Ceramide inhibits pancreatic β-cell insulin production and mitogenesis and mimics the actions of interleukin-1β

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Abstract Ceramide, generated during sphingomyelinase-induced sphingolipid cleavage, is considered an important mediator in cytokine signaling. The effects of endogenously generated and exogenously delivered ceramide on long-term insulin secretion and replication by pancreatic β-cells were investigated, and compared to the effects of interleukin 1β (IL-1β). Generation of β-cell ceramide by exogenous sphingomyelinase, or addition of cell-permeant ceramide analogs C_2-ceramide and C_6-ceramide, caused inhibitory effects on β-cell insulin production and mitogenesis mimicing those evoked by IL-1β. Hence, ceramide may be involved in transducing the cytostatic and cytotoxic actions of IL-1β in the β-cell.

Key words: Ceramide; Sphingolipid; IL-1β; Insulin secretion; Islet

1. Introduction

The ability of the insulin-producing pancreatic β-cell to expand its proliferative capacity to accomodate an increased need for insulin might to be of significance for the development of diabetes mellitus, because this disease in characterized by a reduced β-cell mass [1]. Also in different animal models for both insulin-dependent and non insulin-dependent diabetes does a defect β-cell regeneration seem to be of central importance in the development of glucose intolerance. Thus, it has been shown that administration of β-cell specific toxins, e.g. streptozotocin and alloxan, which results in destruction of the majority of β-cells and subsequent hyperglycemia, is not accompanied by a compensatory regeneration of β-cells [1]. In addition, in the genetic model for insulin-dependent diabetes, the *db/db* mouse, β-cell regeneration is not a noteworthy component [1]. Furthermore, experimental diabetes in rodents may be alleviated by transplantation of pancreatic islet grafts, however requiring a large number of islets [1]. More recently it has been shown that the endogenous β-cell mass can be expanded by autocrine/paracrine factors induced by 'cellophane wrapping' of the pancreas, a maneuver that not only induces a nesidioblastosis-like state but importantly enough also ameliorates experimental diabetes mellitus [2].

Despite the potential importance of an insufficient extent of β-cell replication in diabetes, not much is known about the intracellular mechanisms that normally govern this event, although some extracellular factors stimulating β-cell DNA synthesis in vitro have been identified (reviewed in [1,3,4]). However, far less attention has been paid to the factors that inhibit β-cell function and mitosis, in spite of the fact that such factors may be of regulatory significance. Nonetheless, much interest has been focused on interleukin-1β (IL-1β), a cytokine known to be produced by islet-infiltrating macrophages preceding the onset of type-1 diabetes mellitus [5]. IL-1β exerts cytotoxic and suppressive actions on the β-cell in vitro and is believed to occupy a pivotal role in triggering the outbreak of type-1 diabetes mellitus which is characterized by progressive necrosis of the β-cells by autoimmune processes [5,6].

