Deoxysphingolipids, a novel biomarker for type 2 diabetes, are cytotoxic for insulin-producing cells.

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Running title: Deoxysphingolipids and diabetes

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Abstract

Irreversible failure of pancreatic β-cells is the main culprit in the pathophysiology of diabetes mellitus, a disease that is now a major global epidemic. Recently, elevated plasma levels of deoxysphingolipids, including 1-deoxysphinganine, have been identified as novel biomarkers for the disease. In this study, we analyzed whether deoxysphingolipids directly compromise the functionality of insulin-producing Ins-1 cells and primary islets. Treatment with 1-deoxysphinganine induced dose-dependent cytotoxicity with senescent, necrotic and apoptotic characteristics and compromised glucose-stimulated insulin secretion. In addition, 1-deoxysphinganine altered cytoskeleton dynamics, resulting in intracellular accumulation of filamentous actin and activation of the RhoGTPase Rac1. Moreover, 1-deoxysphinganine selectively up-regulated ceramide synthase 5 expression and was converted to 1-deoxydihydroceramides, without altering normal ceramide levels. Inhibition of intracellular 1-deoxysphinganine trafficking and ceramide synthesis improved the viability of the cells, indicating that the intracellular metabolites of 1-deoxysphinganine contribute to its cytotoxicity. Analyses of signaling pathways identified JNK and p38 MAPK as antagonistic effectors of cellular senescence. Our results revealed that 1-deoxysphinganine is a cytotoxic lipid for insulin-producing cells, suggesting that the increased levels of this sphingolipid observed in diabetic patients may contribute to the reduced functionality of pancreatic β-cells. Thus, targeting deoxysphingolipid synthesis may complement the currently available therapies of diabetes.
Introduction

In the last three decades, the prevalence of diabetes has been rising worldwide at a dramatic rate, with incidence projections approaching 8% of the population by 2030 (1; 2). This remarkable increase is largely due to the epidemic spreading of type 2 diabetes mellitus (T2DM), which accounts for 90% of all cases of diabetes mellitus worldwide (reviewed in (3; 4)). Given the level of complexity associated with the pathophysiology of T2DM, understanding the mechanisms underlying this disease is necessary to design alternative strategies to limit its progression. Recently, substantial improvements occurred in the detection of early stage or undiagnosed T2DM, thus allowing appropriate treatments in high risk populations. One of the latest biomarkers identified in patients with diabetes and metabolic syndromes are increased plasma levels of deoxy-sphingolipids (1-deoxySLs) (5; 6), a type of sphingolipid characterized by an initial condensation of alanine or glycine instead of serine with palmitic acid and the resultant absence of the hydroxyl group in position C1. Consequently, although these deoxy-sphingoid bases can be acylated to deoxy-dihydroceramides, they cannot be further metabolized to complex sphingolipids or efficiently degraded by the canonical degradation pathway, and thus tend to accumulate once produced. Importantly, 1-deoxySLs display toxic properties in vitro toward several cell lines (7-9) and in vivo are thought to impair neuronal functionality in patients with the hereditary sensory and autonomic neuropathy type I (HSAN1) (10).

In light of the increased plasma levels of 1-deoxySLs found in diabetic patients and of the reported cytotoxic effects associated with the exposure to increased 1-deoxySL concentrations, we investigated whether these atypical sphingolipids directly compromise pancreatic β-cells, the dysfunction of which plays an important role in the pathogenesis of both type 1 and type 2 diabetes.
**Research Design and Methods**

**Biochemical reagents**

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL. Inhibitor stock solutions were freshly diluted to the concentrations required for the individual experiment indicated in the figure legends. Lipid stock solutions were prepared as a bovine serum albumin (BSA) complex, as described in (10), and added to the cells at the concentrations indicated in the figure legends. BSA was used as control.

**In vitro cell culture**

The Ins-1 rat insulinoma cell clone 832/13, generously provided by C. Wollheim, was maintained in RPMI 1640 medium, as described (11; 12). Cell metabolic activity was tested with 0.5% tetrazolium salt solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), or WST-1 (Roche), according to the manufacturer’s instructions. Cell death was quantified by trypan blue exclusion or lactate dehydrogenase (LDH) release in the medium (Roche). Cellular senescence was quantified with the β-galactosidase assay kit (Cell Biolabs). Adp21 (rat) and AdGFP adenovirus were purchased from Vector Biolabs, Philadelphia, USA. Rac1 activity was measured with G-LISA Rac1 activation assay (Cytoskeleton, Denver, USA).

**Animal experiments**

Wistar rats, leptin deficient ob/ob mice on C57BL/6J background (B6.V-Lep/OlaHsd) and wild type (WT) C57BL/6J (Harlan Laboratories) were kept under a light-dark regime (16:8 h), constant temperature, and free access to food and water. All animal experiments were performed
in accordance with swiss federal animal regulations and approved by the cantonal veterinary
office of Zurich.

Islets were harvested from pancreata of male Wistar rats (250 to 300 g) by collagenase (NB8
collagenase, Serva, Heidelberg, Germany) followed by trypsin digestion to dissociate them into
single cells, according to (13).

**Insulin secretion**

Dissociated islets cells were seeded in 12-well ECM coated plates (Novamed, Jerusalem, Israel),
treated for 24 h with 5 µM sphinganine, 1-deoxysphinganine or BSA and incubated in RPMI
medium containing 3.3 mM glucose for 1 h. Following sequential 1 h incubations with low (3.3
mM), high (16.7 mM), low (3.3 mM) glucose concentrations, insulin secretion was measured by
radioimmunoassay (Insulin-CT, CIS, Biointernational, Schering AG, Baar, Switzerland),
according to the manufacturer’s instructions.

