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Original Paper

Oxidative Stress Triggers Ca^{2+}-Dependent Lysosome Trafficking and Activation of Acid Sphingomyelinase

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Key Words
Ceramide • Lysosomes • Acid sphingomyelinase • Vesicle • Fusion

Abstract
Recent studies demonstrate that rapid translocation of the acid sphingomyelinase (ASM), a lysosomal hydrolase, to the outer leaflet of the cell membrane and concomitant release of ceramide constitute a common cellular signaling cascade to various stimuli including CD95 ligation, UV-irradiation, bacterial and viral infections. Reactive oxygen species (ROS) were shown to play a crucial role in regulating this signaling cascade at least for some bacterial infections and UV-irradiation. However, the precise role of ROS for regulation of ASM is unknown. Here, by confocal microscopy and flow cytometry analysis, we demonstrate that hydrogen peroxide (H_2O_2), a primary form of ROS in mammalian cells, induces very rapid translocation of ASM and formation of ceramide-enriched membrane platforms in the plasma membrane of Jurkat T cells. In parallel, H_2O_2 triggers lysosome trafficking and fusion with the plasma membrane, i.e. lysosome exocytosis, as detected by exposure of a lysosome-associated protein, LAMP1. Depletion of intracellular Ca^{2+} by cell permeable EGTA-AM inhibits H_2O_2-induced lysosome exocytosis, ASM translocation and formation of ceramide-enriched platforms. Pharmacological inhibition or genetic deficiency of ASM did not affect H_2O_2-induced lysosome exocytosis. These results indicate that ROS-induced membrane translocation of ASM is mediated by exocytosis of lysosomes, which is dependent on intracellular Ca^{2+} release.

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Introduction

Acid sphingomyelinase (ASM) is a hydrolase that degrades sphingomyelin to ceramide and phosphocholine [1-3]. ASM localizes in conventional lysosomes or in specialized lysosomal compartments named secretory lysosomes, which store newly synthesized secretory proteins [4]. In humans, deficiency of ASM activity results in the lysosomal storage disorders Niemann-Pick disease types A and B (NPDA and NPDB) [5]. In NPDA, ASM activity is completely absent and cells accumulated with sphingomyelin are prominent in the central nervous system and throughout the reticuloendothelial system, leading to death in early childhood (less than 3 years) [4]. In NPDB, low levels of residual ASM activity prevent major neurological pathology, allowing survival into late childhood and adulthood. However, type B NPD individuals develop progressive reticuloendothelial system disease. Moreover, type B patients also have progressive pulmonary dysfunction and frequent respiratory infections that may lead to death [6-8].

ASM is activated in response to a variety of stimuli including CD95, DR5 ligation, UV-irradiation, bacterial and viral infections [6-10]. The precise mechanism for ASM activation remains elusive. In many cases, ASM activation is initiated by rapid translocation of the ASM to the outer leaflet of the cell membrane [9, 11]. ASM targets membrane sphingolipids-enriched microdomains, which contains around 70% of total membrane sphingomyelin [12]. ASM then hydrolyzes sphingomyelin and releases ceramide, a hydrophobic sphingolipid that spontaneously forms ceramide-enriched microdomains that fuse to larger ceramide-enriched domains [9, 13]. Ceramide-enriched membrane platforms serve as important signaling platforms [9, 13]. However, the mechanisms that mediate initial ASM activation require definition. Here, we focus on early events of ASM activation, i.e. how ASM externalization is regulated.

Oxidative stress refers to the imbalance of enhanced production of reactive oxygen species (ROS) and/or impaired function of the antioxidant system. ROS include superoxide anions (O_2^-), hydroxyl radicals, and hydrogen peroxide (H_2O_2). O_2^-, once generated, rapidly dismutates into H_2O_2 at low pH or catalyzed by superoxide dismutase (SOD). H_2O_2 can be further converted into highly reactive hydroxyl radicals via iron-catalyzed Fenton reaction under pathological conditions [14]. Since ROS are rapidly produced, short-lived and diffusible, ROS have been proposed as second messengers to mediate cellular responses to many stimuli such as cancer chemotherapeutic agents, UV, ionizing radiation, and TNF [6, 7, 15, 16]. Many of these stimuli also activate ASM and release ceramide in a variety of mammalian cells. Recent studies suggest that ROS generation is an upstream signal to trigger ASM translocation and activation. In U937 cells, ROS scavengers block UV-irradiation-induced ASM translocation, activation and ceramide-enriched membrane platform formation [6]. In splenocytes, TRAIL-induced ASM activation was blocked by ROS scavengers and antioxidants [17]. In macrophages, *Pseudomonas aeruginosa* infection results in ASM translocation and activation, ceramide release and formation of ceramide-enriched platforms, which are blocked by ROS scavengers and inhibitors of NADPH oxidase, a primary ROS generating enzyme [18]. In neutrophils, ROS regulate ASM-derived ceramide production, CD95 clustering and neutrophil apoptosis [19]. However, it is unknown how ROS regulate ASM activity and whether ROS are involved in ASM externalization.