There is compelling recent evidence that ceramide, a product of sphingomyelin hydrolysis, may function as a novel second messenger in conveying cytotoxic signals by effector molecules such as IL-1β in other cell systems (reviewed in [7–9]). In this study, we compared the effects of IL-1β and ceramide, exogenously delivered or endogenously generated, on β-cell insulin production and mitogenesis in vitro.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β was graciously donated by Dr. Klaus Bendtzen, Laboratory of Medical Immunology, Rigshospitalet, University Hospital, Copenhagen, Denmark. The biological activity of the IL-1β was 5 U/pg, as determined by comparison with an interim international standard IL-1β preparation (NIBSC, London, UK) in the mouse thymocyte costimulatory assay and the EL4 murine T-cell line [10]. The endotoxin content after adding the interleukin at the highest concentration (25 U/ml) was <1 pg/ml, as measured in the *Limulus* amoebocyte assay. We have previously found that neither 1 pg/ml nor 10 pg/ml of endotoxin affected the growth or viability of insulin-producing cells [10]. The cell-permeant ceramide analogs C_2-ceramide (D-*erythro*-2-acetamido-4-*trans*-sphingenine) and C_6-ceramide (D-*erythro*-2-hexylamido-4-*trans*-sphingenine) were purchased from Matreya Inc., Pleasant Gap, PA. Sphingomyelinase (from *Bacillus cereus*) and D-sphingosine (from bovine brain) were obtained from Sigma Chemicals, St. Louis, MO, USA. Collagenase type CLS (EC 3.4.24.3) was obtained from Boehringer-Mannheim, Mannheim, Germany. Culture medium RPMI 1640, fetal calf serum, L-glutamine, bensylpenicillin and streptomycin were from Flow Laboratories, Irvine, UK. Antibovine insulin serum was supplied by Miles-Yeda, Rehovot, Israel, whereas crystalline mouse insulin and [^{125}I]insulin were from Novo, Copenhagen, Denmark. [*methyl*-^{3}H]Thymidine (5 Ci/mmol) was purchased from Amersham International, Bucks. UK. Unisolve was from New England Nuclear, Boston, MA, USA and Soluene was provided by Packard Instruments, Downers Grove, IL, USA. All other chemicals of analytical grade were obtained from E. Merck, Darmstadt, Germany.

2.2. Preparation and culture of fetal islets

Pregnant Wistar rats, purchased from B & K Inc. (Sollentuna, Sweden), were killed by cervical dislocation on day 21 of gestation and the fetuses rapidly removed. Fetal rat islets were prepared from pancreatic glands as previously described [11–15]. Briefly, the pancreata were finely chopped and digested for a short time with collagenase. The carefully washed digest was plated in culture dishes allowing cell attach-

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ment (Nunc, Roskilde, Denmark) and cultured for 5 days at 37°C in a humidified atmosphere of 5% CO_2 in ambient air in medium RPMI 1640 containing 11.1 mM glucose, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml bensylpenicillin and 0.1 mg/ml streptomycin. At the end of the culture period, groups of islets were transferred to fresh media containing 1% fetal calf serum and cultured free-floating overnight, a procedure that minimizes fibroblast proliferation. Spherical islets, free of connective tissue, were then selected under a stereo microscope and used for the different analyses listed below. In each experiment, all test groups received the same amount of solvent (EtOH). In order to enhance ceramide solubility and cellular accessibility, the analogs were complexed with BSA at 37°C 60 min prior to addition to culture media. This was done by dissolving C_2-ceramide, C_6-ceramide or sphingosine in absolute ethanol (25 mM) and diluting 1:25 with BSA (4 mg/ml).

2.3. DNA synthesis

Islets in groups of 50 were cultured for 24 h as described above. During the last 5 h, 1 μCi/ml of [*methyl*-^{3}H]thymidine was present in culture media. At the end of the labeling period the islets were washed in PBS, ultrasonically homogenized in redistilled water, and the acid-insoluble material precipitated in ice-cold 10% trichloroacetic acid [12–15]. The precipitate was washed twice in trichloroacetic acid and dissolved in 50 μl of Soluene. The radioactivity incorporated was determined by scintillation counting after addition of 1 ml of Unisolve. Given the long cell cycle of the β-cell [1] and the fact that unsynchronized cells were studied, it was considered necessary to expose the islets for 24 h to the different test substances, a procedure that allows DNA synthesis initiated prior to addition of test substances to be terminated before [^{3}H]thymidine addition.

2.4. Islet insulin content and insulin accumulation

The islet insulin content in homogenates extracted overnight in acid ethanol [12–15] and insulin accumulation in culture media during the last 24 h of culture were determined radioimmunologically [16].

