

## Myocardial Phospholipid Remodeling under Different Types of Load Imposed during Early Postnatal Development

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### Summary

Normal increase in hemodynamic load during early postnatal life is associated with heart growth and maturation of membrane structures that is accompanied by remodeling of membrane protein and lipid components. This review describes remodeling of phospholipids (PL) in rat myocardium during normal postnatal development and during accelerated cardiac growth induced by additional workload (aorta constriction, chronic hypoxia and hyperthyroidism) imposed on the heart early after birth. Normal physiological load after birth stimulates the development of membrane structures and synthesis of PL. While hyperthyroidism accelerates these processes, pressure overload has an inhibitory effect. These changes primarily influence the maturation of mitochondrial membranes as cardiolipin is one of the most affected PL species. The most sensitive part of PL structure in their remodeling process are PL acyl chains, particularly polyunsaturated fatty acids that are the key components determining the basic physicochemical properties of the membrane bilayer and thus the function of membrane-bound proteins and membrane-derived signaling lipid molecules. It is evident that PL remodeling may significantly influence both normal and pathological postnatal development of myocardium.

### Key words

Phospholipids • Cardiolipin • Myocardium • Postnatal development • Pressure overload • Chronic hypoxia • Thyroid status

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### Introduction

Transition from prenatal to postnatal life is associated with profound changes in the circulation followed by restructuring of the cardiovascular system. The newborn heart possesses high capability to cope with an increased load accompanied by neural and endocrine maturation leading to myocardial growth. This high adaptive potential of the myocardium attenuates during maturation (Oštádal *et al.* 1999). Just after birth, the heart has to overcome the rapid switch-over from the right ventricle dominance in the fetus to the left ventricle dominance reflected by different growth pattern of both ventricles (Smolich 1995). In most mammals, ventricular myocytes lose their capability of division shortly after birth, and further growth of the heart is solely due to their hypertrophy and hyperplasia/hypertrophy of other cell types (Clubb and Bishop 1984). It was shown that the switch from hyperplastic to hypertrophic growth of rat ventricular myocytes occurs as early as after the third postnatal day (Li *et al.* 1996). Additional excessive load imposed during the early postnatal period leads to accelerated heart growth response by both myocyte hyperplasia and hypertrophy (Sedmera *et al.* 2003) accompanied by metabolic changes and remodeling of membranes including proteins (Wibo *et al.* 1995, Zheng

*et al.* 1996) and lipids (Mrnka *et al.* 1996, Hamplová *et al.* 2003, Novák *et al.* 2004).

The principal components of the membrane lipid bilayer are phospholipids (PL) and their ontogenetic remodeling contributes to the modification of membrane-bound proteins that influence proper heart maturation. Furthermore, membrane PL are precursors of second messengers involved in a variety of signaling pathways. Hence, changes in the quantity and quality of cardiac PL have an indirect effect on the regulation of cardiac function and growth during postnatal development (Tappia 2007).

The present review summarizes the results dealing with remodeling processes of membrane PL and their fatty acid (FA) composition in rat myocardium during normal postnatal development and during accelerated cardiac growth induced by additional workload (aorta constriction, chronic hypoxia and hyperthyroidism) imposed on the heart early after birth.

## Phospholipid *de novo* synthesis and remodeling

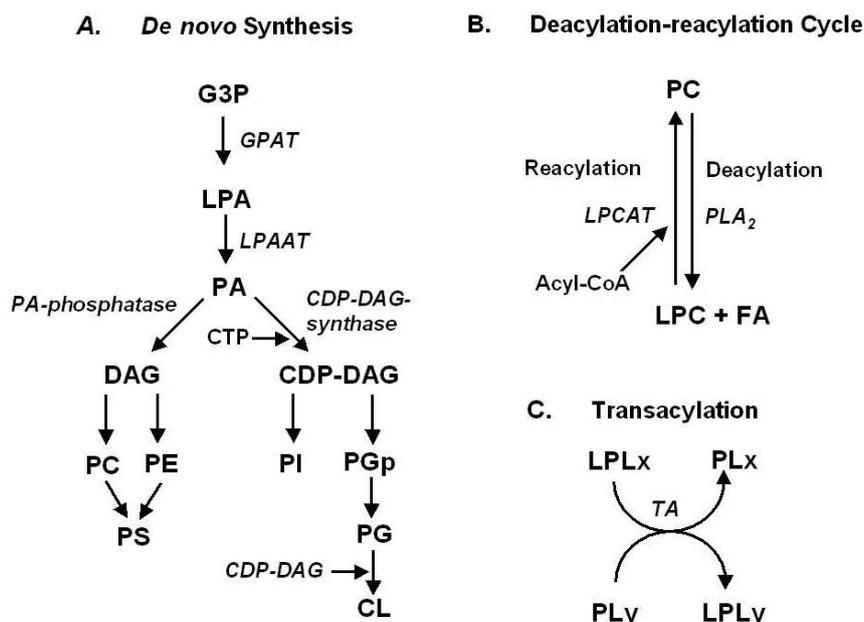
Cellular membranes contain several classes of phospholipids that have numerous structural and functional roles in cells. Heart tissue maintains a distinct content and composition of various PL, such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (cardiolipin, CL), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) (Nováková *et al.* 1994). Glycerophospholipids are first formed by the *de novo* pathway using acyl-CoA as an acyl donor (Kennedy and Weiss 1956). Subsequently, in the remodeling pathway, cycles of deacylation and reacylation of glycerophospholipids modify the FA composition to generate mature membrane PL with asymmetry and diversity (Lands 1958). Polyunsaturated FA (PUFA) are usually located at the *sn*-2 position while saturated FA (SFA) and monounsaturated FA (MUFA) at the *sn*-1 position of glycerophospholipids in an asymmetric manner (Wood and Harlow 1969, Yamashita *et al.* 1997). Biosynthetic pathways of most glycerophospholipids are localized at the endoplasmic reticulum while CL biosynthesis takes place in mitochondria. The precursor for glycerophospholipid *de novo* synthesis is glycerol-3-phosphate (G3P) that is acylated in the first step by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT)