Here, we demonstrate that H_2O_2, a primary form of ROS in mammalian cells, induces rapid translocation of ASM and formation of ceramide-enriched membrane platforms in the plasma membrane. In parallel, H_2O_2 triggers lysosome trafficking and fusion with the plasma membrane, i.e. lysosome exocytosis. Depletion of intracellular Ca^{2+} inhibits H_2O_2-induced lysosome exocytosis, ASM translocation and formation of ceramide-enriched membrane platforms. Thus, our data indicate that ROS promotes membrane lysosome trafficking of ASM, which is dependent on intracellular Ca^{2+} release.

Materials and Methods

Cells culture

Jurkat T cells (clone E6) were from ATCC. All cells were cultured in phenol red-free RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 uM nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin (all Life Technologies) and 50 µM beta-mercaptoethanol.

Flow cytometry

To detect surface ASM or lysosome-associated protein 1 (LAMP1) by flow cytometry, cells were stimulated with H_2O_2 (1 mM) at 37°C, washed with ice-cold PBS supplemented with 1% FCS and 0.1% NaN_3 and blocked for 20 min with the same buffer. Cells were washed and Fc receptors were neutralized by incubation with an irrelevant rabbit IgG (20 µg/mL, Sigma) for 45 min prior to addition of the primary antibodies. Cells were washed again and incubated for 45 min with 1 µg/mL of a polyclonal anti-ASM antibody or a monoclonal PE-conjugated anti-LAMP1 antibodies (both from Santa Cruz Biotech. Inc.) in PBS, 1% FCS and 0.1% NaN_3. For analysis of ASM, cells were re-washed and labeled for 45 min with 1 µg/mL of $F(ab')_2$ fragments of Cy3-coupled donkey anti-goat antibodies (Jackson ImmunoResearch Inc.), washed in PBS and subjected to flow cytometry analysis using a BD-Calibur (BD Biosciences). For LAMP1 staining, cells were re-washed in PBS and directly subjected to flow cytometry.

Fluorescence microscopy

Lymphocytes were washed in HEPES/Saline (H/S, 132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$ and 0.8 mM $MgSO_4$), resuspended in the same buffer, rested for 10 min at 37°C and stimulated with H_2O_2 for indicated time periods. The stimulation was stopped by addition of 1 mL ice-cold PBS with 1% FCS, cells were then washed in PBS and fixed in 1% PFA (w/v) in PBS (pH 7.3) for 15 min. Samples were washed in PBS and blocked with 1% FCS in PBS, 1% NaN_3 for 20 min to reduce unspecific antibody binding. Cells were then washed in PBS and incubated for 45 min at 4°C with mouse anti-ceramide antibodies (1: 50 dilution, Glycobiotech) and/or goat anti-ASM antibodies (500 ng/mL) in PBS, 1% FCS and 0.1% NaN_3. Control samples were incubated with an irrelevant murine IgM, respectively (both from Sigma). Samples were washed three times in PBS and stained for 45 min with Cy3-coupled $F(ab')_2$ fragments of anti-mouse IgM antibodies and/or FITC-labeled $F(ab')_2$ fragments of donkey anti-goat antibodies (all from Jackson ImmunoResearch Inc.) in PBS, 1% FCS and 0.1% NaN_3. Cells were again washed and immobilized on glass cover slips coated with 1% (v/v) poly-L-lysine for 10 min. The cover slips were embedded in Moviol and viewed with a Leica TCS NT scanning confocal microscope. Clustering was defined as one or several intense spots of fluorescence on the cell surface, whereas unstimulated cells displayed a homogenous distribution of the fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in samples of 200 cells was scored by two independent observers. The results are given as percentage of cells showing a cluster.

Isolation of detergent resistant membranes (DRMs)

Sphingolipids-enriched microdomains are biophysically characterized as DRM fractions, which have lower density compared with detergent soluble membrane fractions. The DRM fractions were isolated by sucrose gradient centrifugation as described previously [9]. Briefly, 10^7 cells were lysed in 0.6 mL MES buffer (25 mM morpholinoethane sulfonic acid, 150 mM NaCl, 1 mM EDTA, pH = 6.5) with a cocktail of protease inhibitors (Roche) and 1% Triton X-100. Cell extracts were homogenized by 5 passages through a 25G needle and incubated on ice for 30 min. 0.6 mL homogenates were then adjusted with 60% sucrose to 40% sucrose and overlaid with 1.8 mL 35% sucrose and then 1.8 mL 5% sucrose. Samples were centrifuged at 100,000 × *g* for 18 h at 4°C (rotor: MLS50, Beckman). The gradient was divided into 6 fractions and 0.9 mL/fraction from top to bottom. Fractions 1-3 were mixed and designated as light fractions (enriched in DRMs). Heavy fractions 4-6 were mixed and designated as heavy fractions (none-DRMs).

Immunoblot analyses

10^6 cells were lysed in Laemmli sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 60 mM Tris/HCl, 0.001% Bromophenol Blue, pH 6.8). Samples were sonicated for 15-20 sec and boiled for 5 min at

95°C. The protein samples were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The blots were blocked in 4% BSA in Tris-buffered Saline-supplemented with 0.1% Tween 20 and incubated overnight at 4°C with anti-ASM or anti-Actin antibodies (both in 1:1000 dilution in 4% BSA/PBS and from Santa Cruz Biotech. Inc.). To determine the purity of DRM fractions after sucrose gradient isolation, 20 µl sample of light and heavy fractions (as described above) were applied for SDS-PAGE and the presence of flotillin-1, a marker protein for DRM fractions, was examined using anti-flotillin-1 antibodies (Santa Cruz Biotech. Inc., 1:1000 dilution in 4% BSA/PBS). Blots were labeled with appropriate alkaline phosphatase-coupled secondary antibodies (Santa Cruz Biotechnology Inc.) and developed using the Tropix system (Bedford Inc.).

