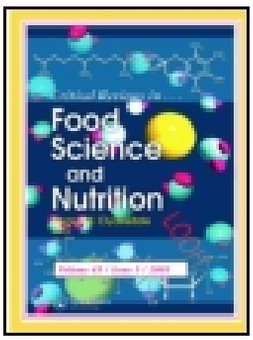

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REVIEW

Lipid rafts as potential mechanistic targets underlying the pleiotropic actions of polyphenols

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ABSTRACT

Polyphenols have attracted a lot of global attention due to their diverse biological actions against cancer, obesity, and cardiovascular diseases. Although extensive research has been carried out to elucidate the mechanisms of pleiotropic actions of polyphenols, this remains unclear. Lipid rafts are distinct nanodomains enriched in cholesterol and sphingolipids, present in the inner and outer leaflets of cell membranes, forming functional platforms for the regulation of cellular processes and diseases. Recent studies focusing on the interaction between polyphenols and cellular lipid rafts shed new light on the pleiotropic actions of polyphenols. Polyphenols are postulated to interact with lipid rafts in two ways: first, they interfere with the structural integrity of lipid rafts, by disrupting their structure and clustering of the ordered domains; second, they modulate the downstream signaling pathways mediated by lipid rafts, by binding to receptor proteins associated with lipid rafts, such as the 67 kDa laminin receptor (67LR), epidermal growth factor receptor (EGFR), and others. This study aims to elaborate the mechanism of interaction between polyphenols and lipid rafts, and describe pleiotropic preventive effects of polyphenols.

KEYWORDS

Chronic disease; lipid rafts; pleiotropic action; polyphenols; signaling pathway

Introduction

Polyphenols are ubiquitous in a wide variety of plant-derived foods which are present in the human diet. With no obvious adverse side effects, the bioactivity and underlying mechanisms of polyphenols have attracted the interest of many food and nutritional scientists (Chen et al. 2013; Gonzalez Arbelaez et al. 2018). Several studies have linked the consumption of polyphenols to a lower risk of chronic diseases (Kwan et al. 2017), such as metabolic syndrome, cardiovascular disease, diabetes mellitus, obesity, and some cancers. Polyphenol-rich diets accelerate glucose absorption in patients with type 2 diabetes mellitus, quickly reducing postprandial hyperglycemia (Xiao and Hogger 2015). Quercetin polyphenols were reported to restore TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis in non-Hodgkin B cells lymphoma, by inhibiting survivin expression and degrading induced myeloid leukemia cell differentiation protein Mcl-1 (Jacquemin et al. 2012). Epigallocatechin gallate (EGCG) was recently shown to ameliorate symptoms of inflammatory bowel disease by negatively regulating Toll-like receptor (TLR) signaling in human intestinal epithelial cells (Byun et al. 2018).

Previous studies have reviewed the effects of polyphenols on human chronic diseases, including cardiovascular disease, neurodegeneration, and cancer (Del Rio et al. 2013). Moreover, a randomized controlled clinical trial study revealed that a diet naturally rich in flavanone (62.1 mg/day) significantly reduced postprandial triglycerides in whole plasma, indicating that a polyphenol-rich diet had a protective effect on individuals having a high cardiovascular risk (Vetrani et al. 2018). The absorption, distribution, metabolism, and excretion of polyphenols after dietary intake have been reviewed by Manach et al. (2005). Their results indicated that phenolic acids, catechins, and isoflavones are well-absorbed polyphenols. However, proanthocyanidins and other polymeric polyphenols, especially those with the polymerization degree over four, are not well-absorbed. The study by Nardini et al. (2002) found that only caffeic acid (CA) was detected in plasma (91.1 ± 33.2 ng/mL) after 200 mL coffee consumption, and Zambonin et al. (2012) showed that CA decreased proliferation of human erythromegakaryocytic leukemia cells at concentrations as low as 5 μM. Lee et al. (2002) reported that EGCG was the only known polyphenol existing in free form in plasma, while other catechins were metabolized into another forms. Moreover, EGCG also has anti-cancer (Huang et al. 2015), anti-inflammatory (Byun et al. 2018), and anti-obesity (Lin and Lin-Shiau 2006) effects. Despite the low bioavailability of these polymeric polyphenols, cranberry proanthocyanidins, which are polymers of epicatechin or flavan-3-ols, reduce urinary tract infections by inhibiting *Escherichia coli* adhesion to the uroepithelium (Micali et al. 2014). Furthermore, the non-absorbed high polymeric polyphenol, persimmon tannin was able to repress the differentiation of 3T3-L1 pre-adipocytes, markedly inhibiting the peroxisome

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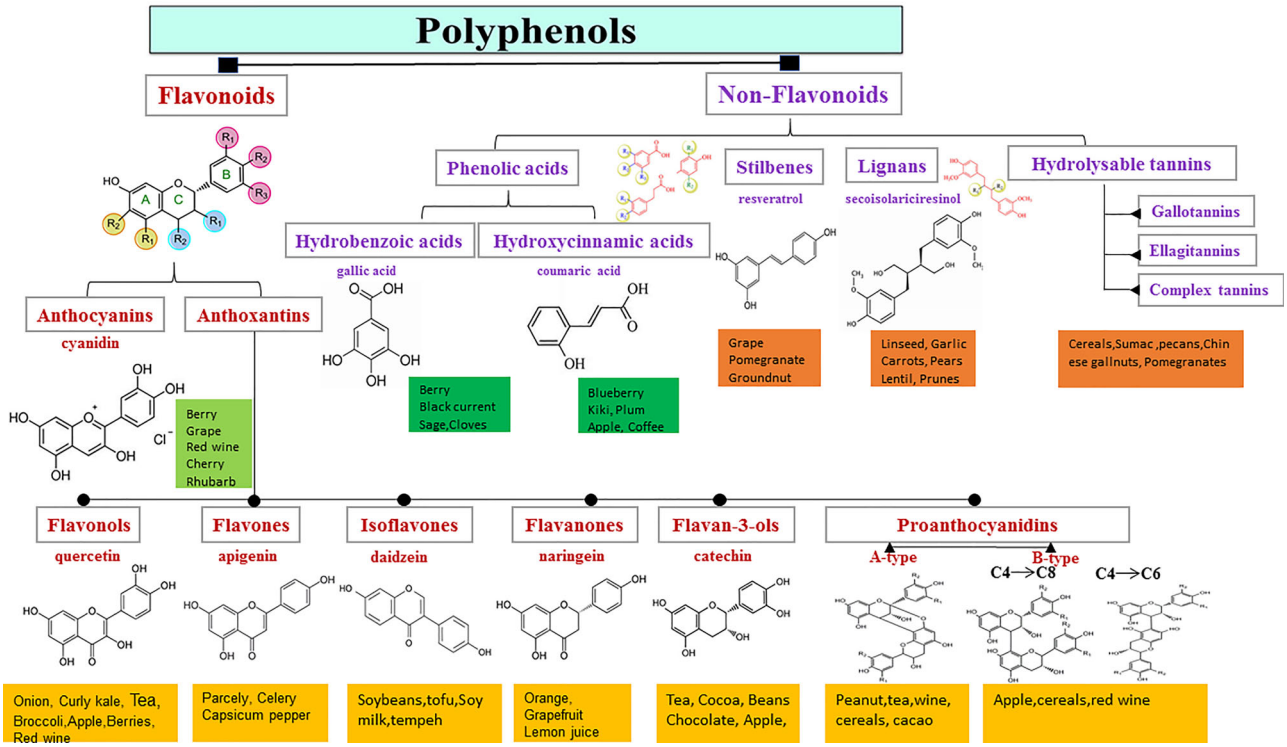

Figure 1. Classification, chemical structure and main sources of polyphenols.

proliferator-activated receptor gamma (PPAR-γ) signaling pathway (Zou et al. 2015). In addition, low absorbed ellagitannins, which are esters of hexahydroxydiphenoic acid with a monosaccharide residue, showed anti-inflammatory, anticancer, and anti-bacterial activities (Lipińska, Klewicka, and Sojka 2014). Although in vivo intervention studies have shown that polyphenols have some pharmacological activities and in vitro mechanistic studies have suggested that polyphenols function in the free form or as metabolites, the mechanism behind the pleiotropic actions of polyphenols at the molecular level remains poorly understood.

