Chapter 8
New Vis-Tas in Lactosylceramide Research

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Introduction

Lactosylceramide (LacCer) is a member of a large family of compounds collectively called the glycosphingolipids (GSL). These molecules are present in all mammalian cells, some bacteria and fungus. GSLs are composed of an amino acid serine, fatty acids and sugars and are usually localized on the cell surface wherein they serve as receptors for diverse physiologically relevant molecules, bacteria and viruses. However, LacCer is predominantly stored within cytoplasmic vesicles located in the perinuclear area though some LacCer is present on the cell surface. The dynamics of these two pools of LacCer is not known. Nevertheless, recent efforts by several groups of investigators have opened up new vis-tas in LacCer research. The present article is to bring to forth these findings for further experimental validation and for use in translational research to develop better diagnostics and therapeutics for use humans and certain veterinary purposes. In particular, this article will focus on two areas: 1. Inflammation and the LacCer–phospholipase-A-2 (PLA2) connection and 2. Implications of LacCer modulation on cardiac hypertrophy.

Briefly, the biosynthesis of GSL begins upon the condensation of L-serine with palmitoyl-CoA to form sphingosine (Fig. 8.1). In mammalian cells, sphingosine is then metabolized in a sequential manner to synthesize several intermediates leading up to the synthesis of ceramide. Ceramide forms the non-polar tail of all GSL (presumed to be buried within the cell membrane) to which glucose and galactose (from respective nucleotide sugars) is added consecutively to yield glucosylceramide and LacCer, respectively.

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A. Chakrabarti, A. Surolia (eds.), *Biochemical Roles of Eukaryotic Cell Surface Macromolecules*, Advances in Experimental Medicine and Biology 842,
DOI 10.1007/978-3-319-11280-0_8

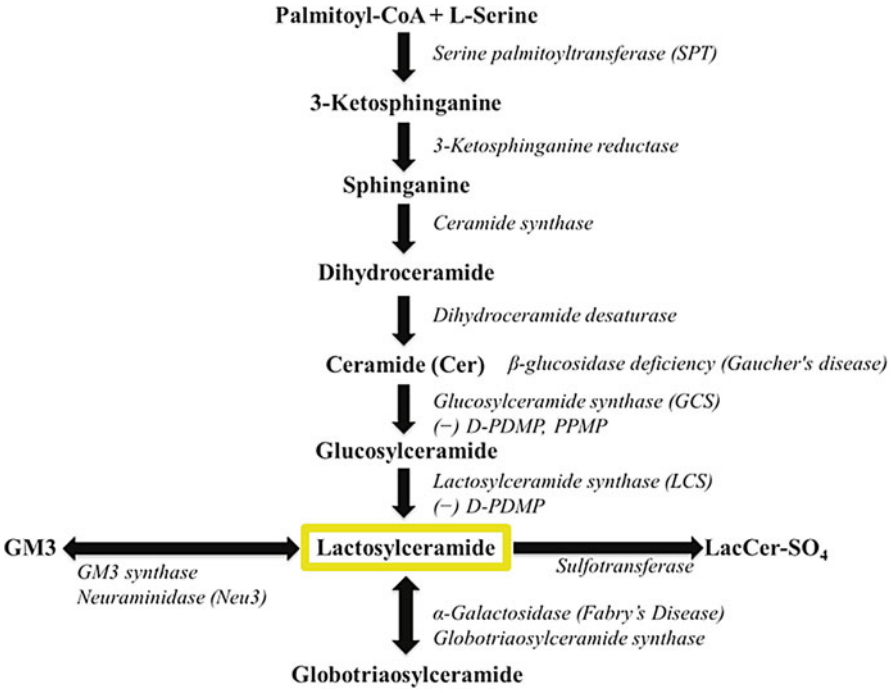

Fig. 8.1 Metabolic pathways involved in lactosylceramide biosynthesis and its role as a precursor to complex glycosphingolipids