3. Results

As shown in Fig. 1, the addition of IL-1β (25 U/ml) caused a marked suppression of long-term (24 h) accumulation of insulin in the culture medium, thus confirming previous reports [6,17]. There was a corresponding slight decrease in the islet insulin content, an effect which however did not attain statistical significance in this series (Fig. 2). Moreover, as demonstrated in earlier work [13,18], IL-1β also decreased β-cell mitogenesis, as monitored by measurements of [^{3}H]thymidine into DNA (Fig. 3). This effect of IL-1β, and the inhibited secretion, were mimiced by exogenous sphingomyelinase (1 U/ml; Figs. 1 and 3). By contrast, the effect of sphingomyelinase on the islet content of insulin was not significantly different from that of controls (Fig. 2). Addition of the cell-permeant ceramide analogs (C_2-ceramide and C_6-ceramide, 15 μM each) complexed with BSA to the culture media resulted in more pronounced cytotoxic effects, as evidenced by increased accumulation of insulin in culture media (taken to reflect cell leakage; Fig. 1), decreased cellular insulin content (Fig. 2) and inhibition of cell proliferation (Fig. 3). In addition, light microscopical inspection of islets after 24 h of culture with C_2-ceramide and C_6-ceramide revealed signs of gross cytotoxicity (not shown). In contrast, equimolar concentrations of sphingosine caused small stimulatory effects, which, however, were not entirely consistent (not shown).

4. Discussion

The present study shows that exogenously delivered ceramide analogs capable of entering the cell, as well as generation of endogenous ceramide by addition of exogenous sphingomyelinase, evoke cytotoxic effects and inhibits β-cell function. Similar effects were recorded in this study, and in many previous reports [1,5,6,10,13,17,18], by exposure to the cytokine IL-1β. There is much converging evidence to support a key effector role for IL-1β in the development of type-1 diabetes mellitus. Hence, IL-1β is secreted from leukocytes infiltrating the islets early in the development of insulin-dependent diabetes mellitus [5,6] and IL-1 levels in serum are elevated in newly diagnosed diabetic patients [19]. In addition, IL-1β has been shown to exert both inhibitory and cytotoxic actions on pancreatic islet cells in vitro [1,5,6,10,13,17,18], findings reproduced in the present study.

A humongous amount of work has been done to pin down the mechanisms by which IL-1β works, and according to the

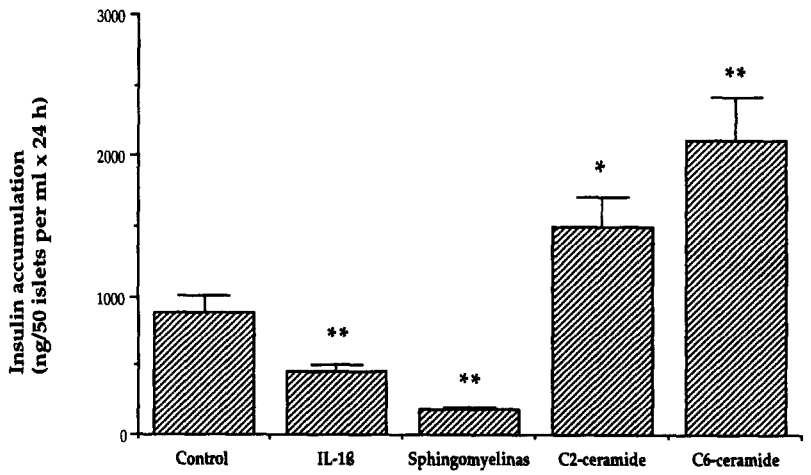

Fig. 1. Effects of IL-1β, sphingomyelinase and ceramide analogs on insulin accumulation. Groups of 50 fetal rat islets were cultured free-floating for 24 h in medium RPMI 1640 containing 1% fetal calf serum and supplemented as indicated in the figure. The accumulation of insulin in the culture medium was determined radioimmunologically. Concentrations used were: IL-1β, 25 U/ml; sphingomyelinase 1 U/ml; C_2-ceramide and C_6-ceramide, 15 μM. Bars represent means ± S.E.M. for 6 experiments. * and ** denote $P < 0.05$ and $P < 0.01$ for chance differences vs. controls using Student's paired *t*-test.

