Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling

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Contributed by Lutz Birnbaumer, December 13, 2016 (sent for review November 14, 2016; reviewed by Hugo Maccioni and Arohan Subramanya)

Soluble klotho, the shed ectodomain of the antiaging membrane protein α-klotho, is a pleiotropic endocrine/paracrine factor with no known receptors and poorly understood mechanism of action. Soluble klotho down-regulates growth factor-driven PI3K signaling, contributing to extension of lifespan, cardioprotection, and tumor inhibition. Here we show that soluble klotho binds membrane lipid rafts. Klotho binding to rafts alters lipid organization, decreases membrane’s propensity to form large ordered domains for endocytosis, and down-regulates raft-dependent PI3K/Akt signaling. We identify α2-3-sialylactose present in the glycan of monosialogangliosides as targets of soluble klotho. α2-3-Sialyllactose is a common motif of glycans. To explain why klotho preferentially targets lipid rafts we show that clustering of gangliosides in lipid rafts is important. In vivo, raft-dependent PI3K signaling is up-regulated in klotho-deficient mouse hearts vs. wild-type hearts. Our results identify ganglioside-enriched lipid rafts to be receptors that mediate soluble klotho regulation of PI3K signaling. Targeting sialic acids may be a general mechanism for pleiotropic actions of soluble klotho.

Significance

Soluble klotho is the shed ectodomain of the antiaging membrane protein α-klotho that exhibits pleiotropic actions, including down-regulation of growth factor-driven PI3K signaling, contributing to lifespan prolongation, cardioprotection, and tumor inhibition. Whether membrane receptors exist for soluble klotho is unknown. We identify lipid rafts as receptors for soluble klotho. We show klotho binds specific sialic acid residues of gangliosides highly enriched in the outer leaflet of lipid rafts. Klotho binding to gangliosides modulates lipid organization and inhibits lipid raft-dependent PI3K signaling. In vivo, klotho-deficient mouse hearts have heightened raft-dependent PI3K signaling vs. wild-type hearts. We reveal a novel physiological regulator of lipid raft formation and function and provide a potential general mechanism for pleiotropic actions of SKL.


[Reviews: H.M., National University of Cordoba; and A.S., University of Pittsburgh School of Medicine.]

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620301114/-/DCSupplemental.

Results

sKL Binds Lipid Rafts and Modulates Lipid Organization Within Rafts. sKL is pleiotropic. We posit that multifunctional lipid rafts may be receptors for sKL. sKL comigrated with markers of lipid rafts (Fig. 1A), indicating affinity for isolated lipid rafts. To examine whether klotho binds lipid rafts in intact cells and the potential effects of binding, we used colocalization analysis of two different fluorophore-labeled gangliosides in cell membranes. HeLa cells were stained with CTxB-Alexa Fluor-594 conjugate (to label endogenous GM1) followed by exogenous BODIPY-FL-C5-GM1 and imaged by dual-color confocal microscopy. Correlation between pixel intensity histogram of CTxB-labeled red channel and BODIPY-GM1 green channel was analyzed by Pearson’s correlation (coefficient values “1” and “0” correspond to perfect colocalization and completely random uncorrelated distribution, respectively). As shown, mean Pearson’s coefficient (p.c.) for CTxB-labeled GM1 and BODIPY-GM1 is 0.53 ± 0.02 (Fig. 1B and C; P < 0.001 vs. 0), indicating highly significant colocalization. Disrupting lipid rafts by methyl-β-cyclodextrin (MβCD) markedly reduced p.c. for CTxB and BODIPY-GM1 to a value not significantly different from 0, supporting that the two fluorescence-labeled gangliosides are highly colocalized in lipid rafts (Fig. 1B and Fig. S1A). Caveolin-1, a marker for lipid rafts, also showed significant colocalization with CTxB, whereas the non-raft protein CD71 (transferrin receptor) did not show colocalization with CTxB (Fig. 1B and Fig. S1B and C). Treatment with sKL markedly decreased p.c. for CTxB and BODIPY-GM1 (Fig. 1B and Fig. S1D; see Fig. S1 legend for comments).