**qRT-PCR**

Total RNA was extracted from Ins-1 cells cultured in 1 µM sphinganine or 1-deoxysphinganine
for 24 h. Quality of RNA was assessed by 2100 bioanalyzer (Agilent Technologies, Basel,
Switzerland). cDNA was obtained with the RT2 First Strand Kit and profiled using the Rat Cell
Death Pathway Finder PCR Array (both from SABiosciences, Hombrechtikon, Switzerland),
according to the manufacturer’s instructions. CerS primers for SYBR green qPCR are listed in
Supplementary Materials and Methods.

**Immunohistochemistry and flow cytometry analyses**

Pancreas specimens were fixed in 4% formalin and paraffin embedded according to standard
procedures (14). Ins-1 cells were fixed in 3.6% formaldehyde and permeabilized with 0.2%
Triton X-100 in PBS. Primary antibodies used in this study are listed in Supplementary Materials
and Methods. Apoptosis detection was performed with an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France). Immunofluorescence analysis and image data collection were performed on a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany), or on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using a glycerol immersion objective lens (Leica, HCX PL APO CS 63x 1.3 Corr). Image z-stacks were collected with a pinhole setting of Airy 1 and twofold oversampling. Image stacks of optical sections were processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL). Three-dimensional reconstruction, volume rendering, and quantification of signal overlap in the 3D volume model were done with the Imaris software suite (Version 7.2.1, Bitplane, Zurich, Switzerland). The degree of signal overlap in the 3D volume models is shown graphically as scatterplots by plotting the intensity of two fluorescent signals in each voxel of the 3D model. Voxels with similar signal intensity for both signals appear in the area of the diagonal. Single-cell quantification of stained cells by flow cytometry was performed using a FACSDiva flow cytometer (BD Bioscences, Allschwil, Switzerland).

**Western blotting**

Ins-1 cells cultured in 1 µM sphinganine, 1-deoxysphinganine or BSA for 24 h were lysed as described (15). Aliquots corresponding to 35 µg of proteins were separated by SDS-PAGE electrophoresis, blotted and probed over night at 4°C. Primary antibodies used in this study are listed in Supplementary Materials and Methods. Immunoreactive bands from at least 3 independent experiments were quantified by densitometry and normalized to β-actin or GAPDH levels.

**Analysis of sphingoid bases and ceramides by LC–MS/MS**
The sphingoid base profile was analyzed as described earlier (5). Ceramide species were extracted by adding 1 ml methanol/chloroform (2:1) (including 200 pmole of C12 ceramide internal standard (Avanti Polar Lipids) to 100 µl of re-suspended cells followed by the addition of 0.5 ml of chloroform and 200 µl alkaline water (10). Interfering phospholipids were hydrolyzed by re-extracting the dried lipids with methanol-KOH:chloroform (4:1) as described earlier (10). Lipids were separated on a C18 column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, France) and analyzed on a TSQ Quantum Ultra (Thermo, Reinach, Switzerland) using atmospheric pressure chemical ionization (APCI) (10). Ceramides and deoxy-Ceramides were identified by precursor ion scan (20mV collision energy) with fragments of (m/z 264.3) and (m/z 268.3) respectively. Levels were normalized to ISTD and cell numbers.

**Statistical analyses**

Results are expressed as means ± SEM. Significance was assessed using Student’s unpaired, two-tailed t tests or one-way analysis of variance. A probability value <0.05 was considered significant. When the overall probability value was <0.05, the Bonferroni multiple-comparison test was used to determine whether there was a significant difference between values of control (reference sample) and samples of interest.

**Results**

**1-deoxySL treatment is cytostatic and cytotoxic for Ins-1 cells**

As 1-deoxySLs were found elevated in the plasma of diabetic patients in the low µM range (5; 6), we analyzed whether 1-deoxySLs can directly affect the viability of insulin-secreting cells. To this aim, the rat insulinoma cell line Ins-1 was treated at 50% confluence (Fig. 1A, L: low density) for 24 h with 1-deoxysphinganine or sphinganine as control. 1-deoxysphinganine incubation reduced both the metabolic activity, as measured by MTT (Fig.1A) and WST-1
reduction (Fig. S1A), and the number of live cells (Fig. 1B) in a dose-dependent manner. Treatment with 5 µM caused cell round up (Fig. 1C) and death, as shown by robust trypan blue inclusion (Fig. 1D) and LDH release (Fig. 1E). However, cells treated with 1 µM 1-deoxyxypinganine did not increase in number compared with the initial seeding but showed modest levels of lethality (Fig. 1D, E), or up-regulation of genes involved in cell death pathways (Fig. S1B-D), suggestive of a cytostatic effect of the lipid at this concentration (Fig. 1B). When the lipid treatment was performed on 90% confluent cells (Fig. 1A, H: high density), the metabolic activity was reduced only at the highest concentration of 1-deoxyxypinganine tested, further indicating that 5 µM 1-deoxyxypinganine is cytotoxic for both dividing and quiescent cells, while lower lipid concentrations are cytostatic. In addition, treatment for only one hour, followed by washout and subsequent 23 h incubation was sufficient to reduce the metabolic activity of the cells comparably to a continuous 24 h incubation with 1-deoxyxypinganine (Fig. 1F), suggesting a rapid effect of the lipid.

1-deoxyxypinganine triggers p21-mediated senescence and multiple cell death pathways in Ins-1 cells

We then investigated whether the reduction of replication following low 1-deoxyxypinganine concentration is mediated by induction of senescence. 1 µM 1-deoxyxypinganine increased β-galactosidase activity (Fig. 2A) and nuclear p21WAF1/Cip1 expression (Fig. 2B, C), a hallmark (16) and inducer of senescence (17), respectively. To investigate if increased p21 expression was sufficient to trigger the senescence pathway in Ins-1 cells, we used adenovirus infection overexpressed p21 (Adp21) or GFP (AdGFP) as a control (Fig 2D). Adp21 infection decreased Ins-1 cell replication and increased β-galactosidase activity, while both parameters were unchanged following AdGFP incubation (Fig 2E, F). In addition, the increased β-galactosidase
activity following 1 µM 1-deoxysphinganine treatment or Adp21 infection was accompanied by increased MTT reduction/cell (Fig. S2), suggesting increased mitochondrial dehydrogenase activity, a parameter associated with cellular senescence (18). These data suggest that up-regulation of p21 induced by 1 µM 1-deoxysphinganine treatment contributes to the decreased Ins-1 replication by activating a senescence program.

