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Interleukin-1β-induced apoptosis through adenylyl cyclase and ERK1/2 inhibition in primary cultured thyroid cells ☆

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Abstract

The programmed cell death plays a crucial role in the regulation of numerous physiological and pathological phenomena. In this study, we show that interleukin-1β (IL-1β) induces an early production of endogenous ceramides via N-sphingomyelinase (N-Smase) as well as an inhibition of adenylyl cyclase activity in pig thyroid cells. This effect is followed by a down-regulation of the extracellular signal-regulated protein kinase (ERK1/2) phosphorylation, an activation of caspase-3, and ends by setting up the programmed cell death. The permeable exogenous C_2-ceramide reproduces IL-1β effects on: (i) inhibition of adenylyl cyclase activity, (ii) down-regulation of ERK1/2 phosphorylation, (iii) activation of caspase-3, and (iv) apoptosis in pig thyroid cells. Cell treatment with a PKA inhibitor down-regulates ERK1/2 phosphorylation. Furthermore, inhibition of ERK1/2 signaling pathway by U-0126 enhances caspase-3 activity and sets up programmed cell death. Both IL-1β and exogenous C_2-ceramide effects are reproduced by U-0126 so illustrating the implication of ERK1/2 down-regulation in both caspase-3 activation and apoptosis induction. Our study shows for the first time that endogenous ceramides are important second messengers in IL-1β-induced apoptosis in pig thyroid cells through inhibition of adenylyl cyclase and ERK1/2 activities.

Keywords: Thyroid; Ceramide; Apoptosis; ERKs; cAMP; Caspase; N-Smase

Apoptosis, a specific type of cell death, is involved in the regulation of cell number in multicellular organisms, and in the pathogenesis of various diseases including tumor progression, neurodegenerative disorders, as well as many viral infections [1,2]. Morphological changes pointing out cells that undergo apoptosis, such as cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation are well described in the literature [3]. Apoptosis activation under different physiological and pathological conditions is well documented in matter of extracellular effectors [4–8]. Yet, the forwarding of signals from the activated cellular receptor to apoptotic process, the intracellular enzymatic activities intercalating these events, and the intracellular messengers lack many precisions in thyroid.

Ceramides, natural membrane sphingolipids, have been evidenced as important second messengers in many signaling pathways regulating various cellular responses [9–13]. Ceramide generation may involve hydrolysis of sphingomyelin or de novo synthesis [14–16] and emerges as an important mediator of apoptosis [17–19]. Among the main intracellular targets of ceramides, a serine/threonine protein kinase [20], a serine/threonine protein phosphatase [21,22], and a protein kinase Cζ [23,24] have been identified.

In primary cultured pig thyroid cells, incubation with the physiological thyroid stimulating hormone (TSH) allows the reconstitution of follicle-like structures similar

☆ *Abbreviations:* IL-1β, interleukin-1β; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; PKA, protein kinase A; TSH, thyroid stimulating hormone; cAMP, cyclic AMP; N-Smase, neutral sphingomyelinase.

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to those found in vivo. This cellular organization is clearly associated with expression of thyroid cell functions and can be achieved upon stimulation of adenylyl cyclase by forskolin [25–27].

By contrast, hormone deprivation or IL-1β treatment promotes the destabilization of the follicular structures [28] and the activation of a sphingomyelinase resulting in an increase of intracellular ceramide level [24,29].

In the present study, we address the importance of sphingomyelin pathway, and particularly the role of intracellular ceramide level in the induction of apoptosis in porcine thyrocytes. We demonstrate that the increase of endogenous ceramide level by IL-1β down-regulates adenylyl cyclase activity and ERK1/2 phosphorylation. Then, we provide direct evidence that ERK1/2 down-regulation leads to apoptosis in primary cultured pig thyroid cells.