The resolution of confocal microscopy is limited by the diffraction of light to ~250 nm. To support that klotho targets lipid rafts, we conducted Förster resonance energy transfer (FRET) studies. FRET detects molecular interactions on a scale of 1–10 nm, smaller than the lowest size limit of rafts (10–200 nm). FRET was measured by fluorescence lifetime imaging microscopy (FLIM) and analyzed by the phasor approach (12). As a control, lifetime for

![Fig. 1. Klotho binds lipid rafts and alters lipid organization.](image-url)
GM1 (donor) in the absence of cholesterol (acceptor) has longer values, and sKL treatment did not affect lifetime for GM1 alone (Fig. 1D, Top; blue color ≈ longer lifetime values). Lifetime for GM1 shifted to the shorter values (purple) in the presence of cholesterol, indicating quenching of GM1 by cholesterol (i.e., FRET occurrence between GM1 and cholesterol; Fig. 1D, Middle). Klotho treatment decreased FRET between GM1 and cholesterol – lifetime for GM1 shifted back to the longer values (Fig. 1D, Bottom). Phasor plot analysis supported the validity of FLIM-FRET data (Fig. 1E). The results indicate that klotho treatment decreased FLIM-FRET occurrences between GM1 and cholesterol, supporting an effect of klotho binding on lipid organization within rafts. Lipid rafts feature a high degree of membrane order. sKL decreases membrane order (SI Text and Fig. S2), further supporting that it binds lipid rafts to modulate lipid organization.

Klotho Selectively Down-Regulates Lipid Raft-Dependent PI3K/Akt Signaling and Thereby Inhibits TRPC6 Channel Function. sKL inhibits growth factor (GF)-driven PI3K signaling (2, 9). PI3K is present in lipid rafts and in nonraft membranes depending on context (13). Supporting that lipid rafts are receptors for sKL and mediate its effect to inhibit PI3K signaling, sKL treatment down-regulated PI3K/Akt signaling associated with lipid rafts (fractions 4 and 5, marked by caveolin-1), but not in nonraft regions (fractions 9–12; Fig. 2 A and B). The specificity of sKL on lipid raft-dependent, GF-driven PI3K/Akt signaling is further evidenced by findings that whereas IGF1 restored PI3K/Akt signaling in raft and nonraft membranes, IGF1 treatment only affected IGF1-stimulated PI3K/Akt signaling in the rafts (Fig. S3). For comparison, pharmacological inhibition of PI3K by wortmannin (Wmn) abrogated PI3K/Akt signaling in both raft and nonraft regions (Fig. S5; see legend for comments). sKL inhibits PI3K-dependent diacylglycerol (DAG)-stimulated exocytosis of TRPC6 channels, underlying its cardioprotective action (9). TRPC6 channel is activated by DAG via dual mechanisms: (i) direct activation of channel gating and (ii) stimulation of channel exocytosis (Fig. 3A and refs. 14 and 15). We used DAG-stimulated TRPC6 function as an additional experimental system to study the regulation of PI3K signaling by sKL. As reported before, klotho inhibited DAG-stimulated TRPC6 currents, an effect not additive to the effect of Wmn or to tetanus toxin (TTX)-mediated blocking of channel exocytosis (Fig. 3B). Because klotho down-regulates PI3K signaling to inhibit DAG-stimulated TRPC6 exocytosis but does not affect direct channel gating by DAG, the maximal inhibition of TRPC6 by klotho is only partial (Fig. 3 A and B). Treatment with the cholesterol binder filipin decreased DAG-stimulated TRPC6 currents and prevented further inhibition of currents by klotho (Fig. 3C). There was no additivity among inhibition of TRPC6 caused by klotho, filipin, TTX, and Wmn (Fig. 3C,Inset and Fig. S4). Thus, lipid rafts mediate down-regulation of PI3K-dependent TRPC6 exocytosis by sKL. Inhibition of TRPC6 by klotho was reversed after klotho washout (Fig. S5).

