D609, an inhibitor of phosphatidylcholine-specific phospholipase C (29, 30) whose stimulation activates the pathway specifically involving Ac SMase. The contribution of N SMase on TNFα action cannot be extrapolated from the present findings, as no pharmacological tools are currently available to specifically modulate this pathway. However, an increased GT1–7 cell proliferation after TNFα treatment has never been observed. It is important to underline that activation of the sphingolipid metabolism leads to the production of sphingosine-1-phosphate, a second messenger involved in the regulation of cellular proliferation and survival (45), and sphingosine, whose role as an intracellular regulator of cellular differentiation and apoptosis is now emerging (46, 47). It is, then, possible that the involvement of the sphingomyelin pathway in TNFα-induced apoptosis may result from a balance between the generation of different messengers (ceramide, sphingosine, and sphingosine-1-phosphate), all able to regulate cellular viability. In the present study we have focused our attention primarily on the role of ceramide, as ceramide analogs were able to mimic the TNFα effects. In addition, the involvement of ceramide in the action of TNFα in GT1–7 cells is further supported by the complete prevention of the TNFα effect by treatment with low concentrations of okadaic acid. However, an involvement of sphingosine in TNFα-induced apoptosis in GT1–7 cells cannot be ruled out and requires further investigation. In the context of a complete picture of TNFα action in GT1–7 cells, although this would open a new, vast issue, well beyond the confines of the present study, a role for the caspase cascade could be envisaged. Caspases may, in fact, take part in the action of TNFα merely as executioners of apoptosis (48), but they may also be more deeply involved in TNFα signaling events by regulating intracellular ceramide production (49, 50).

In conclusion, in immortalized hypothalamic neurons, TNFα exerts a cytotoxic effect, presumably through an increased production of ceramide. This activity on hypothalamic neuronal viability together with the modulation of neurohormone secretion may critically contribute to the impairment of gonadal axis function occurring during infectious states.

References

1. **Spangelo BL, Judd AM, Call GB, Zumwalt J, Gorospe WC** 1995 Role of the cytokines in the hypothalamic-pituitary-adrenal and gonadal axes. Neuroimmunomodulation 2:299–312
2. **Vassilopoulou-Sellin R** 1994 Endocrine effects of cytokines. Oncology 8:43–46
3. **Kalra PS, Edwards TG, Xu B, Jain M, Kalra SP** 1998 The anti-gonadotropic effects of cytokines: the role of neuropeptides. Dom Anim Endocrinol 15:321–332
4. **Rivier C, Vale W** 1989 In the rat, interleukin 1-α acts at the level of the brain and the gonads to interfere with gonadotropin and sex steroid secretion. Endocrinology 124:2105–2111
5. **Yoo MJ, Nishihara M, Takahashi M** 1997 Tumor necrosis factor-α mediates endotoxin induced suppression of gonadotropin-releasing hormone pulse generator activity in the rat. Endocr J 44:141–148
6. **Rivier C, Vale W** 1990 Cytokines act within the brain to inhibit luteinizing hormone secretion and ovulation in the rat. Endocrinology 127:849–856
7. **Gaillard RC, Turnill D, Sappino P, Muller AF** 1990 Tumor necrosis factor α inhibits the hormonal response of the pituitary gland to hypothalamic releasing factors. Endocrinology 127:101–106
8. **Kaipia A, Chun S-Y, Eisenhauer K, Hsueh AJW** 1996 Tumor necrosis factor-α and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. Endocrinology 137:4864–4870
9. **Fiers W** 1991 Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS Lett 285:199–212
10. **Pan W, Zadina JE, Harlan RE, Weber JT, Banks WA, Kastin AJ** 1997 Tumor necrosis factor-α: a neuromodulator in the CNS. Neurosci Biobehav Rev 21:603–613
11. **Chao CC, Molitor TW, Hu S** 1993 Neuroprotective role of IL-4 against activated microglia. J Immunol 151:1473–1481
12. **Gelbard HA, Dzenko KA, DiLoreto D, del Cerro C, del Cerro M, Epstein LG** 1993 Neurotoxic effects of tumor necrosis factor alpha in primary human neuronal cultures are mediated by activation of the glutamate AMPA receptor subtype: implications for AIDS neuropathogenesis. Dev Neurosci 15:417–422
13. **Talley AK, Dewhurst S, Perry SW, Dollard SC, Gummuluru S, Fine SM, New D, Epstein LG, Gendelman HE, Gelbard HA** Tumor necrosis factor α-induced apoptosis in human neuronal cells: protection by the antioxidant *N*-acetylcysteine and the genes *bcl-2* and *crmA*. Mol Cell Biol 15:2359–2366
14. **Bodgan I, Leib SL, Bergeron M, Chow I, Tauber MG** 1997 Tumor necrosis factor-α contributes to apoptosis in hippocampal neurons during experimental group B streptococcal meningitis. J Infect Dis 176:693–697
15. **Heneka MT, Loschmann PA, Gleichmann M, Weller M, Schulz JB, Wullner U, Klockgether T** 1998 Induction of nitric oxide synthase and nitric oxide-mediated apoptosis in neuronal PC12 cells after stimulation with tumor necrosis factor-α/lipopolysaccharide. J Neurochem 71:88–94
16. **Jeohn GH, Kong LY, Wilson B, Hudson P, Hong JS** Synergistic neurotoxic effects of combined treatments with cytokines in murine primary mixed neuron/glia cultures. J Neuroimmunol 85:1–10

