Hyaluronan synthase-2 upregulation protects smpd3-deficient fibroblasts against cell death induced by nutrient deprivation, but not against apoptosis evoked by oxidized LDL

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A R T I C L E   I N F O

Article history:
Received 26 November 2014
Received in revised form 10 December 2014
Accepted 12 December 2014
Available online 16 December 2014

Keywords:
Neutral sphingomyelinase-2
Nutrient starvation
Hyaluronan synthase-2
Oxidized LDL
Apoptosis
Hsp72

A B S T R A C T

The neutral type 2 sphingomyelinase (nSMase2) hydrolyzes sphingomyelin and generates ceramide, a major bioactive sphingolipid mediator, involved in growth arrest and apoptosis. The role of nSMase2 in apoptosis is debated, and apparently contradictory results have been observed on fibroblasts isolated from nSMase2-deficient fragilits ossium (homozygous fro/fro) mice. These mice exhibit a severe neonatal dysplasia, a lack of long bone mineralization and delayed apoptosis patterns of hypertrophic chondrocytes in the growth plate. We hypothesized that apoptosis induced by nutrient deprivation, which mimics the environmental modifications of the growth plate, requires nSMase2 activation. In this study, we have compared the resistance of fro/fro fibroblasts to different death inducers (oxidized LDL, hydrogen peroxide and nutrient starvation). The data show that nSMase2-deficient fro/fro cells resist to apoptosis evoked by nutrient starvation (fetal calf serum/glucose/pyruvate-free DMEM), whereas wt fibroblasts die after 48 h incubation in this medium. In contrast, oxidized LDL and hydrogen peroxide are similarly toxic to fro/fro and wt fibroblasts, indicating that nSMase2 is not involved in the mechanism of toxicity evoked by these agents. Interestingly, wt fibroblasts treated with the SMase inhibitor GW4869 were more resistant to starvation-induced apoptosis.

The resistance of fro/fro cells to starvation-induced apoptosis is associated with an increased expression of hyaluronan synthase 2 (HAS2) mRNAs and protein, which is inhibited by ceramide. In wt fibroblasts, this HAS2 rise and its protective effect did not occur, but exogenously added HA exhibited a protective effect against starvation-induced apoptosis.

The protective mechanism of HAS2 involves an increased expression of the heat-shock protein Hsp72, a chaperone with antiapoptotic activity. Taken together, these results highlight the role of nSMase2 in apoptosis evoked by starvation nutrition that could contribute to the delayed apoptosis of hypertrophic chondrocytes in the growth plate, and emphasize the antiapoptotic properties of HAS2.

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Introduction

Sphingomyelinases are a family of enzymes implicated in the catabolism of sphingomyelin, a major sphingolipid present in cellular membranes, rafts and caveolae [1,2]. Several sphingo-

References

[3–6] A variety of factors, such as nature of the stressors, stress duration, cell and tissue specificity, subcellular localization and metabolism of ceramide can influence its biological effects [6,7]. Ceramide can be catabolized by ceramidases into sphingosine, which can be converted by sphingosine kinases into sphingosine 1-phosphate (S1P), another sphingolipid mediator exhibiting survival and mitogenic properties [6,8].

Several evidences indicate that the balance ceramide/S1P (ceramide/S1P rheostat) is an important determinant of cell fate towards survival or apoptosis depending on the ability of cells to generate S1P from ceramide [9]. The neutral sphingomyelinase 2 (nSMase2), encoded by the smpd3 gene, is a redox-sensitive [6] enzyme that plays a key role in ceramide generation upon

mediator involved in cell growth arrest, apoptosis, autophagy and cell differentiation [3–6]. A variety of factors, such as nature of the stressors, stress duration, cell and tissue specificity, subcellular localization and metabolism of ceramide can influence its biological effects [6,7]. Ceramide can be catabolized by ceramidases into sphingosine, which can be converted by sphingosine kinases into sphingosine 1-phosphate (S1P), another sphingolipid mediator exhibiting survival and mitogenic properties [6,8].

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Abbreviations: nSMase2, neutral sphingomyelinase type 2; HAS2, hyaluronan synthase 2; HA, hyaluronan; LDL, low density lipoprotein
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http://dx.doi.org/10.1016/j.redox.2014.12.004
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stimulation by stress-inducing agents, including hydrogen peroxide ($H_2O_2$) and oxidized LDL [10–13]. We have recently reported that nSMase2 activation by low $H_2O_2$ concentrations mediates the proliferation and migration of smooth muscle cells (SMC) and fibroblasts, via a ceramide-dependent sequential signaling cascade implicating the activation of src, and the subsequent phosphorylation and activation of the PDGF receptor, that is implicated in the activation of sphingosine kinase and the generation of S1P [12]. In contrast, high oxidative stress inhibits sphingosine kinase and triggers apoptotic cell death [14].