Sphingolipids including gangliosides are important components of lipid rafts. Pretreatment of cells with N-butyldeoxyglucosylactonoyrriymycin (NB-DGJ), which blocks biosynthesis of gangliosides (16), decreased DAG-stimulated TRPC6 currents and prevented further decreases by sKL (Fig. 3 D and E). Specificity of NB-DGJ-mediated depletion of membrane gangliosides is supported by findings that application of exogenous GM1, but not asialo-GM1 (a GM1 analog missing sialic acid), reversed NB-DGJ-induced decreases of TRPC6 currents to the control level without NB-DGJ (Fig. 3E). In cells rescued from the effect of NB-DGJ by GM1, klotho treatment decreased TRPC6 currents. GM3 is a more abundant monosialoganglioside than GM1 in many nonneuronal tissues (17). Thus, we examined the role of GM3. Exogenous GM3, but not lactosylceramide (LacCer; a GM3 analog missing sialic acid), also reversed the inhibition of TRPC6 caused by NB-DGJ and restored the effect of klotho to inhibit TRPC6 after NB-DGJ (Fig. 3F). Thus, gangliosides and cholesterol-enriched lipid rafts mediate klotho regulation of PI3K signaling and TRPC6 function. Klotho Specifically Targets α2-3-Sialyllactose in the Glycan of Gangliosides to Regulate Lipid Rafts. Lipid rafts also contain glycoproteins with α2-3-sialic acid in the glycan structure. We next address whether sKL targets α2-3-sialic acids present in gangliosides or in raft-associated glycoproteins to regulate TRPC6 function, by using disialoganglioside GD3 that has an additional α2-8-sialic acid linkage to the underlying α2-3-sialic acid (Fig. 3D). Exogenous GD3 rescued PI3K-dependent TRPC6 activity after inhibition by NB-DGJ (Fig. 3G), indicating it can substitute endogenous gangliosides for klotho regulation of TRPC6. Interestingly, klotho treatment failed to inhibit TRPC6 in cells rescued by GD3 after NB-DGJ. The fact that whereas both GD3 and GM3 rescue TRPC6 currents klotho only inhibited TRPC6 in cells rescued by GM3, but not by GD3, indicates that klotho targets exposed α2-3-linked sialic acid in gangliosides, rather than α2-8-linked sialic acid. Because glycoproteins in lipid rafts are not expected to be different in GD3-rescued vs. GM3-rescued cells, the results argue against klotho’s targeting α2-3-sialic acid-containing glycoproteins. This notion is further extended by competition experiments. When preincubated with GM1, but not asialo-GM1, klotho failed to inhibit TRPC6 (Fig. S6A). Moreover, preincubation with GM3, but neither LacCer nor GD3, neutralized the ability of klotho to inhibit TRPC6 (Fig. S6 A–C). Inability of GD3 to neutralize the effect of klotho lends additional support to the
finding in Fig. 3G that accessibility of α2-3-linked sialic acid is critical for klotho binding. Together, the results in Fig. 3G and Fig. S6A–C point to the structure of GM3 glycan, α2-3-sialyllactose (Neu5Acα2-3Galβ1-4Glc; also known as 3'-sialyllactose), as the minimal motif for klotho binding and regulation of TRPC6. Indeed, 3'-sialyllactose at a concentration (3 mM) much higher than that for GM1 and GM3 in the above experiments (10 μM) fully neutralized the effect of klotho, whereas lactose at the same concentration had no effect (Fig. S6D). The apparent $K_d$ determined similarly for 3'-sialyllactose binding to klotho is ~1 mM (Fig. S6F) and agrees with the $K_d$ for 3'-sialyllactose binding with sialic acid-binding Ig-like lectin (Siglec) family proteins (18). GM3 forms micelles in solution, likely explaining differences in the apparent $K_d$ for GM3 vs. free 3'-sialyllactose (Discussion).

Next, we used biolayer interferometry to analyze binding of klotho to 3'-sialyllactose in vitro. Klotho bound to streptavidin-immobilized biotin-labeled 3'-sialyllactose, but not to lactose (Fig. S6 G and H). $K_d$ for the
Klotho Binds Lipid Rafts in Live Cells by Interacting with Raft-Associated GM1. To further support that klotho indeed binds to monosialogangliosides clustered in lipid rafts, we examined klotho interaction with GM1 in live cells by studying FRET between fluorophore-labeled klotho and BODIPY-GM1. Addition of fluorophore-labeled klotho causes fluorescence quenching of cell membrane BODIPY-GM1 analyzed by FLIM-FRET in 10 min (Fig. 4 A and B), indicating klotho and GM1 interact within a 10-nm distance. In cells pretreated with MβCD to disrupt lipid rafts, BODIPY-GM1 remained partitioned into cell membranes, but addition of fluorophore-labeled klotho failed to quench GM1 fluorescence (Fig. 4 C and D). As a control, addition of unlabeled klotho to normal cells did not cause quenching of BODIPY-GM1 (Fig. 4E). The results provide direct evidence to support that klotho targets raft-associated GM1. In addition, klotho prevents cells from forming large ordered domains but does not cause global membrane disorder (SI Text and Fig. S8).

