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Sphingolipids are involved in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced epidermal growth factor receptor clustering

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Abstract

Previously we have found that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), an alkylating agent, can induce the clustering of cellular surface receptors including tumor necrosis factor receptor (TNFR) and epidermal growth factor receptor (EGFR). Since sphingolipids, especially ceramide, have been suggested as major players in ligand-induced receptor clustering, their involvement in this ligand-independent, chemical-induced receptor clustering was evaluated. It was shown that MNNG-induced EGFR clustering occurred primarily at lipid rafts, as nystatin, which can disrupt lipid raft structure, significantly decreasing MNNG-induced EGFR clustering. Lipidomic studies revealed that MNNG treatment induced profound changes in sphingolipids metabolism, which were not the same as those induced by EGF treatment. Acid sphingomyelinase (ASM) is responsible for hydrolyzing sphingomyelin to generate ceramide, and it was demonstrated that MNNG treatment caused ASM distribution changing from diffused state to concentrated area of cells, which colocalized with lipid rafts. Nystatin treatment also abolished the redistribution of ASM. In addition, blockage of ceramide production by ASM inhibitor imipramine interrupted MNNG-induced receptor clustering. Taken together, these data suggested that sphingolipids are involved in MNNG-induced receptor clustering; however, the specific species involved may be different from those involved in EGF-mediated receptor clustering.

Keywords

Sphingolipids; Epidermal growth factor receptor; Lipid raft; Lipidomics

Receptor clustering or oligomerization is a prerequisite for the activation of cellular signaling pathways for many types of receptors, including receptor tyrosine kinases (RTKs, such as epidermal growth factor receptor, EGFR), death receptors (such as tumor necrosis factor receptor (TNFR) and Fas/CD95), platelet-derived growth factor receptor (PDGFR), the TCR-CD3 complex, B cell receptor (BCR), CD2, CD28, CD40, CD44, FcεRI, FcγRII, integrins, L-selectin, and probably more [1–9]. Upon ligand binding, these receptors can cluster in specific cell membrane regions termed lipid rafts, which are rich in sphingolipids and cholesterol [10–12]. Intensive studies have revealed that ceramide, the structural backbone of sphingolipids, plays vital role in this clustering process [13,14]. It has been demonstrated that acid sphingomyelinase (ASM), an enzyme responsible for hydrolyzing sphingomyelin to

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generate ceramide at the outer leaflet of the cell membrane, could be activated within a few seconds to minutes following ligand binding to receptors; a translocation of ASM also occurs simultaneously [7,15,16]. These events are central in the transformation of rafts in unstimulated, resting cells into active signaling domains transmitting stimuli from the outside into cells [3,13].

Besides ligand-induced receptor clustering, accumulating evidences indicate that many stress stimuli can also induce the clustering of various surface receptors. Among the different stimuli, UV probably is the best documented one. For example, it has been reported that UV light induces clustering of CD95 receptors in cell membrane in the absence of CD95L or anti-CD95 Abs, resulting in the recruitment of the death adaptor molecule FADD and the induction of caspase 8-mediated apoptosis [17–19]. In vivo study of UV has also found similar effects [20]. Cytochalasin B (CyB) disrupts the actin cytoskeleton and causes apoptosis; furthermore, it enhances the apoptotic effect of UV [21]. Detailed studies showed that CyB itself induced CD95 clustering, and it could also enhance UV-induced CD95 clustering. Disruption of CD95 signaling by a dominant negative mutant of FADD or using CD95(–/–) cells protected cells from CyB-induced apoptosis and prevented the UV-enhancing effect [21]. These data point to an essential role of receptor clustering in UV-mediated cellular responses. However, the underlying mechanism for this non-ligand-induced receptor clustering is mostly unknown.

In our laboratory, a similar phenomenon of receptor clustering has also been observed. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), an alkylating agent, is a potent carcinogen and is able to induce various cellular responses, including the activation of intracellular signal transduction pathways and changes in protein expression profile [22–24]. One unique response induced by MNNG treatment is the clustering of TNFR and EGFR in both African green monkey kidney Vero cells and human amnion FL cells [24,25]. Furthermore, the clustering of receptors is independent of the DNA damaging property of MNNG, as it can still occur in enucleated cells [24]. However, the mechanism for MNNG-induced receptor clustering is not known. Since sphingolipids are regarded as the major player in ligand-induced receptor clustering, we were interested to know if sphingolipids were also involved in this ligand-independent, chemical-induced receptor clustering. In this study, it was found that MNNG-induced receptor clustering did occur in lipid rafts; however, it may not be the same as EGF-induced receptor clustering, since MNNG and EGF showed different effects on sphingolipids metabolism.