Cell membranes consist of an extremely complex mix of lipids and proteins, necessary for normal cell functioning. Lipid rafts are distinct nanodomains enriched in cholesterol (CHOL) and sphingolipids, present in the inner and outer leaflets of cell membranes, forming functional platforms for the regulation of cellular processes (Simons and Ikonen 1997). Lipid rafts segregate specific elements to regulate their interaction with other membrane components and activities. Effectively, they act as a gate for extracellular-to-intracellular signal transduction (Simons and Toomre 2000). Interactions of exogenous compounds (such as polyphenols) with CHOL and other lipids from lipid rafts may lead to disruption of raft domains or change the conformation of raft-resident proteins, which in turn can help regulate cellular signaling pathways (Neves, Nunes, and Reis 2016). EGCG was reported to prevent the binding of epidermal growth factor (EGF) to its receptor (EGFR), inhibiting the phosphorylation of EGFR (found only within the lipid raft fractions) (Adachi et al. 2007). Similarly, EGCG and Epicatechin-3-gallate (ECG) limited the growth and pervasion of DU145 prostate tumor cells, by disrupting the structure of lipid rafts (Duhon et al. 2010). Moreover, phenolic acids could target the lipid raft domains and affect the lateral membrane organization, suggesting that changing in raft structure and dynamics may be the core mechanism of phenolic acid activity in vivo (Filipe et al. 2018). Furthermore, Erlejman et al. (2008) reported that hexameric procyanidins were not absorbed at the gastrointestinal tract, but could exert biological activities, such as inhibiting tumor necrosis alpha (TNFα)-induced NF-κB activation in Caco-2 intestinal cells. Subsequently, Verstraeten et al. (2013) found that the mechanism of action of hexameric procyanidins in Caco-2 cells was related to its interaction with lipid rafts by binding to cholesterol. This result providied new insights into the intestinal health benefits of large procyanidins. Thus, lipid rafts may fill the gap between well-absorbed or not-absorbed polyphenols and its metabolites, and affect downstream disease risk factors.

Recent evidences suggest that lipid rafts could be a mechanistic target of polyphenols. An understanding of this interaction between polyphenols and lipid rafts might provide new insights on the pleiotropic actions of polyphenols. Therefore, in the current review, we focused on the effects of polyphenols on the structure of lipid rafts, as well as on the respective downstream effects on cellular signaling pathways, and aimed to provide a new perspective on the biological activities of polyphenols.

Structural classification of polyphenols

Based on their chemical structure, polyphenols are divided into two main categories: flavonoids and non-flavonoids (Figure 1). Flavonoids are the most common type and account for two-thirds of the daily intake of polyphenols

(Scalbert and Williamson 2000). They share a common C6-C3-C6 skeleton structure, and are categorized as anthocyanidins, flavonols, flavones, isoflavones, flavanones, flavan-3-ols, and proanthocyanidins (Del Rio et al. 2013). Proanthocyanidins (also known as condensed tannins) are oligomers or polymers of flavan-3-ols and can be subdivided into B-type (with C4-C8 or C4-C6 linkage) or A-type procyanidins, which are characterized by a C2-O-C5 (or C7) linkage in addition to the basic C4-C8 linkage (Smeriglio et al. 2017). The non-flavonoid polyphenols mainly consist of phenolic acids, stilbenes, lignans, and hydrolyzed tannins (Figure 1). Phenolic acids can further classified as hydroxybenzoic and hydroxycinnamic acids. Hydrolyzed tannins are further classified as gallotannins, ellagitannins, and complex tannins.

Although there are differences in the bioavailability of polyphenols, existing studies showed that when rats were administered 250 mg/kg body weight of grape seed proanthocyanidins, the plasma levels of proanthocyanidins were found to be about 23 μM (Baselga-Escudero et al. 2012). Hexameric procyanidins could disrupt the lipid rafts structure at 10 μM. The lipid rafts disruption effect was detected with as little as 2 μg/ml of EGCG (4.3 μM) within 5 minutes. Our previous studies revealed that the IC_{50} value of proanthocyanidin dimer (A-ECG dimer) for inhibiting the differentiation of 3T3-L1 pre-adipocytes was about 20.36 μM, indicating that concentrations at the experimental level can be achieved in vivo (Zhu et al. 2015). Lipid rafts may fill the gap between well-absorbed or not-absorbed polyphenols and their biological activities. However, the effect of low-dose polyphenols (at physiological levels) on lipid rafts structure needs further study.

Structure and function of lipid rafts

Cell membranes can be separated into detergent-soluble and detergent-resistant fractions (Lingwood and Simons 2010). The former fraction is rich in unsaturated lipids with a low melting temperature (also known as liquid-disordered [Ld] phase). The latter fraction mostly consists of saturated lipids and CHOL, which have higher melting temperatures (also known as liquid-ordered [Lo] phase or lipid rafts) (Kaiser et al. 2009). This observation suggested that multiple domains are present in cellular membranes, and led to the origin of the membrane rafts (or lipid rafts) hypothesis. The membrane rafts hypothesis aimed to explain this lateral membrane inhomogeneity and claimed that lipid rafts were formed by dynamic clustering of sphingolipids and CHOL, which were able to move within the fluid bilayer and be used as a functional platform for attaching signaling proteins (Simons and Ikonen 1997). The hypothesis remained controversial, as the existence of these proposed membrane raft domains could not be easily proved in vivo, due to a lack of direct observation tools. A consensus definition of lipid rafts was formulated in 2006, with available evidence showing that lipid rafts are small heterogeneous and dynamic nanodomains (10-200 nm), consisting of sphingolipids and CHOL, which can form larger functional platforms through protein-protein and protein-lipid interactions (Pike 2006). This concept was supported by new biochemical and biophysical techniques such as giant plasma membrane vesicles (GPMVs), fluorescence probes, confocal microscopy, photoactivated localization microscopy (PALM), and molecular dynamics (MD) simulations. Together, these techniques have provided increasing evidence for the presence of lipid raft domains in cell membranes, and have suggested key roles for membrane heterogeneity in various cellular functions (Klymchenko and Kreder 2014; Niemela et al. 2007; Sengupta et al. 2011). It had been proposed that the balance of a strong attraction between saturated lipid species and CHOL and a strong repulsion between unsaturated lipids and saturated lipids/CHOL is the driving force for lipid rafts formation (Wang, Krause, and Regen 2015). Furthermore, the hydrogen bond between sphingomyelin (SM) and CHOL also plays an important role in the formation of lipid rafts (Slotte 2016). This view is also supported by Bera and Klauda (2017), who showed that CHOL tends to cluster near the SM, through a simulation model of lipid rafts with mixed lipid bilayers (consisting of SM, CHOL and glycerophospholipids). Caveolae are small bulb-shaped plasma membrane invaginations (50-80 nm), which form due to the accumulation of caveolins on the plasma membrane (Parton and Simons 2007). They exhibit microdomains similar to lipid rafts, and are also known as caveolar lipid rafts. Because caveolin may not be found or presents at very low levels in some cell lines (James et al. 2002; Pleasant-Jenkins et al. 2017), this review just focused on non-caveolar lipid rafts.

