Fc γ RII Activation Induces Cell Surface Ceramide Production which Participates in the Assembly of the Receptor Signaling Complex

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Key Words

Ceramide • Fc receptors • Microdomains • Sphingomyelinase • Tyrosine phosphorylation

Abstract

We studied an involvement of various cellular ceramide pools in signaling of immunoreceptor Fc γ RII (Fc γ RII). The cell surface ceramide level was assessed by a technique based on binding of ceramide probes to intact cells. Total cellular ceramide was estimated by radioactive measurements. The activity of sphingomyelinases was measured by NBD-ceramide release while immunoprecipitation and immunoblotting were applied to analyze protein tyrosine phosphorylation. A complex pattern of protein phosphorylation was found to accompany Fc γ RII activation and the phosphorylation was either diminished by imipramine or increased by B13, modulators of acid sphingomyelinase and acid ceramidase activity. The effects of the drugs on the phosphorylation of Fc γ RII and NTAL were prominent

and correlated with a reduction of the cell surface ceramide production by imipramine and an augmentation of the ceramide generation by B13. The ceramide generation followed activation of acid sphingomyelinase and preceded that of neutral sphingomyelinase. The level of cell surface ceramide was additionally elevated by exogenous bacterial sphingomyelinase, but only at later stages of the receptor activation. The total mass of ceramide was diminished in the course of receptor activation pointing to an engagement of enzymes metabolizing ceramide. The data indicate that Fc γ RII activates enzymes of the sphingomyelin cycle which affect various sphingomyelin/ceramide pools in a cell.

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Introduction

Fc γ receptors bind IgG and initiate a wide array of immunoresponses, including phagocytosis of IgG-opsonized pathogens [1]. Transduction of signals by

Fc γ RII is initiated by phosphorylation of tyrosine residues of the signaling motif located in the cytoplasmic part of the receptor [2]. Our previous data demonstrated that Fc γ RII phosphorylation requires receptor clustering and its association with plasma membrane microdomains, known as rafts [3]. Rafts are enriched in cholesterol and sphingolipids, including sphingomyelin, and create an environment that facilitates a local accumulation of dual-acylated tyrosine kinases of the Src family [4]. Lyn, one of the Src family members, is a likely candidate kinase catalyzing the Fc γ RII phosphorylation which triggers the downstream signaling cascades [3, 5]. However, the mechanisms which control the association of Fc γ RII with rafts for signaling complex assembly are poorly known.

The enrichment of sphingomyelin in the outer leaflet of the plasma membrane [6] turned our attention to the so-called sphingomyelin cycle [7] as a possible factor playing a role in Fc γ RII signaling. The cycle begins with sphingomyelin hydrolysis by acid or neutral sphingomyelinases, yielding ceramide [6, 8]. Neutral sphingomyelinase (NSMase) is associated mainly with the plasma membrane while acid sphingomyelinase (ASMase) is attributed to late endosomes and lysosomes. However, it was found recently that ASMase could be also extruded to the cell surface and generate exofacial ceramide upon activation of TNF receptor family members and UV-irradiation of cells. The ceramide formed was required for efficient clustering of death receptors adding a new aspect to pro-apoptotic functions of ceramide [9-11]. Aside from the role of ceramide in induction of apoptosis, the lipid was recently shown to be essential for clustering of immunoreceptor Fc γ RII in rafts and, hence, for Fc γ RII signal generation [12]. In this report we continue our studies on Fc γ RII-triggered ceramide production and analyze how Fc γ RII clustering affects the level of various cellular ceramide pools by activation of ASMase and NSMase.

Materials and Methods

Cell culture and Fc γ RII activation

U937 cells were cultured as described [3]. For Fc γ RIIA cross-linking, cells were incubated with mouse anti-Fc γ RII antibody (IgG2b clone IV.3, 30min, 4°C) followed by goat anti-mouse F(ab)₂ IgG (1-20min, 4°C; ICN). The stage immediately after incubation of the cells with anti-Fc γ RII only, which does not induce receptor activation [3,13], was set as time point "0" of receptor cross-linking. Imipramine at 50-70 μ M or N,N-dimethylformamide up to 0.03% as imipramine carrier (both Sigma) were applied for 1h (37°C) prior to incubation of the

cells with anti-FcRII. Bacterial sphingomyelinase (bSMase; Sigma) was used at 40-70 mU/ml for 4min at 4°C. B13 at 10 μ M and 0.05% dimethyl sulfoxide, a B13 carrier, were used overnight at 37°C in the growth medium.