Materials and methods

Cell culture and treatment

Human amnion FL cells were cultivated in Eagle's minimum essential medium (EMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 0.03% L-glutamine in a humidified incubator at 37 °C with 5% CO₂. MNNG (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock. For MNNG treatment, cells were treated with 10 μM MNNG for 20 min. EGF (Sigma)–, DMSO-treated or untreated cells were used as positive control, solvent control or blank control, respectively.

Immunofluorescent microscopy

EGFR clustering was observed using immunofluorescent microscopy. Briefly, 1×10^5 FL cells were seeded into a six-well culture plate (Corning) containing a glass coverslip in each well. After MNNG (10 μM) or EGF (100 ng/ml) treatment for 20 min, cells were fixed and permeated with 100% ice-cold methanol for 5 min, followed by blocking in a blocking solution (Zymed Laboratories, South San Francisco, CA) for 2 h. The plate was washed with PBS for three

times, and a rabbit-anti-human polyclonal anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:250 dilution) was added and incubated for 90 min. An FITC-labeled goat-anti-rabbit secondary antibody (Sigma, 1:160 dilution) was then added to the plate and incubated for another 60 min. The glass coverslip was then removed from the plate and mounted onto a glass slide, and the images were observed with an Olympus AX70 fluorescent microscope (Olympus, Japan) and analyzed using Image-Pro Plus software. For nystatin or imipramine treatment, cells were pre-incubated with either 60 µg/ml nystatin or 50 µM imipramine for 1 h before adding MNNG or EGF.

The translocation of ASM was also observed by immunofluorescent microscopy. The procedures were basically the same as described above, except that after MNNG or EGF treatment, cells were stained with a polyclonal rabbit anti-ASM antibody (1:200 dilution, Santa Cruz) for 2 h, followed by another hour of incubation with a Cy3-labeled goat-anti-rabbit secondary antibody (1:200 dilution, Boster Biological Technology). These cells were then washed and stained with 500 ng/ml of FITC-Cholera Toxin B (Sigma) for 1 h.

Ceramide and sphingomyelin extraction

After various treatments, approximately 4×10^7 cells were resolved in 500 µl chloroform-methanol (2:1, v/v) after treatment. One milliliter of H₂O was then added to each sample. The samples were then centrifuged at 6500 rpm for 15 min, and the lower phase was dried by vacuum centrifugation in a centrifugal evaporator (Speed-Vac, Thermo Savant, Holbrook, NY). Five hundred microliters of methanol containing 0.1 M NaOH was added to each tube at 55 °C for 1 h to decompose glycerophospholipids. After neutralization with 100 µl methanol containing with 1 M HCl, 500 µl hexane, and one drop of water were added to each sample. The samples were then centrifuged again at 6500 rpm for 15 min, the lower phase dried in a centrifugal evaporator after the upper phase was removed. The residue was mixed with 0.8 ml theoretical lower phase (chloroform:methanol:water, 86:14:1, v/v/v) and 0.2 ml theoretical upper phase (chloroform:methanol:water, 3:48:47, v/v/v) for the Folch partition, and centrifuged at 6500 rpm for 15 min. The lower phase was evaporated in a centrifugal evaporator after removing the upper phase to discard the salt. The residue as crude sphingomyelin and ceramide was stored at -70 °C for MALDI-TOF-MS (matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry) analysis.

MALDI-TOF-MS

Each sample obtained from the above procedure was dissolved in 5 µl chloroform-methanol (v/v, 2:1), followed by the addition of 5 µl matrix solution (0.5 M of 2,5-dihydroxybenzoic acid (2,5-DHB) (Sigma) solution in ethylacetate containing 0.1% TFA) in a 0.5 ml Eppendorff tube. The tube was agitated vigorously on a vortex mixer and then centrifuged in a microcentrifuge for 1 min. One microliter of mixture was directly added onto the sample plate and rapidly dried under a moderate warm stream of air in order to remove the organic solvent within seconds.