Next, we further explored the cytotoxic effect of high 1-deoxysphinganine concentrations. Five µM 1-deoxysphinganine abrogated the expression of p21 and induced cells with condensed pyknotic nuclei and high levels of activated caspase-3, hallmarks for the execution phase of apoptosis (Fig. 3A, S3A). In addition, FACS analyses of cells co-stained with annexin V and propidium iodide (PI) revealed that 5 µM 1-deoxysphinganine treatment increased the amount of both apoptotic and necrotic cells (Fig. 3B, C, S3B), and induced the cells to arrest in the G0/G1 phase of the cell cycle (Fig. S4), suggesting that the lipid triggers multiple cell death pathways in Ins-1 cells.

1-deoxysphinganine intracellular metabolites contribute to cytotoxicity in Ins-1 cells

As treatment with exogenous 1-deoxy-dihydroceramides (m18:0,24:1 and m18:0,16:0) and 1-deoxy-methylsphinganine, where alanine is replaced by glycine, reduced the cell replication similarly to 1-deoxysphinganine treatment (Fig. 4A), we then tested whether exogenous 1-deoxysphinganine was also metabolized by the cells to the deoxy forms of ceramide. Incubation with 1-deoxysphinganine significantly increased the cellular levels of deoxy-dihydroceramide with different acyl chain length (Fig. 4B) without altering normal ceramide levels (Fig. S5A), and selectively up-regulated the expression of ceramide synthase 5 (Fig. 4C), suggesting that 1-deoxysphinganine is readily metabolized in Ins-1 cells and accumulates in the acylated form. In addition, pre-treatment of Ins-1 cells with the class 2 amphiphile U-18666A, a well-established
inhibitor of NPC1 (Niemann–Pick C) protein that prevents intracellular trafficking of sphingolipids and cholesterol (19; 20), partially rescued 1-deoxysphinganine-mediated cytotoxicity (Fig. 4D, S5B). Moreover, pharmacological inhibition of ceramide synthesis with fumonisin B1 was unique in reducing the toxicity of 5 µM 1-deoxysphinganine (Fig.4D, S5C), while inhibition of either the first step of sphingolipid synthesis with myriocin or glucosylceramide synthesis with 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) had no effect on cell viability (not shown). Collectively, these data indicate that intracellular uptake followed by metabolic conversion to 1-deoxy-dihydroceramides is responsible, at least in part, for 1-deoxysphinganine toxicity.

1-deoxysphinganine increases the phosphorylation of selected kinases in Ins-1 cells

To further characterize the biochemical components of 1-deoxysphinganine-induced cytotoxicity, we analyzed the phosphorylated status of key proteins involved in major signaling pathways. Western blot quantification revealed increased phosphorylation of JNK and p38 MAPK in 1-deoxysphinganine treated Ins-1 cells. In addition, the ratio of AKT phosphorylation was unchanged upon lipid treatment but the total protein amount increased in presence of 1-deoxysphinganine (Fig. 5A-C). As kinase activation by phosphorylation suggests a possible role of these proteins in 1-deoxysphinganine-mediated phenotype, we tested this hypothesis by treating the cells with specific kinase inhibitors. Pre-treatment with the p38 MAPK inhibitor Birb796 partially rescued cell replication (Fig. 5D) and reduced 1-deoxysphinganine-induced senescence (Fig. 5E). Conversely, the JNK inhibitor SP600125 potentiated cytotoxicity and senescence (Fig. 5D, E), and the combined treatment partially rescued the JNK inhibitor effect at 1 µM 1-deoxysphinganine incubation. These data suggest that p38 MAPK activation is an effector of 1-deoxysphinganine cytotoxicity and senescence, while JNK activation plays a protective role in Ins-1 cells.
1-deoxysphinganine treatment promotes re-organization of the actin cytoskeleton in Ins-1 cells

The observed changes in cell morphology observed following 1-deoxysphinganine treatment (Fig. 1C) prompted us to further analyze whether the lipid induced cytoskeletal alterations. To detect early cytoskeletal re-arrangements, cells were imaged after 5 h of treatment. Phalloidin-stained actin filaments were mainly concentrated in the cortical area and in filopodia following control BSA or sphinganine treatment. However, upon 1-deoxysphinganine incubation, actin staining accumulated in punctated structures mainly located in the peri-nuclear area of the cells. Different from the actin phenotype, tubulin staining showed a similar microtubule pattern in all treatments, suggesting that 1-deoxysphinganine preferentially interferes with the organization of actin cytoskeleton in Ins-1 cells (Fig. 6A). Quantitative analysis of members of the Rho family of GTPases that regulate intracellular actin dynamics (reviewed in (21)), showed increased levels of Rac1 activity (Fig. 6B) and expression of Rac1 and Rho, albeit the latter did not reach significance, upon 1-deoxysphinganine treatment (Fig. 6C), suggesting that Rho family GTPases may be involved in 1-deoxysphinganine-mediated changes in actin cytoskeleton re-organization. Of note, actin alterations were not accompanied by apoptosis at this time point (data not shown). In addition, pre-treatment with JNK, p38 MAPK and ceramide synthase inhibitors did not prevent actin remodeling (Fig. S6A), suggesting that cytoskeletal and cell cycle effects are modulated by different signaling pathways.

To further characterize whether aberrantly localized actin may intersect with the secretory apparatus of the cells, we performed co-staining for actin and insulin. FACS-based single cell quantification revealed that the lipid treatment did not alter the cellular content of actin and insulin (Fig. S6B). However, confocal analyses showed that filamentous actin accumulated
intracellularly in close proximity to and partially co-localized with insulin-containing vesicles (Fig. 6D).