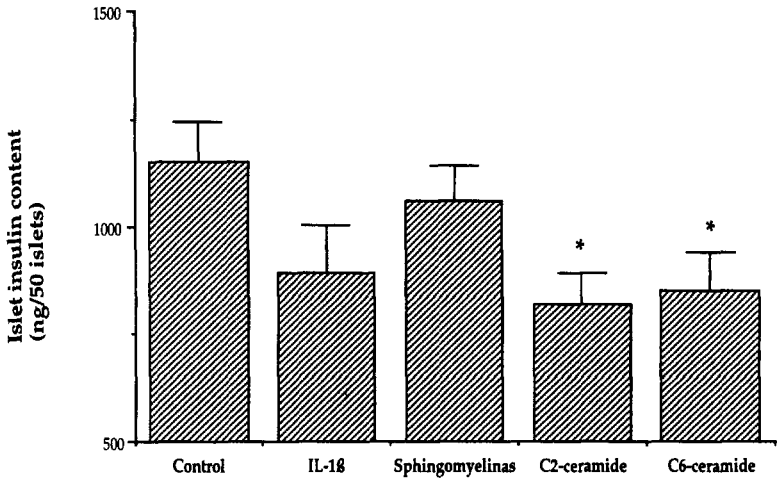

Fig. 2. Effects of IL-1β, sphingomyelinase, and ceramide analogs on islet insulin content. Groups of 50 fetal rat islets were cultured free-floating for 24 h in medium RPMI 1640 containing 1% fetal calf serum and supplemented as indicated in the figure. The insulin concentration in islet homogenates extracted in acid ethanol was determined radioimmunologically. Concentrations used were: IL-1β, 25 U/ml; sphingomyelinase 1 U/ml; C_2-ceramide and C_6-ceramide, 15 μM. Bars represent means ± S.E.M. for 6 experiments. * denotes $P < 0.05$ for a chance difference vs. controls using Student's paired t-test.

contemporary conceptual framework a primary event is generation of the noxious gas nitric oxide shutting down ATP production through inhibition of Krebs cycle enzymes [17]. Additionally, however, generation of ceramide through the action of sphingomyelinase is a rapid and consistent event after exposure to IL-1β and other cytokines, e.g. interferon-γ and tumor necrosis factor α [8,9,20–22]. In contrast to the large wealth of information accumulating regarding products of phosphoinositide hydrolysis as regulatory molecules, the sphingolipids have been poorly appreciated and were until recently merely perceived as structural components of the plasma membrane. However, a rapidly growing body of evidence implicate ceramide, generated through sphingomyelin hydrolysis, as a novel second messenger, conveying antiproliferative signals by cytotoxic molecules such as IL-1 (reviewed in [7–9,22]). It is noteworthy that ceramide seems to counteract the effects of the phosphoinositide cleavage product diacylglycerol, the latter being an activator of protein kinase C and linked to growth promotion [23,24]. Thus, although ceramide reportedly does not inhibit protein kinase C, it has emerged as a second messenger mediating growth suppression and apoptosis [7–9,22] and may well prove to be as paramount as diacylglycerol in regulation of cell signaling. Additionally, specific targets for ceramide have been identified, including activation of a serine/threonine protein phosphatase of the 2A subfamily [25,26], the latter recently being identified by us in β-cells [27,28]. Entirely consistent with our present results are the previous findings that specific inhibition of serine/threonine protein phosphatase 2A with low concentrations of okadaic acid [28] or protein kinase C stimulation with phorbol ester [24,28,29] promotes insulin secretion. Conversely, nanomolar concentrations of okadaic acid or phorbol ester prevent apoptosis and c-*myc* downregula-