All the samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (ABI Applied Biosystem, Framingham, MA) with a 337 nm N₂ UV laser. The mass spectra of the samples were obtained in positive ion mode. Mass/charge ratios (M/S) were measured in the reflector/delayed extraction mode with an accelerating voltage of 20 kV, grid voltage of 67%, and delay time of 100 ns. C2-dihydroceramide (MW 343.6, Sigma) was used to calibrate the instrument. All sample lipid spectra were acquired using a low-mass gate at 400 Da.

Results

MNNG-induced receptor clustering occurs at lipid rafts

Since CD95 ligand-induced CD95 clustering has been shown to occur at lipid rafts, we were interested to know whether MNNG-induced receptor clustering also occurred at lipid rafts. As shown in Fig. 1, upper panel, both EGF (100 ng/ml) and MNNG (10 μ M) treatment, but not DMSO treatment, induced the formation of bright spots around the cells, indicating the clustering of EGFR. However, when cells were pre-incubated with nystatin, which can deplete cholesterol from lipid rafts thus leading to the disruption of lipid rafts [15], neither EGF nor MNNG treatment can induce the clustering of EGFR (Fig. 1, lower panel). This observation suggested that both EGF and MNNG-induced receptor clustering occurred primarily at lipid rafts.

MNNG induces the redistribution of ASM

One important aspect of ligand-induced receptor clustering is the translocation of ASM. Therefore, the distribution of ASM in MNNG-treated cells was evaluated using immunofluorescent microscopy. As shown in Fig. 2A, in both control and DMSO-treated cells, ASM existed in a diffused state of distribution. However, after either EGF or MNNG treatment, ASM translocated to a concentrated area of the cell, forming a “cap-like” structure. Furthermore, this cap-like structure resided in lipid rafts, as it colocalized with FITC-labeled cholera toxin B, which binds GM1 gangliosides enriched in rafts and therefore represents lipid rafts [15]. In addition, nystatin pre-incubation abolished the formation of cap-like structure (Fig. 2B), implying that the integrity of lipid rafts might be important for the translocation of ASM.

MNNG induces the generation of novel ceramide species

The consequence of ASM translocation and activation is the generation of ceramide through the hydrolysis of sphingomyelin. The traditional techniques for measuring ceramide or sphingomyelin (such as isotope labeling or HPLC) are either inaccurate or labor-intensive, and are unable to distinguish the various sphingolipid species; therefore, a new lipidomic approach using MALDI-TOF was applied for the rapid identification of sphingolipid species [26,27]. As listed in Table 1, there were several sphingomyelin and ceramide species in untreated cells. The treatments of either EGF or MNNG caused profound changes in sphingolipids metabolism. The effect of MNNG on sphingolipids metabolism was more dramatic, besides the increases in amount of some sphingolipid species, there was also the generation of some new sphingomyelin or ceramide species. For example, the concentrations of ceramide species at m/z of 571 and 572 were increased by both EGF and MNNG treatments (compare the ratio between peaks 571 or 572 and 610, Fig. 3A). On the other hand, MNNG treatment caused the generation of a new ceramide species at m/z ratio of 604, while the effect of EGF treatment on the induction of this new species was marginal (Fig. 3B). Part of the identified ceramide species are listed in Table 1, and some of the above-mentioned sphingolipids structures are presented in Table 2. These data provided strong evidence for the effects of EGF and MNNG on sphingolipids metabolism; furthermore, these data also reflected the differences between EGF and MNNG on sphingolipids metabolism.

Inhibition of ceramide generation abolishes MNNG-induced receptor clustering and ASM translocation

To explore the role of ceramide in MNNG-induced receptor clustering, imipramine, an inhibitor of ASM activity [28], was used to inhibit the generation of ceramide. Imipramine itself caused changes in sphingolipids metabolism, which was expected (data not shown). When cells were preincubated with imipramine before EGF or MNNG treatment, the increases

of peaks 571 and 572 were significantly decreased (data not shown). In addition, the generation of ceramide species 604 was also strongly inhibited by EGF or MNNG treatment (data not shown). Furthermore, EGFR clustering was abolished by imipramine preincubation (Fig. 4). The translocation of ASM was also observed using immunofluorescent microscopy, and the distinct cap-like structure was not observed after imipramine preincubation (data not shown). Taken together, these observations pointed to an important role of ceramide in MNNG-induced receptor clustering.