In nature, at least two LacCer synthases (LCS) have been reported. According to the recent nomenclature, they are termed GalT-V and GalT-VI. Before the human genome was unraveled, only one LCS was known and was termed GalT-2, now referred to as GalT-VI. While GalT-V is a constitutively expressed in most tissues, GalT-VI is expressed in a tissue specific manner-in the brain (Lo et al. 1998). In this context, the readers are referred to another chapter in this series where a detailed description of the biosynthesis of complex GSL and nomenclature of these enzymes are described by Basu and co-workers (Ref). The important feature about GalT-V is that it is the major LCS in human endothelium (Chatterjee et al. 2008). Therefore, it plays a critical role in the biosynthesis of LacCer and LacCer-regulated phenotypes and diseases (Chatterjee and Alsaeedi 2012). For example, LacCer plays a critical role in vascular endothelial growth factor (VEGF)/fibroblast growth factor (FGF)-induced angiogenesis (Rajesh 2005; Kolmakova et al. 2009), a phenotype central to tumor metastasis, and tumor growth. Thus, the use of siRNA to ablate GalT-V gene in vitro and in vivo was found to mitigate angiogenesis and tumorigenic potential in B16-F10 mouse melanoma cells, respectively (Rajesh 2005; Wei et al. 2010; Furukawa et al. 2014). Also, the use of inhibitor's of LCS such as D-PDMP can reverse VEGF and FGF-induced angiogenesis. Further, the observation that the tumor necrosis factor (TNF) induced expression of intercellular cell adhesion molecule-1 (ICAM-1) requires the activation of the endothelial cell derived GalT-V which may be central to both inflammation and atherosclerosis. Also ICAM-1 serves as a receptor for Mac-1/CD11b present on the surface of monocytes

and neutrophils. Thus, the adhesion of these blood cells to the endothelium and their subsequent intravasation is a first critical step in the initiation of inflammation and atherosclerosis seen below (Bhunia et al. 1997).

Another major source of LacCer production is due to the action of a sialidase termed Neu3 on a ganglioside GM3 (Miyagi and Yamaguchi 2012). This enzyme is highly enriched with the plasma membrane in cancer cells. This reaction seems to be utilized largely in human cancer cells and cancer tissue noted for its highly malignant properties as its contribution to an induction of phenotypes, e.g. cell migration and invasion. Studies using colon cancer cells have revealed that Neu3 activates Wnt receptor by phosphorylation of Ras/MAPK upon stimulation by EGF (Miyagi et al. 2012). Thus, Neu-3 induced LacCer production may well partake in signaling pathways leading to tumor metastasis. Neu3 activation was also shown to occur upon exposure of human dermal fibroblast to elastin that can activate this enzyme to generate LacCer (Rusciani et al. 2011). Furthermore, LacCer is shown to generate reactive oxygen species (ROS): a superoxide to activate the phosphorylation of mitogen activated protein kinase. In a cancer cell line, this helps increase in cell proliferation (Miyagi and Yamaguchi 2012), and in elastic tissue, the phosphorylation of MAPK facilitates its elasticity (Rusciani et al. 2011). These findings are summarized diagrammatically (Fig. 8.2).