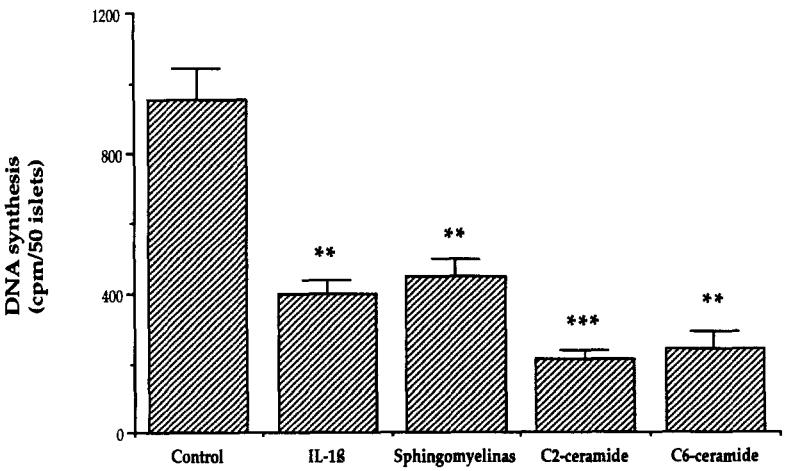

Fig. 3. IL-1β and ceramide decrease β-cell proliferation. Groups of 50 fetal rat islets were cultured free-floating for 24 h in medium RPMI 1640 containing 1% fetal calf serum and supplemented as indicated in the Figure. During the final 5 h of culture, 1 μCi/ml [^{3}H]thymidine was present in culture media and DNA synthesis rates were determined by measuring the radioactivity incorporated into trichloroacetic acid-precipitable material. Concentrations used were: IL-1β, 25 U/ml; sphingomyelinase 1 U/ml; C_2-ceramide en C_6-ceramide, 15 μM. Bars represent means ± S.E.M. for 6 experiments. ** and *** denote $P < 0.01$ and $P < 0.001$ for chance differences vs. controls using Student's paired t-test.

tion evoked by ceramide [9]. Thus, ceramide and diacylglycerol appear to be the Yin and Yang of cellular signaling through their opposing effects on protein phosphorylation.

The recent development of cell-permeant ceramide analogs has made elucidation of the role of ceramide in living cells become amenable and conclusive [9]. Hence, one of the analogs employed in this study, C_2-ceramide, was shown to inhibit growth of leukemia cells and it appears that ceramide regulates the expression of *c-myc* levels and the transcription factor NF-KB, and is also able to interact with phospholipase A_2 and cyclo-oxygenase [9], which are also affected by IL-1 [17]. Whether any of these elements are targeted by ceramide in the β-cell remains to be seen, and it surely also will be of interest to look into the possible interaction between ceramide and nitric oxide, i.e. whether ceramide acts uphill or downhill of nitric oxide. In this context, it is noteworthy that serum from patients with type I diabetes mellitus, whose β-cells are succumbed by autoimmune assault, not only contains elevated levels of IL-1 [19] but also causes exaggerated Ca^{2+} influx in insulin-secreting cells leading to DNA fragmentation characteristic of apoptosis [30], and that IL-1β activates apoptosis in these cells through nitric oxide generation [31], thus mimicing the actions of ceramide presently and previously [32] recorded.

Our present findings not only show that exogenous ceramide suppresses β-cell growth and function, but also that artificially stimulating generation of ceramide in the β-cell by addition of the normally membrane-bound enzyme sphingomyelinase gave rise to qualitatively similar results. This also mimicked the suppressive actions of IL-1β, a pathophysiological effector of ceramide synthesis and β-cell death, thus adding further credence to a role of ceramide as a second messenger mediating cytotoxic signals by molecules such as IL-1β. It should be noted that in another system [9], exogenous sphingomyelinase mimicked the actions of a cell-permeant ceramide analog, thus showing that ceramide can be formed intracellularly when sphingomyelinase is added to the outside of the cell.

It is concluded that ceramide, either delivered exogenously or generated endogenously, causes inhibitory and cytotoxic actions on the insulin-secreting pancreatic β-cell in vitro, effects that are mimicked by IL-1β. Hence, ceramide may be an important intracellular second messenger, conveying the suppressive and cytotoxic actions of IL-1β on the β-cell.

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