Lipid rafts have great biological significance as functional platforms for the regulation of cellular processes and diseases (Simons and Ehehalt 2002). Pretreatment with methyl-β-cyclodextrin (MβCD), a CHOL extraction reagent, which disrupts the structure of lipid rafts, inhibited the growth of triple negative breast cancer (TNBC) cells, suggesting that the integrity of lipid rafts structure was required for the survival of TNBC cells (Badana et al. 2016). A similar study in 3T3-L1 pre-adipocytes found that the disruption of lipid rafts by MβCD blocked insulin-like growth factor 1 receptor (IGF-1R) signaling and inhibited cellular differentiation (Huo et al. 2003). Another study done in MDA-MB-231 human breast cancer cells by the Field group showed that the n-3 fatty acids could embed into lipid rafts, modifying the composition of lipid rafts; thus, interfering with EGFR signaling and slowing the growth of breast cancer cells (Schley, Brindley, and Field 2007). In addition, the integrity of the lipid raft structure plays a crucial role in the activation of B-cell, T-cell, and IgE signaling, providing the therapeutic potential for autoimmune disorders and inflammatory diseases (Varshney, Yadav, and Saini 2016).

Therefore, this review indicates that lipid raft domains play critical roles in various cellular functions by regulating many biological processes and may be a potential target for the treatment of certain diseases, using drugs or polyphenols. This review also explains the mechanisms and structure-activity relationship of polyphenols.

Lipid rafts-dependent molecular mechanisms of polyphenols

Increasing evidence indicates that polyphenols exhibit pleiotropic effect through lipid rafts. Polyphenols can affect lipid rafts by interfering with their structure or competitively binding to specific proteins, which are critical for biological processes. Both modes of action can directly interfere with cellular signaling pathways and have been described in detail below.

Biological actions of polyphenols by disrupting lipid raft structure

The interference of polyphenol with the structural integrity of lipid rafts is protective in many diseases, such as cancer (Mollinedo and Gajate 2015), cardiovascular disease (Das et al. 2008), inflammation (Maceyka and Spiegel 2014), and allergic reactions (Maceyka and Spiegel 2014; Schauberger et al. 2016). MβCD, a cholesterol-depleting agent, is widely used in studying lipid rafts (Zidovetzki and Levitan 2007). For example, the disruption of lipid raft structure by MβCD could induce breast cancer cell apoptosis by attenuating the expression of survivin (Badana et al. 2018). Studies have shown that polyphenols exert a similar lipid raft disruption effect and inhibit proliferation and invasive migration of several cancer cells, such as chronic myeloid leukemia (Huang et al. 2015), colon carcinoma (Delmas et al. 2004; Psahoulia et al. 2007), prostate cancer (Duhon et al. 2010), head and neck squamous (Zhang et al. 2008) and multiple myeloma cells (Tsukamoto et al. 2012). Polyphenols can influence lipid rafts structure by reducing or clustering the ordered domains of membrane rafts.

Reduction of membrane rafts ordered domains

A direct binding study of EGCG and human basophilic KU812 cell through surface plasmon resonance biosensor revealed that EGCG bound to membrane lipid rafts and influenced IgE-mediated allergic reactions, by inhibiting the phosphorylation of the extracellular signal-regulated kinases (ERKs) 1 and 2 (Fujimura, Tachibana, and Yamada 2004). Adachi et al. (2007) suggested that EGCG could be incorporated into the membrane ordered domains of colon cancer cells to produce a marked reduction in the ordered lipid domains in the plasma membrane; thus, inhibiting the growth of cancer cells. Similar results were found in mammary and epidermoid carcinoma cells, with EGCG disrupting the location of 67 kDa laminin receptor (67LR) in lipid rafts and inducing cell death in these cell types (Mocanu et al. 2014). Furthermore, membrane CHOL was important in maintaining the stabilization of raft domains and the barrier function of epithelial monolayers in Caco-2 cells (Lambert, O'Neill, and Padfield 2005). Verstraeten et al. (2013) suggested that in Caco-2 cells, the intestinal health benefits of non-absorbed hexameric procyanidins can be attributed to their binding with CHOL in lipid rafts and alleviating the activation of ERK1 and ERK2. Our previous studies have demonstrated that A-type EGCG and ECG dimers were capable of inhibiting the differentiation of 3T3-L1 pre-adipocytes via inhibiting lipid biosynthesis-related target genes including stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1), and fatty acid synthase, in a way that was related to their ability to disrupt lipid rafts (Zhu et al. 2017). Further studies revealed that A-type ECG and EGCG dimers could bind with CHOL of lipid rafts in a 3T3-L1 cells assay, leading to disruption of lipid raft structure (Zhu et al. 2018). Therefore, the disruption of lipid raft structure and inhibition of downstream lipid biosynthesis-related target genes is a possible mechanism through which A-type ECG and EGCG dimers exert their lipid-lowering activity. While it is not clear how ECG and EGCG dimers are associated with CHOL in lipid rafts at the molecular level, this is currently being researched in our laboratory.

Clustering of membrane rafts ordered domains

Research done in "lipid raft-like" liposomal systems, composed of egg L-α-phosphatidylcholine (EPC), CHOL, and sphingomyelin (SM) revealed the clustering of ordered raft domains induced by resveratrol and could partly explain the molecular mechanism of resveratrol on inhibition of tumorigenesis, cardiovascular, and neurodegenerative diseases (Neves, Nunes, and Reis 2015; Neves, Nunes, and Reis 2016). Ajdzanovic et al. (2013) suggested that the clustering of raft domains by genistein, induced anti-prostate carcinoma activity, which significantly increased the order and decreased the fluidity of the membrane; thus reducing the invasion of cancer cells. Furthermore, clustering of lipid raft ordered domains induced by polyphenols led to aggregation, accumulation, or redistribution of many death receptors in lipid rafts, including CD95, DR4, and DR5, which could enhance TRAIL-mediated apoptosis in tumor cells. This could potentially explain the anti-cancer activity of polyphenols, including resveratrol (Delmas et al. 2004; Reis-Sobreiro, Gajate, and Mollinedo 2009), quercetin (Jacquemin et al. 2012; Psahoulia et al. 2007) and tannic acid (Cosan et al. 2009). In addition, Tarahovsky et al. (2012) found that phosphatidylcholine artificial membranes model would aggregate and fuze in the presence of divalent iron and flavonoids (quercetin, taxifolin, catechin, and morin) by freeze-fracture electron microscopy, suggesting that flavonoid-iron complexes function as a bridge to link two adjacent bilayers, thus facilitating artificial membranes model aggregation. Subsequently, Tarahovsky et al. (2014) summarized the physicochemical properties of flavonoid-metal complexes and hypothesized that the complexes were involved in lipid rafts signaling. Research on flavonoid-metal complexes and rafts should be further implemented to support their hypothesis.

