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Manipulation of Lipid Rafts in Neuronal Cells

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Abstract: Lipid rafts are specialized plasma membrane micro-domains highly enriched in cholesterol, sphingolipids and glycosylphosphatidylinositol (GPI) anchored proteins. Lipid rafts are thought to be located in the exofacial leaflet of plasma membranes. Functionally, lipid rafts are involved in intracellular trafficking of proteins and lipids, secretory and endocytotic pathways, signal transduction, inflammation and in cell-surface proteolysis. There has been substantial interest in lipid rafts in brain, both with respect to normal functioning and with certain neurodegenerative diseases. Based on the impact of lipid rafts on multitude biochemical pathways, modulation of lipid rafts is used to study related disease pathways and probably offers a target for pharmacological intervention. Lipid rafts can be targeted by modulation of its main components, namely cholesterol and sphingolipids. Other approaches include the modulation of membrane dynamics and it has been reported that protein-lipid interactions can vary the occurrence and composition of these membrane micro-domains. The present review summarizes the possibilities to modulate lipid rafts with focus on neuronal cells.

Keywords: Lipid raft, cholesterol, membrane fluidity, statin, cyclodextrine, docosahexaenoic acid.

IMPACT OF LIPID RAFTS FOR PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL FUNCTIONS IN THE CNS

Lipid rafts are specialized plasma membrane micro-domains highly enriched in cholesterol, sphingolipids, sphingomyelin [SM], gangliosides (GM)) and glycosylphosphatidylinositol (GPI) anchored proteins [1]. Alternatively, lipid rafts have been named as detergent insoluble glycolipid enriched membranes or detergent‐resistant membranes [2]. Caveolae are defined as membrane invaginations enriched in cholesterol and glycosphingolipids containing the protein caveolin, which play a role in endocytosis [3]. The reader is referred to recently published reviews for a comprehensive and detailed discussion of lipid rafts [1, 4-6]. The present review summarizes the impact and possibilities to modulate lipid rafts with focus on neuronal cells.

Lipid rafts are thought to be located in the exofacial leaflet of plasma membranes [7], although it has been suggested that lipid rafts may also be found in the cytofacial leaflet [8]. Potential explanations for raft formation include the preference of cholesterol for the saturated acyl chains of sphingolipids compared with glycerophospholipids and the dynamic interactions of protein–lipid and protein–protein linkages [6, 9]. An additional mechanism has been proposed whereby proteins are encased in a "lipid-shell" of cholesterol and sphingolipids [10, 11].

The majority of studies on lipid rafts have used methods that takes advantage of the insolubility of the lipid fraction of membranes in detergents such as Triton X-100 [12-15]. There is evidence indicating that the composition of the isolated lipid rafts may differ depending on whether a detergent is used [16-18]. It has been argued that the use of detergents may actually induce formation of deter-gent‐resistant lipid domains and not to be representative for physiological membrane structures [19]. In addition, it has also been observed that different detergent/lipid ratios and starting materials can influence the lipid and protein composition, making comparisons among studies difficult [4]. Since it was suspected that the presence of detergent could produce artifacts, the use of non-detergent methods has been suggested [8, 20]. Accordingly, studies on brain synaptosomes revealed differences both qualitatively and quantitatively in proteins and lipids when detergent and non-detergent methods were applied [20, 21]. Beside the biochemical characterization of isolated membrane fractions, different techniques for studying lipid rafts in situ such as functional imaging, cytometry, two-photon microscopy, electron microscopy, fluorescent-quenching and resonance energy transfer techniques have been developed [22-29].

Functionally, lipid rafts are involved in intracellular trafficking of proteins and lipids, secretory and endocytic pathways, inflammation and in cell-surface proteolysis [16, 30-34]. In the brain, there has been substantial interest in lipid rafts, with respect to both normal functioning and certain neurodegenerative diseases. It is well accepted that lipid rafts play an important role for signaling processes in the central nervous system [7]. Synaptic proteins such as synaptophysin or synaptotagmin associate with lipid rafts [35-38] and lipid rafts play a role in the control of post synaptic membrane viscosity [39]. Moreover, brain-derived neurotrophic factor, which exerts multiple biological functions in the CNS increased the levels of presynaptic proteins in lipid rafts of neurons [40]. Regulation of the glutamateric neurotransmission, which is involved in the formation of spatial memory, represents one example for the impact of lipid rafts on classical signaling processes [41].

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Accordingly, NMDA-, AMPA- and metabotrobic glutamate receptors are regulated by lipid raft related pathways [42-44]. However, excess of extracellular glutamate induced by cerebral ischemia leads to neuronal cell death, which is accompanied by increased phosphorylation and redistribution of NMDA receptors between synaptic lipid rafts and post-synaptic densities in the rat brain [45].