Acid sphingomyelinase activity assay

Activity of the acid sphingomyelinase in light and heavy fractions was measured as previously described [17]. Briefly, after sucrose gradient isolation, 100 µl sample from light or heavy fractions were mixed with 100 µl reaction buffer containing 100 mM sodium acetate (pH 5.0), 0.2% Triton X-100 and a cocktail of protease (Roche) and phosphatase inhibitors (Sigma). The reaction mixture was incubated with 0.02 µCi [^{14}C]-sphingomyelin for 30 min at 37°C. [^{14}C]-sphingomyelin as a substrate for ASM was dried prior to use and resuspended in 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100 followed by 10 min bath sonication to promote the formation of micelles. The reaction was stopped by addition of 1 mL of $CHCl_3$:CH_3OH (v/v). Phases were separated by 5 min centrifugation at 14,000 rpm and an aliquot of the aqueous phase was applied for liquid scintillation counting. Hydrolysis of [^{14}C]-sphingomyelin by ASM results in release of [^{14}C]-choline chloride into the aqueous phase, whereas ceramide and intact [^{14}C]-sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]-choline chloride (pmol/10^7 cells/h) serves to determine the activity of the ASM.

Isolation of murine spleen T-cells

Murine spleen T cells were obtained from sphingomyelin phosphodiesterase 1-deficient (*Smpd1*$^{-/-}$) mice lacking Asm activity or normal wild-type mice as previously described [7]. Briefly, single cell suspension of splenocytes was obtained by mechanical disruption of the spleen, followed by Ficoll gradient centrifugation (Histopaque, Sigma) to remove erythrocytes and cellular debris. Untouched murine spleen T-cells were enriched by depleting splenocytes with MHC II MicroBeads by MACS (Miltenyi Biotec. Inc.). The cells were maintained in RPMI-1640 supplemented as described above. *Smpd1*$^{-/-}$ mice (on a C57BL/6 background) were kindly provided by Dr. R. Kolesnick (Memorial Sloan-Kettering Cancer Center, NY, USA); syngenic wild-type mice were from the same heterozygous breeding.

Results

ROS induce ASM translocation and platform formation in Jurkat Cell

We first investigated whether hydrogen peroxide (H_2O_2) induces ASM externalization in the membrane of human Jurkat T cells. The results reveal a very rapid externalization of ASM (Fig. 1A and 1C) and ceramide clustering in the membrane of human Jurkat T cells (Fig. 1B) upon treatment with H_2O_2. Quantitative analysis shows that ~50% of all cells formed ceramide-enriched membrane platforms within 10 min treatment (Fig. 1D). Since the optimum response of ASM externalization was obtained at 10 min, the same treatment was used in all experiments of the present study if not otherwise mentioned.

ROS-induced ASM translocation and activation in ceramide-enriched lipid raft microdomains

As shown in Fig. 2A, stimulation with H_2O_2 had no effect on ASM expression level in Jurkat T cells. To investigate the spatial relation between ASM and ceramide, Jurkat cells were stimulated with H_2O_2 and live cells were stained with FITC-labeled anti-ASM and Cy3-labeled anti-ceramide antibodies. Confocal microscopy revealed that ASM co-localized in ceramide-enriched membrane platforms after H_2O_2 stimulation (Fig. 2B). Further, we determined the activity of ASM in isolated sphingolipids-enriched microdomains after subcellular