Although a previous review indicated that the formation of raft domains may be due to the localization of polyphenols in the hydrophobic region of the membrane and the disruption of rafts may be due to polyphenols in the polar interface region (Tarahovsky, Muzafarov, and Kim 2008). However, Filipe et al. (2018) conducted a study combining large unilamellar vesicle, fluorescence spectroscopy, and MD simulations, and demonstrated that CA, a low lipophilicity

compound, targeted liquid-ordered domains to decrease the order and induced the formation of rigid gel-like phases. This implies that further studies to explore the effect of polyphenols with different structures on lipid rafts, using experimental and complementary experimental approaches, are needed.

Polyphenols influence signaling pathways mediated by specific receptors in lipid rafts

Signaling proteins localized to lipid rafts, include 67LR, epidermal growth factor receptor (EGFR), cellular mesenchymal to epithelial transition factor (c-Met), high-affinity IgE receptor (FcεRI), insulin receptor (IR), and IGF-1R. The modulation of their activities and downstream signaling pathways is upstream of the anti-cancer (Firdous et al. 2014; Shimizu et al. 2005), anti-inflammatory (Fujimura, Yamada, and Tachibana 2005), and anti-obesity (Hong et al. 2004) properties of polyphenols. The schematic diagram of specific receptors and corresponding pathways modulated by polyphenols are shown in Figure 2.

67LR

Since 67LR is usually overexpressed in neoplastic cells and is localized in lipid rafts, it is thought to be a molecular target in cancer therapy (Negri et al. 2018; Pesapane et al. 2017). Polyphenols, especially EGCG, exert their anti-cancer activity by inducing apoptosis and decreasing tumor cell growth rates by inhibiting signaling pathways of 67LR. The specific binding of EGCG to 67LR in the plasma membrane of multiple myeloma cells led to apoptosis by nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) production, translocation of acid sphingomyelinase (ASM) to lipid rafts, and the phosphorylation of protein kinase Cδ (PKCδ) at Ser^{664}. All these processes were necessary for ASM/ceramide signaling (Tsukamoto et al. 2012). Similar results were also obtained in chronic myeloid leukemia (CML) cells by Huang et al. (2015). Fujimura et al. (2012) suggested that residues 161-170 of 67LR could be a possible binding site of EGCG. The expression of eukaryotic translation elongation factor 1 A (eEF1A) was increased upon the binding of EGCG to 67LR. Subsequently the dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr^{696} was promoted, activating myosin phosphatase and reducing the phosphorylation of myosin regulatory light chain (MRLC) at Thr^{18}/Ser^{19}. This suggested that the 67LR/eEF1A/MYPT1/MRLC axis was part of a growth inhibition signaling pathway involved in the anti-cancer activity of EGCG (Umeda et al. 2008). The all-*trans*-retinoic acid (ATRA) potentiated the ability of EGCG to inhibit cancer cell growth (in B16 melanoma cells) through the 67LR signaling pathway, suggesting a synergistic anti-cancer effect of ATRA and EGCG (Lee et al. 2010). A schematic diagram of pathways modulated by polyphenols are shown in Figure 2.

Other beneficial effects of EGCG include its anti-allergic, anti-inflammatory and cardioprotective effects, all of which are potentially downstream of EGCG binding to 67LR. In this regard, the binding of EGCG to 67LR downregulated FcεRI expression to prevent IgE-mediated allergic reactions (Fujimura, Yamada, and Tachibana 2005); it also upregulated the expression of Toll interacting protein (TOLLIP) in HT-29 cells, which is a negative regulator of TLR signaling, which explained the anti-inflammatory properties of EGCG (Byun et al. 2018). Hsieh et al. (2013) suggested that 67LR might also be a key target for EGCG in myocardial protection. The interaction of EGCG with 67LR led to increased of endothelial nitric oxide synthase (eNOS) and sirtuin 1 (SIRT1) expression and activation of various kinases, such as ERK1 and ERK2, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) complex, and glycogen synthase kinase-3β (GSK-3β). In addition, proanthocyanidins (Hu et al. 2014) and resveratrol (Fourny et al. 2019) also activated similar signaling pathways to protect myocardial cells and type 2 diabetic female rat heart from anoxia-reoxygenation injury and ischemia-reperfusion injury, respectively. A schematic diagram of the anti-allergic, anti-inflammatory, and cardioprotective signaling pathways mediated by 67LR is shown in Figure 3. Whether polyphenols other than EGCG can also bind to 67LR and exert different biological activities remains unknown.

EGFR

EGFR is another receptor, which is localized in lipid rafts. Elevated levels of EGFR correlate with aberrant growth of colon cancer (Shimizu et al. 2005), prostate cancer (Firdous et al. 2014) and head and neck squamous carcinoma cells (Masuda, Suzui, and Weinstein 2001). EGCG has been shown to block the progression of colon cancer cells by disrupting the structure of lipid rafts (Figure 2). It also hinders the binding of EGF to EGFR and consequent dimerization and phosphorylation of this receptor, preventing the activation of the PI3K/Akt pathway (Adachi et al. 2007), which is important in cancer therapy (Liu et al. 2009). Furthermore, other polyphenols, such as quercetin (Firdous et al. 2014), and curcumin (Boven et al. 2019), can also halt the growth of carcinoma cells through the EGFR signaling pathway, but their mechanism is unclear.

C-Met

C-Met is the receptor for the hepatocyte growth factor (HGF) and was found within lipid rafts (Duhon et al. 2010). Activation of the HGF/c-Met pathway correlated with the growth of carcinoma cells and activation of c-Met promoted the growth of prostate cancer cells (Duhon et al. 2010). A study on four green tea catechins' effect on HGF/c-Met pathway in prostate cancer cells showed that EGCG and ECG disrupted the structural integrity of lipid rafts and inhibited the HGF-induced phosphorylation of $Tyr^{1234/1235}$ residues within the kinase domain of the c-Met receptor and the activation of PI3K/Akt (Figure 2). However, epicatechin (EC) and epigallocatechin (EGC) showed little anti-cancer activity and had no effect on the structural integrity of lipid rafts or on the HGF/c-Met signaling pathway mentioned above, implying that the anti-cancer effect of galloylated