The expression of nSMase2 is high in the brain and bones [15,16]. Mice knockout for nSMase2 and nSMase2-deficient fro/fro mice (homozygous $smpd3fro/smpd3fro$ with fragilis ossium phenotype) exhibit bone deformations and neonatal growth retardation [16–18]. A number of studies have been carried out on cells and tissues isolated from these mice, to decipher the physiological role of nSMase2 and its implication in apoptosis evoked by cell death inducers. We recently reported that mutant fibroblasts isolated from fro/fro mouse undergo apoptosis similarly to wild fibroblasts, when exposed to stress-inducing agents such as cytokines (TNF-α), $H_2O_2$ or oxidized LDL [11]. Likewise, the hepatotoxicity resulting from TNF-α injection to mice, is similar in fro/fro and wild type mice, indicating that the nSMase2 mutation does not confer any resistance to these acute stress-inducing agents [11]. However, other studies, including experiments done on fro/fro cells, indicate an apoptotic role for nSMase2 [19,20]. Recently Kavandhgar et al. [21] reported that the defect in bone mineralization in fro/fro mouse is associated with an accumulation of hypertrophic chondrocytes in the growth plate and a reduced number of TUNEL positive cells, indicating a defective apoptosis, which is necessary for bone mineralization [22]. These reports point out the complicated role of nSMase2 in apoptosis, which may differ function of the state of development, the tissue specificity and the nature of the stress inducer. The terminal apoptotic differentiation of hypertrophic chondrocytes, is necessary for bone mineralization and involves environmental modifications, including nutrient deprivation [23], which is a known sphingolipid pathway trigger [24]. The present study was carried out to decipher whether nSMase2 is involved in cell death induced by nutrient starvation and to characterize the mechanisms of resistance evoked by nSMase2 mutation in fro/fro cells.

### Materials and methods

#### Chemicals and reagents

Anti-HAS2 mouse monoclonal antibody (sc-365263) was from Santa Cruz Biotechnology (Texas, USA); anti-hsp72/73 mouse antibody was from Calbiochem (Merck Millipore, United Kingdom). Anti Akt-phospho (Ser473) rabbit was from Cell Signalling. Secondary antibodies anti-mouse and anti-rabbit were from Cell Signalling Technology (Denver, USA), SYTO-13, propidium iodide, alexa-Fluor 488 (green) and Alexa-Fluor 546 (red)-conjugated secondary antibody were from Invitrogen, Cergy-Pontoise, France. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), GW4869, KN437 were from Sigma-Aldrich. RPMI 1640, DMEM, fetal calf serum (FCS) were from Invitrogen (France). Dulbecco’s Modified Eagle Medium (DMEM) without $p$-glucose and Sodium pyruvate was from Gibco/Life Technology (Paisley, United Kingdom). Hyaluronan (High Molecular Weight > 950 kDa) was from RD System (Minneapolis, USA). C2-ceramide was from Biomol, Laboratory Research. Acrylamide-4 × bisacrylamide-2 × solution was from Euromedex (Souffleursheim, FR). The ECL chemiluminescence kit was from Amersham Pharmacia (Velizy-Villacoublay, France).

#### Cell culture

Primary cultures of fibroblasts were obtained by skin biopsies from newborn control and fro/fro mice. Briefly, skin samples were minced and put in Petri dishes, dermis facing down. After 15 min of dry contact with the dishes, DMEM culture medium containing 10% FCS penicillin, streptomycin, amphotericin A was added, and the skin preparation was cultured at 37 °C, 5% CO2. After 1–3 weeks, cells growing around the tissue pieces were expanded.

Control (wt) or fro/fro fibroblasts were grown in DMEM Glutamax culture medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) in a 5% CO2 humidified incubator, at 37 °C. At sub-confluence, this medium was removed and replaced by serum/glucose/pyruvate-free DMEM (nutrient-starvation conditions), or by serum-free RPMI-1640 containing oxidized LDL (200 μg/ml), or $H_2O_2$ (100 μM), for 48 h.

#### Animals

The genetic background of fro/fro and wt mice was 129/SV. Homozygous mice, harboring a truncating mutation in nSMase2 and fragilis ossium (fro) phenotype were genotyped by PCR, as previously described [17], using the following primers: 5'-GCCGCGAGCCATGTATAGTA-3', 5'-CTCAATGGAGGCACACAG-3' and 5'-CAGGTITTAGGACCCGAC-3'.

#### TUNEL assay

For detecting apoptosis in cells and tissues, we used the ApopTag® In Situ Apoptosis Detection Kit (Millipore). Apoptotic cells were detected by labeling and by modifying DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT). Total and apoptotic cells were counted after immunoperoxidase and DAPI staining.