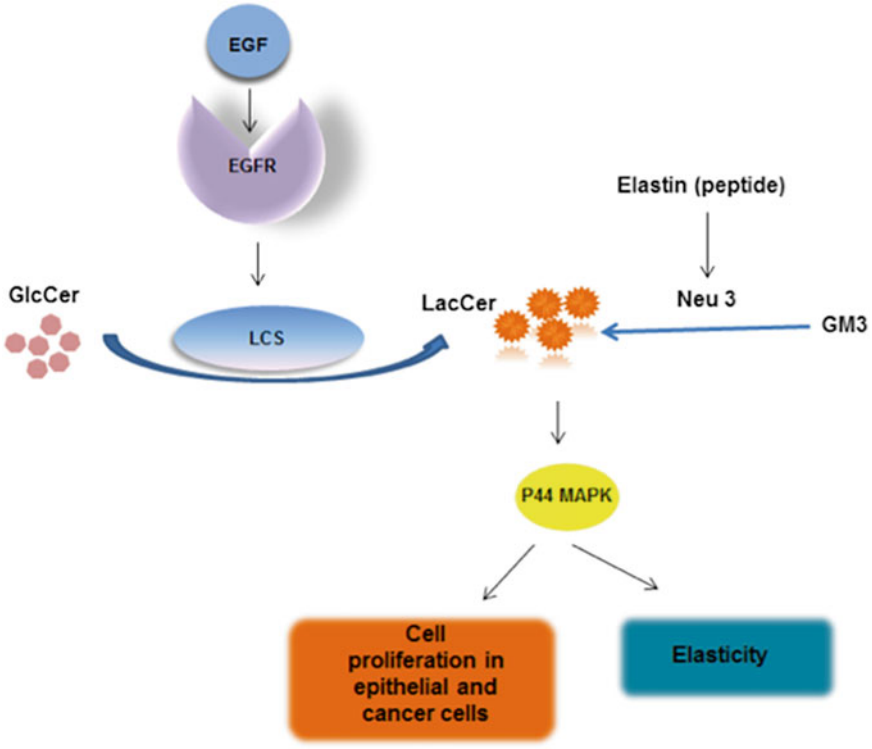

Fig. 8.2 Signaling pathways by which epidermal growth factor recruits LacCer to induce cell proliferation in cancer cells and elasticity

Inflammation and the LacCer: Phospholipase-A-2 (PLA2) Connection

Inflammation is probably the earliest process leading to the two major killers of mankind: heart disease and cancer, perhaps other inflammatory diseases as well. This involves the participation of cells in the circulation notably platelets, macrophages, neutrophils, leukocyte/monocytes, and the vascular cells such as arterial smooth muscle cells and the endothelium. Infections and other stressor's allow the release of various growth factors and pro-inflammatory cytokines from the various blood cells and smooth muscle cells. Since endothelial cell surface forms a barrier between blood cells and its components and the vascular wall, pro-inflammatory cytokines such as tumor necrosis factor (TNF-α, inflammatory cytokines, growth factors etc.) bind to their receptors on the surface of the these cells, thus activating them and producing signaling molecules such as LacCer (via activation of LacCer synthase) (Chatterjee and Alsaeedi 2012). In turn, LacCer specifically induces the expression of a cell adhesion molecule-intercellular cell adhesion molecule (ICAM-1) through an "oxygen-sensitive" signaling pathway. Studies in vitro and in vivo demonstrate that ICAM-1 serves as a receptor for another protein Mac-1/CD11b expressed on the surface of neutrophils and monocytes. This allows the capture of circulating neutrophils and monocytes and their intravasation into the sub-endothelial space. Herein, the monocytes undergo proliferation and differentiation into macrophages due to the action of several growth factors. Since macrophages express scavenger receptors e.g. SRB-1, CD-36 etc., it allows them to take up oxidized LDL. Studies show that oxidized LDL not only contribute to the deposition of cholesterol esters but also can inhibit their hydrolysis contributing to fatty streaks in the arterial wall, plaque development, and its subsequent pathological pathway. Additional studies show that LacCer can directly interact with neutrophils and monocytes to activate phospholipase-A-2 to increase the expression of Mac-1/CD-11b to facilitate adhesion to the endothelium (Fig. 8.3) (Arai et al. 1998).

The emerging view is that LacCer taken up by cells from lipoproteins, other cell membranes due to cell–cell interaction or simply by an exogenous supply which may form a LacCer membrane microdomain (Fig. 8.4). And such microdomains are involved in generating superoxides and/or activating phospholipase to bring about profound phenotypic changes in vitro. First, using human arterial smooth muscle cells it was shown that LacCer dose and time dependently raised the cellular levels of superoxides by activating NAD(P)H oxidase activity (Bhunia et al. 1997). Next, LacCer was shown to facilitate the migration of several components of the NAD(P)H complex such as c47phox and c67phox from the cytosol to the plasma membrane to bind with the other components of NAD(P)H oxidase, generating superoxides (Martin et al. 2006). Additional studies revealed that the LacCer microdomain together with a Src kinase; Lyn, expressed on the neutrophil plasma membrane, may well be implicated in innate immune response (Yoshizaki et al. 2008).