Ceramide determination

Cell surface ceramide: cells (3x10⁶/sample) were fixed with 1% formaldehyde and incubated with 1 μ g/ml anti-ceramide IgM (clone MID 15B4, Alexis; 45min, 20°C). Cell-bound antibody was desorbed for 30 s with ice-cold 100 mM glycine-HCl, pH 3.0, neutralized, spotted onto nitrocellulose and revealed with goat anti-mouse IgM conjugated with peroxidase. Immunoreactive spots were visualized by chemiluminescence (Pierce) and quantified densitometrically in a Fluor MultiImager using Quantity One software (BioRad) [11]. Alternatively, exofacial ceramide level was analyzed using FACScan and Cell Quest software (Becton Dickinson) after incubation of the cells with anti-ceramide and anti-mouse IgM goat IgG-FITC (Jackson ImmunoResearch).

Total cellular level of ceramide: [³H]serine (1.5 μ Ci/ml, Perkin Elmer) was administrated to the cells (1x10⁶/ml) for 48h in RPMI containing 6% dialyzed FBS. Lipids were extracted (1x10⁷cell/sample) [14], subjected to alkaline hydrolysis [15] and analyzed by thin layer chromatography on silica gel 60 F₂₅₄ using chloroform/acetic acid (9:1) mixture. Ceramide was identified by iodine vapor staining, in reference to a ceramide standard (Sigma), scraped off and quantified by liquid scintillation counting in a Beckman counter.

ASMase and NSMase activity assays

For ASMase activity assay, cells (3x10⁶/sample) were lysed in 120 μ l of 0.5% Triton X-100, 50 mM acetate buffer, pH 5.0. After 20min, lysates were centrifuged (14000g, 5min, 4°C) and 100 μ l of supernatant was mixed with 150 μ l of 2 mM EDTA, 200 mM acetate buffer, pH 5.0, containing liposomes composed of 12 μ M NBD-sphingomyelin (Molecular Probes), 24 μ M sphingomyelin, 24 μ M cholesterol and 108 μ M 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (all from Sigma). The liposomes were prepared by sonication of lipids suspended in 70 mM NaCl. For determination of NSMase activity, cells were lysed in 0.5% Triton X-100 in the presence of 10 mM MgCl₂, 2 mM EDTA, 1 mM ATP, 30mM p-nitrophenylphosphate, 0.2 mM Na₃VO₄, 10 mM β -glycerophosphate, 5 mM DTT, 20 mM Hepes, pH 7.4, and protease inhibitor cocktail [16]. After preclearing, 100 μ l of lysate was mixed with 150 μ l of 100 mM NaCl, 2 mM MgCl₂, 50 mM Hepes, pH 7.4, containing liposomes as described above. After 1h at 37°C, NBD-ceramide was isolated [17] and measured in a fluorimeter (460 nm /534nm).

Electron microscopy

Plasma membrane sheets were obtained by mechanical cleavage of cells [18]. The cytoplasmic surface of the plasma membrane was probed for 2h at 20°C with rabbit anti-Fc γ RII antibody and goat or donkey anti-rabbit IgG-gold (10 nm gold; Sigma, Aurion). In a series of experiments, prior to cell cleavage the cells were incubated with 4 μ g/ml probe containing the cysteine-rich domain of kinase suppressor of RAS fused with glutathione S-transferase (GST) which specifically recognizes