#### Real time quantitative PCR assay

TRI Reagent RT (Molecular Research Center) was added to cell pellets for RNA extraction according to the manufacturer’s instructions. RNA was quantified with Xpose (Trinean). One microgram of RNA was used for reverse transcription with a high-capacity cDNA reverse transcription kit (Applied Biosystems/Life Technologies). Fast SYBR green master mix (Applied Biosystems/Life Technologies) and the ABI StepOne+ real-time PCR system (Applied Biosystems) were used to evaluate mRNA levels according to the manufacturer’s recommendations. The following primers were used: For HAS2, forward, 5'-GAACTCTCCTCACGACCC-3' and reverse 5'-GCACCGTACAGTCAAATGAG-3'. For Beclin 1, forward 5'-AATCTAGAGTGGCTTATAC-3' and reverse 5'-CCAGTGCTTCAATCTGGCC-3'; for LC3b, forward, 5'-ATTGGTGTCCGCCAATGTCTC-3' and reverse 5'-CCTCCTGGCAACAAGGACT-3'; for HNRT, forward 5'-TTGCTTAGTTGATGAAAGA-3' and reverse 5'-CCAGCCGGTCGAAAGAATT-3'. Incubation was 95 °C for 20 s, followed by 40 cycles of 3 s at 95 °C and annealing/extension for 30 s at 60 °C. Each sample was done in duplicate and data were analyzed using StepOne+ software version 2.3. Expression was normalized to HPRT.
Cell viability and apoptosis

The cell viability was evaluated by the MTT assay [11]. Apoptotic/necrotic cells were counted by fluorescence microscopy after staining by fluorescent DNA intercalating agents SYTO-13 and propidium iodide (PI). Cells grown in 6-multiwell plates were incubated with permeant DNA intercalating green fluorescent probe SYTO-13 (0.6 μM) and the non-permeant DNA intercalating red fluorescent probe PI (15 μM), using an inverted fluorescence microscope (Fluovert FU, Leitz). Intact, apoptotic and necrotic cells were characterized on the basis of their morphological features.

Fig. 1. Resistance of fro/fro fibroblasts to apoptosis induced by serum starvation, but not to oxidized LDL and H2O2. Sub-confluent fibroblasts from fro/fro (fro) or wt mice (wt) were incubated for 48 h in standard medium DMEM containing 10% FCS, 4.5 g/l glucose, pyruvate and GlutaMAX (ref. 61965-026, Life Technologies) (compl) or in serum/glucose/pyruvate-free DMEM culture medium containing L-glutamine (ref. 11966-025, Life Technologies) (nutrient deprivation condition) (starv, stripped bars), or in serum free RPMI, supplemented with oxidized LDL (200 μg apoB/ml) (oxLDL) or H2O2 (100 μM). At the end of 48 h incubation, cell viability was evaluated by the MTT assay (A).

(B) Counting of apoptotic vs living cells after staining with Syto13/PI fluorescent probes which allow to distinguish between living cells (green bars), and apoptotic cells (primary apoptosis and post-apoptotic necrosis) (orange bars for wt, red bars for fro/fro fibroblasts). Apoptotic cells were counted, and are expressed as percent of the unstimulated control. C, Representative pictures of syto13/PI-stained wt and fro/fro fibroblasts. (D) TUNEL positive wt and fro/fro fibroblasts. Cells were incubated for 48 h in serum-free medium, and stained with the ApopTag In Situ Apoptosis Detection Kit (Millipore), and counterstained with DAPI. The number of TUNEL positive cells is expressed as percent of the total cell number counted as DAPI stained fibroblasts. (E) Western-blot showing the degradation of procaspase-3, indicative of its activation. These data are a mean ± SEM of 5 separate experiments, *p < 0.05.
Western blot analysis

Cultured cells were washed and scrapped in PBS, then disrupted at 4 °C in the extraction buffer (20 mM HEPES, 1 mM EDTA, 1 mM Na3V04, 250 mM sucrose, 5 μM digitonin, 1 mM DTT, and 1 mM PMSF) for 15–30 min on ice. Cell extracts were centrifuged at 12,000g for 15 min (Beckman Optima) and the supernatant was used for Western-blot experiments. Protein concentration was determined using the Bradford reagent (Biorad). 50 μg of protein cell extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes (Millipore). Then membranes were probed with the primary antibodies and revealed with secondary antibodies coupled to horseradish peroxidase using the ECL chemoluminescence kit (Amersham). β-actin was used to control equal loading of proteins.

SiRNA transfection

Murine fibroblasts were transfected with murine HAS2 or control siRNA using OptiMEM (Invitrogen) and HiPerFect reagent according to the manufacturer’s recommendations.

Statistical analysis

Data are given as mean ± SEM. Estimates of statistical significance were performed by One Way Anova followed by multiple comparison analysis by Holm-Sidak method (SigmaStat software). Values of p < 0.05 were considered significant.