Discussion

Ligand-induced receptor clustering is essential for the activation of many receptors as well as the downstream signaling pathways. The fundamental mechanisms for the ligand-induced receptor clustering have been under intensive investigation. Through the detailed studies of ligand/specific antibody-induced CD95 clustering, a general scheme has been elucidated: the binding of ligand to receptors induces the translocation and activation of ASM; activated ASM then hydrolyzes sphingomyelin to generate ceramide. As a major component of lipid raft, the generated ceramide mediates the clustering of receptors [15,16,29,30]. This mechanism of receptor clustering has been extended towards other ligand-induced receptor clustering, including CD14 and CD40 [7,31,32], and may very well be a general principle for most types of ligand-induced receptor clustering [3,13,14].

A number of extracellular/intracellular factors, including UV, reactive oxygen species (ROS), and some chemicals, have also been shown to induce the clustering of certain cellular receptors, including CD95, TNFR, and EGFR [17,18,20,24,25,33–35]. The mechanism for this type of non-ligand-induced receptor clustering has also been explored based on the finding from studies of ligand-induced receptor clustering. For example, recently Lacour et al. [35] found that cisplatin induced clustering of CD95 at the surface of human colon cancer cell line HT29; while ASM inhibitor imipramine and cholesterol sequestering agent nystatin could prevent cisplatin-induced CD95 clustering. It was concluded that cisplatin activated ASM and induced ceramide production, which triggered the redistribution of CD95 into the plasma membrane rafts [35]. This is the first report that deciphered the molecular mechanism for a non-ligand, chemical-induced receptor clustering, which indicates that both ligand- and non-ligand-induced receptor clustering might share a common mechanism. In our study, we presented a similar result regarding the mechanism for an alkylating agent MNNG-induced EGFR clustering, e.g., clustering occurred at lipid rafts and ceramide was involved. Taken together, these results strongly support the idea that sphingolipids may be involved in both ligand- and non-ligand-induced receptor clustering.

However, one major difference exists between MNNG-induced EGFR clustering and cisplatin-induced CD95 clustering. In the cisplatin study, as well as in many other UV studies, the clustering of CD95 is functional, which is at least partially responsible for these stimuli-induced cell killing [17,20,35]. In MNNG-treated cells, although EGFR exhibited the same pattern of clustering as EGF treatment by immunofluorescent microscopy (Fig. 1 and [25]), and the dimerization of EGFR was further proved by Western blot (data not shown), EGFR lacked the characteristic phosphorylation for activation [25]. In addition, the downstream signal molecule Ras was also not activated [25]. Therefore, it seems that although different reagents can induce receptor clustering using the same mechanism, the consequences are quite different. The cause for this difference is worth further investigation.

The identification and quantification of different sphingolipid species has long been a bottleneck in the study of sphingolipids. The traditional methods, such as isotope labeling or HPLC, could provide some useful information, but are far from enough. Nevertheless, until the application of mass spectrometry in sphingolipid study does a great amount of information

is generated. Recently, Han and Gross [36,38] and Han and Cheng [37] have proposed a new concept as “lipidomics,” in which multiple techniques, particularly mass spectrometry, are utilized to quantitate the hundreds of chemically distinct lipids in cells and determine the molecular mechanisms through which they facilitate cellular function. Previously, using isotope labeling technique, we have shown that MNNG treatment can cause the increase in cellular ceramide concentration [39]. Here, using MALDI-TOF, we were able to show the increase in the concentrations of some ceramide species, as well as the generation of some new ceramide species following either MNNG or EGF stimulation (Table 1). The results obtained using this lipidomic approach clearly showed its advantage: the different species of ceramide were individually identified, which was not possible using the traditional method. However, the importance of these different ceramides needs to be clarified.

Taken together, we have shown that MNNG-induced EGFR clustering shared a common mechanism with EGF-induced receptor clustering, in which both EGF and MNNG induced the redistribution of ASM and the generation of ceramide. The integrity of lipid rafts also is essential for EGFR clustering. Nonetheless, EGF and MNNG had different effects on sphingolipids metabolism, and these differences might be responsible for the different activation status of EGFR.

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Fig. 1.

MNNG and EGF induce EGFR clustering at lipid rafts. Upper panel, FL cells were either untreated (blank), treated with DMSO (solvent control), EGF (100 ng/ml, positive control), or 10 μ M MNNG for 30 min. The clustering of cell surface EGFR was observed using immunofluorescent microscopy. Lower panel, cells were preincubated with 60 μ g/ml nystatin for 1 h and then were treated as described above for immunofluorescent microscopy.