Another instance of direct LacCer protein interaction is the case with phospholipase-A-2. The roles of phospholipase-A-2 are many including initiation and propagation of inflammation, cellular damage, modulation of chemotaxis, phagocytosis

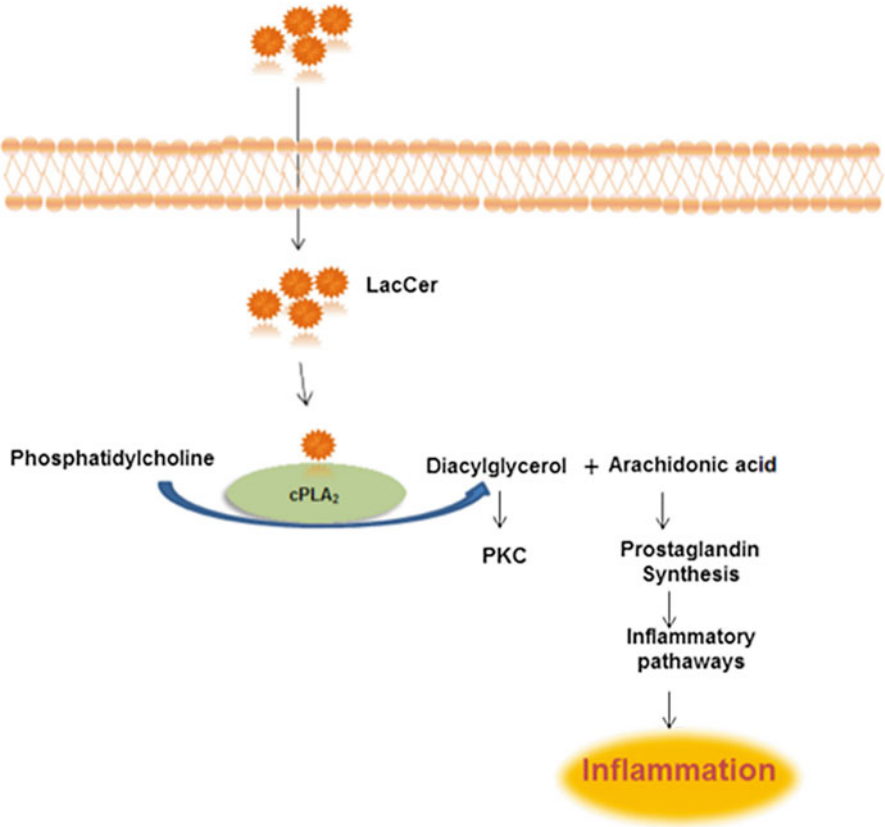

Fig. 8.3 Signaling pathways by which exogenous LacCer is involved in the activation of phospholipase-A-2, leading to inflammation

and superoxide generation. It also modulates vascular tone, enhances vascular permeability and may also impact T cell function. The substrate for PLA-2 is a phospholipid phosphatidylcholine wherein it cleaves the sn-2 fatty acid, arachidonic acid (Fig. 8.3). While LacCer can directly activate PLA-2, studies show that FcER1 cross linking may well activate PLA-2 via a receptor independent tyrosine kinase (src, lyn, yes, syk etc.). The IP3 generated interacts with the Ca2+ channel on the endoplasmic reticulum to increase the concentration of cytosolic Ca2+. In turn, this allows the translocation of cPLA2 from the cytosol to cell membrane compartment thus cleaving arachidonic acid. The phosphorylation of Ser 505 in PLA-2 increases intrinsic enzyme activity.

The proof of concept that indeed LacCer directly activated PLA2 arrived from several experimental observations. First, treatment of cells with PPMP an inhibitor of glucosylceramide synthase and LacCer synthase, reduced the level of LacCer and the release of arachidonic acid. This was bypassed by treating cells with just LacCer