Results

nSMase2 and Cer are involved in the apoptotic process triggered by nutrient starvation, but not by hydrogen peroxide or oxidized LDL; fro/fro fibroblasts resist to cell death induced by nutrient deprivation

Wt and fro/fro fibroblasts were exposed to toxic concentrations of oxidized LDL (200 μg apoB/ml), hydrogen peroxide (H2O2, 100 μM) in serum-free RPMI (5 g/l glucose) or to nutrient deprivation in (glucose/pyruvate/serum)-free DMEM culture medium, for 48 h. As shown in Fig. 1A, cytotoxicity experiments, using the MTT assay, indicates that the cytotoxic effect of oxidized LDL or H2O2 is comparable in wt and in fro/fro cells (Fig. 1B and C). TUNEL staining (Fig. 1D) and procaspase 3 cleavage (Fig. 1E), confirmed that nSMase2-deficient fro/fro fibroblasts are more resistant to nutrient starvation induced-apoptosis than wt fibroblasts. It is to note that i/ the serum-free RPMI medium, in which oxidized LDL and H2O2 were added, was not toxic to wt fibroblasts for the period of the experiment, indicating that glucose and pyruvate starvation is the main trigger of cell death in wt fibroblasts, and ii/ oxidized LDL were toxic for fro/fro fibroblasts in the nutrient-free culture medium (data not shown).

The role of nSMase2 in cell death induced by nutrient starvation was confirmed by the effect of the sphingomyelinase inhibitor GW4869, which prevented (or delayed) the apoptotic effect of nutrient deprivation of wt fibroblasts (Fig. 2A). In contrast, GW4869 had no effect on cell death evoked by oxidized LDL in agreement with previously reported data [11]. In the same way, the resistance of fro/fro fibroblasts was reversed by the addition of the permeant C2-ceramide (5 μM) to the nutrient-starved medium (less than 25% cell viability) (Fig. 2B). No toxicity of C2-ceramide was observed in complete culture medium (Fig. 2B).

Altogether, these data indicate that i/ nSMase2 and ceramide are involved in cell death of wt fibroblasts evoked by nutrient starvation, but not by oxidized LDL, ii/ nSMase2-deficient fro/fro fibroblasts resist to nutrient-starvation induced cell death, but not to oxidized LDL.

Autophagy is not involved in the resistance to apoptosis induced by nutrient starvation in fro/fro fibroblasts

Autophagy and apoptosis processes are often associated, either through a cross inhibitory signaling, or sometimes through interconnected pathways [25,26]. Generally, autophagy is a survival system that blocks the induction of apoptosis, whereas activated caspases inhibit the autophagic process [26]. Moreover, ceramide is a potent autophagy inducer [27], via a downregulation of nutrient transporters [28]. This led us to investigate whether Cer generated by nSMase2 plays a role in autophagy induced by nutrient deprivation and in the mechanism of resistance of fro/fro fibroblasts.

As reported in Fig. 3, in fro/fro and wt cells, nutrient starvation elicited a strong conversion of LC3-I into LC3-II and an increase of Beclin-1 expression, both parameters being characteristic markers of autophagy activation. In contrast, the autophagic machinery was not activated by oxidized LDL, in fro/fro and wt cells (Fig. 3A and B). No difference was observed between fro/fro and wt fibroblasts, in the induction of autophagy parameters by nutrient starvation, this indicating that autophagy evoked by nutrient starvation does not require the nSMase2 activity.

Finally, autophagy markers are similarly induced by nutrient deprivation in wt and fro/fro cells, while these cells exhibit a great difference in their resistance to apoptosis induced by starvation. Thus, it may be concluded that, in this model, i/ ceramide is not required for autophagy marker induction by nutrient deprivation, ii/ autophagy alone plays no major role in the resistance of fro/fro cells to nutrient deprivation.
Hyaluronan synthase 2 (HAS2) and hyaluronan mediate the resistance to apoptosis induced by nutrient deprivation in fro/fro fibroblasts

Fibroblasts from fro/fro mouse secrete high amounts of hyaluronan (HA), due to an increased expression and activity of the hyaluronan synthase 2 (HAS2), resulting from PP2A inhibition and Akt phosphorylation [29]. As HA and HAS2 protect fibroblasts against environmental stress-induced apoptosis [30], we checked whether HA, HAS2 and Akt are involved in the resistance of fro/fro cells to apoptosis induced by nutrient starvation.

As expected, HAS2 expression was much higher in fro/fro fibroblasts than in wt fibroblasts (Fig. 4A and B), in agreement with Qin et al. [29], but HAS2 expression was not (or only slightly) dependent on culture conditions, since HAS2 was high in fro/fro cells grown in complete medium and in nutrient starvation conditions, while HAS2 of wt fibroblasts was low in both culture media (Fig. 4A and B). HAS2 expression was dependent on Cer generated by nSMase2, as shown by treatment with C2-ceramide (5 μM) that decreased HAS2 expression in fro/fro fibroblasts (Fig. 4B).