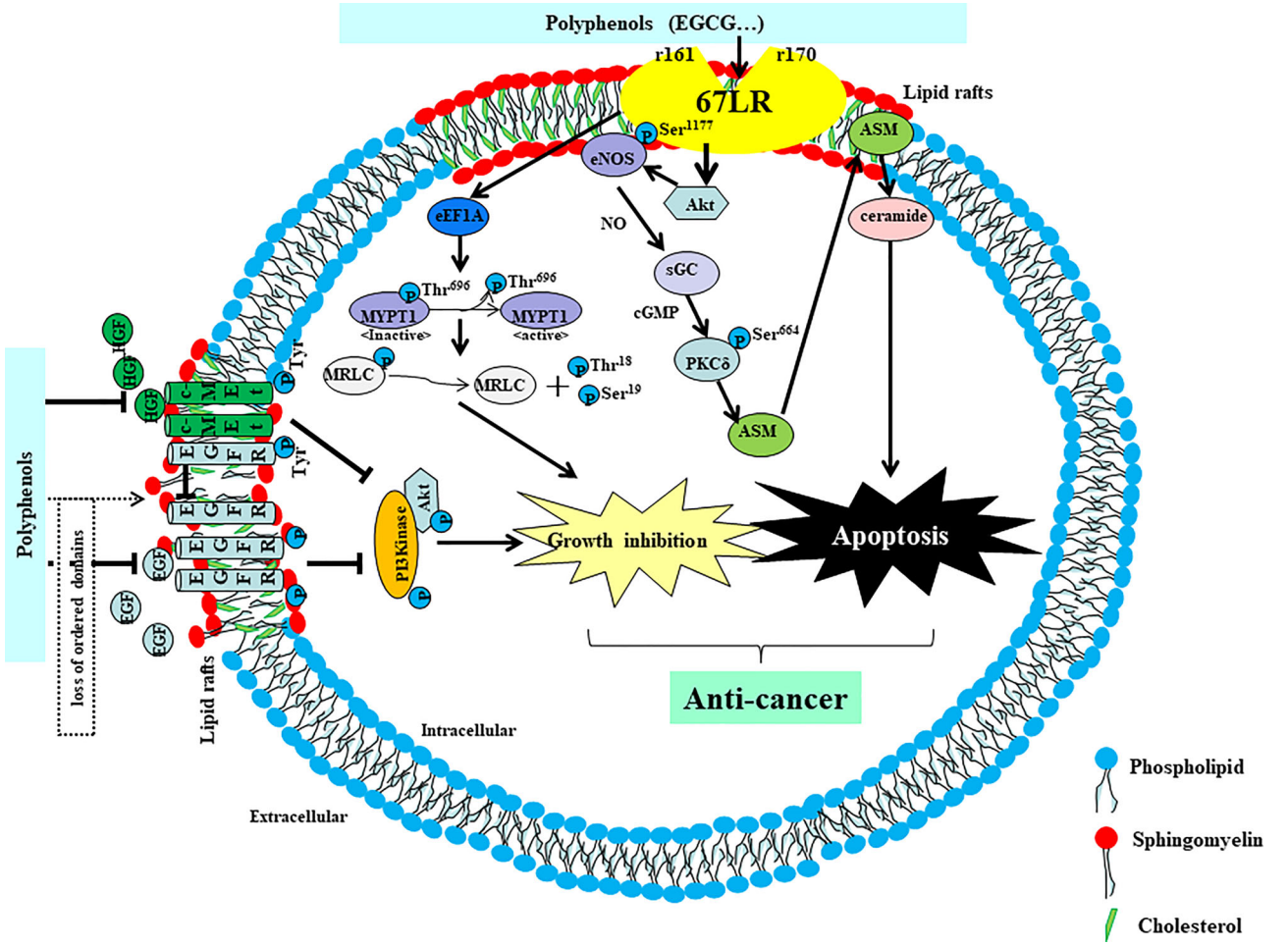

Figure 2. Polyphenols regulate growth inhibition and the apoptosis pathway in cancer cells via lipid rafts. EGCG prevents the binding of EGF to EGFR and inhibits the activation of EGFR by interfering with the structure of lipid rafts, thus affecting the progression of colon cancer cells. Furthermore, EGCG curbs the growth of prostate cancer cells via inhibition of the HGF-induced phosphorylation of tyrosines residues in the kinase domain of c-Met. EGCG binds to 67LR to upregulate the expression of eEF1A and decrease the phosphorylation of MRLC, inhibiting the growth of cancer cells. EGCG binds to residue 161-170 of 67LR and promotes apoptosis, through the enhancements of NO and cGMP production and PKCδ and ASM activation.

catechins (EGCG and ECG) depends on the blockage of c-Met signaling through a mechanism involving lipid rafts (Duhon et al. 2010). Nevertheless, how EGCG and ECG affect the integrity of the lipid rafts structure remains unclear and needs further elucidation by high resolution microscopy and other tools. Whether other polyphenols also exert their anti-cancer activity through the modulation of HGF/c-Met signaling remains poorly understood and must be further studied systematically.

FcεRI

FcεRI, which is present on the surface of cells and can be recruited into lipid rafts, is a high-affinity immunoglobulin E (IgE) receptor (Fujimura, Tachibana, and Yamada 2004). The binding of IgE to FcεRI is involved in the development of allergy (Fujimura, Tachibana, and Yamada 2004). Many studies showed that plant polyphenols, including EGCG, chrysin, and apigenin, inhibit the expression of FcεRI; therefore, possessing anti-allergic activity (Tachibana, Fujimura, and Yamada 2004; Yano, Tachibana, and Yamada 2005). It was also shown that the strong inhibition of FcεRI expression by EGCG correlated with the inhibition of the phosphorylation of ERK1 and ERK2. Furthermore, EGCG disrupted lipid rafts similar to MβCD, suggesting that the anti-allergic effects of EGCG are a result of its disruption of the structural integrity of lipid rafts (Fujimura, Tachibana, and Yamada 2004). Some other polyphenols, such as genistein, quercetin, luteolin, and apigenin, were also reported to reduce both acute and chronic inflammation by blocking the FcεRI signaling pathway (Kumazawa, Kawaguchi, and Takimoto 2006). However, whether they did so by acting at the level of lipid rafts is currently unknown.

IGF-1R

The binding of insulin or insulin-like growth factor 1 (IGF-1) to IGF-1R activates signaling pathways downstream of IGF-1R, promoting the terminal differentiation of 3T3-L1 pre-adipocytes to adipocytes (Jin et al. 2000). Through double immunofluorescence staining and western blot techniques, it has been confirmed that IGF-1R resides in lipid rafts/caveolae (Huo et al. 2003). Using double-stranded RNA interference (RNAi) to selectively suppress the formation of caveolae without disturbing lipid rafts, it was possible to confirm that lipid rafts (but not caveolae) play an