Fig. 2.

MNNG and EGF induce the redistribution of ASM. (A) After various treatments for 30 min, cells were stained with anti-ASM antibody and Cy3-labeled secondary antibody. After washing, cells were further stained with FITC-cholera toxin B (CtxB) for 1 h. The images were captured with an Olympus AX70 fluorescent microscope and analyzed using Image-Pro Plus software. (B) Cells were preincubated with nystatin for 1 h before MNNG or EGF treatment, the distribution of ASM was then observed as described above.

Fig. 3.

MNNG and EGF induce changes in sphingolipids metabolism. After 30 min of treatment, lipids were extracted and subjected to mass spectrometry analysis as described under Materials and methods. (A) Representative mass spectrometry data for ceramide species m/z 571 and 572. The proposed molecular for m/z 571 was (t18C16h:0) [M]. And for m/z 572 the proposed molecule was either (t18C16h:0) [M + H]⁺; or (t18C16:1) [M + H + H₂O]⁺; or (d18C16h:0) [M + H + H₂O]⁺. Mass range was from 560 to 625. (B) Representative mass spectrometry data for ceramide species m/z 604. The proposed molecular for m/z 604 was either (t18C18:1) [M + Na]⁺; or (d18C18h:0) [M + Na]⁺; or (t18C17h:0) [M + H + H₂O]⁺. Mass range was from 582 to 615.

Fig. 4.

Imipramine inhibits EGFR clustering. Upper panel, immunofluorescent microscopy for FL cells with various treatments. Lower panel, cells were preincubated with imipramine for 1 h and then were treated as described above for immunofluorescent microscopy.

Table 1

The effects of MNNG and EGF on ceramide metabolism

<i>m/z</i>	Blank	DMSO	EGF	MNNG
571 (t18C16h:0) [M]	+	+	^a	^a
572 (t18C16h:0) [M + H] ⁺	+	+	^a	^a
(t18C16:1) [M + H + H ₂ O] ⁺				
(d18C16h:0) [M + H + H ₂ O] ⁺				
576 (t18C16:1) [M + Na] ⁺	+	+	^a	^a
(d18C16h:0) [M + Na] ⁺				
581 (t18C18:1) [M]				
(d18C18h:0) [M]				+
604 (t18C18:1) [M + Na] ⁺			±	+
(d18C18h:0) [M + Na] ⁺				
(t18C17h:0) [M + H + H ₂ O] ⁺				
610 (d18C20:1) [M + H + H ₂ O] ⁺	+	+	+	+
(t18C20:1) [M + H] ⁺				
669 (t18C23h:0) [M]		+	+	+
670 (d18C24:1) [M + Na] ⁺	+	+	+	+
(t18C23:1) [M + H + H ₂ O] ⁺				
(d18C23h:0) [M + H + H ₂ O] ⁺				
(t18C23h:0) [M + H] ⁺				

^a Increase in concentration.

Table 2
Proposed molecular structures of some identified ceramide species

<i>m/z</i>	Proposed structure
571 (t18C16h:0) [M]	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}-\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH-OH} \\ \\ (\text{CH}_2)_{13} \\ \\ \text{CH}_3 \end{array} $
572 (t18C6:1) [M + H + H ₂ O] ⁺	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}-\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH} \\ \\ \text{CH} \\ \\ (\text{CH}_2)_{12} \\ \\ \text{CH}_3 \end{array} $
(d18C16h:0) [M + H + H ₂ O] ⁺	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}=\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \\ \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH-OH} \\ \\ (\text{CH}_2)_{13} \\ \\ \text{CH}_3 \end{array} $
581 (t18C18:1) [M]	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}-\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH} \\ \\ \text{CH} \\ \\ (\text{CH}_2)_{14} \\ \\ \text{CH}_3 \end{array} $
(d18C18h:0) [M]	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}=\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \\ \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH-OH} \\ \\ (\text{CH}_2)_{15} \\ \\ \text{CH}_3 \end{array} $
670 (t18C23h:1) [M + H + H ₂ O] ⁺	$ \begin{array}{c} \text{CH}_3(\text{CH}_2)_{13}\text{CH}-\text{CH}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{NH} \\ \\ \text{C=O} \\ \\ \text{CH} \\ \\ \text{CH} \\ \\ (\text{CH}_2)_{19} \\ \\ \text{CH}_3 \end{array} $