**1-deoxysphinganine reduces metabolic activity, insulin secretion and modulates actin cytoskeleton in primary islets**

To confirm the relevance of our results in primary cells, we tested whether 1-deoxysphinganine affects the functionality of isolated islets. Lipid delivery to the cells was improved by dissociation of 1400 islets from 6 Wistar rats into single cells, these were seeded in ECM-coated plates and treated for 24 h with 5 µM sphinganine, 1-deoxysphinganine, or BSA as control. Of note, dissociated islets plated on ECM are virtually quiescent (R. Zuellig, personal communication). Under these experimental conditions, 1-deoxysphinganine treatment induced cellular vacuolization (Fig. 7A), and reduced the metabolic activity (Fig. 7B) and, to a lesser extent, the number of live cells (Fig. 7C). However, senescence was not observed, as indicated by comparable βgal activities (Fig. S7A) and absence of p21 staining (not shown). Similarly to the effect in Ins-1 cells, 1-deoxysphinganine treatment induced re-arrangement of actin cytoskeleton with the resulting accumulation of actin in intracellular punctated structures (Fig. 7D). 1-deoxysphinganine did not alter the cellular content of insulin (Fig. S7B) or selectively reduce the number of insulin-producing cells (Fig. S7C). However, following incubation with the lipid, isolated β cells were unable to regulate insulin secretion in response to glucose stimulation (Fig. 7E, S7D). Collectively, these data indicate that 1-deoxysphinganine is cytotoxic to isolated primary islets and compromises both functionality and cyto-architecture of β cells.

**Increased glucose levels potentiate 1-deoxysphinganine toxicity**

Our *in vitro* results showed 1-deoxySL-mediated cytotoxicity in insulin-producing cells, suggesting that raised levels of these lipids may contribute to the failure of β-cells during the
development of diabetes. However, elevated 1-deoxySLs levels were also found in the plasma of patients with metabolic syndrome that did not present hyperglycemia and overt diabetes (5), raising the question that increased amount of 1-deoxySLs are not the sole cause of β-cell toxicity and that additional factors contribute to the diabetic phenotype. To further investigate the causal network of atypical sphingolipids and β-cell toxicity, we analyzed pancreatic islets in leptin deficient ob/ob mice. Kept on a normal chow diet, these mice develop obesity and a mild hyperglycemia that reverts with aging, as pancreatic β-cell compensation occurs and increased insulin levels improve glucose homeostasis (22; 23). 60 week old ob/ob mice had only slightly higher plasma glucose levels compared with age matched WT animals, but a robust increase in plasma HDL, cholesterol and ALT levels (Fig. S8A), the latter reflecting steatotic liver damage (Fig. S8B). As previously shown (23), ob/ob pancreata were characterized by pronounced islet hyperplasia, vascularization and robust insulin production (Fig. S8B), suggesting that β-cells could compensate for the increased insulin demand without reaching exhaustion. Despite the evident hyperplasia, islets did not show active replication at the analyzed age (Fig. S8B). Interestingly, quantification of the plasma lipid profiles in ob/ob mice revealed a moderate but significant increase of 1-deoxysphinganine levels (Fig. 8A), which was not associated with increased senescence or apoptosis (Fig. S8B). In addition, like in human samples (5), sphingosine was the most abundant species in the mouse plasma and significantly increased in ob/ob mice (Fig. S8C). While it is likely that plasma 1-deoxySLs did not reach a critical concentration threshold to induce β-cell failure, absence of other pathological parameters, including hyperglycemia, may account for the normal viability of β-cells. To test this hypothesis, we incubated Ins-1 cells with 1-deoxysphinganine in presence or absence of glucose. Treatment with 30mM glucose for 24 h potentiated 1-deoxysphinganine-induced toxicity (Fig. 8B, S8D),
suggesting that hyperglycemia and 1-deoxySLs synergize to induce glucolipotoxicity in insulin-producing cells.

**Discussion**

Elevated levels of 1-deoxySLs have been found in the blood of patients suffering from metabolic syndrome and diabetes mellitus (5; 6), raising the question of the role of atypical lipids in the development of these pathologies. Our results showed that exposure to 1-deoxySLs in the low \( \mu \text{M} \) range compromised insulin secretion and triggered senescence and cell death in insulin-producing cells, indicating that 1-deoxySLs are indeed toxic for these cells. In our experimental approach we elucidated 1-deoxysphinganine toxicity at three distinct levels, namely i) changes in cellular structure, ii) engagement of signaling molecules and iii) activation of effector proteins. A major finding of our study is that 1-deoxysphinganine-mediated toxicity is a complex phenomenon and triggers multiple pathways, including cytoskeletal remodeling, senescence, necrosis and apoptosis.

*1-deoxysphinganine triggers the re-organization of actin cytoskeleton in insulin-producing cells*

1-deoxysphinganine treatment selectively altered cytoskeleton organization both in Ins-1 cells and primary islets, inducing accumulation of filamentous actin in intracellular punctated structures juxtaposed to insulin-containing vesicles. Similar but transient actin fiber alterations have been observed previously in 1-deoxysphinganine-treated Vero cells (7). In addition, 1-deoxysphinganine impaired cytoskeleton dynamics in sensory and motoneurons without inducing cell death (10). Our study showed not only that the lipid induced actin rearrangements before the appearance of apoptotic markers, but also that inhibitors shown to mitigate cytotoxicity did not prevent cytoskeletal remodeling. These data suggest that cytoskeleton rearrangements are a direct effect of 1-deoxysphinganine incubation rather than a consequence of cell lethality and that
cytoskeleton and cell cycle effects are regulated by distinct signaling pathways. In this context, it is currently under investigation whether alterations in Rho GTPase activation, reported by (7) and our study, are the key conserved molecular mechanism also behind cytoskeletal alteration in neurons.