Lipid rafts have been attracted attention in neurodegeneration, such as Prion diseases, Parkinson`s disease and especially in AD [46-48]. Recently, alterations in lipid rafts isolated from AD brain were reported [49], including the localization of active γ-secretase in lipid rafts in human brain [50]. Active γ-secretase is involved in the pathological proce-ssing of the AD related amyloid precursor protein (APP). Neurotoxic β-amyloid peptide (Aβ) is a product of the secretase cleavage of APP and both proteins have been located in lipid rafts [26, 51-54]. Moreover, it was demonstrated that the presenilin-1 protein, which is part of the γ-secretase complex, induces lipid raft formation *in vivo* [55].

Gradual changes in steady-state levels of Aβ in brain are considered as an initial step in the amyloid cascade hypothesis of AD. There is evidence that the membrane lipid environment may modulate secretase activity and alters its function. Cleavage of APP strongly depends on membrane properties and it was shown that Aβ-oligomers from AD brains associate with a detergent-resistant membrane fraction in a cholesterol-dependent manner [56]. Since Aβ perturbs cell membrane fluidity, the cell membrane may be the location where the neurotoxic cascade of A is initiated. Based on the observation that Aβ binds to lipid raft related ganglioside GM-1 [57] the effects of oligomeric Aβ on membrane fluidity of whole living cells, the impact of exogenous and cellular Aβ on the processing of APP and the role of GM-1 ganglioside was tested recently [58]. Evidence was presented that oligomeric Aβ stimulates the amyloidogenic processing of APP by reducing membrane fluidity and complexing with GM-1 ganglioside. It was concluded that this dynamic action of Aβ might start a vicious circle, where endogenous Aβ stimulates its own production [58].

In brains of AD patients, abundance of raft related flotillin proteins was reported to increase with progression of A deposition [59, 60]. However, abundance of flotillin-1 was reduced in lipid rafts isolated from mice harboring human apoE4, which represent an AD model [61]. Although, the function of flotillin is not well understood, it was proposed that it plays a role for neuronal regeneration [62, 63]. Sphingomyelin represent one of the major glycosphingolipids present in lipid rafts [17]. Changing sphingomyelin levels modify lipid raft structure and function [5, 64, 65]. Sphingomyelin levels were significantly lower in the lipid rafts isolated from synaptosomes of 12- and 24- month-old apoE4 mice in contrast to the 2-month-old apoE4 mice [61].

Based on the impact of lipid rafts on multitude biochemical pathways, modulation of lipid rafts is used to study related disease pathway and probably offers a target for pharmacological intervention. Lipid rafts can be targeted by modulation of its main components, namely cholesterol, sphingo- and ganglioside lipids. Other approaches include the modulation of membrane biophysical parameters such as fatty acid composition.

MEMBRANE CHOLESTEROL AND SPHINGO-MYELIN – TARGETS FOR THE MODULATION OF LIPID RAFTS

Cholesterol is known to be essential for the functional activity of physiological membranes [66, 67] and plays an essential role in the regulation of synaptic function and plasticity [68, 69]. The highest cellular cholesterol load is found within the plasma membrane (PM). Levels and distribution of PM cholesterol are tightly regulated by the cell [70]. The capacity of the PM to incorporate cholesterol is largely a function of its sphingomyelin content [67]. PM polarity is conditioned by the asymmetric insertion of cholesterol as well as functional proteins and phospholipids. Inside the PM, about 70-85% of free cholesterol resides in the cytofacial bilayer leaflet, whereas only about 15-30% join the exofacial leaflet [67, 71, 72]. Even in this outer membrane domain the intra-membrane distribution of cholesterol follows a strict organization into structural pools and is altered during aging [73]. Cholesterol builds up lateral membrane domains or kinetic pools that probably mediate cellular cholesterol efflux and participate in the formation lipid raft domains [67].

Cholesterol is highly enriched in lipid rafts [17] and evidence suggest that cholesterol condenses the packing of the sphingolipid molecules and thus cholesterol-sphingolipid microdomains form a separate lipid ordered phase in the exofacial leaflet of the membrane [74, 75]. Cholesterol levels and consequently lipid raft structure can be modulated either by physical extraction from the plasma membrane *in vitro* or by inhibition of the cellular biosynthesis using specific enzyme inhibitors *in vivo*. Changing membrane cholesterol domains also affect the cellular cholesterol homeostasis. Non-raft cholesterol pool within the plasma membrane primarily senses the amount of cellular bulk cholesterol [76]. Changing sphingomyelin levels modify lipid raft structure and function [5, 64, 65]. Raft destruction with sphingomyelinase shuttles cholesterol into the non-raft pool, which probably flows back to the endoplasmatic reticulum and thus blocks the intracellular translocation of the SREBP-SCAP complex to the Golgi and further cholesterol synthesis [76, 77].