We then checked whether HAS2 plays a role in the mechanism of fro/fro fibroblast resistance to nutrient deprivation. HAS2-specific siRNA transfected in fro/fro fibroblasts reduced the expression of HAS2 and decreased the resistance of fro/fro cells to apoptosis evoked by starvation (Fig. 4C and D). Likewise, methylumbelliferone (MU), a classical HAS2 inhibitor (1 mM), reversed the resistance of fro/fro fibroblasts to apoptosis induced by nutrient starvation (Fig. 4D).

Conversely, the addition of HA to wt fibroblasts significantly improved their resistance to apoptosis induced by nutrient starvation (Fig. 5A–C). However, HA did not protect wt fibroblasts against cell death evoked by oxidized LDL, in accordance to the lack or resistance of fro/fro fibroblasts to oxidized LDL toxicity.

Finally, these data suggest that the increased resistance of fro/fro cells to nutrient starvation results from the rise of HAS2 expression and subsequent increased synthesis of HA induced by the deficiency of Cer generated by nSMase2 (deficient in fro/fro cells).

Hsp72 is involved in the protective effect of HAS2 in fro/fro fibroblasts

Previous report from Xu et al. [31] had shown that synovial cell death evoked by stress conditions (including serum starvation) in an arthritis model, is suppressed by hyaluronan via upregulation of stress-inducible heat-shock proteins of the HSP70 family. In agreement with this report, we found that Hsp72 expression is increased in fro/fro fibroblasts both in standard and nutrient-starved conditions (Fig. 6A). In contrast, Hsp72 expression was strongly decreased in cells transfected with the HAS2-specific siRNA, thus confirming that Hsp72 expression in fro/fro fibroblasts depends on HA and HAS2 (Fig. 6B). The protective role of Hsp72 was supported by the effect of the pharmacological Hsp70 inhibitor KNK437, which reversed the resistance of fro/fro fibroblasts to apoptosis evoked by serum starvation. No increased expression of Hsp72 was observed in wt fibroblasts and in fro/fro fibroblasts incubated with oxidized LDL (data not shown).

Taken together, these data indicate that HAS2 expression and HA secretion in fro/fro fibroblasts, resulting from Akt activation, are protective against cell death evoked by nutrient starvation.

Discussion

In this article, and as summarized in the graphical abstract, we show that nSMase2 is involved in apoptosis evoked by nutrient starvation, and this is protected in fro/fro fibroblasts mutant for nSMase2, via an increased expression of HAS2 and of Hsp72.

Role of nSMase2 in apoptosis induced by nutrient starvation

A first important point is that nSMase2 is involved in apoptosis induced by nutrient starvation, but not by oxidized LDL or H₂O₂. A number of studies have been focused on the apoptotic signaling of nSMase2, with controversial responses depending on the cell type, or the nature of the stressors [6]. We recently reported that nSMase2-deficient fro/fro mice, characterized by a strong neonatal growth retardation [17], do not resist to TNFα-induced hepatotoxicity, and fibroblasts isolated from these mice, do not resist to apoptosis evoked by oxidized LDL or TNFα ([11] and present article), suggesting that nSMase2 is not involved in acute stress-induced cell death. Here we show that fro/fro fibroblasts resist to cell death evoked by nutrient starvation in contrast to wt fibroblasts. Apoptotic cell death of wt cells is evidenced by the increased number of TUNEL positive cells, and by the morphological features of apoptosis observed using Syto13/PI staining, all these parameters being reduced in fro/fro fibroblasts. These data point out the different mechanisms of apoptosis evoked by nutrient starvation (protected in fro/fro fibroblasts) or by oxidized LDL (not protected in these cells). We previously reported that ceramide is not involved in apoptosis induced by oxidized LDL in endothelial cells, SMC and fibroblasts [32,33], which mainly depends on the deregulation of cytosolic calcium and the subsequent activation of the intrinsic mitochondrial apoptotic pathway [34,35]. In contrast, nSMase2 activation by oxidized LDL, and subsequent ceramide

Fig. 3. Autophagy markers are similarly up-regulated in fro/fro and wt fibroblasts by nutrient deprivation. Fibroblasts were incubated for 48 h under nutrient starvation condition, or with oxidized LDL, as reported in the legend to Fig. 1. (A) Western-blot experiments showing the expression of Beclin-1 and the conversion of LC3-I to LC3-II, in complete medium (C), nutrient deprivation (Dp) and oxidized LDL (oxL) conditions. B. qPCR experiments showing the expression of LC3b and beclin-1, normalized to HPRT. The results are mean ± SEM of 3 separate experiments. *p < 0.05.
generation, are involved in SMC proliferation, via the activation of a signaling cascade leading to the activation of sphingosine kinase-1 and the generation of the survival and mitogenic sphingolipid mediator, S1P [12]. Indeed, fro/fro fibroblasts and nSMase2-silenced SMC do not proliferate in the presence of oxidized LDL [11].