Materials and methods

Materials. TSH, human recombinant IL-1β, and H-89 were purchased from Calbiochem. C_2-ceramides, Fumonisin B1, and Hoechst-33258 were from Sigma. Thin-layer chromatographic plates (silica gel 60) were obtained from Merck. U-0126 and colorimetric caspase-3 assay (CaspACE assay) were purchased from Promega. Rabbit anti-extracellular Signal-Regulated Kinase 1 and 2 polyclonal antibodies were obtained from Tebu. PhosphoPlus ERKs1/2 antibody kit and anti-rabbit immunoglobulin G conjugated to horseradish peroxidase were obtained from Cell Signaling Technology. Annexin V-FITC apoptosis detection kit was purchased from BD PharMingen. Bradford assay was purchased from Bio-Rad. ECL detection system was purchased from Amersham.

Primary culture of thyroid cells. The preparation of thyroid cells has been described in detail elsewhere [30]. Briefly, thyroid epithelial cells were isolated from adult porcine glands by discontinuous trypsin–EGTA treatment. Freshly isolated cells suspended at a concentration of 10^6 cells/ml in Eagle's minimum essential medium (pH 7.4) containing 10% (v/v) fetal calf serum, penicillin (200 U/ml), streptomycin sulfate (0.05 mg/ml), and TSH (1 mU/ml) were plated directly onto poly (L-lysine) treated culture dishes and incubated at 37 °C in a 95% air–5% CO_2 atmosphere.

Ceramide quantification. Two days after the onset of cultures, the medium was withdrawn and replaced with fresh serum-free medium containing IL-1β (10 ng/ml) with or without GSH (3 mM) or FB1 (1 μM) from 0 to 60 min. After scrapping and centrifugation, cells were collected in phosphate-buffered saline (PBS, pH 4.7). Total cellular lipids were then extracted according to Bligh and Dyer [31], and the organic phase was dried under a nitrogen stream. Ceramide levels were quantified by the diacylglycerol (DAG) kinase assay [32]. Briefly, Total lipids were resuspended in 20 μl solubilization buffer (cardiolipin 5 mM, diethylenetriaminepentaacetic acid 1 mM, and octylglucopyranoside 7.5% w/v). To the solubilized lipids, 80 μl imidazole buffer (50 mM, pH 6.5) containing NaCl (50 mM), EGTA (1 mM), and $MgCl_2$ (12.5 mM), 50 μCi [^{32}P-γ]ATP and DAG kinase (10 μl/63 mU) were added. After incubation for 30 min at 25 °C, the reaction was stopped by extraction with 3 ml methanol/chloroform (2:1 v/v), 1.7 ml of 1% (w/v) perchloric acid, and 1 ml chloroform. The organic phase was washed twice with 2 ml $HClO_4$ 1% (w/v) and an aliquot (0.5 ml) was dried under nitrogen stream. The lipids were then resuspended in 100 μl chloroform/methanol (100:5 v/v). An aliquot (20 μl) of the lipid solution was then applied to a thin-layer chromatographic plate (silica gel 60) and developed in chloroform/methanol/acetic acid (65:15:5 v/v). The dried plate was autoradiographed and ceramide-1-phosphate ($R_f = 0.14$) spots were scraped off and counted.

Detection of apoptosis. Thyroid cell monolayers were cultured during two days onto dishes in the presence of TSH (1 mU/ml). After washes, cells were incubated with either IL-1β alone or in the presence of GSH, C_2-ceramide or MEKs inhibitor (U-0126) for 24 h. For annexin V-FITC labeling, the medium was removed and cells were incubated for 10 min at room temperature with 200 μl Hepes buffer (Hepes 10 mM, NaCl 140 mM, and $CaCl_2$ 5 mM) containing annexin V-FITC (3 μg/ml) and propidium iodide (PI) (1 μg/ml). Annexin V-FITC labeled cells were visualized by confocal microscopy using a 510 nm emission filter for green FITC fluorescence and a 580 nm filter for red PI staining. For Hoechst reagent (33258) labeling, the cells were incubated for 15 min at 37 °C with 200 μl Hoechst-33258 (20 μg/ml in PBS) and observed under fluorescence microscope. Apoptotic cells, which contained condensed chromatin fragments, were scored and results expressed as percentage of the total cell number.