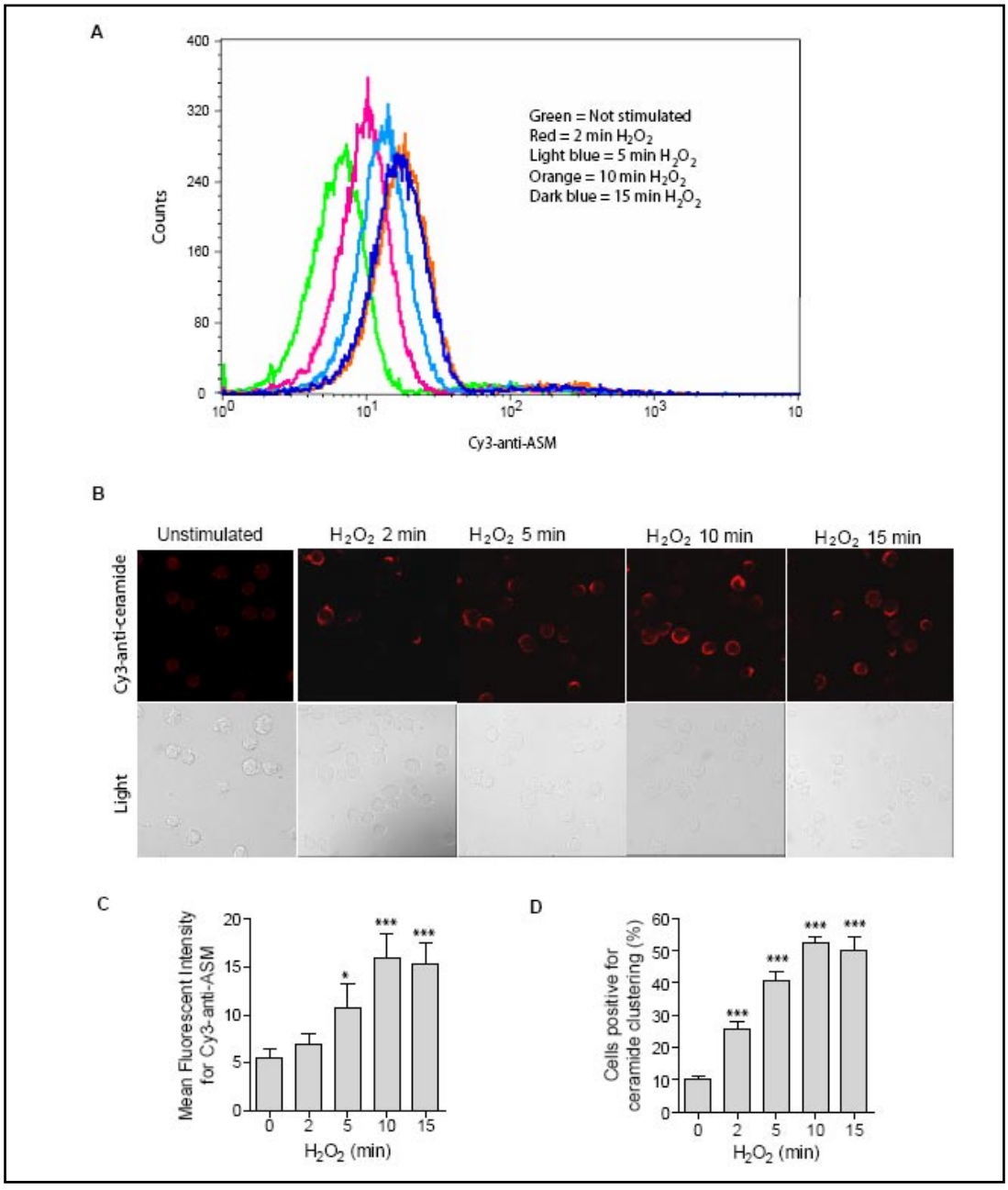

Fig. 1. Oxidative stress induces ASM externalization and formation of ceramide-enriched membrane platforms. (A) Oxidative stress by H_2O_2 (1 mM) induces a translocation of ASM onto the cell surface. Surface ASM was detected by flow cytometry analysis of cells incubated with Cy3-labeled goat anti-ASM antibodies. Shown is a representative flow cytometry analysis from four independent experiments. (B) H_2O_2 (1 mM)-induced ASM translocation correlates with rapid clustering of ceramide in the membrane and formation of ceramide-enriched membrane platforms. Representative confocal fluorescence microscopy and light images from three independent experiments are shown. (C) Displayed are the summarized data showing the mean fluorescence intensity for Cy3-anti-ASM staining from four independent experiments. (D) The quantitative analysis of the data from four independent experiments, which each included analysis of 200 cells/time point, shows the formation of ceramide-enriched membrane platforms in a large proportion of the cells. Displayed is the percentage of cells with a ceramide-enriched membrane platform at indicated time points. Panel C and D show the mean ± S.D. Significant differences between treated and non-treated controls were determined by *t*-test and are indicated by *** (P<0.001, *t*-test) or * (P<0.05, *t*-test), respectively.