Fig. 1. Fc γ RII cross-linking induces protein tyrosine phosphorylation that is sensitive to modulators of ASMase and acid ceramidase activity. (A, B) Time-course of protein tyrosine phosphorylation during Fc γ RII cross-linking (4°C) in U937 cells preincubated without and with 50 μ M imipramine (A) and 10 μ M B13 (B). Molecular mass standards are shown in kDa on the left. (C-D) Densitometric quantification of phosphoproteins revealed in (A and B) without (closed symbols) and with drug pretreatment (open symbols) after normalization against actin content. The phosphorylation of a particular band is expressed in relation to its maximum value in control cells non-exposed to the drugs, that was arbitrarily set at 100. (E, F) Tyrosine phosphorylation of Fc γ RIIA (doublet) and NTAL revealed after immunoprecipitation of the proteins from whole cell lysates. Prior to Fc γ RII cross-linking, cells were preincubated with 50 μ M imipramine or 10 μ M B13. The immunoprecipitates were probed for the presence of tyrosine phosphoproteins (upper panels) and either biotin-labeled IV.3 anti-Fc γ RII reflecting the receptor content or NTAL (lower panels). Results of one of three experiments are shown.

ceramide, as shown previously [11]. The probe was followed by goat anti-GST (Rockland) and donkey anti-goat IgG-gold (6 nm, Aurion). Samples were fixed with 2.5% glutaraldehyde in 50 mM cacodylate buffer (15min, 20°C), postfixed with 1% OsO₄ in the buffer (10min, 20°C) and counterstained with 1% uranyl acetate in water. The samples were examined under a JEM-1200 EX (JEOL) electron microscope. Gold particles marking ceramide were scored from micrographs (2 μ m² of the membrane surface). The calculations were performed for two independent

experiments; at least 10 micrographs were analyzed for each set of experiments.

SDS-PAGE and immunoblotting

To obtain whole cell lysates, cells (5x10⁵/sample) were lysed in ice-cold 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 30 mM Hepes, pH 7.4, with protease and phosphatase inhibitors. After 10min, SDS-sample buffer was added, the samples were boiled and proteins were separated

by 10% SDS-PAGE. After transfer to nitrocellulose sheets, they were probed with mouse anti-phosphotyrosine, clone PY66 (Santa Cruz Biotechnology) and goat anti-mouse IgG-peroxidase (ICN). The membranes were reprobed, after stripping, with mouse anti-actin IgG (Roche). Immunoreactive bands were visualized with the chemiluminescent substrate and quantified densitomerically as described above.

Immunoprecipitation of FcγRII was performed from cell lysates obtained in the presence of 0.2% Triton X-100 and 1.5% N-octylglucoside essentially as described earlier [3]. In this set of experiments, biotin-labeled IV.3 and rabbit anti-mouse IgG (Jackson ImmunoResearch) were used for FcγRII cross-linking. For immunoprecipitation of NTAL, rabbit-anti-NTAL was applied. The precipitates were probed with mouse anti-phosphotyrosine and, in parallel, either with goat anti-biotin peroxidase conjugated IgG (Sigma) or with mouse anti-NTAL antibodies to reveal amounts of the precipitated receptor and NTAL.

Data were evaluated by one-way ANOVA test with $p < 0.05$ considered significant.

Results

FcγRII cross-linking induces activation of ASMAse, NSMAse and asynchronous protein phosphorylation

FcγRII of U937 monocytes was activated by cross-linking with mouse anti-FcγRII antibody followed by anti-mouse IgG. The receptor clustering induced tyrosine phosphorylation of several proteins (Fig. 1A, B), although a detailed densitometric analysis revealed that the time-course of the phosphorylation of individual bands varied. A 130 kDa band and a prominent 120 kDa band were phosphorylated progressively during receptor cross-linking in an approximately linear fashion, while a 65 kDa band underwent abrupt phosphorylation reaching the maximal phosphorylation level after about 5 min of the receptor clustering (Fig. 1C, D, closed circles). On the other hand, a 30 kDa band, identified as an adaptor protein NTAL by immunoprecipitation (see below), displayed a significantly delayed tyrosine phosphorylation which started at 5 min of the receptor cross-linking (Fig. 1A).