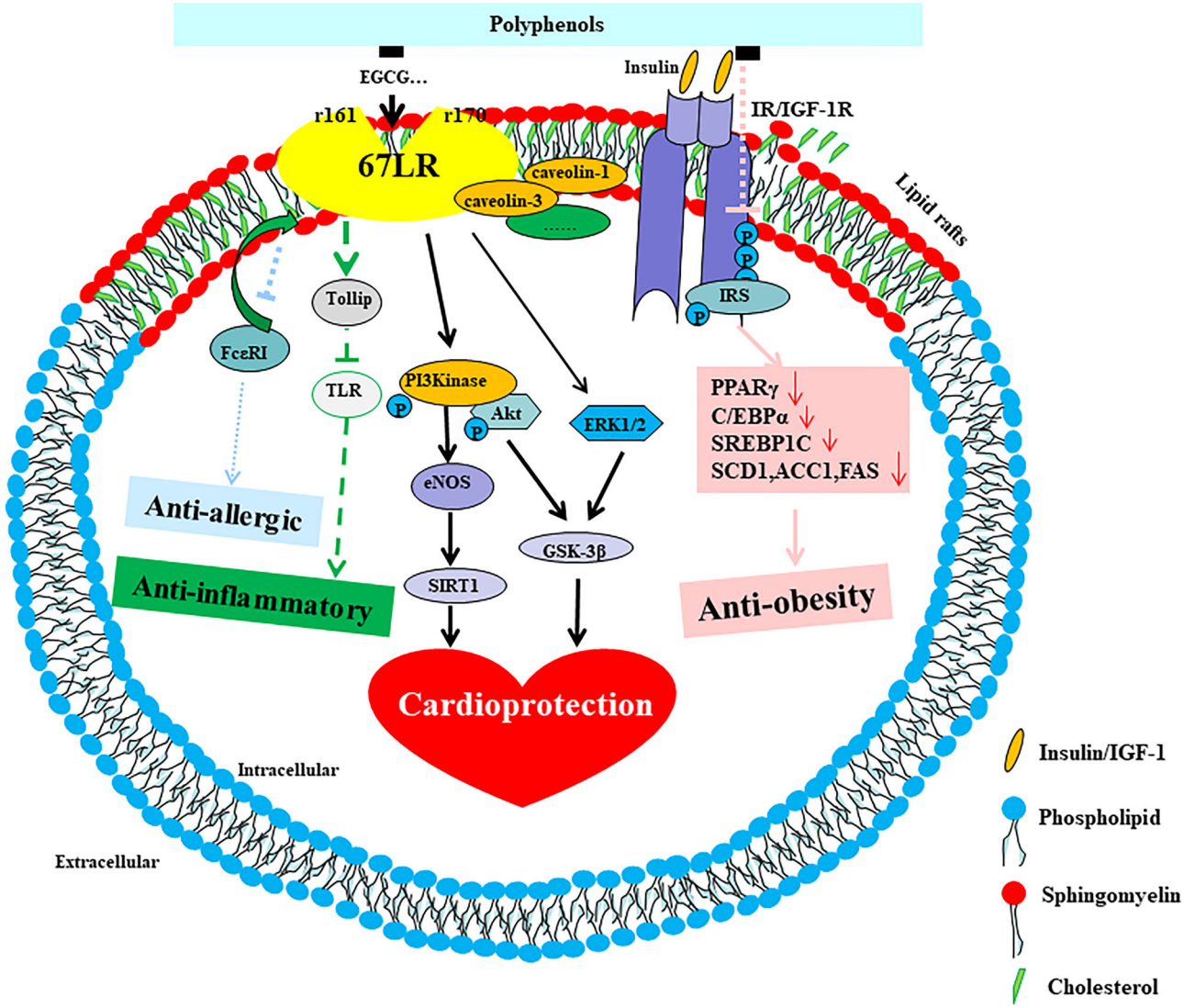

Figure 3. A schematic illustration of polyphenols exerting their pleiotropic actions through lipid rafts. 67LR, IR, and IGF1R, localized in lipid rafts, may be key receptors responsible for the pleiotropic actions (such as anti-allergic, anti-inflammatory, anti-obesity and cardioprotective) of polyphenols. The binding of EGCG to 67LR downregulates FcεRI expression to prevent IgE-mediated allergic reactions, and also upregulates the expression of TOLLIP in HT-29 cells, which is a negative regulator of TLR signaling, helping explain the anti-inflammatory properties of EGCG. In addition, the interaction of EGCG with 67LR leads to the increase of eNOS and SIRT1 expression and to the activation of various kinases, such as ERK1 and ERK2, PI3K/Akt, and GSK-3β. A-type EGCG and ECG dimers significantly inhibit the differentiation of 3T3-L1 pre-adipocytes by disrupting lipid rafts structure to inhibit the phosphorylation of IR/IGF-1R receptor and expression of downstream involving lipid synthesis proteins.

indispensable role in the differentiation of 3T3-L1 pre-adipocytes mediated by IGF-1R (Hong et al. 2004). Treatment with MβCD also led to a significant inhibition in IGF-1R-mediated differentiation in these cells (Delle Bovi et al. 2019; Huo et al. 2003). Additionally, the study by Zhu et al. (2017) revealed that A-type EGCG and ECG dimers significantly inhibited the differentiation of 3T3-L1 pre-adipocytes by binding to CHOL; thus, disrupting the structural integrity of lipid rafts. Further, unpublished data from our laboratory suggest that these dimers also inhibit IGF-1R receptor phosphorylation. Their effects on lipid raft structures and downstream signaling pathways are summarized in Figure 3.

Glucose transporter type 4 (GLUT4)

GLUT4, which relies on the IR signaling pathway, transports extracellular glucose into the cytosol of cells, and helps in regulating glucose homeostasis (Thong, Dugani, and Klip 2005). Insulin-stimulated GLUT4 translocation required lipid rafts, but not caveolae (Yuan et al. 2007). Instead, caveolae appeared to stimulate GLUT4 internalization in the absence of insulin (Yuan et al. 2007). A higher degree of GLUT4 clustering was observed in insulin-resistant adipocytes. With the disruption of lipid rafts, the clustering of GLUT4 in the plasma membrane of 3T3-L1 adipocytes was reduced, suggesting a potential method to reverse insulin resistance via the modulation of lipid rafts (Gao et al. 2017). Treatment with EGCG (Xu et al. 2019), anthocyanin (Luna-Vital, Weiss, and de Mejia 2017), resveratrol (Li et al. 2018), and *Chimonanthus nitens* leaf extract (Chen et al. 2018) activated the insulin-dependent IRS1/PI3K/AKT/GLUT4 signaling cascade; thus, promoting glucose uptake, which can be due to an increased translocation of GLUT4 to the plasma membrane. However, it is unclear how polyphenols activate the signaling cascade itself. In a model of streptozotocin-induced diabetes, resveratrol increased GLUT4 migration