Caspase-3 activity. Thyroid cell monolayers were cultured during 2 days onto dishes at the density of 10^6 cells/ml per well in the presence of TSH (1 mU/ml). After washes, cells were treated by either IL-1β, C_2-ceramide or MEK1/2 inhibitor (U-0126) from 0.5 to 8 h. To determine a possible implication of N-Smase-dependent pathways, cells were treated by IL-1β in the presence of GSH for 4 and 8 h. Cell pellets were washed with ice-cold phosphate-buffered saline and resuspended in 100 mM Hepes buffer (pH 7.4) containing protease inhibitors (5 mg/ml aprotinin and pepstatin, 10 mg/ml leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride). Cells were lysed by sonication and the cytosolic fraction was obtained by centrifugation at 12,000*g* for 20 min at 4 °C. Cytosolic fraction (50 μg protein measured by the Bradford assay) [33] was added to the colorimetric caspase-3 substrate, containing the *p*-nitroaniline (*p*NA) moiety that absorbs at 405 nm upon cleavage by activated caspase-3. The absorbance was read using a Genios Tecan multifunctional plate reader.

Cyclic AMP assay. Thyroid cell monolayers were cultured onto dishes at the density of 10^6 cells/ml per well in the presence of TSH (1 mU/ml). Two days after the onset of culture, medium was withdrawn and replaced by serum-free medium with or without IL-1β (10 ng/ml) or C_2-ceramide (20 μM) from 0 to 60 min. To evaluate the role of N-Smase-dependent pathways, cells were incubated with IL-1β and GSH for 30 and 60 min.

Cells were then washed with 25 mM Earle's Hepes buffer (pH 7.4) and incubated at 37 °C in 350 μl final volume of the same buffer with or without TSH (10 mU/ml) for 10 min. The reaction was stopped by addition of 39 μl of 10 N $HClO_4$. Cells were scraped and cyclic AMP content was assayed by radioimmunological method according to Cailla et al. [34], except that bound and free ligands were separated by precipitation of bound ligands with a mixture of γ-globulin (2.5 mg/ml) in 0.1 M citrate buffer (pH 6.2) and polyethylene glycol 6000 (20% w/v in water).

Western blot of ERK1/2. Thyroid cell monolayers were cultured during 2 days onto dishes at the density of 10^6 cells/ml per well in the presence of TSH (1 mU/ml). After washes, cells were incubated with either IL-1β (10 ng/ml) alone or in the presence of GSH, C_2-ceramide (20 μM) or H-89 (5 μM) from 0 to 120 min. Cells were lysed in 0.5 ml lysis buffer, pH 7.4 (50 mM β-glycerophosphate, 1.5 mM EGTA, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM benzamidine, and 1% NP-40 (v/v)). Insoluble material was removed by centrifugation (10,000*g*, 45 min at 4 °C). Protein quantification was performed by the Bradford assay. Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membrane. Western blots were probed with phosphorylated ERK1/2 specific antibodies. The membrane was then incubated for 30 min with horseradish peroxidase-conjugated secondary antibody. The peroxidase reaction was developed with an ECL detection system. The same membrane was stripped and reprobed with specific ERK1/2 antibody.

Statistical analysis. The data presented are means ± SEM of triplicate samples from three independent experiments. Statistical analysis was performed using Student's *t* test. A level of $p < 0.05$ was accepted as statistically significant.

Results

IL-1β induces pig thyroid cell apoptosis via ceramide production

To study thoroughly the role of IL-1β on thyroid cells, we first examined the time-course effects of IL-1β on