In contrast, our data show that in our model, the mechanism of apoptosis evoked by nutrient starvation involves nSMase2 since i) fro/fro fibroblasts mutant for this nSMase2, resist to apoptosis, ii) the addition of GW4869, an inhibitor of neutral SMases, protects wt fibroblasts against cell death induced by nutrient privation, but not that induced by oxidized LDL, iii) the addition of C2-ceramide to fro/fro fibroblasts, reverses their resistance to cell death. Thus it can be hypothesized that ceramide released via the degradation of sphingomyelin by nSMase2, elicits apoptosis in nutrient-starved conditions.

Our data show that autophagy activation is not efficient in fro/fro fibroblasts, thus is probably not involved in the mechanism of cell death mediated by nSMase2. Apoptosis induced by serum or nutrient starvation, is often associated to autophagy, which is a survival mechanism, able to mediate a non-apoptotic cell death, when apoptotic pathways are blocked [36]. Ceramide is a potent autophagic cell death inducer, via a downregulation of nutrient transporters [28], thus it was hypothesized that autophagy could be defective in fro/fro fibroblasts, in which the ceramide generation is reduced. However, our data do not show any defect in the activation of autophagy markers such as the conversion LC3-I to LC3-II, or beclin-1 mRNA and protein expression, which suggests that ceramide is either not involved in the autophagic process evoked by nutrient starvation in these fibroblasts, or is generated by another SMase, such as the acidic SMase which modulates autophagy in several pathophysiological models for Alzheimer’s disease, steatosis or atherosclerosis [37–39].

The resistance of fro/fro fibroblasts to nutrient starvation involves HAS2 increased expression

HAS2 is highly expressed in fro/fro fibroblasts, leading to an increased secretion of HA in the extracellular medium [29]. HAS2

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**Fig. 4.** HAS2 expression is dependent on nSMase2/Cer and is involved in the resistance of fro/fro to nutrient deprivation-induced apoptosis. (A) Western blot of HAS2 in wt and fro/fro fibroblasts grown in complete medium (compl) and nutrient deprivation conditions (depriv). (B) HAS2 mRNA expression evaluated by q-PCR experiments, and normalized to HPRT, in fro/fro and wt fibroblasts grown in complete medium (compl), nutrient deprivation conditions (depriv). The effect of C2-ceramide (C2Cer, 5 µM), was tested in fro/fro fibroblasts, as indicated. (C) Western blot of HAS2 in fro/fro fibroblasts grown in complete medium and treated with scrambled siRNA or with specific HAS2 siRNA (upper panel) or in complete (compl) or nutrient deprivation (depriv) medium with or without methylumbelliferone (MU, 1 mM) (lower panel). (D) After 48 h incubation, the cytotoxicity was evaluated by the MTT test. Data are mean ± SEM of 5 separate experiments, *p < 0.05.
Nutrient deprivation + HA

In the same way, the addition of HA protects against apoptosis evoked by environmental stress, such as serum starvation [30]. High molecular weight HA are antiapoptotic and protective in various pathophysiological conditions, such as human corneal epithelial cells [40], cornea protection and oxidative stress [41], trophoblasts [42], chondrocytes and synovial lesions [43,44]. We hypothesized that HAS2 contributes to the resistance of fro/fro fibroblasts in nutrient deprivation conditions.

In fro/fro cells, the high HAS2 expression depends on Akt activation, itself resulting from the decreased activity of PP2A, due to a reduced ceramide content [29]. Indeed, ceramide is a potent activator of PP2Ac/ceramide-activated protein phosphatases [45,46], which in turn inhibits Akt [47]. In fro/fro fibroblasts, the nSmase2/Cer deficiency in fro/fro cells prevents PP2A activation, thereby impeding Akt inactivation. Thus, Akt activation persists and supports the expression of HAS2 [29]. Under nutrient deprivation conditions, we found that HAS2 expression remains high in fro/fro fibroblasts. This high HAS2 expression is required for survival of fro/fro fibroblasts under deprivation conditions, as shown by the reversion of this resistance to deprivation by silencing of HAS2 by specific siRNAs and by inhibiting HAS2 with MU, in agreement with Wang et al. [30]. In the same way, the addition of HA protects in part wt fibroblasts against apoptosis induced by nutrient starvation. However this protective effect was not effective against the toxicity of oxidized LDL, which inhibited HAS2 expression in fro/fro fibroblasts.