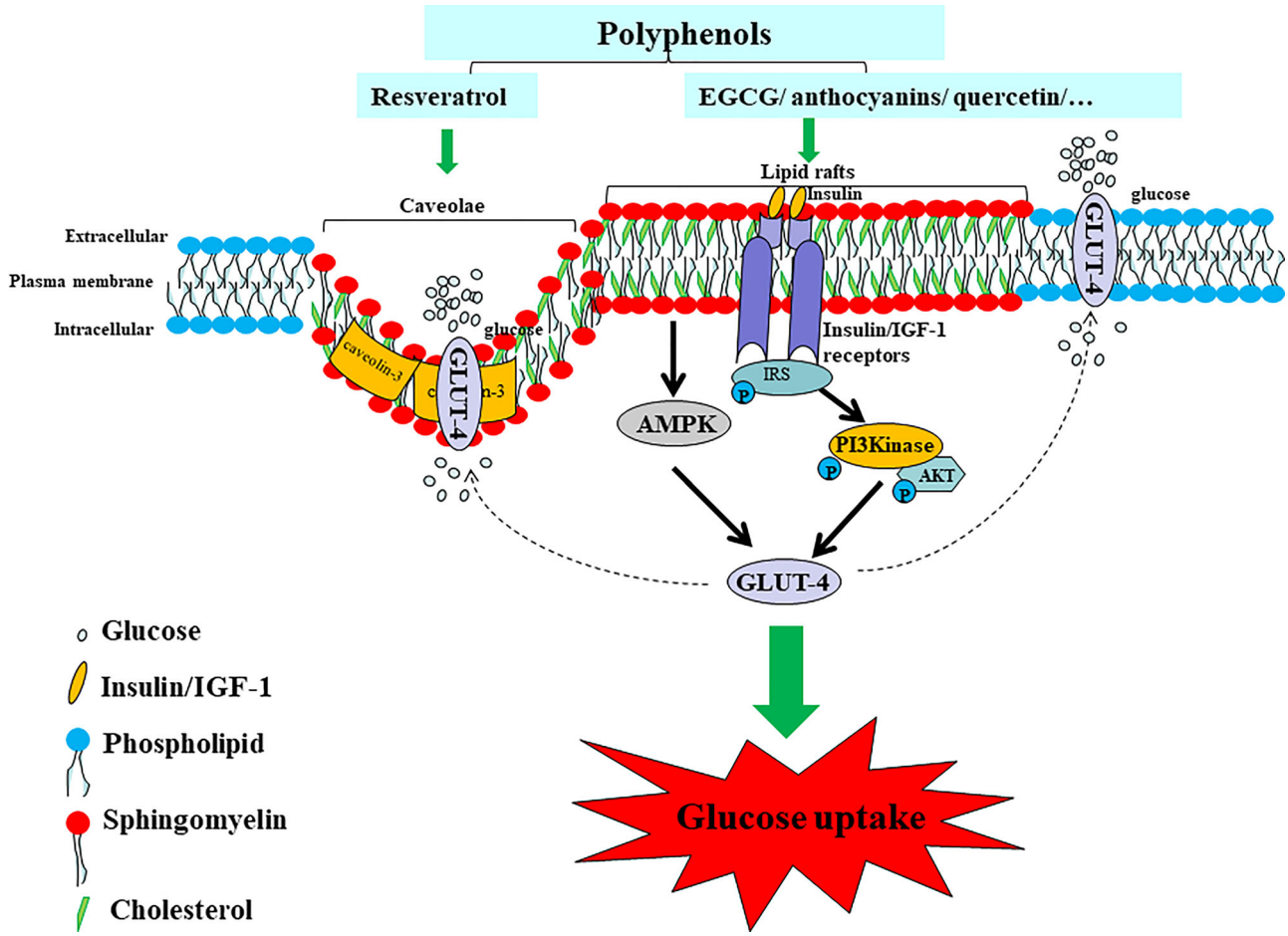

Figure 4. Polyphenols promote glucose uptake via lipid rafts. Polyphenols activate the AMPK or insulin-related signaling pathways to accelerate GLUT4 migration to the plasma membrane, transporting glucose from the extracellular space to the cytoplasm. Moreover, resveratrol promotes the migration of GLUT4 to caveolae, assisting in glucose uptake.

into caveolae in diabetic myocardial cells, and helped in regulating glucose homeostasis (Penumathsa et al. 2008). Tan et al. (2012) demonstrated that the beneficial effects of resveratrol on GLUT4 translocation and glucose uptake were significantly attenuated by knocking out the caveolin-3 protein, indicating that the effect of resveratrol depends on the activity of this protein . The available data show that polyphenols of different structures promote glucose uptake in different ways, but the precise mechanisms are poorly understood. A schematic representation showing several polyphenols promoting glucose uptake via lipid rafts is presented in Figure 4.

State-of-the-art methodology employed to study the interaction between polyphenols and lipid rafts

Lipid rafts have been recognized as hotspots in life sciences since the introduction of the rafts concept by Simons and Ikonen (1997). With the development of novel techniques and tools, more methodologies can be explored to research lipid rafts. The advent of novel biochemical, biophysical, super-resolution visualization, and MD simulation tools makes it possible to answer new questions pertaining to the biology of lipid rafts. This new range of possibilities open the path toward a full understanding of these biological structures. We have attempted to summarize them below.

Biochemical tools

Detergent resistance is the most significant feature of lipid rafts. Treatment of cell membranes with nonionic detergents under cold temperatures (4 °C) wields fractions named detergent-resistant membranes (DRMs). This is the most widely used method to assess the composition of lipid rafts (Chamberlain 2004). Detergents such as 1% Triton X-100, Lubrol WX, Lubrol PX, Brij 58, Brij 96, Brij 98, Nonidet P40, CHAPS, and octylglucoside are routinely used in the separation of lipid rafts (Schuck et al. 2003). Although DRMs can be extracted by these detergents, the conventional extraction process is time-consuming, taking about 16-20 h. Moreover, Triton X-100, one of most widely used detergents, promoted the formation of DRMs, making the evaluation of the size, structure, and composition of lipid rafts in living cells imprecise (Heerklotz 2002). Newer, cheaper, and faster methods, which do not rely on the addition of detergents for the extraction of lipid rafts, have been reported. For example, Macdonald and Pike (2005) established a rapid lipid rafts purification method, with a lower centrifugation time (90 minutes) via an OptiPrep gradient centrifugation to

obtain detergent-free lipid rafts. Nevertheless, DRMs or detergent-free lipid raft fractions are not fully representative of lipid rafts in intact cells, and is the inherent shortcomings of this indirect method.

Although DRM assays can provide primary evidence on the propensity of some compounds associated with specialized membrane regions (Tachibana, Fujimura, and Yamada 2004), results from these assays must be confirmed by more robust and consistent methods. The combination of this method with other techniques such as Western Blotting (WB) makes it possible to explore the underlying mechanisms of a compound. For example, a previous study demonstrated the high affinity of EGCG for DRMs by high performance liquid chromatography, concomitantly with an inhibition of FcεRI expression (detected by WB). Taken together, their results might partially explain the anti-allergic mechanism of this polyphenol (Fujimura, Tachibana, and Yamada 2004). In addition, unpublished data from our previous studies, generated by combining the OptiPrep gradient centrifugation and WB techniques, hinted that A-type ECG dimer inhibited the differentiation of pre-adipocytes by disrupting the structure of lipid rafts and interfering with the pathways downstream of IR signaling.

Biophysical tools

Artificial membrane systems consisting of lipids and CHOL (Lingwood and Simons 2010; Zhao et al. 2007) have been developed as a simple method to study lipid rafts formation. Importantly, due to the artificial nature of the method, results obtained this way should ideally be validated with other models, such as DRMs. Owing to the difference in size and shape of model membranes, artificial model membranes are divided into biomimetic monolayers and bilayers (Tamm and McConnell 1985), giant unilamellar vesicles (Veatch and Keller 2003), and nanoscopic bilayer vesicles (Feigenson and Buboltz 2001). These models are simple, economic and can reveal the phase behavior of model membranes (Simons and Vaz 2004; Zhao et al. 2007). Artificial rafts models constituting phosphatidylcholine, CHOL, and SM were constructed to study the interaction of resveratrol with lipid rafts (Neves, Nunes, and Reis 2016). Similarly, the "lipid raft-like" artificial membranes model was used to explore the molecular mechanism of A-type ECG/EGCG dimers on the inhibition of 3T3-L1 pre-adipocytes differentiation (Zhu et al. 2017).

Artificial membrane models partially reflect the characteristics of lipid rafts; however, some important differences exist between them and cell membranes. For example, the lipid composition of artificial rafts membranes is limited and does not fully represent the composition of cell membranes. Another important difference relates to the lack of transmembrane proteins and peptides, which represent up to 25% of the cross-sectional area of the native membranes (Dupuy and Engelman 2008). Both differences can lead to erroneous results that are not translatable to live cells. GPMVs are directly isolated from live cells and maintain the physiological lipid and protein complexity of intact plasma membranes, avoiding some caveats of artificial membrane models (Sezgin et al. 2012). Nevertheless, GPMVs have some limitations, such as loss of membrane asymmetry, lack of assembled cytoskeleton, unknown enzymatic activity of the remaining transmembrane proteins, and downstream contamination due to residual chemicals used in the process to obtain GPMVs. Despite these caveats, GPMVs are considered to be a methodological breakthrough in the field of lipid rafts research (Levental and Levental 2015; Sezgin et al. 2012). Since this technique has not yet been applied in the study of lipid rafts and polyphenols, there is potential to unravel novel mechanisms using this techniques in the future studies.