The level of the phosphorylation was diminished to various extents in cells pretreated with imipramine (Figs. 1A, and 1C, open circles), an ASMAse inhibitor with recently discovered activity against acid ceramidase as well [19,20]. Notably, imipramine reduced tyrosine phosphorylation of FcγRII and affected also phosphorylation of NTAL, as found after immunoprecipitation of the proteins (Fig. 1E, F). An estimation of sphingomyelinase activity in whole cell lysates revealed that FcγRII cross-linking induced fast

Fig. 2. FcγRII cross-linking activates ASMAse and NSMAse differently. Activity of ASMAse (circles) and NSMAse (squares) in whole lysates of U937 cells during FcγRII cross-linking. Open circles indicate activity of ASMAse in cells pretreated with 70 μM imipramine prior to FcγRII activation. The enzyme activities are expressed in relation to their maximum values equalized to 100. The data shown are mean ± SEM from four experiments.

and transient elevation of ASMAse activity with the maximum at 5 min of the clustering. In contrast, the activity of NSMAse rose progressively, peaking at a later stage of the receptor cross-linking, at 11 min (Fig. 2). As expected, pretreatment of cells with imipramine strongly inhibited the activity of ASMAse in derived cell lysates (Fig 2, compare open and closed circles). On the contrary, imipramine slightly activated (by 15-20%) rather than inhibited the activity of bSMAse in vitro (not shown). This is in agreement with earlier data that the drug exerts its inhibitory effect on ASMAse in intact cells by induction of proteolytic degradation of the enzyme in lysosomes [19].

To further explore the involvement of ceramide in FcγRII-induced protein phosphorylation, the cells were exposed to B13, a potent blocker of acid ceramidase [21], a critical scavenging enzyme of ceramide. An analysis of overall protein tyrosine phosphorylation revealed that, in contrast to the imipramine action, pretreatment of cells with B13 accelerated and augmented the FcγRII-induced phosphorylation. The reinforcing effect of B13 was clearly manifested at early stages of FcγRII cross-linking, at 5-10 min, as seen for a 65 kDa and a 75 kDa phosphoproteins (Fig. 1B, D). The data were supported

Fig. 3. Fc γ RII cross-linking induces cell surface ceramide production. (A, B) Ceramide detected on the surface of intact U937 cells by anti-ceramide IgM binding and dot-blotting assay during Fc γ RIIA cross-linking (A) and after incubation of cells with 40-70 mU/ml bSMase (4min, 4°C) without receptor cross-linking (B). The upper panels show representative dot-blots, while lower panels display profiles of ceramide generation. (C, D). Quantification of cell surface ceramide production as a function of concentration of anti-Fc γ RII. The quantification is based on dot-blotting (C) and FACSscan analysis (D) of cell-surface bound anti-ceramide IgM. Anti-Fc γ RII was followed by 10 μ g/ml goat anti-mouse F(ab) $_2$. (E, F) Cell surface ceramide production in cells treated with 70 μ M imipramine (E) and 10 μ M B13 (F) prior to Fc γ RIIA cross-linking. The data in (A, C) are the mean $\pm$ SEM from two-three experiments, in (B, D-F) results of representative experiments are shown.

by examining phosphorylation of immunoprecipitated Fc γ RII and NTAL. Under the influence of B13 both proteins demonstrated a marked increase of phosphorylation at early steps of Fc γ RII clustering and the phosphorylation of NTAL sustained at the elevated level later on (Fig 1E, F).

Fc γ RII cross-linking triggers generation of cell surface ceramide and affects the level of total cellular ceramide

To examine whether the Fc γ RII-induced protein phosphorylation correlated with ceramide production in the cells we first evaluated the level of cell surface ceramide in an assay based on immunodetection of an anti-ceramide antibody bound to intact cells. This approach revealed that the exofacial ceramide level increased transiently by about 3-fold between 5-10min of Fc γ RII

cross-linking (Fig. 3A). The adequacy of the ceramide detection by the anti-ceramide-binding procedure was confirmed by treatment of the cells with exogenous bSMase, without Fc γ RII cross-linking. Under these conditions, the amounts of ceramide detected on the cell surface were proportional to the amounts of the exogenous bSMase applied (Fig. 3B). A transient elevation of exofacial ceramide during Fc γ RII cross-linking was also detected when surface-bound anti-ceramide was analyzed by FACSscan. However, the magnitude of the changes was lower due to the high background of fluorescence found in cells prior to Fc γ RII cross-linking (see Fig. 4A).