Evidence for Effect of sKL on Lipid Rafts in Vivo. Cardioprotection by klotho is mediated by circulating sKL through down-regulating PI3K-dependent activation of TRPC6 channels (9). To investigate the role of klotho on lipid rafts in vivo, we first showed that lipid rafts mediate sKL’s effect to down-regulate PI3K-dependent TRPC6 channel function in freshly isolated cardiac myocytes: Filippin, Wmn, and klotho each decreased TRPC6-mediated currents by a similar degree, and the combined effects from individual agents were not additive (Fig. 5A). Together with the recent report that TRPC6 is localized to lipid rafts of isolated myocytes (19), the results support that raft-dependent PI3K signaling in the heart is a useful readout for in vivo effects of sKL on lipid rafts. We then compared raft-associated PI3K signaling in klotho-deficient hearts vs. wild-type hearts. Raft-dependent PI3K signaling was significantly up-regulated in klotho-deficient hearts (from mice rescued from death by dietary phosphate restriction) compared with wild-type hearts (Fig. 5 B and C). The increase in PI3K signaling was restricted to lipid rafts; no differences in PI3K signaling were observed in nonraft membranes between klotho-deficient and wild-type hearts. Thus, circulating sKL plays an important role in regulating lipid rafts in vivo.

Discussion
Monosialogangliosides are dynamically distributed between raft and nonraft membranes as lipid rafts undergo self-assembly and disassembly. sKL normally circulates at very low levels (30–100 pM) (2). The low affinity of sKL for α2-3-sialyllactose (KD ~1 mM) suggests that isolated α2-3-sialyllactose monomers in nonraft membranes may not be effective physiological targets of sKL. Glycan clustering is a common mechanism for enhancing binding of low-affinity glycans to multivalent glycan-binding proteins (11). sKL is likely multivalent due to the fact that sKL forms dimers and each unit contains two highly homologous KL1 and KL2 domains with potential glycan-binding activity (4, 20). Results of in vitro binding assays and competition experiments using TRPC6-based functional assays show that the apparent binding is estimated ~40 nM. High density of 3-sialyllactose immobilized on the binding surface likely explains the apparently higher binding affinity of klotho determined by this method.

Fig. 4. Klotho–GM1 interaction live cell membranes analyzed by FLIM-FRET. (A) HEK cells were stained with BODIPY FL-505/510-C5-GM1 (donor; 100 nM). Lifetime for GM1 alone has longer values (blue color cursor = regions with longer lifetime values). Lifetime for GM1 shifted to the shorter values (purple color cursor) in the presence of fluorophore-labeled sKL (acceptor; 300 pM) over 10 min, indicating quenching of GM1 by sKL and FLIM-FRET occurrence between GM1 and klotho. (B) Phasor plot analysis of FLIM-FRET data showed FRET efficiency ~20%, with fractional contribution of lifetimes ~36% from quenched donor, 55% from unquenched donor, and 9% from background. In the trajectory, blue circle marks lifetime for donor plus unquenched klotho, purple circle for donor plus labeled klotho, and green circle for background autofluorescence. (C and D) Cells were pretreated with 5 mM MβCD for 3 h at 37 °C to disrupt lipid rafts. GM1 lifetime after addition of fluorophore-labeled sKL was comparable to GM1 only, indicating little to no FRET occurrence between GM1 and klotho. Shown is representative of three separate experiments with similar findings. (E) Addition of unlabeled klotho does not cause shift to shorter values (vs. A), indicating no quenching of BODIPY-GM1 fluorescence by unlabeled klotho. (Scale bars, 5 μm in A, C, and E.)
binding affinity of sKL for clustered α2-3-sialyllactoses is >300-fold higher than for free α2-3-sialyllactoses, strongly supporting the notion that lipid rafts highly enriched in α2-3-sialyllactose–containing gangliosides are effective physiological targets of sKL. The notion of selective targeting of sKL to lipid rafts is further supported by FRET experiments in live cells showing that klotho selectively interacts with raft-associated GM1, and by experiments using the highly sensitive electric probe C6TPP showing that sKL does not affect disordered membranes that reflect nonraft membrane regions (Fig. S8). Moreover, raft-dependent PI3K signaling is selectively up-regulated in klotho-deficient hearts compared with wild-type hearts. The precise stoichiometry of klotho subunits and sialyllactose motifs required for effective binding in vivo remains to be determined.