**Hsp72 expression is involved in HAS2-induced resistance of fro/fro fibroblasts to nutrient starvation**

Heat-shock proteins (Hsps) are induced in response to various stressors including heat stress, toxic chemicals or modifications of cell environment, to suppress apoptosis [48]. HA are known to up-regulate the expression of heat-shock proteins from the Hsp70 family, particularly Hsp72, which may suppress cell degeneration and apoptosis in various models, such as canine arthritis [49], K562 cells exposed to hyperthermia and PC12 cells in serum deprivation conditions serum deprivation [31]. Our results show that Hsp72 expression is increased in fro/fro fibroblasts, and its inhibition, either by the specific pharmacological inhibitor KNK437, or by siRNA directed against HAS2, reversed the resistance of fro/fro cells, indicating that Hsp72 expression depends on HAS2 and is anti-apoptotic in nutrient-starved conditions, as reported [31].

In conclusion, the reported data show that nSmase2 is involved in cell death induced by nutrient deprivation, through a ceramide-dependent activation of PP2A that negatively regulates Akt activity, thereby reducing HAS2 and Hsp72 expression. In contrast, the data on fro/fro cells confirm that oxidized LDL-induced apoptosis occurs through ceramide-independent mechanisms, as previously reported [35,50–52]. Moreover, nutrient starvation triggers a robust increase of autophagy markers, which is independent of nSmase2/Cer and plays no major role in cell death induced by nutrient deprivation.

Finally, the functional link between nSmase2/Cer, Akt and HAS2 suggests that this pathway is involved in the protection against cell death induced by nutrient deprivation, by regulating not only the classical anti-apoptotic mechanisms mediated by Akt, but also the additional protective pathway mediated by HAS2, which is apparently required to prevent the apoptotic effect of nutrient deprivation.

**Acknowledgments**

The authors wish to thank M.H. Grazide and C. Bernis for their excellent technical assistance. This work was supported by...
**References**

[1] A.E. Cremesti, F.M. Goni, R. Kolesnick, Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Letters 531 (1) (2002) 47–53. http://dx.doi.org/10.1016/S0014-5793(02)03489-0

[2] C.J. Clarke, C.F. Snook, M. Tani, N. Matmari, N. Marchesini, Y.A. Hannun, The extended family of neutral sphingomyelinases, Biochemistry 45 (38) (2006) 11247–11256. http://dx.doi.org/10.1021/bi61037v.10881685.

[3] R. Kolesnick, Z. Fulek, Ceramide: a signal for apoptosis or mitogenesis? Journal of Experimental Medicine 181 (6) (1995) 1949–1952. http://dx.doi.org/10.1084/jem.181.6.1949.17759991.

[4] W.D. Jarvis, S. Grant, R.N. Kolesnick, Ceramide and the induction of apoptosis, Clinical Cancer Research 2 (1996) 1–6. 9816082.

[5] C.A. Hetz, M. Hunn, P. Rojas, V. Torres, L. Leyton, A.F. Quest, Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: onset of necrosis is associated with delayed ceramide increase, Journal of Cell Science 115 (23) (2002) 4671–4683. http://dx.doi.org/10.1242/jcs.00153. 12415011.

[6] Y.A. Hannun, L.M. Obeid, Principles of bioactive lipid signalling: lessons from sphingolipids, Nature Reviews Molecular Cell Biology 9 (2) (2008) 139–150. http://dx.doi.org/10.1038/nrm2329. 18216770.

[7] M. Nikolova-Karakashian, A. Karakashian, K. Rutkute, Role of neutral sphingomyelinases in aging and inflammation, Subcellular Biochemistry 49 (2008) 469–486. http://dx.doi.org/10.1007/978-1-4020-8831-5_18 18751823.

[8] M. Maceyka, K.B. Harikumar, S. Milstien, S. Spiegel, Sphingosine-1-phosphate signaling and its role in disease, Trends in Cell Biology 22 (1) (2012) 50–60. http://dx.doi.org/10.1016/j.tcb.2011.09.003. 22001186.

[9] O. Cuvillier, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, S. Gutkind, S. Spiegel, Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate, Nature 381 (6585) (1996) 800–803. http://dx.doi.org/10.1038/381800a0. 8657285.

[10] B. Liu, Y.A. Hannun, Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione, Journal of Biological Chemistry 272 (26) (1997) 16281–16287. http://dx.doi.org/10.1074/jbc.272.26.16281. 9195931.

[11] R. Devillard, S. Galvani, J.C. Thiers, J.L. Guenet, Y. Hannun, J. Bielawski, A. Nègre-Salvayre, R. Salvayre, N. Augé, Stress-induced sphingolipid signalling: role of type-2 neutral sphingomyelinase in murine cell apoptosis and proliferation, PLoS One 5 (3) (2010) e9826. http://dx.doi.org/10.1371/journal.pone.0009826. 20352118.