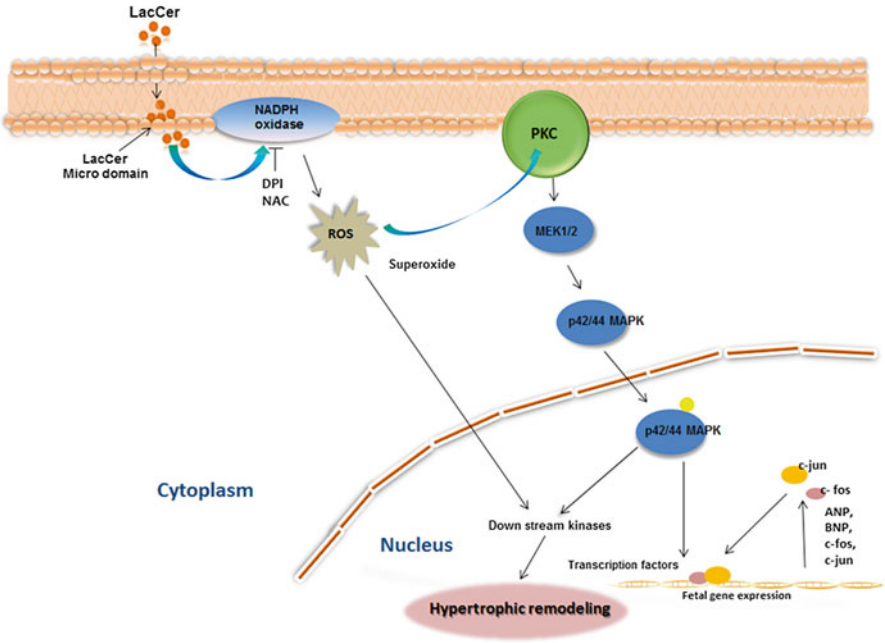

Fig. 8.4 Lactosylceramide induces hypertrophy in cardiomyocytes via ROS generation and activation of P44 MAP kinase (Mishra and Chatterjee 2014)

and no other glycosphingolipids (Nakamura et al. 2013). Second, silencing the enzyme cPLA2 or the use of an inhibitor of cPLA-2 also mitigated LacCer induced cPLA2 activation. These studies conducted using Chinese hamster ovary cells (CHO-W11A) showed that LacCer translocated D43N mutant of cPLA-2. Additional studies in a human monocytic cell line (U-937) have revealed that LacCer recruited PKC-α/ε to activate PLA-2 and the intrinsic expression of platelet endothelial cell adhesion molecule (PECAM-1). Since COX-2 inhibitors mitigated arachidonic acid-induced PECAM-1 expression, prostaglandins may mediate PECAM-1 expression in monocytes (Gong, NL 2004 PNAS). Previous studies show that PECAM-1 plays a critical role in the trans-endothelial migration of monocytes into the sub-endothelial space thus initiating atherogenensis and involving LacCer in the pathology in this disease (Chatterjee and Pandey 2007).

Implications of LacCer Modulation on Cardiac Hypertrophy

Lipids are required by all organs, including heart, for its function. They consist of fatty acids (FA) that supply calories required for numerous cellular activities and are also the important structural component of cells. The majority of plasma fatty acid constitutes triglycerides and phospholipids that exist in esterified form. Oxidation

of various substrates like FA, glucose, lactate and ketone bodies generate ATP in normal adult hearts. Among these substrates, glucose and fatty acids are the most important for ATP production in the heart. Approximately 70 % of the ATP essential for regular cardiac function is provided by the FA. There are multiple pathways that modulate the attainment of FA by the cardiomyocytes, and any shift in these pathways affects cardiac metabolism and function (Taegtmeyer 1994). High blood cholesterol is a major risk factor for heart diseases, and hyperlipidemia for atherosclerosis and cardiovascular disease, respectively, including coronary heart disease. Epidemiologic studies have shown that hypercholesterolemia is associated with increased left ventricular mass and cardiac hypertrophy (Jung et al. 2010; Luo et al. 2010; Miguel-Carrasco et al. 2010; Planavila et al. 2011; Singh and Krishan 2010; Takayama et al. 2011; Wang et al. 2010; Wojciechowski et al. 2010). Hypertrophic cardiomyopathy is a pathological hypertrophy of the heart due to an increase in the size of myocytes in various heart diseases including long-term hypertension, myocardial infarction, chronic pressure overload, valvular defects and endocrine disorders (Frey et al. 2004; Grossman et al. 1975; Hood et al. 1968; Sandler and Dodge 1963). Myocardial hypertrophy is an adaptive response of the heart to increased workload. Cardiac hypertrophy is one of the main responses of cardiomyocytes to mechanical and neuro-hormonal stimuli. Although cardiac hypertrophy may initially represent an adaptive response of the myocardium, it often progresses to ventricular dilatation leading to heart failure, one of the leading causes of mortality in the world. Increased left ventricular mass (LVM) and decreased fractional shortening (FS) are risk factors in cardiac morbidity and mortality in the general population (Baumgartner et al. 2007; Lorell and Carabello 2000; Movahed and Saito 2009). Cardiac hypertrophy and fibrosis, which are the most common responses of the heart to all forms of injury, are the major determinants of morbidity and mortality from cardiovascular disease in both developing and developed countries.