We measured the exofacial ceramide generation as a function of the amount of activated Fc γ RII. Dot-blotting and FACSscan analysis yielded very consistent results showing that the ceramide production was triggered

Fig. 4. Exogenous bSMase intensifies cell surface ceramide production at later stages of Fc γ RII cross-linking. (A, B) Fc γ RII was cross-linked in U937 cells for 7 and 12 min (4°C) and during the last 4 min of the procedure 70 mU/ml bSMase was applied. Cell surface ceramide was estimated by binding of anti-ceramide IgM and FACSscan analysis (A), while the total cellular ceramide level was analyzed by thin layer chromatography (B). The data in (A, B) are the mean $\pm$ SEM of three experiments. Differences significant at $p < 0.05$ are marked by bars, differences statistically insignificant are not marked. (C) Dot blotting analysis of cell surface ceramide production during Fc γ RII cross-linking with and without bSMase.

sharply by low doses of anti-Fc γ RII used for receptor cross-linking and reached a plateau at 0.5–1 μ g/ml of the antibody (Fig. 3C, D). Therefore, in the course of the work, 3 μ g/ml of anti-Fc γ RII was used routinely to ensure maximal receptor activation.

The level of cell surface ceramide produced during Fc γ RII activation was altered in cells pretreated with imipramine and B13, the modulators of ASMase and acid ceramidase activity. Imipramine reduced the amount of the exofacial ceramide while B13-treated cells had

significantly increased cell surface ceramide level (Fig. 3E, F). The increase was detectable at 5 min of Fc γ RII cross-linking and the amount of the ceramide in B13-exposed cells sustained elevated for 20 min compared with a transient ceramide accumulation in the control (Fig. 3E, F). The opposed influence of imipramine and B13 on cell surface ceramide generation during Fc γ RII activation correlated well with the inhibition and the augmentation of Fc γ RII-induced protein tyrosine phosphorylation seen in cells exposed to the drugs (see Fig. 1).

Having established that Fc γ RII cross-linking is correlated with a transient rise of cell surface ceramide, we examined whether these changes were reflected by modulation of the total cellular ceramide level estimated by radioactive measurements of [^{3} H]ceramide. It was found that the total mass of ceramide in the cells was progressively diminishing in the course of Fc γ RII cross-linking. After 12 min of the receptor activation, the total ceramide level fell to 63% of its value found in the cells before receptor activation (Fig. 4B).

Bacterial SMase augments ceramide production only at later stages of Fc γ RII cross-linking

Since the exofacial ceramide is generated from sphingomyelin most likely residing in the outer leaflet of the plasma membrane, we assumed that treatment of cells with bSMase could augment this process. FACSscan analysis revealed that bSMase applied for 4 min without Fc γ RII cross-linking elevated the cell surface ceramide level by 1.4-fold. For comparison, receptor cross-linking led to a 1.8-fold and 1.6-fold increase of the exofacial ceramide level after 7 and 12 min, respectively (Fig. 4A). It is of note that treatment of the cells with bSMase for the last 4 min in the course of 7 minute-long receptor cross-linking did not affect the level of surface ceramide (Fig. 4A). However, application of the exogenous enzyme during the last 4 min of receptor cross-linking lasting for a total of 12 minutes markedly elevated the amount of cell surface ceramide. The combined treatment resulted in a 1.7-fold increase of the exofacial ceramide level above that found after Fc γ RII cross-linking alone (Fig. 4A). The FACSscan analysis was supported by dot-blotting which showed an intensification of ceramide production when bSMase was added for 4 min and followed by 15–20 min of Fc γ RII cross-linking (Fig. 4C). This additive effect of Fc γ RII activation and bSMase action on the ceramide level was also detectable when the level of total cellular ceramide was estimated. bSMase applied for 4 min at the end of a 12 min period of receptor cross-linking doubled the amount of the total ceramide (Fig. 4B).