[12] C. Cinq-Frais, C. Coatrieux, M.H. Grazide, Y.A. Hannun, A. Nègre-Salvayre, R. Salvayre, N. Augé, A signaling cascade mediated by ceramide, src and FGRF1 coordinates the activation of the redox-sensitive neutral sphingomyelinase-2 and sphingosine kinase-1, Biochimica et Biophysica Acta 1831 (8) (2013) 1344–1356. http://dx.doi.org/10.1016/j.bbalip.2013.04.014. 23604957.

[13] P.P. Dotson, A.A. Karakashian, M.N. Nikolova-Karakashian, Neutral sphingomyelinase-2 is a redox sensitive enzyme: role of catalytic cysteine residues in regulation of enzymatic activity through changes in oligomeric state, Biochemical Journal (2014). http://dx.doi.org/10.1042/BJ20140665. 25287744.

[14] D. Pechetevski, O. Kunduzova, A. Dayon, D. Calise, M.H. Seguelas, N. Leducq, I. Seif, A. Parini, O. Cuvillier, Oxidative stress-dependent sphingosine kinase-1 inhibition...
mediates monoamine oxidase A-associated cardiac cell apoptosis, Circulation Research 100 (1) (2007) 41–48. http://dx.doi.org/10.1161/01.RES.0000239400.66661.34 17158540.
[15] K. Hofmann, S. Tomomura, C. Wolff, W. Stoffel, Cloning and characterization of the mammalian brain-specific, Mg2+–dependent neutral sphingomyelinase, Proceedings of the National Academy of Sciences of the United States of America 97 (11) (2000) 5895–5900. http://dx.doi.org/10.1073/pnas.100005997 102353.
[16] W. Stoffel, B. Jenke, B. Block, M. Zumbansen, J. Koebel, Neural sphingomyelinase 2 (smpd2) in the control of postnatal growth and development, Proceedings of the National Academy of Sciences of the United States of America 102 (12) (2005) 4524–4529. http://dx.doi.org/10.1073/pnas.0406380102 15764706.
[17] I. Aushin, P.C. Adams, S. Opsahl, D. Septier, C.E. Bishop, N. Auge, R. Salvayre, A. Negre-Salvayre, M. Goldberg, J.L. Guenet, C. Poirier, A deletion in the gene encoding sphingomyelin phosphodiesterase 3 (smpd3) results in osteogenics and dentino-genesis imperfecta in the mouse, Nature Genetics 37 (8) (2005) 803–805. http://dx.doi.org/10.1038/ng1603 16025116.
[18] W. Stoffel, B. Jenke, B. Holz, E. Binczek, R.H. Günter, J. Knifka, J. Koebel, A. Niehoff, Neural sphingomyelinase 2 (SMPD3) deficiency causes a novel form of chondrodysplasia and dwarfism that is rescued by Col2A1-driven smpd3 transgene expression, American Journal of Pathology 171 (1) (2007) 153–161. http://dx.doi.org/10.2353/ajpath.2007.06125S 17595162.
[19] S. Filosto, S. Castillo, A. Danielson, L. Franzi, K. Kenyon, J. Last, K. Pinkerton, M. Astudillo, J. Balsinde, P. Garcia-Rovés, M. Elena, I. Bergheim, S. Lotersztajn, C. Trautwein, H. Appelquist, A.W. Patton, J.C. Paton, M.J. Caza, N. Kaplowitz, J.C. Fernandez-Checa, C. García-Ruiz, ASMA regulates apoptosis and early stage non-alcoholic steatohepatitis. Journal of Hepatology 61 (5) (2014) 1126–1134. http://dx.doi.org/10.1016/j.jhep.2014.06.009 24946279.
[20] B. Fazi, D. Santin, P. Viel, A. Carasso, M. Calabrese, M. Nishimura, J.M. Kraveka, Z. Khavandgar, Y.A. Hannun, H. Nakauchi, J.E. Carter, X. He, E. H. Schuchman, J.S. Bae, Acid sphingomyelinase modulates the autophagic process by controlling lysosomal biogenesis in Alzheimer’s disease. Journal of Experimental Medicine 211 (8) (2014) 1551–1570. http://dx.doi.org/10.1084/jem.20122451 25408335.
[21] R. Fucho, L. Martínez, A. Báules, S. Torres, N. Tarrats, A. Fernandez, V. Ribas, A. M. Astudillo, J. Balsinde, P. Garcia-Rovés, M. Elena, I. Bergheim, S. Lotersztajn, C. Trautwein, H. Appelquist, A.W. Patton, J.C. Paton, M.J. Caza, N. Kaplowitz, J.C. Fernandez-Checa, C. García-Ruiz, ASMA regulates apoptosis and lysosomal membrane permeabilization and its inhibition prevents early stage non-alcoholic steatohepatitis. Journal of Hepatology 61 (5) (2014) 1126–1134. http://dx.doi.org/10.1016/j.jhep.2014.06.009 24946279.