Visualization tools

Microscopy is a powerful tool to directly visualize changes in lipid rafts. Lipid rafts are small nanoscopic domains (10-200 nm) and cannot be observed by conventional optical microscopy, which has a diffraction resolution limit of approximately 250 nm (Pike 2006). To overcome this limitation, optical microscopy with high temporal and spatial resolution has been used to directly observe lipid rafts in the plasma membrane of living cells. They include stimulated emission depletion microscopy (STEDM) (Eggeling et al. 2009), total internal reflection fluorescence microscopy (LG-TIRFM) (Asanov, Zepeda, and Vaca 2010), and PALM (Sengupta et al. 2011). For more spatiotemporally heterogeneous measurements, such as the dynamic diffusion and clustering of raft lipids in living cells, fluorescence correlation spectroscopy (FCS) could be used in combination with STEDM (Honigmann et al. 2014). Most of the aforementioned methods rely on the usage of lipid raft-specific fluorescent probes (Klymchenko and Kreder 2014).

A study on prostate cancer cell lipid rafts, which labeled membrane-ordered domains with the $DiIC_{16}$ fluorescent probe, showed that EGCG interfered with the structure of lipid rafts and affected the activation of HGF/c-Met signaling pathway, ultimately inhibiting the proliferation and invasion of prostate cancer cells (Duhon et al. 2010). Similarly, using $DiIC_{16}$ to label pre-adipocyte membrane lipid rafts, it was shown that A-type ECG/EGCG dimers interfered with the structure of lipid rafts, inhibiting downstream signaling pathways involved in 3T3-L1 pre-adipocyte differentiation (Zhu et al. 2017). Tsukamoto et al. (2012) used fluorescein isothiocyanate (FITC)-tagged cholera toxin subunit B to label lipid raft constituents in the presence and absence of EGCG, showing that EGCG induced the clustering of lipid rafts. However, the cholera toxin probe *per se* has been reported to interfere with the clustering of the lipid membrane (Sezgin et al. 2017), suggesting caution in the interpretation of the aforementioned results and highlighting the urgent need for non-interfering labeled fluorescent probes for raft and non-raft domains. In this regard, the non-interfering probe, FITC-conjugated glycol chitosan localized to lipid rafts through electrostatic attractive and hydrophobic interactions, and could be used to visualize lipid rafts in live cells (Jiang et al. 2016). Further research is required to find

more suitable fluorescent probes, which can be combined with super resolution microscopy to visualize the effects of different polyphenols on lipid raft structures.

MD simulation tools

MD simulations apply Newton's equation of motion to describe the spatial motion of atoms. An inherent advantage of MD techniques is the ability to visualize molecular behavior at a high spatial and temporal resolution. Thus, all-atom (AA) and coarse-grained (CG) MD simulations can provide supplementary information to complement in vitro and in vivo experimental studies. Due to the limitations of processing power of computers, AA MD simulations are only able to simulate short processes (nanoseconds to microseconds). To extend this spatiotemporal scale, CG MD simulations based on the Martini force field have been introduced (Saunders and Voth 2013). Nonetheless, the difficulty of constructing such a simulation model and high degree of specialization may limit the widespread usage of MD.

Currently, MD simulation techniques can be used to infer the nature of lipid-lipid and lipid-protein interactions at an atomic level within lipid rafts (Bennett and Tieleman 2013; Bera and Klauda 2017; Niemela, Hyvonen, and Vattulainen 2009; Niemela et al. 2007). Furthermore, they can also simulate the interaction between polyphenols and membrane lipids (Sirk et al. 2008). Using AA MD simulations of *E.coli* and yeast membrane models, curcumin, a natural antimicrobial polyphenol molecule, readily inserted into the biological membrane mimics, parallel to the membrane surface. This resulted in membrane thinning and could explain curcumin's antimicrobial activity (Lyu et al. 2018). Our group constructed a 3T3-L1 pre-adipocyte membrane lipid rafts model to study the interaction between A-type ECG dimer and lipid rafts by AA MD simulations. Our results indicated that the A-type ECG dimer penetrated deeper within the lipid rafts to bind with CHOL and interfered with the hydrophobic chain of raft lipids (unpublished data). The results were in agreement with those obtained in cell systems, showing that the MD simulation tools are an effective supplementary method, which complements biological studies. Additionally, CG MD simulations have also been performed to test the ability of polyphenols to interfere with cell membranes and different membrane proteins (Ingólfsson et al. 2014). In this study, the tested polyphenols mainly interfered with cell membranes and promiscuously affected the function of different proteins, suggesting that the bioactivity of polyphenols might occur due to perturbations of the cell membrane itself and not of specific proteins (Ingólfsson et al. 2014). Naturally, *in silico* tools such as MD simulations are a great tool to study lipid rafts, since they can provide molecular details that are not readily observed or measured via classical in vitro or in vivo techniques. The disadvantage of MD simulations is the incomplete simulation environment; combination of MD simulations with other experimental techniques discussed in the previous chapter will be crucial to study the mechanisms governing the interaction between polyphenols and lipid rafts.

Conclusions and future perspectives

The structure and function of lipid rafts play an important role in both prevention and treatment of chronic diseases that endanger human health. Recent studies suggest that the pleiotropic actions of polyphenols can be mediated through their interaction with lipid rafts. Here, polyphenols are thought to act by modulation of the structural integrity of lipid rafts and interference with the activity of proteins localized in lipid rafts, such as EGFR, c-Met, FcεRI, IR, IGF-1R, and 67LR. Presently, the tools used to study the interaction between polyphenols and lipid rafts are mainly divided into four categories: biochemical, biophysical, visualization, and MD. A multidisciplinary approach based on combination of different existing tools will help elucidating the details of the molecular interaction between polyphenols and lipid rafts.

Although increasing evidence points toward lipid rafts as a mechanistic target of polyphenols, our understanding is still quite limited and current studies on the interaction of polyphenols and lipid rafts are unsystematic. Therefore, further studies should focus on the following aspects: (1) the research on polyphenols or their metabolites and lipid rafts in vivo; (2) the direct evidence that the physiological effects of polyphenols are due to changes in the structure of lipid rafts or signal pathways mediated by lipid rafts, especially the effects of low concentration of polyphenols present in the body on lipid rafts; (3) the comprehensive application of multiple new technologies to understand the interaction of polyphenols with lipid rafts; (4) the precise effects promoted by polyphenols on the structure of lipid rafts and conformational changes to signaling proteins present in lipid rafts; (5) the relationship between the effects of polyphenols on lipid rafts structure and downstream signaling pathways, as well as biological activities; (6) development of novel techniques, such as high resolution microscopy combined with online tracking of molecular motion equipment, non-interfering lipid rafts fluorescent probes etc. We believe that these developments should promote the generation of invaluable knowledge in this area.

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