Fig. 5. Distribution of FcγRII and ceramide in the plasma membrane during receptor cross-linking. (A) FcγRII in cells exposed to anti-FcγRII only. (B-C) Formation of electron-dense structures accommodating FcγRII after 5 min (B) and 20 min (C) of receptor cross-linking. (D) Colocalization of FcγRII (10 nm gold) and cell surface ceramide (6 nm gold) after 5 min of FcγRII cross-linking. (E) Quantitation of gold particles reflecting ceramide on the external surface of the plasma membrane in cells exposed to anti-FcγRII only and after 5 min of FcγRII cross-linking. The data are the mean ± SEM, from two experiments. Bars, 100 nm.

Cell surface ceramide associates with clusters of activated FcγRII

For ultrastructural analysis of the distribution of cell surface ceramide during FcγRII cross-linking we used large plasma membrane sheets obtained by mechanical cleavage of cells. As seen in Fig. 5A, the receptor was uniformly dispersed in the plane of the plasma membrane of unstimulated cells. Upon FcγRII cross-linking, electron-dense structures were formed in the membrane; the structures were distinct at 5 min and their size increased with time of receptor activation. These clusters

accommodated the vast majority of the activated FcγRII (Fig. 5B, C).

When the external surface of the plasma membrane was examined for ceramide presence, prominent labeling of the lipid was found at FcγRII clusters after 5 min of receptor cross-linking (Fig. 5D). Quantitation of gold particles attributed to the cell surface ceramide demonstrated that the vast majority of the exofacial ceramide was associated with the electron-dense structures, whereas minute amounts of the lipid were detected outside the structures. Only traces of ceramide

were seen on the surface of resting cells (Fig. 5E).

Discussion

It has been established that activation of FcγRII triggers signaling cascades which require protein tyrosine phosphorylation [13, 22, 23]. Our present analysis demonstrates differences in the time course of the phosphorylation concerning various proteins suggesting that these proteins may be involved at different stages of FcγRII signaling. Electron microscopy studies revealed that the receptor signaling was accompanied by formation of electron-dense structures in the plasma membrane accommodating FcγRII. Since FcγRII is known to associate with rafts for phosphorylation [3, 11, 22], and since Lyn kinase, CD55 and phosphoproteins have been shown to occupy the electron-dense structures [18], the formation and progressive enlargement of the structures is likely to reflect raft coalescence during FcγRII signaling complex formation. The electron-dense structures were found to accommodate cell surface ceramide (Fig. 5D, E).

An immunochemical analysis of the exofacial ceramide revealed that the ceramide level was transiently elevated upon FcγRII activation. We found that two various modulators of the sphingomyelin cycle affected FcγRII-induced ceramide production in an opposite manner. Imipramine diminished the level of the ceramide and concomitantly inhibited tyrosine phosphorylation of proteins accompanying FcγRII activation. On the contrary, B13 accelerated and augmented accumulation of ceramide on the cell surface during FcγRII cross-linking. Simultaneously, under these conditions the protein tyrosine phosphorylation underwent also rapid elevation in a manner reflecting the profile of exofacial ceramide appearance. We obtained similar data (not shown) with an use of LCL204, a new B13 analogue considered as an acid ceramidase inhibitor [24]. The exofacial ceramide seems to affect strongly the phosphorylation of FcγRII and NTAL, judging from the influence of imipramine and B13 on the process. This may suggest that transmembrane proteins are especially sensitive to the presence of ceramide in the outer leaflet of the plasma membrane. The present data are in line with our earlier observations that administration of exogenous C₁₆-ceramide facilitated clustering of cross-linked FcγRII and an association of the receptor with rafts [12]. Taken together, the data indicate that once generated upon FcγRII activation, the ceramide can facilitate translocation of proteins in the

plane of the plasma membrane and govern coalescence of rafts into FcγRII signaling platforms where protein phosphorylation occurs. These processes are modulated by ceramide due to tendency of the lipid to self-aggregate and laterally separate into domains [25].