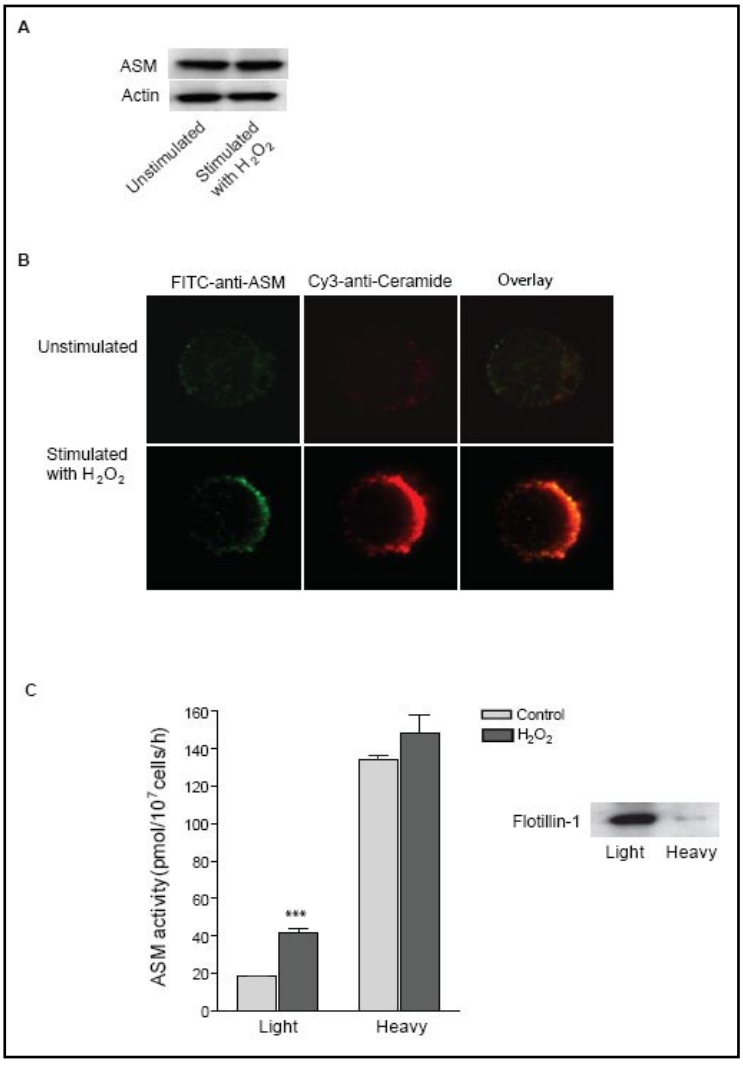

Fig. 2. Oxidative stress induces ASM translocation and activation of ASM onto membrane lipid rafts. (A) Western blot analysis of ASM and Actin in Jurkat cells stimulated with or without H_2O_2 (10 min, 1 mM). The experiments were repeated three times with similar results. (B) Confocal microscopy of Jurkat cells revealed a translocation of ASM onto the surface of the cell membrane and a co-localization of ASM with ceramide-enriched membrane platforms. Shown is a representative results from three independent experiments. (C) ASM activity in DRM fractions is increased upon H_2O_2 stimulation. Low density membrane fractions were collected and designated as DRMs (Light). High density membrane fractions were collected in parallel and designated as none-DRM fraction (Heavy). The DRM marker protein flotillin-1 was detected by Western blot analysis. Shown is the mean ± S.D. of three independent experiments. Significant differences between treated and non-treated controls were determined by *t*-test and are indicated by ***, respectively (P<0.001, *t*-test).

fractionation across a sucrose density gradient. H_2O_2 increased ASM activity in isolated DRMs (light fractions) whereas only minor increase was detected in non-DRM fractions (heavy fractions) (Fig. 2C). The efficiency of DRM isolation was indicated by detection of flotillin-1, which was primarily present in light membrane fractions (Fig. 2C inset). We decided to detect the ASM by enzyme assays, since these assays are more sensitive than western blots and, most important, at present the molecular size of the ASM form in the heavy fraction is not clear. Thus, at present they are the only way to unambiguously prove the presence or absence of the ASM in lipid rafts.

ROS induce lysosomal trafficking and fusion (exocytosis)

To test whether ASM-translocation onto the cell membrane is mediated by lysosomal fusion with the plasma membrane, cells were treated with H_2O_2 and surface staining was performed in live cells at 4°C with PE-conjugated anti-LAMP1, a lysosomal marker protein. Cells were analyzed by flow cytometry. The results reveal a time-dependent increase in membrane staining for LAMP1 upon H_2O_2 treatment (Fig. 3A and 3B). Plasma membrane exposure of LAMP1 exposure was maximized at around 10 min H_2O_2 stimulation.

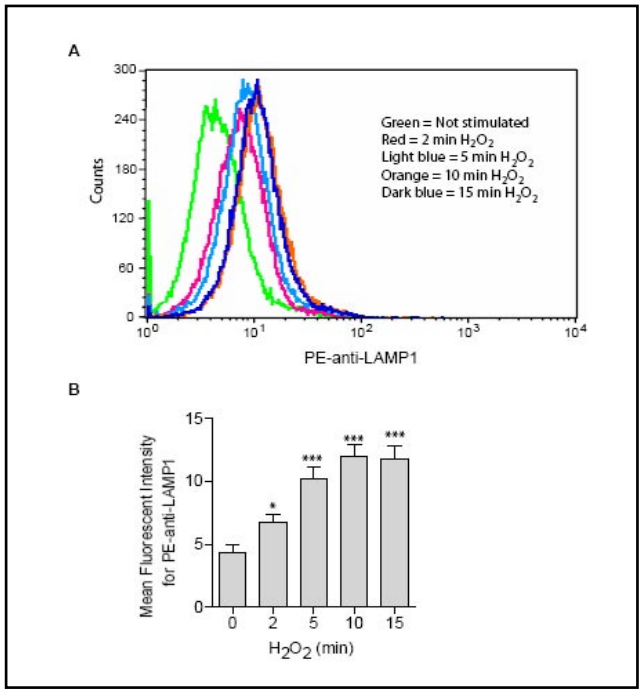

Fig. 3. H_2O_2 induces lysosome exocytosis. The translocation of LAMP1 onto the cell surface served as marker for lysosome fusion with the plasma membrane and was detected by flow cytometry analysis using PE-conjugated goat anti-LAMP1. (A) Shown is a representative flow cytometry analysis from four independent experiments. (B) Summarized data showing the mean fluorescence intensity for PE-anti-LAMP1 staining (n=4). Shown are the mean ± SD. Significant differences between treated and non-treated controls were determined by *t*-test and are indicated by *** (P<0.001, *t*-test) or * (P<0.05, *t*-test), respectively.