17. **Viviani B, Corsini E, Galli CL, Marinovich M** 1998 Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-α. Toxicol Appl Pharmacol 150:271–276
18. **Cheng B, Christakos S, Mattson MP** 1994 Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron 12:139–153
19. **Barger SW, Horster D, Furukawa K, Goodman Y, Krieglstein J, Mattson MP** 1995 Tumor necrosis factors α and β protect neurons against amyloid β-peptide toxicity: evidence for the involvement of a κB-binding factor and attenuation of peroxide and Ca^{2+} accumulation. Proc Natl Acad Sci USA 92:9328–9332
20. **Shen Y, Li R, Shiosaki K** 1997 Inhibition of p75 tumor necrosis factor receptor by antisense oligonucleotides increase hypoxic injury and β-amyloid toxicity in human neuronal cell line. J Biol Chem 272:3550–3553
21. **Tartaglia LA, Goeddel DV** 1992 Two TNF receptors. Immunol Today 13:151–153
22. **Kim MY, Linardic C, Obeid L, Hannun Y** 1991 Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α and γ-interferon. Specific role in cell differentiation. J Biol Chem 266:484–489
23. **Kolesnick R, Golde DW** 1994 The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cells 77:325–328
24. **Mellon PL, Windle PC, Padula CA, Roberts JL, Weiner RI** 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1–10
25. **Sortino MA, Canonico PL** 1996 Neuroprotective effect of insulin-like growth factor I (IGF-I) in immortalized hypothalamic cells. Endocrinology 137:1418–1422
26. **Tsai P-S, Werner S, Weiner RI** 1995 Basic fibroblast growth factor is a neurotropic factor in GT1 gonadotropin-releasing hormone neuronal cell lines. Endocrinology 136:3831–3838
27. **Preiss J, Loomis CR, Bishop WR, Stein R, Niedel JE, Bell RM** 1986 Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. J Biol Chem 261:8597–8600
28. **Wiegmann K, Schutze S, Machleidt T, Witte D, Kronke M** 1994 Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell 78:1005–1015
29. **Muller-Decker D** 1989 Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of phospholipase C-type reaction. Biochem Biophys Res Commun 162:198–205
30. **Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M** 1992 TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell 71:765–776
31. **Dobrowsky RT, Hannun YA** 1992 Ceramide stimulates a cytosolic protein phosphatase. J Biol Chem 267:5048–5051
32. **Wolff RA, Dobrowsky RT, Bielawska A, Obeid LM, Hannun YA** 1994 Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. J Biol Chem 269:19605–19609
33. **Cohen P, Cohen PTW** 1989 Protein phosphatases come of age. J Biol Chem 264:21435–21438
34. **Florio T, Perrino BA, Stork PJS** 1996 Cyclic 3,5 adenosine monophosphate and cyclosporin A inhibit cellular proliferation and serine/threonine protein phosphatase activity in pituitary cells. Endocrinology 137:4409–4418
35. **Smith CA, Farrah T, Goodwin RG** 1994 The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76:959–962
36. **Liu T, Clark RK, McDonnel PC, Young PR, White MS, Barone FC, Feuerstein GZ** 1994 Tumor necrosis factor-α in ischemic neurons. Stroke 25:1481–1488
37. **Hannun YA** 1994 The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269:3125–3128
38. **Brugg B, Michel PP, Agid Y, Ruberg M** 1996 Ceramide induces apoptosis in cultured mesencephalic neurons. J Neurochem 66:733–739
39. **Ito A, Horigome K** 1995 Ceramide prevents neuronal programmed cell death induced by nerve growth factor deprivation. J Neurochem 65:463–466
40. **Goodman Y, Mattson MP** 1996 Ceramide protects hippocampal neurons against excitotoxic and oxidative insults, and amyloid β-peptide toxicity. J Neurochem 66:869–872
41. **Riboni L, Prinetti A, Bassi R, Caminiti A, Tettamanti G** 1995 A mediator role of ceramide in the regulation of neuroblastoma Neuro2a cell differentiation. J Biol Chem 270:26868–26875
42. **Schwarz A, Futerman AH** 1997 Distinct roles for ceramide and glucosylceramide at different stages of neuronal growth. J Neurosci 17:2929–2938
43. **Obeid LM, Linardic CM, Karolak LA, Hannun YA** 1993 Programmed cell death induced by ceramide. Science 259:1769–1771
44. **Testi R** 1996 Sphingomyelin breakdown and cell fate. Trends Biol Sci 21:468–471
45. **Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind JS, Spiegel S** 1996 Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 381:800–803
46. **Sakakura C, Sweeney EA, Shirahama T, Hagiwara A, yamaguchi T, Takahashi T, Hakomori S, Igarashi Y** 1998 Selectivity of sphingosine-induced apoptosis. Lack of activity of DL-erythro-dihydrosphingosine. Biochem Biophys Res Commun 246:827–830
47. **Ohta H, Sweeney EA, Masamune A, Yatomi Y, Hakomori S, Igarashi Y** 1995 Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. Cancer Res 55:691–697
48. **Nicholson DW, Thornberry NA** 1997 Caspases: killer proteases. Trends Biochem Sci 22:299–306
49. **Dbaibo GS, Perry DK, Gamard CJ, Platta R, Poirier GG, Obeid LM, Hannun YA** 1997 Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-α: crmA and bcl-2 target distinct components in the apoptotic pathway. J Exp Med 185:481–490
50. **Bourteele S, Hausser A, Doppler H, Horn-Muller J, Ropke C, Schwarzmann G, Pfizenmaier K, Muller G** 1998 Tumor necrosis factor induces ceramide oscillations and negatively controls sphingolipid synthases by caspases in apoptotic Kym-1 cells. J Biol Chem 273:31245–31251