Imipramine and closely related desipramine were considered for a long time as drugs inducing proteolytic degradation of ASMase [19]. However, it was demonstrated recently that acid ceramidase is also susceptible to the proteolysis [20]. Without further analysis it is hard to reconcile whether effect of imipramine on cells can be ascribed either to lack of ceramide generation (ASMase inhibition) or accumulation of ceramide and lack of its derivatives, such as sphingosine and sphingosine-1-phosphate (inhibition of acid ceramidase). Indeed, we found that pretreatment of cells with imipramine led to 70% inhibition of ASMase (Fig. 2) but also diminished activity of acid ceramidase by nearly 50% (not shown). It is of note that, regardless of this potentially dual action of imipramine, in our hands the drug reduced both the level of cell surface ceramide and protein tyrosine phosphorylation during FcγRII cross-linking, as opposite to the B13 action. Therefore, we assume that the assembly of FcγRII signaling complex requires, at least at the onset, the presence of cell surface ceramide generated presumably by ASMase, the target of imipramine. In agreement with this assumption, the peak of the ceramide generation during FcγRII cross-linking in cells not exposed to the drugs followed activation of ASMase and preceded activation of NSMase. The supposition that imipramine restrained the assembly of the FcγRII signaling complex by inhibition of ASMase activity is supported by our earlier findings that exogenous C₁₆-ceramide partially reversed the inhibitory effect of imipramine exerted on FcγRII clustering and phosphorylation [12].

An involvement of NSMase in FcγRII-induced protein phosphorylation might be also considered, especially in view of the data that imipramine can augment the activity of this enzyme, while inhibiting ASMase [26]. Previously, ceramide was suggested to regulate the level of protein phosphorylation by activation of a kinase and a phosphatase, both specific toward serine/threonine residues. Production of intracellular ceramide controlling these enzymes was attributed to the activity of plasma-membrane-associated NSMase [8]. A joint participation of ASMase and NSMase in the signaling cascades triggered by TNF in U937 cells was demonstrated. However, the two enzymes controlled distinct, non-overlapping pathways of the TNF receptor signaling [16].

The findings presented here indicate that ASMase is responsible for production of the cell surface ceramide during FcγRII clustering, while the role of the intracellular ceramide generated by NSMase at later stages of the receptor activation remains unknown. Yet another source of cell surface ceramide can be hydrolysis of glycolipids, although participation of this process in signaling pathways remains obscure [27].

The exofacial ceramide is presumably not the predominant fraction of total cellular ceramide since despite a temporal increase of its level, the total mass of the lipid was diminished in the cells (Fig. 4). Moreover, these data indicate that FcγRII cross-linking triggers activation of enzymes of the sphingomyelin cycle which generate the exofacial ceramide and simultaneously metabolize the intracellular pool of the lipid. This supposition is in line with earlier suggestions that the membrane sphingomyelin cycle imposes a quantitative control on the interplay between T-cell receptor and death receptors affecting eventually T cell activation and survival [28].

The level of ceramide was synergistically elevated by bSMase and FcγRII cross-linking at later stages of receptor activation. This suggests that at the beginning of FcγRII clustering, exogenous bSMase and endogenous receptor-activated ASMase can target a common pool of exofacial sphingomyelin. As a result, at the initial stage of FcγRII activation, the effect of these enzymes on ceramide production is not additive. Later on, two separate pools of sphingomyelin become available for the enzymes and one can assume that endogenous ASMase is coupled to the raft-based signaling complexes of FcγRII while bSMase utilizes sphingomyelin exposed/synthesized in the plasma membrane during assembly of the complex. This sphingomyelin fraction located outside the receptor

signaling platforms is likely to acquire a fluid state that has been found as a requirement for sphingomyelin hydrolysis by bSMase [29]. The existence of distinct sphingomyelin pools in the plasma membrane has been suggested before. However, the “signaling” pool of the lipid utilized upon prolonged activation of TNFα receptor and bSMase treatment was ascribed to sphingomyelin located in the inner leaflet of the plasma membrane [30], in contrast to the results of the present work implicating the outer leaflet.

Abbreviations

ASMase (acid sphingomyelinase); bSMase (bacterial sphingomyelinase); GST (glutathione S-transferase); NSMase (neutral sphingomyelinase).

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