ROS-induced lysosomal trafficking and fusion with and ASM translocation to plasma membrane are Ca^{2+}-dependent

Previous studies have indicated that the increase in $[Ca^{2+}]_i$ triggers lysosome exocytosis [20]. H_2O_2 triggers a rapid and transient increase in $[Ca^{2+}]_i$ in Jurkat T cells [21]. Thus, we tested the hypothesis that ROS induced a transient increase in $[Ca^{2+}]_i$ that is involved in the lysosomal fusion with and ASM externalization to the plasma membrane. To examine if extracellular Ca^{2+} is involved in the increase in $[Ca^{2+}]_i$, cells were incubated with PBS buffer lacking Ca^{2+}. The absence of extracellular Ca^{2+} had no effect on H_2O_2-induced ASM externalization (Fig. 4A left panel). Further, to deplete intracellular Ca^{2+}, cells were incubated in PBS buffer in the presence of cell membrane permeable Ca^{2+} chelator EGTA-AM (100 µM). In contrast, chelation of intracellular Ca^{2+} by EGTA-AM abolished ASM externalization (Fig. 4A right panel and 4C). EGTA-AM also inhibited surface exposure of LAMP1 (Fig. 4B and 4D) and formation of ceramide-enriched membrane platforms (Fig. 4E). These data suggest that Ca^{2+}-dependent lysosome trafficking is an important mechanism for translocation of ASM to the membrane.

Effect of ASM inhibition and deficiency on lysosome exocytosis

To address whether ASM is critical for lysosome exocytosis, we performed experiments in spleen T cells freshly isolated from wild type and *Smpd1*$^{-/-}$ mice lacking Asm activity. In addition, we inhibitied ASM by treatment with amitriptyline, a known pharmacological blocker of ASM. Inhibition of ASM activity by treatment of Jurkat cells with amitriptyline (10 µM) did not affect H_2O_2-induced LAMP1 translocation (Fig. 5A and 5C). Likewise, Asm deficiency did not alter H_2O_2-induced LAMP1 translocation in freshly isolated spleen T cells (Fig. 5B and 5D). These data indicate that lysosome trafficking is independent of ASM activity upon H_2O_2 stimulation.

Discussion

Here, we show that oxidative stress triggers intracellular Ca^{2+}-dependent lysosome trafficking to and fusion with the plasma membrane, i.e. lysosome exocytosis, that results in surface expression of the ASM. Depletion of intracellular Ca^{2+} abolished ASM externalization

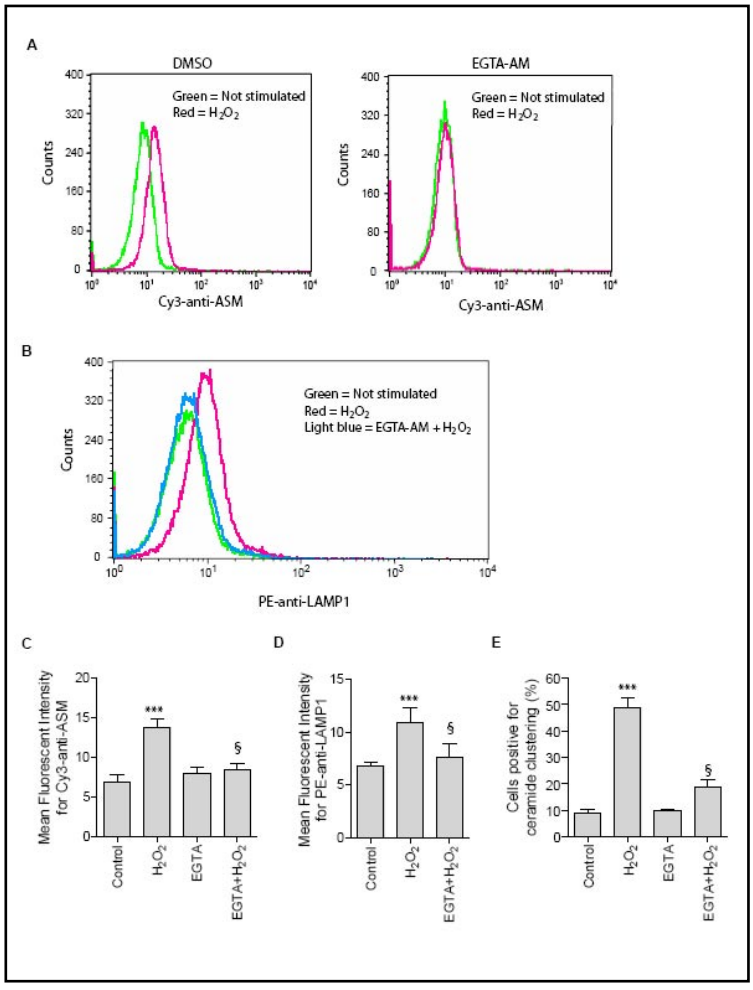

Fig. 4. H_2O_2-induced lysosomal excytosis and ASM externalization are Ca^{2+}-dependent. (A,B) Jurkat cells were stimulated with or without H_2O_2 (1 mM, 10 min) in the absence (DMSO) or presence of EGTA-AM (100 µM) in a Ca^{2+}-free PBS buffer. H_2O_2-induced ASM externalization and lysosomal excytosis were detected by flow cytometry analysis using Cy3-conjugated anti-ASM (A) and PE-conjugated goat anti-LAMP1 (B). Shown is a representative flow cytometry analysis from four independent experiments. (C) and (D) summarized data of four independent studies and show the mean fluorescence intensity for Cy3-anti-ASM or PE-anti-LAMP1 staining. (E) Attenuation of H_2O_2-induced ceramide-enriched membrane platforms by EGTA-AM. Displayed is the percentages of cells with a ceramide-enriched membrane platform in the cells treated with 1 mM H_2O_2 for 10 min (n = 4). Panel C-E show the mean ± S.D. Significant differences were determined by *t*-test (***, H_2O_2 *vs.* Control, P<0.001; §, EGTA+H_2O_2 *vs.* H_2O_2, P<0.001).

and concomitant formation of ceramide-enriched membrane platforms. In addition, ROS induced lysosome exocytosis in the absence of ASM activity. These data suggest a novel role for Ca^{2+}-dependent lysosome exocytosis in oxidative stress-induced ASM externalization, activation and formation of ceramide-enriched membrane platforms.