METHYL-β-CYCLODEXTRIN – A BIOPHYSICAL TOOL TO MODULATE MEMBRANE CHOLESTEROL LEVELS

Cyclodextrins are torus-shaped cyclic oligosaccharides containing at least six glucose units attached by glycosidic bonds. They possess a hydrophilic outer surface and a hydrophobic inner cavity. Cyclodextrins enhance the solubility of non-polar substances (e.g., cholesterol) by incorporating them into their hydrophobic cavity and forming non-covalent water-soluble inclusion complexes. Cyclodextrins comprised of 6, 7, and 8 glucose units (α-, β- and γ-forms, respectively) were used to alter the lipid composition of cells. Among those, β-cyclodextrins and derivatives thereof such as methyl-β-cyclodextrin (MβCD) or 2-hydroxypropyl-β-cyclodextrin were found to selectively extract cholesterol from the plasma membrane, in preference to other membrane lipids [78]. MβCD affects membrane raft domains and modulates the location of raft-related proteins

[23, 79, 80]. Hence, MβCD is commonly used to study lipid raft related processes in neuronal cells such as receptor mediated signaling [81-83] or processes related to neurodegeneration [66, 84-88]. Recently, the mechanism how MβCD affect lipid rafts was studied in more detail: Treatment of synaptosomal plasma membranes (SPM) isolated from mouse brains with MβCD significantly lowered SPM free cholesterol levels and the opposite was observed when cholesterol inclusion complexes were used [89]. Interestingly, MβCD treatment resulted in significantly reduced exofacial percent cholesterol values in the membrane bilayer of SPM, leading to decreased exofacial to cytofacial cholesterol ratio values [89]. The idea that MβCD preferentially extracts cholesterol from the detergent insoluble areas of the plasma membrane, is supported by the stronger reduction in cholesterol content of the lipid raft fractions from human T-cell lymphoblastic leukaemia cells after MβCD incubation (~88% reduction) compared to the membrane preparations (~50% reduction) and crude cell lysate (~30%) preparation [90]. Hence, it could be concluded that MβCD exclusively extracts exofacial membrane cholesterol pools. Reversely, cholesterol enrichment with MβCD-cholesterol complexes induces contagious effects in SPM: Percent exofacial cholesterol levels are clearly enhanced. Accordingly, exofacial to cytofacial cholesterol ratio values were significantly increased in SPM of mice [89]. These data indicate that exofacial membrane cholesterol pools are most sensitive to external cholesterol manipulations using MβCD complexes, where lipid raft domains reside [91].

STATINS – INHIBITORS OF THE CHOLESTEROL BIOSYNTHESIS

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase the rate-limiting enzyme in cholesterol biosynthesis [92]. The inhibition of HMG-CoA reductase not only prevents cholesterol biosynthesis and induce significant plasma cholesterol reductions [93], but also affects the isoprenoid pathway, which accounts for statin´s pleiotropic effects [94, 95]. Simvastatin and lovastatin are prodrug lactone forms that are transformed to the active acid forms mainly by hepatocytes. Lipophilicity is further characterized by the behavior of compounds on the octanol/water phase. Based on the logarithm of the partition coefficient simvastatin, lovastatin, cerivastatin, fluvastatin, pitavastatin and atorvastatin are lipophilic, while pravastatin is hydrophilic [96]. Rosuvastatin is a relatively new statin, having a polar methane sulphonamide group, and it can be placed between cerivastatin and pravastatin [96]. Lactone and active acid forms of simvastatin and lovastatin were determined in picomolar levels in mouse brains after oral administration [97].

Recently, the effects of statins including lovastatin and simvastatin as lipophilic agents as well as pravastatin as a hydrophilic compound was studied focussing on their efficiency to affect subcellular membrane cholesterol pools in synaptosomal plasma membranes (SPM) of mice [72]. In contrast to the hydrophilic pravastatin, the lipophilic lovastatin and simvastatin strongly reduced the levels of free cholesterol in SPM, confirming earlier data [98]. Interestingly, statins significantly reduced cholesterol levels in the exofacial membrane leaflet. These changes were accom-

panied by modified membrane bulk fluidity. All three statins reduced the expression of the raft marker protein flotillin-1, which indicates that statins modulate lipid rafts *in vivo* [72]. Accordingly, Burns *et al.* demonstrated that changes in the distribution of cholesterol between the cyto- and exofacial membrane leaflet were directly related to lower levels of Aβ, a lipid raft related process with impact on AD pathology (see above) [99].