In the case of β-cells, the early cellular morphological alterations may impair cellular functionality, including insulin secretion. Indeed, earlier studies demonstrated that insulin secretion in pancreatic β-cells is coupled with re-organization of the filamentous actin web located beneath the plasma membrane, thus allowing docking of insulin-containing granules to the cell membrane and consequent secretion. Importantly, glucose stimulation directly induces re-arrangement of the actin web (24; 25), to which insulin-containing granules are in tight contact (26). Thus impaired remodeling of actin cytoskeleton resulting from 1-deoxysphinganine treatment may contribute to the defective insulin secretion observed in our primary β-cells cultures. However, we cannot exclude that additional lipid-induced changes, including altered gene transcription, may contribute to the phenotype.

*1-deoxy-dihydroceramide contributes to 1-deoxysphinganine-induced cytotoxicity*

Ceramide is a key intracellular signaling molecule involved in several cellular functions, including cell death (reviewed in (27)). Importantly, both cell-permeant analogues of ceramide (28) and *de novo* ceramide synthesis (29) impair insulin secretion and mitogenesis in pancreatic β-cells and induce apoptosis (reviewed in (30)), supporting a critical regulatory role for ceramide in the metabolic dysfunction of these cells. In the search for the molecular signaling generated by 1-deoxysphinganine treatment, we explored the hypothesis that 1-deoxysphinganine uptake and conversion to 1-deoxy-dihydroceramide is necessary to exert its toxicity. Our mass spectrometry and inhibitor analyses supported this hypothesis. In addition, 1-deoxysphinganine increased the expression of ceramide synthase 5 (CerS5), while β-cell lipotoxicity resulting from palmitate
treatment stimulated the synthesis of CerS4 (31), suggesting that different lipids stimulate specific CerS isoforms. Collectively, our results suggest that some of the cytotoxic effects of 1-deoxysphinganine occur after its intracellular uptake and metabolism to 1-deoxy-dihydroceramide. However, we cannot exclude that 1-deoxysphinganine triggers death receptors on the cell surface. Given the similarity of the molecular structure of 1-deoxysphinganine and sphingosine, it is worthy of further investigation to determine if 1-deoxysphinganine can engage or antagonize the same membrane receptors, as previously suggested (8).

**1-deoxysphinganine activates multiple intracellular pathways**

In addition to cytoskeletal remodeling, 1-deoxysphinganine treatment induced a complex dose-dependent pattern of toxicity in Ins-1 cells, characterized by the appearance of p21-induced senescence at low doses and apoptosis and necrosis at high doses. Of note, the senescence growth arrest was limited to replicating cells, as demonstrated previously (32-34), and quiescent primary islets were devoid of senescence markers upon lipid treatment. These data imply that 1-deoxysphinganine triggers multiple signaling pathways. Indeed, the lipid was shown to selectively activate JNK, MAPK, Erk1/2, PKC but not AKT in NIH-3T3, RH-7777, PC-3 and LNCaP cell lines (8; 9), while we showed that JNK, p38 MAPK phosphorylation and AKT levels increased in Ins-1 cells, indicating that the intracellular signaling effectors stimulated by 1-deoxysphinganine depend on the cellular context. As JNK and MAPK are known to be activated by intracellular ceramide (35), it is possible that the increased 1-deoxy-dihydroceramide synthesis observed in Ins-1 cells activates these kinases. In addition, the fact that the CerS inhibitor FB1 was the only compound able to rescue the toxicity of high 1-deoxysphinganine doses, suggests that increased ceramide (or its deoxy form) synthesis is upstream of or a prerequisite for the activation of different signaling effectors.
While a more in depth analysis of the set of kinases activated by 1-deoxysphinganine is needed to elucidate the precise downstream signaling cascade, our inhibitor studies revealed an intriguing antagonistic role of JNK and p38 MAPK in the context of 1-deoxysphinganine-induced senescence. Interestingly, a similar dose-dependent senescence-apoptosis transition and opposite effect of p38 MAPK and JNK on senescence has been reported in endothelial progenitor cells upon doxorubicin treatment (36), further confirming that different kinases activated by the same stimulus may exert opposite cellular effects.

In conclusion, our work shows that 1-deoxysphinganine treatment and its conversion to 1-deoxy-dihydroceramide compromises the viability of insulin-producing cells via multiple pathways. This indicates that, similar to free fatty acids (reviewed in (30)), 1-deoxySLs induce lipotoxicity but with higher efficiency (low µM for 1-deoxySLs versus low mM for free fatty acids (31; 37; 38)). However, our in vivo study, together with the fact that HSAN1 and metabolic syndrome patients have elevated 1-deoxysphinganine levels without overt diabetes, suggests that the raised amount of 1-deoxysphinganine observed in vivo is not sufficient to directly induce β-cell failure but would require additional pathological parameters, for instance an established chronic hyperglycemic state, to promote β-cell toxicity (39). In this context, targeting 1-deoxySL synthesis as a combination therapeutic strategy for diabetes mellitus warrants further investigations.

Acknowledgments

We thank Heidi Seiler for excellent technical assistance and Amedeo Caflisch for providing the Birb796 inhibitor. This research was supported by the Zurich Center for Integrative Human Physiology (ZIHP) and Rare Disease Initiative Zurich (radiz), Clinical Research Priority Program
for Rare Diseases, University of Zurich. No potential conflicts of interest relevant to this article were reported. All the authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. R.A.Z., T.H., A.O., A.B.H., E.S., K. G., H.B., Y.W., T.G., O.O.O., J-H.J., U.U. researched data and reviewed/edited manuscript, A.v.E., R.G. contributed to discussion, reviewed/edited manuscript. S.S. wrote manuscript, researched data. S.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References


**Figure legends**

**Figure 1.** Deoxysphingolipids decrease the replication of Ins-1 cells. A. Metabolic activity tested by MTT assay of Ins-1 cells treated at 50% (low density, L) and 90% (high density, H) confluence with sphinganine (SA) and 1-deoxysphinganine (dSA) at the indicated concentrations and incubated for 24 h. B. Enumeration of live cells treated with the indicated dSA concentrations for 24 h. Note that the number of cells treated with 1 µM dSA is comparable to the initial seeding density. C. Bright field images showing cell rounding up upon 24 h dSA treatment. D. Quantification of trypan blue positive cells after treatment with the indicated concentrations of dSA for 24 h. Data are expressed as percentage of total cell number. E. Quantification of LDH released in the medium after 24 h dSA treatment. Data are normalized to the number of cells. F. MTT assay of Ins-1 cells treated with dSA for the indicated time and assessed 24 h after adding the lipid. Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 µm.