Local lipid and protein interactions and interactions of raft proteins or lipids with cortical actin cytoskeleton play important roles in lipid raft formation (10, 21). Physiological soluble factors that control the formation and function of lipid rafts are much less known. We identify sKL as a physiological circulating regulator of lipid rafts by targeting sialogangliosides. Physiological levels of sKL likely modulate the function of lipid rafts acting as a rheostat rather than as an on-off switch. Interestingly, a recent paper reports that soluble FLT1 (the shed ectodomain of VEGF receptors) also reorganizes lipid rafts with functional consequences by binding to GM3 in lipid rafts (22). We have also found that klotho is expressed in podocytes and protects podocytes against injury (23). Thus, gangliosides-enriched lipid rafts are important targets of extracellular factors with potentially important biological and disease significance.

sKL is a pleiotropic hormone with poorly understood mechanism of action (2, 6–9). Lipid rafts may mediate multiple cellular effects of sKL. The physiological effect of klotho through targeting sialic acid, however, is not limited to glycolipids. Previously, we showed that sKL targets terminally exposed α2-6-sialic acids in the N-glycan of TRPV5 to prevent its endocytosis via caveolae (24). Here, we show that α2-3-sialyllactose also binds klotho, supporting that klotho targets both α2-3- and α2-6-linked sialic acids. Klotho’s effect on N-glycans of TRPV5 is different from that on lipid rafts. Klotho’s effect on lipid rafts is reversible on washout. Further, the effect of klotho on TRPC6 function is mediated by raft-dependent PI3K/Akt signaling, whereas klotho acts directly on the TRPV5 channel protein (9, 24, 25). Regarding klotho’s effect on α2-6-sialyllactose of TRPV5 N-glycans, the binding affinity may be enhanced by the channel’s tetrameric structure and concentration in caveolae. Ultimately, the crystal structure of klotho will help elucidate the molecular basis of klotho’s specificity for α2-3- and α2-6-sialic acid and the role of underlying lactose.

In summary, sialic acids in the context of α2-3- or α2-6-sialyllactose are low-affinity targets of sKL. Through enrichment of gangliosides containing terminal α2-3-sialyllactoses, lipid rafts form effective binding sites for physiologically low circulating levels of sKL. sKL binding to lipid rafts modulates lipid organization and down-regulates PI3K signaling, perhaps by affecting function of raft-associated GF receptors and/or recruitment of PI3K to rafts or its activity.

Materials and Methods

Expanded methods are available in SI Materials and Methods. Animal use was approved by the institutional animal care and use committee of the University of Texas Southwestern Medical Center.

Lipid rafts were fractionated using sucrose gradient ultracentrifugation. Confocal imaging of fluorescein-labeled CTxB, GM1, cholesterol, and FLIM-FRET studies were performed using a Zeiss LSM510 laser scanning confocal microscope system. GP studies were performed by staining cells with Di-4-ANEPPDHQ. Binding studies were performed using biolayer interferometry. Whole-cell patch-clamp recording was performed in HEK or Hela cells transfected with TRPC6 plasmid.

ACKNOWLEDGMENTS. We thank Moosé Levi and Tim Lei for discussion and support, Amberlyn Wands and Janet McCombs for participation in the early stage of the project, and Marc Diamond and Jaime Vaquer-Alcea for provision of the Octet and helpful discussions. This work was supported in part by NIH Grants DK05726, DK100665, and HL11893, the Intramural Research Program of the NIH, Project Z01-ES101684 (L.B.), Welch Foundation Grant 1-1686, and NIH/National Center for Advancing Translational Sciences Colorado Clinical and Translational Science Institute Grant UL1 TR001082.