Our data suggest that lysosome exocytosis mediates ASM externalization. Several recent studies support a role of lysosome exocytosis in ASM externalization. In cytotoxic T lymphocytes, exocytosis of secretory granules, the only lysosome type organelles present in the cells, trafficks Asm to the plasma membrane [22]. In endothelial cells, disruption of lysosome functions by glycyl-L-phenylalanine 2-naphthylamide, a lysosome-disrupting cathepsin C substrate, abolished CD95 ligation-induced ASM translocation [23]. Upon stimulation, lysosomes, especially secretory lysosomes, can traffick and fuse with plasma membrane and release its acid hydrolases to the extracellular milieu [24]. Fusion of lysosomes with the plasma membrane results in the exposure of ASM on the outer leaflet of the cell membrane [25]. We previously demonstrated that ASM externalization is required to induce ceramide-mediated death receptor clustering, DISC formation and apoptosis initiation [9, 26]. Here, we found inhibition of lysosome exocytosis also abolished the clustering of membrane ceramides. Thus, these data support the view that ROS induce lysosome trafficking of ASM, most likely by secretory lysosomes, to plasma membrane leading to ceramide release and formation of ceramide-enriched membrane platforms.

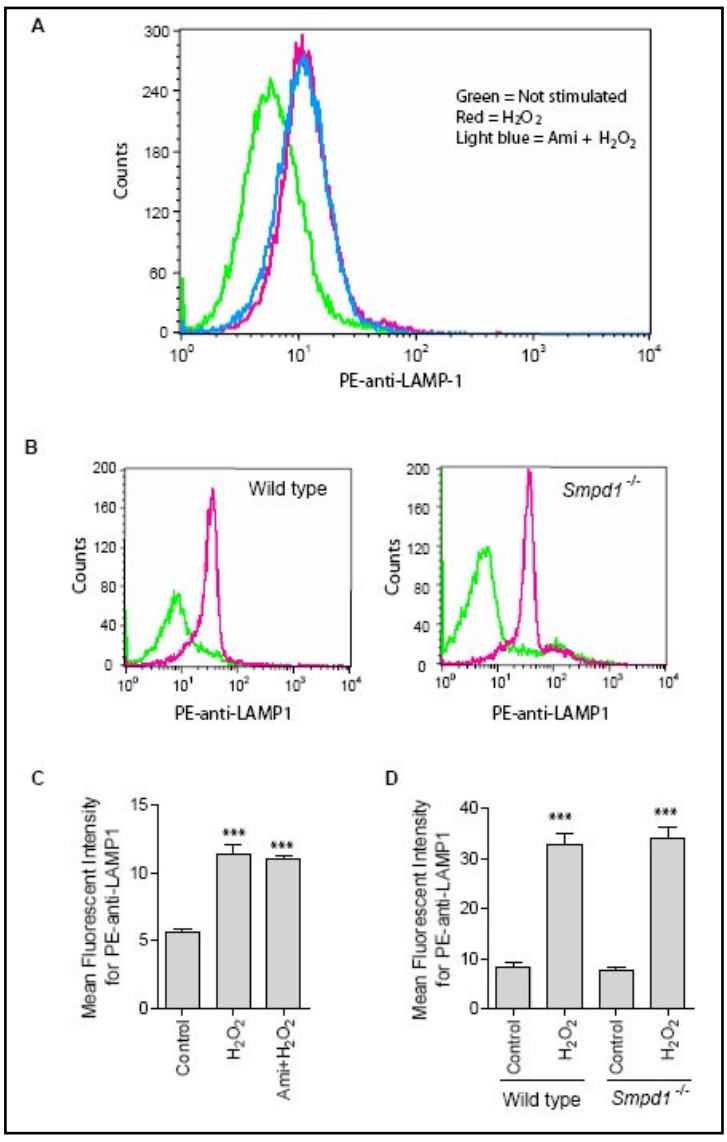

Fig. 5. Effect of ASM inhibition or deficiency on H_2O_2-induced lysosomal exocytosis. (A) Shown is a representative flow cytometry analysis diagram using PE-conjugated goat anti-LAMP1 antibodies in Jurkat cells with or without ASM inhibitor amitriptyline (10 μM) (n = 4). (B) Spleen T cells were isolated from wild-type and Asm deficient (*Smpd1*$^{-/-}$)mice,stimulatedwithout (green) or with H_2O_2 (red) and stained with PE-conjugated goat anti-LAMP1 antibodies. Shown are representative flow cytometry analyses from 3 independent experiments. Summarized data from 3 independent studies show the mean fluorescence intensity for PE-anti-LAMP1 staining in Jurkat cells (C) or spleen T cells (D). Displayed is the mean ± S.D. Significant differences between treated and non-treated controls were determined by *t*-test and are indicated by *** (P<0.001, *t*-test).