**Figure 2.** 1-deoxysphinganine triggers senescence in Ins-1 cells. A. Quantification of β-galactosidase activity following incubation for 24 h with 1 µM sphinganine (SA), 0.5 and 1 µM 1-deoxysphinganine (dSA), or BSA as control (cntl). Values are normalized to the number of live cells. B. Immunofluorescence imaging showing nuclear expression of p21 following incubation for 24 h with 1 µM SA, 1 µM dSA, or BSA as control (cntl). Nuclei are stained with DAPI (blue). Lower panels show nuclear localization of p21. C. Western blot and densitometric quantification of p21/GAPDH levels following incubation for 24 h with 1 µM SA, 1 µM dSA or BSA as control. D. Immunostaining of p21 (upper panels) or live imaging (lower panel) of Ins-1 cells infected with p21 (Adp21) and GFP (AdGFP) adenoviruses at multiplicity of infection.
(MOI) of 50. E. Enumeration of live Ins-1 cells 24 h after Adp21 or AdGFP infection. Note how Adp21 decreased the replication of Ins-1 cells in a dose response manner without inducing cell death. F. Quantification of β-galactosidase activity 24 h after Adp21 or AdGFP infection. Note how senescence is induced only in presence of Adp21. Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 µm.

**Figure 3.** 1-deoxysphinganine triggers apoptosis and necrosis in Ins-1 cells. A. Left panel. Quantification of cleaved caspase 3 (CC-3) and p21 positive cells after 24 h incubation with 5 µM sphinganine, 1 and 5 µM 1-deoxysphinganine (dSA) or BSA as control. Right panel. Immunofluorescence imaging showing cytosolic expression of cleaved caspase 3 (CC-3). Note the pyknotic nuclei in CC-3 positive cells (arrows). Nuclei are stained with DAPI (blue). Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 µm. B. FACS analyses of Ins-1 cells after 24 h incubation with 1 and 5 µM dSA or BSA as control and staining with PI and annexin V. C. Quantification of cells PI/ annexin V negative (live cells), PI positive (necrotic cells), annexin V positive (apoptotic cells) and double positive. Total population consisted of 20’000 cells. Note the increased lethality of cells treated with 5 µM 1-dSA.

**Figure 4.** 1-deoxy-dihydroceramide contributes to 1-deoxysphinganine-induced cytotoxicity in Ins-1 cells. A. MTT assay of Ins-1 cells incubated for 72 h with sphinganine (SA), sphingosine (SO), 1-deoxysphinganine (dSA), deoxy-methylsphinganine (dmethSA), deoxy-dihydroceramides (1-deoxy-dh-Cer m18:0,24:1 and m18:0,16:0). B. Mass spectrometry quantification shows the increased formation of 1-deoxy-dh-Cer with different acyl chain length upon 24 h treatment with 3 µM 1 dSA. SA, SO and BSA incubation were used as control. C. RNA expression levels of different ceramide synthase (CerS) isoforms following lipid incubation as in B. Transcript levels were normalized using GAPDH RNA as a reference. D. Enumeration
of live cells following incubation for 24 h with dSA in presence or absence of 5 μM U-18666A or 35 μM fumonisin B1 (FB1). Results are average ± SEM (n=3), *p<0.05.

**Figure 5.** 1-deoxysphinganine increases the phosphorylation of selected kinases in Ins-1 cells. A. Western blot of phosphorylated and total levels of JNK, p38 and AKT following incubation for 24 h with 1 μM sphinganine (SA), 1 μM 1-deoxysphinganine (dSA) or BSA as control. B. Densitometric quantification of protein phosphorylation/total protein/actin levels. Data are expressed as percentage of control (cntl). C. Densitometric quantification of total protein/actin levels. Data are expressed as percentage of control (cntl). D. Enumeration of live cells treated with the indicated dSA concentrations for 24 h in presence of 10 μM of inhibitors of p38 MAPK (Birb796, Birb), JNK (SP600125, SP) or a combination of Birb796 and SP600125 (B/SP). Cells were pre-treated for 1 hour with the indicated inhibitors before addition of 1-deoxySA. E. Quantification of β-galactosidase activity following incubation for 24 h with 1 μM dSA in presence of 10 μM of inhibitors of p38 MAPK (Birb796, Birb), JNK (SP600125, SP) or a combination of Birb796 and SP600125 (B/SP). Values are normalized to the number of live cells. Results are average ± SEM (n=3), *p<0.05.

**Figure 6.** 1-deoxysphinganine incubation induced actin cytoskeleton rearrangements. A. Ins-1 cells treated with 5 μM 1-deoxysphinganine (dSA), sphinganine (SA) or BSA as control for 5 h and stained with phalloidin (green, left panels) or anti-tubulin antibody (red, right panels). Nuclei are stained with DAPI (blue). Note the actin staining in punctated structures following dSA treatment (arrows in left panel). In the right panels, arrows indicate tubulin midbody in recently divided cells and arrowhead the mitotic spindle. Scale bars: 50 μm. B. Rac1 activity following 5 h treatment with 5 μM dSA, 5 μM SA or BSA as control. NC, negative control. Constitutively active Rac1 (RCCA) was used as a positive control. C. Western blotting quantification of Rac1
and RhoA expression upon 24 h treatment with 1 µM dSA, 1 µM SA or BSA as control. Results are average ± SEM (n=3), *p<0.05. D. Confocal images of Ins-1 cells treated with 5 µM dSA or BSA as a control for 5 h and stained with phalloidin (green) or anti-insulin antibody (red). Nuclei are stained with DAPI (blue). Signal intensities representing the voxel space of the reconstructed image stacks are depicted as scatterplots showing partial signal overlap of actin and insulin in cntl and dSA-treated samples. Robust overlap of actin and β-catenin signals (dots accumulated in the mid-diagonal of the plot) and limited overlap of actin and DAPI (dots preferentially distributed along the axes) were used as a control. Nuclei are stained with DAPI (blue). Scale bars: 10 µm.