The role of diet is crucial in the development and prevention of cardiovascular disease. It also impacts all other cardiovascular risk factors. Previous studies have demonstrated that dyslipidemia, hypercholesterolemia and cardiac lipotoxicity are associated with cardiac hypertrophy (Balakumar et al. 2011; Berger et al. 2005; Borradaile and Schaffer 2005; Lopaschuk et al. 2007; Poornima et al. 2006; Semeniuk et al. 2002; Smith and Yellon 2011; Unger and Orci 2001; Yang and Barouch 2007).

Hypertrophy induced by fat diet intake is steadily becoming one of the primary causes of myocardial infarction, morbidity, and stroke and is a major clinical concern in cardiovascular medicine. Increased levels of FAs from fatty diets can impact the heart harmfully due to the formation of toxic derivatives of glucose and lipid metabolism (Bayeva et al. 2013). Epidemiological studies showed that hypercholesterolemia is associated with higher left ventricular mass and that dyslipidemia is an independent determinant of increased left ventricular mass (Lee et al. 2005). In patients with Fabry's disease, glycosphingolipid deposition in heart causes progressive left ventricular hypertrophy that mimics the morphological and clinical picture of hypertrophic cardiomyopathy, with dyspnea on effort, palpitation and angina as the typical symptoms (Nakao et al. 1995). A close association between GSLs level

and cardiac hypertrophy in vivo in apoE -/- mice fed a western diet was suggested by us recently (Chatterjee et al. 2013). We have observed that feeding a high fat and cholesterol diet to apoE-/- mice results in marked increase in the level of GSL e.g. glucosylceramide (GlcCer) and LacCer in heart tissue accompanied by an increase in the activity of glycosphingolipid glycosyltransferases (GTs) (Chatterjee et al. 2013). However, these in vivo studies did not elaborate whether one or more GSLs were implicated in cardiac hypertrophy. To address this issue, we used cultured neonatal rat cardiomyocytes and H9C2 cells and sought to determine whether GSL's affect cardiac hypertrophy. The cardiomyocytes were treated with different glycosphingolipids and their effect on hypertrophy was measured using multiple biochemical molecular and morphological parameters (Mishra and Chatterjee 2014). Among several glycosphingolipids examined, Lactosylceramide specifically stimulated hypertrophic parameters to a similar extent as PE (Phenylephrine) in these cells. Cardiac hypertrophy *in vivo* involves the enlargement of the heart caused by an overload of blood volume and increased blood pressure. Cardiac hypertrophy *in vitro* is induced by the use of agonists such as PE that binds to its cognate receptors and transduces downstream components of a ROS-mediated signal transduction pathway to eventually induce hypertrophy.

In this study, PE was used as a positive control. We demonstrated that at a similar concentration (100 μM), LacCer could serve as a bonafide agent to induce cardiac hypertrophy in H9c2 cells and freshly cultured primary rat cardiomyocytes. In contrast, the other classes of GSL, such as sulfatides, complex gangliosides and other neutral GSLs, failed to induce hypertrophy in cardiomyocytes. This shows that an intact molecule of LacCer is required to induce cardiac hypertrophy. Importantly, the catabolic or anabolic products of LacCer failed to induce this phenotype. At the cellular level, hypertrophy is characterized by an increase in the size of cells, protein synthesis, reactivation of fetal genes e.g. ANP (Atrial natriuretic peptide) and BNP (Brain natriuretic peptide), changes in the signal transduction pathways and reorganization of sarcomere structure. The increase in cell size is mainly accompanied by an increase in protein synthesis. Our studies employed multiple criteria to assess hypertrophy in these cardiomyocytes e.g. increased cell volume, increased protein synthesis using [^{3}H]-Leucine as a precursor, determination of cell size and the measurement of mRNA levels of ANP and BNP-established biomarkers of cardiac hypertrophy. These studies suggest that LacCer specifically induced hypertrophy in cardiomyocytes.