Neuroprotective effects of statins have been reported, including protection from NMDA-induced neuronal death [100, 101]. Excess of brain extracellular glutamate after cerebral ischemia over-activates NMDA receptors, which subsequently leads to neuronal death [102]. Since NMDA receptors have been reported to be associated with lipid rafts, the effect of simvastatin on levels on excitotoxicity and on association of NMDA receptors to lipid rafts was investigated recently. The data demonstrated that reduction of membrane cholesterol levels protects from NMDA-induced neuronal damage probably by reducing the association of NMDA receptors from lipid rafts [102].

SEQUESTRATION OF CHOLESTEROL BY FILIPIN

Filipin represents a polyene antibiotic with macrolide structure and amphipatic nature, which forms a fluorescent complex with cholesterol and is commonly used to visualize the cellular distribution of free cholesterol [78]. Since filipin sequesters cholesterol it is also used to disrupt lipid raft structures [103-105]. For its action filipin requires a sterol partner with a free 3-OH group and it was speculated that filipin may form large planar aggregates between the two layers of the membrane, it may be absorbed at the membrane surface or located at the upper layer of the membrane [106]. However, different models have been generated to explain the organization of filipin–sterol complexes within the membrane bilayer [78]. Filipin is a cytotoxic compound and disrupts the integrity of sterol-containing membranes. Thus, sequestering of cholesterol using filipin should only be employed in fixed cells or tissues [78].

LONG-CHAIN POLYUNSATURATED FATTY ACIDS MODULATE LIPID RAFTS

Omega-3 fatty acids are taken up by virtually all body cells and affect membrane composition, eicosanoid biosynthesis, cell signaling cascades, and gene expression [107]. Long-chain polyunsaturated fatty acids (LC-PUFA) like eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are especially important during human brain development. Maternal deficiency of omega-3 fatty acids leads to deficits in neurogenesis, neurotransmitter metabolism, and altered learning and visual function in animals [108] and may result in several neurological disorders [107]. Among various organs, in the brain omega-3 fatty acids are most extensively studied. In fact, the brain is the organ richest in lipids and it was shown that the differentiation and functioning of cultured brain cells requires LC-PUFA [109].

A number of studies have demonstrated that dietary LC-PUFA are incorporated into diverse cell types and appear to uniquely modulate cell membrane micro-domains [110-113].

Using a T-cell model, Stulnig *et al.* showed the ability of PUFA enrichment to selectively modify the cytoplasmic layer of lipid rafts [113, 114]. Accordingly, Chapkin and co-workers showed that dietary n-3 PUFA reduced lipid raft sphingolipid content and altered raft fatty acid composition [110, 115, 116]. Recently, the same group studied the effects of DHA on the size and distribution of lipid rafts in living HeLa cells [117]. Selected PUFA can increase the clustering of proteins in cholesterol-dependent micro-domains, whereas non-raft mirco-domains were insensitive to DHA modulation [117]. The impact of these findings for neurodegenerative diseases is underlined by reports that DHA enhanced synaptic membrane fluidity in aged mice [118] and decreased Aβ levels in cells and in brains of murine AD models [119-123]. Moreover, lipid rafts from AD brains displayed abnormally low levels of LC-PUFA, as well as reduced unsaturation and peroxidation indexes [49].

LC-PUFA alter the basic properties of cell membranes and enhances membrane viscosity [124]. It was suggested that because of its polyunsaturation, PUFA are sterically incompatible with sphingolipid and cholesterol and, therefore appear to alter lipid raft behavior and protein function [125-127].

CONCLUSIONS

In summary, lipid raft signaling is involved in multitude biochemical pathways. Modulation of cholesterol using physical extraction by methyl-β-cyclodextrine, filipin or inhibition of cholesterol biosynthesis by statins is most commonly used to change lipid rafts in membranes. Size and distribution of lipid rafts depend also on the membrane environment, which could be chanced by long-chain polyunsaturated fatty acids. The alteration of lipid rafts represents a useful tool to study related disease pathways and probably offers a target for pharmacological intervention. However, it is not defined yet if targeting lipid rafts *in vivo* might impair physiological functions. For instance, it is not clear whether dietary LC-PUFA are incorporated into raft lipids or whether their low affinity to cholesterol disallows this and causes phase separation from rafts and displacement of raft proteins [128]. Moreover, it was shown that depletion of cholesterol leads to instability of surface AMPA-glutamate in lipid rafts, which was accompanied by gradual loss of synapses and dendritic spines [129].

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