**Figure 7.** 1-deoxysphinganine treatment is cytotoxic for rat primary islets. Dissociated islets were plated on ECM plates and treated for 24 h with 5 µM sphinganine (SA), 5 µM 1-deoxysphinganine (dSA), and BSA as control. A. Quantification of vacuolized cells following lipid treatment. Data are expressed as percentage of total cell number. Right panels: bright field images of treated cells showing cell vacuolization (arrows and inset) upon dSA treatment. B. Metabolic activity tested by MTT assay. Data are expressed as percentage of BSA-treated control cells. C. Enumeration of live cells following lipid treatment. D. Quantification of insulin secretion stimulated with 3.3 mM (Low I), 16.7 mM (High) and 3.3 mM (Low II) glucose following incubation for 24 h with the lipids. Secreted insulin was normalized to the cell number. E. Filamentous actin (phalloidin, green) and insulin (red) co-staining of treated islets. Nuclei are stained with DAPI (blue). Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 µm.

**Figure 8.** A. Mass spectrometry quantification of 1-deoxysphinganine (dSA) serum levels of 60 week old wild type (WT) and ob/ob mice. Results are average ± SEM (n=5), *p<0.05. B. Enumeration of live Ins-1 cells treated with the indicated dSA concentrations for 24 h in presence (Gluc) or absence (Cntl) of 30 mM glucose. Results are average ± SEM (n=3), *p<0.05.
Fig. 1
Fig. 2

203x222mm (300 x 300 DPI)
Fig. 4

204x231mm (300 x 300 DPI)
Fig. 5
Fig. 6
Fig. 7

197x223mm (300 x 300 DPI)
Fig. 8
Supplementary Online Material

List of antibodies

Primary antibodies used for immunostaining were: Alexa Fluor 594 phalloidin (Life Technologies), anti-α–tubulin (Cell Signaling), anti-Rac1 (Abcam), anti-cleaved caspase-3 (Cell Signaling), anti-p21 (Abcam), anti-insulin (Dako), anti-glucagon (Sigma), fluorescein-conjugated Annexin V (BD Pharmingen). Fluorophore-conjugated secondary antibodies were from Alexafluor. Nuclei were visualized with 4’, 6-diamidino-2-phenylindole (DAPI).

Primary antibodies used for western blotting were: anti-p21 (Abcam), anti-GAPDH (Santa Cruz), anti-RhoA (Santa Cruz), anti-Rac1 (BD Biosciences), anti-phospho JNK and anti-JNK (R&D System), anti-phospho p38MAPK and anti-p38MAPK, anti-phospho AKT and anti-AKT (all from Cell Signaling) and anti-β-actin (Sigma). Secondary HRP-conjugated antibodies were from Jackson ImmunoResearch.

Ceramide synthase primers for qPCR

Rat CerS1: rat CerS1_LC_F: AGTGCCTGGAAGCTTCTGTT,
rat CerS1_LC_R: GACCTCCAGCCG TAGAAGAC

Rat CerS2: rat CerS2_LC_F: TTGAGGAAAGTTTGGGAAGG,
rat CerS2_LC_R: AAACCAGGAAGCAGAGGA

Rat CerS3: rat CerS3_LC_F: TGCCACACCTCTAGCCAATG,
rat CerS3_LC_R: CCTGGCGCTCTGTCAAGTTA

Rat CerS4: rat CerS4_LC_F: TCACTCTGCCCTTTGACATC,
rat CerS4_LC_R: TGACGCTGTAGGAGAAGACG

Rat CerS5: rat CerS5_LC_F: CACACAGCTGGCCTTCTACT,
rat CerS5_LC_R: ACTCGCACCATGTGTTGAT
Rat CerS6: CerS6_F: GGGATCTTAGCCTGGTTCTGG,
CerS6_R: GCCTCCTCCGTGGTCTTCAG
Rat(Mouse)GAPDH: rat GAPDH_LC_F: GGTCGGTGTGAACGGATTTG,
rat GAPDH_LC_R: TTGCCGTGGGTAGAGTCATA

**Figure legends**

**Figure S1.** A. Comparison of Ins-1 metabolic activity by MTT and WST-1 reduction following 24 h incubation with 1-deoxysphinganine (dSA) at the indicated concentrations. BSA incubation was used as a control. Results are average ± SEM (n=3). B. Expression profile of genes involved in different cell death pathways following 1 μM dSA compared to 1 μM sphinganine incubation by Rat Cell Death Pathway Finder PCR Array (SABiosciences). 1 μM 1-deoxysphinganenine treatment moderately up-regulated genes involved in necrosis, autophagy and apoptosis (Olr1583, Ctss, Esr1, Casp1, Fas), while anti-apoptotic genes (Bel2a1d, Tnfrsf11b) were down-regulated. C. Analysis of extracted RNA integrity by Agilent 2100 Bioanalyzer. Examples of electrophoresis runs and assigned RNA integrity number (RIN) are shown. D. List of genes present in the array.

**Figure S2.** Analysis of MTT reduction /cell number following 24 h of 1-deoxysphinganine (dSA) treatment (A) or Adp21 and AdGFP infection (B). Data are expressed as percentage of control. Results are average ± SEM (n=5), *p<0.05.