We observed that in cardiomyocytes LacCer induces the generation of superoxides in a time and concentration-dependent manner. This was mitigated by the use of antioxidants such as N-acetyl cysteine, a scavenger of free oxygen radicals and diphenylamine iodonium (DPI), an inhibitor of NAD(P)H oxidase (Hsieh et al. 2013; Yang et al. 2013a, b). Use of these inhibitors also mitigated LacCer induced cardiac hypertrophy biomarkers mRNA levels e.g. ANP and BNP. This observation suggests that, by activating NAD(P)H oxidase, LacCer generates superoxide radicals which in turn activates a downstream signaling cascade leading to cardiac hypertrophy (Fig. 8.4).

The immediate early genes activated during hypertrophic stimulus include c-jun, c-fos, c-myc etc. In our study we found that, LacCer induced hypertrophy also

involved the upregulation of both c-fos and c-jun genes. We also demonstrated that the activation of these immediate early genes involves oxidative stress.

Subsequent studies have shown the effects of LacCer on Protein Kinase C (PKC) activation and cardiac hypertrophy. The involvement of PKC in cardiac hypertrophy has been reported previously (Bowman et al. 1997; Braz et al. 2002; Vijayan et al. 2004). We observed marked inhibition of LacCer-induced ANP and BNP mRNA levels in cardiomyocytes in the presence of PKC inhibitor, suggesting that PKC plays a central role in LacCer induced hypertrophy.

Previous studies have placed p44 MAPK activation as a central component in agonist induced cardiac hypertrophy (Araujo et al. 2010; Dai et al. 2011; Fahmi et al. 2013; Ferguson et al. 2013; Lopez-Contreras et al. 2013; Ruppert et al. 2013; Sbroggio et al. 2011). Also transforming growth factor-β 1 induces hypertrophy and fibrosis via activation of p44 MAPK (Bujak and Frangogiannis 2007). Therefore, we examined the effects of LacCer on p44 MAPK and cardiac hypertrophy. LacCer induced the rapid phosphorylation of p44 MAPK and this activation process was required to induce cardiac hypertrophy (Fig. 8.4). Our study suggests that LacCer alone can induce hypertrophy in cardiomyocytes and therefore exposes both LacCer and LacCer synthase as novel drug targets to mitigate this phenotype. This emphasizes the need for a better understanding of GSLs and GTs in cardiac hypertrophy and other cardiovascular diseases.

Perspectives

As of to date, it is still unclear whether or not PLA 2 is a bonafide inflammatory marker for cardiovascular risk due to the lipoprotein-PLA2 possibly playing a dual role as a pro-atherogenic and anti-atherogenic molecule. On one hand, Lp-PLA2 generates arachidonic acid, a precursor for prostaglandins and relevant to the inflammatory pathway contributing to atherosclerosis. On the other hand, LP-PLA 2 is implicated in the degradation of platelet activating factor (PAF), a potent mediator of inflammation. These characteristic of Lp-PLA 2 raises a burning question ,under what conditions does the PLA2 become atherogenic vs. anti atherogenic. Another aspect of PLA2 action is the generation of oxidized phospholipids and lysoPC. While the oxidized phospholipids could render LDL prone to oxidation and consequently contribute to atherosclerosis, the lysoPC is a potent fusogenic compound and may also serve in accelerating atherosclerosis.

Literature on the role of LacCer in vascular stiffness and in cardiac hypertrophy is just beginning to unravel. These studies must be validated using large mammals. Since GalT-V gene ablation is embryo - lethal alternative experimental models designed to deplete LacCer and or the use of highly specific inhibitors of LacCer synthesis are needed to explore this area of research. Since one in three people worldwide suffers from high blood pressure which can contribute to cardiac hypertrophy, research in this area could be lucrative for the industry and to the NIH effort to bolster their program of excellence in Glycosciences.

Acknowledgments This work was supported by grants from the NIH, PO-1-HL-107-153 and 3PO1HL 107153-03S1.

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