The present data provide evidence that ROS-induced lysosome exocytosis is Ca^{2+}-dependent. Several studies showed that lysosomes may traffick to and fuse with the plasma membrane in response to an increase in $[Ca^{2+}]_i$ [20, 27] and lysosome exocytosis is regulated by the Ca^{2+} sensor synaptotagmin-VII, which restricts both the kinetics and the extent of Ca^{2+}-dependent fusion [25]. In T cells, Ca^{2+} signaling initiated by TCR activation and ionomycin, a Ca^{2+} ionophore, has been implicated in lysosome exocytosis [22]. However, the role of Ca^{2+} in ROS-induced lysosome exocytosis and ASM externalization is undefined. $[Ca^{2+}]_i$ can be elevated via influx of extracellular Ca^{2+} through membrane Ca^{2+} channels or mobilization of intracellular Ca^{2+} release from Ca^{2+} stores such as sarco-endoplasmic reticula and mitochondria [28]. In the present study, intracellular chelation of Ca^{2+} by cell-permeable EGTA-AM but not extracellular Ca^{2+} removal abolished ROS-induced lysosome exocytosis indicating that increase in $[Ca^{2+}]_i$ is primarily originated from intracellular Ca^{2+} stores. Most interestingly, ASM externalization and formation of ceramide-enriched platform were also blocked by EGTA-AM indicating that increase in $[Ca^{2+}]_i$ serves as driving force for lysosome exocytosis resulting in ASM externalization and consequent ASM activation and ceramide release.

Some stress and bacterial stimuli such as UV-irradiation and *Pseudomonas aeruginosa* induce ROS-dependent plasma membrane trafficking of ASM as well as increase in $[Ca^{2+}]_i$ [6, 8, 29, 30]. In this respect, our findings suggest a novel role of ROS as secondary messengers to these stimuli to trigger Ca^{2+}-dependent lysosome trafficking of ASM resulting in formation of ceramide-enriched membrane platforms. However, activation of death receptors including CD95 and DR4/5 induces ROS-dependent ASM trafficking without alteration in $[Ca^{2+}]_i$ suggesting that trafficking of ASM by ROS can be also independent of Ca^{2+} [17, 31]. The precise mechanism for Ca^{2+}-independent ASM externalization is not well defined, which may involve PKCδ. ROS activate PKCδ, which then may interact with ASM-containing vesicles and translocate ASM to plasma membrane [11, 32].

Another important conclusion from this study is that ROS-induced lysosome exocytosis is independent of ASM expression and activity. This is consistent with a recent study showing TCR-triggered fusion of lysosome-type lytic granules is not altered in Asm-deficient cytotoxic T lymphocytes compared to wild-type cells, while in contrast, Asm deficiency results in impaired expulsion of vesicular contents [22]. The role of Asm and ceramide have also been investigated in other fusion events. Asm is required for fusion of late phagosomes with lysosomes since transfer of lysosomal fluid phase markers and lysosomal proteases cathepsin B, D and L into bacteria containing phagosomes are severely impaired in Asm-deficient macrophages [33]. Moreover, ceramide has been shown to trigger macroautophagy, another form of cell death and is probably involved in late formation of autophagolysosomes [34]. Thus, it seems that ASM activity is required for intracellular fusion processes, while lysosome exocytosis is independent of ASM activity.

The present study did not attempt to explore whether ceramide derived from other pathways such as *de novo* ceramide synthesis plays a role in ROS-induced lysosome exocytosis. *De novo* ceramide synthesis occurs primarily in the endoplasmic reticulum, where newly synthesized ceramide is transported to the Golgi apparatus via vesicular trafficking or by the action of the ceramide transfer protein. In the Golgi apparatus, ceramides are converted to other sphingolipids (i.e. sphingomyelin or glycosphingolipids) [35]. Previous studies have demonstrated that ceramide release via sphingomyelin hydrolysis is a much faster event than the *de novo* ceramide synthesis [36, 37]. In this regard, the present study revealed a very rapid lysosome exocytosis triggered by ROS (2 min). Therefore, our data support the view that the location and time period required for *de novo* ceramide synthesis may not favor its role in membrane fusion process and thereby mediate rapid signaling events. Nonetheless, the precise role of *de novo* ceramide synthesis in lysosome exocytosis requires future investigation.

In conclusion, we propose that Ca^{2+}-dependent lysosome trafficking of ASM mediates ASM externalization under oxidative stress. The implication of our findings may also explain the action of ROS as a secondary messenger mediating ASM externalization, activation, ceramide release and formation of ceramide-enriched platforms in response to external stimuli, at least, for UV-irradiation and some bacterial infections.

Abbreviations

ASM (acid sphingomyelinase); DRM (detergent resistant membranes); H_2O_2 (hydrogen peroxidase); LAMP1 (lysosome associated protein 1); NPDA and NPDB (Niemann-Pick disease type A and B); O_2^- (superoxide anions); SMPD1 (sphingomyelin phosphodiesterase 1); ROS (reactive oxygen species); SOD (superoxide dismutase).

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