**Figure S3.** 1-deoxysphinganine triggers apoptosis in Ins-1 cells. A. Co-staining with cleaved caspase 3 (CC-3, red) and p21 (green) showing the mutually exclusive expression of the proteins upon incubation for 24 h with 5 μM sphinganine, 1 and 5 μM 1-deoxysphinganenine or BSA as control. Nuclei are stained with DAPI (blue). Scale bars: 50 μm. B. FACS based quantification of
dead cells following 24 h incubation with 1 and 5 µM dSA or BSA as control as determined by FSC and SSC parameters shown in Fig. 3.

**Figure S4.** Cell cycle analysis by quantification of DNA content in Ins-1 cells after 24 h incubation with 1 and 5 µM 1-deoxysphinganine (dSA) or BSA as control. The amount of cells in different cell cycle phases is plotted in the lower panel. Total population consisted of 20’000 cells. Note the decreased amount of cells in G2/M phase and accumulation in G0/G1 phase following 5 µM 1-deoxysphinganine treatment.

**Figure S5.** A. Mass spectrometry quantification of ceramides in Ins-1 cells treated for 24 h with 3 µM of sphinganine (SA), sphingosine (SO), 1-deoxysphinganine (dSA) and BSA as control. B. MTT assay following incubation for 24 h with SA or dSA in presence or absence of 5 µM U-18666A (U). C. Bright field images of Ins-1 cells showing reduced toxicity following 5 µM dSA treatment in presence of FB1. Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 µm.

**Figure S6.** A. Ins-1 cells were treated with 10 µM of inhibitors of JNK (SP600125, SP), p38 MAPK (Birb796, Birb) or 35 µM ceramide synthase inhibitor FB1 for 1 h, followed by 5 h treatment with 5 µM 1-deoxysphinganine (dSA), sphinganine (SA) or BSA and phalloidin staining. Nuclei are stained with DAPI (blue). Scale bars: 50 µm. B. FACS-based quantification of actin and insulin cellular content following 5 h treatment with 5 µM dSA, SA or BSA.

**Figure S7.** Dissociated islets were plated on ECM plates and treated for 24 h with 5 µM sphinganine (C18SA), 1-deoxysphinganine (dSA), and BSA as control. A. Quantification of β-galactosidase activity normalized by the cell number. B. Quantification of cellular insulin content. Cells were lysed and total insulin level was normalized by the cell number. C. Enumeration of insulin-expressing cells. Data are expressed as percentage of total cell number.
D. Quantification of insulin secretion stimulated with 3.3 mM (Low I), 16.7 mM (High) and 3.3 mM (Low II) glucose following incubation for 24 h with the lipids. Secreted insulin was normalized by the total insulin content. Results are average ± SEM (n=3), *p<0.05.

Figure S8. A. Weight and blood parameters of WT and ob/ob mice. B. Histological morphology (HE staining) of paraffin embedded liver and pancreas sections from WT and ob/ob mice. Note the accumulation of lipid deposit and steatotic phenotype in the latter. Staining of insulin/glucagon, replicating (Ki67), senescent (p21) and apoptotic cells (TUNEL) in islets of 60 week old wild type (WT) and ob/ob mice. Nuclei are stained with DAPI (blue). Scale bars: 50 µM. D. Mass spectrometry quantification of serum levels of total sphingoid bases after hydrolyzing the head group and N-linked fatty acid. Results are average ± SEM (n=5), *p<0.05. E. MTT assay of Ins-1 cells incubated for 24 h with 1-deoxysphinganine in presence (Gluc) or absence (Cntl) of 30 mM glucose. Results are average ± SEM (n=3), *p<0.05.
Fig. S1

A

Metabolic activity (% of cntl) vs dSA concentration (µM)

B

Magnitude of log2

C

RNA expression levels

D

Well Gene symbol Well Gene symbol
A01 A01 2001 2002
A02 A01 2002 2003
A03 A01 2003 2004
A04 A01 2004 2005
A05 A01 2005 2006
A06 A01 2006 2007
A07 A01 2007 2008
A08 A01 2008 2009
A09 A01 2009 2010
A10 A01 2010 2011
A11 A01 2011 2012
A12 A01 2012 2013
B01 B01 2013 2014
B02 B01 2014 2015
B03 B01 2015 2016
B04 B01 2016 2017
B05 B01 2017 2018
B06 B01 2018 2019
B07 B01 2019 2020
B08 B01 2020 2021
B09 B01 2021 2022
B10 B01 2022 2023
B11 B01 2023 2024
B12 B01 2024 2025
C01 C01 2025 2026
C02 C01 2026 2027
C03 C01 2027 2028
C04 C01 2028 2029
C05 C01 2029 2030
C06 C01 2030 2031
C07 C01 2031 2032
C08 C01 2032 2033
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C10 C01 2034 2035
C11 C01 2035 2036
C12 C01 2036 2037
D01 D01 2037 2038
D02 D01 2038 2039
D03 D01 2039 2040
D04 D01 2040 2041
D05 D01 2041 2042
D06 D01 2042 2043
D07 D01 2043 2044
D08 D01 2044 2045
D09 D01 2045 2046
D10 D01 2046 2047
D11 D01 2047 2048
D12 D01 2048 2049

Fig. S1
Fig. S2
**Fig. S3**

A

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B

- **Live (P1)**
- **Dead (P2)**

Cell number (% of tot)

- Cntl
- 1 μM dSA
- 5 μM dSA

**Fig. S3**
Fig. S4
Fig. S5
Fig. S6
Fig. S7
**A**

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TG, triacylglycerol; HDL, high density lipoprotein; Chol, cholesterol; ALT, alanine aminotransferase. Values are expressed as means ± SEM (N=7)

**B**

![HE liver](image1)

WT

ob/ob

**C**

![Lipid serum levels](image2)

**D**

![Metabolic activity](image3)

*Fig. S8*