(Kennedy and Weiss 1956) (Fig. 1A). The resulting lysophosphatidic acid (LPA) is further acylated to PA by acyl-CoA:monoacylglycerol-3-phosphate acyltransferase (LPAAT). PA is converted either to cytidinediphosphate-diacylglycerol (CDP-DAG) by CDP-DAG synthase or to DAG by the PA phosphatase. CDP-DAG can be further incorporated into PS, PI, PG and CL while DAG is transferred to CDP-choline or CDP-ethanolamine resulting in PC and PE (Fig. 1A). It is necessary to mention that both acyltransferases, involved in *de novo* PL synthesis in the heart, possess broad substrate affinity for different SFA and MUFA (Zaror-Behrens and Kako 1976) that does not correspond to characteristic acyl chain composition of mature PL in specific cellular membranes with preference for PUFA in *sn*-2 position of individual PL. Thus, the acyl chain remodeling pathways have been proposed to account for the characteristic profile of acyl chains in phosphoglyceride species. Lands' cycle involves a two-step deacylation-reacylation process whereby non-specific acyl chains are cleaved from appropriate PL by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and generated lyso-PL is reacylated by lyso-PL acyl-CoA acyltransferases with high specificity for unsaturated FA (Fig. 1B) (Lands 1958). Another pathway, participating in PL remodeling, is the transacylation reaction (Yamashita *et al.* 1997), which involves transfer of acyl chain from various diacyl-PL to lyso-PL (Fig. 1C).

## Normal postnatal development of myocardial phospholipids

### *Phospholipid species*

The mass of cardiac membrane structures rises dramatically during early postnatal development; the biosynthesis of protein and PL components predominates over its degradation, whereas both processes are more in a dynamic equilibrium in the adult heart under physiological conditions (Girard *et al.* 1992, Nováková *et al.* 1994). The time course of changes in the myocardial concentration of total PL is generally proportional to ventricular growth. Nevertheless, there are two exceptions (Fig. 2). In the rat heart, the increment of total PL between postnatal days d2-d5 and d20-d40 is higher than that of ventricular weight (Novák *et al.* 2006). These periods are considered to be the most critical and stressful periods of rat postnatal life (Olivetti *et al.* 1980). The first one is characterized by an abrupt increase of cardiac workload and concomitant transition from predominantly anaerobic fetal metabolism of carbohydrates to aerobic



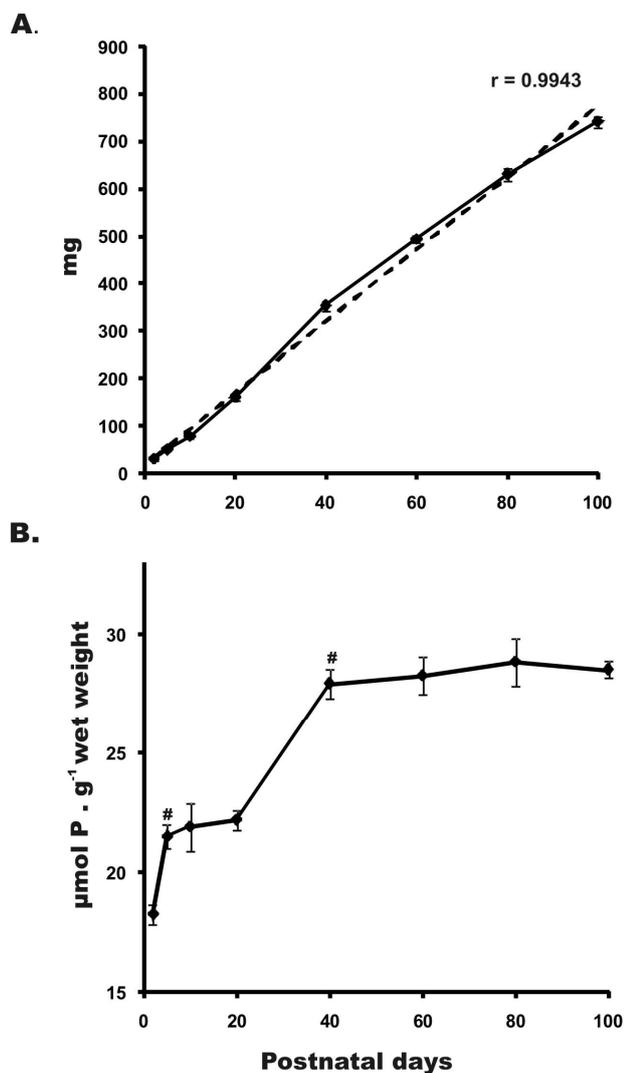
**Fig. 1.** Simplified scheme of phospholipid *de novo* synthesis and remodeling pathways. **A.** G3P (glycerol-3-phosphate), GPAT (acyl-CoA:glycerol-3-phosphate acyltransferase), LPA (lysophosphatidic acid), LPAAT (acyl-CoA:monoacylglycerol-3-phosphate acyltransferase), PA (phosphatidic acid), CTP (cytidinetriphosphate), CDP-DAG (cytidinediphosphate-diacylglycerol), DAG (diacylglycerol), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PI (phosphatidylinositol), PGp (phosphatidylglycerol phosphate), PS (phosphatidylserine), PG (phosphatidylglycerol), CL (cardiolipin, diphosphatidylglycerol). **B.** PC (phosphatidylcholine), LPCAT (lysophosphatidylcholine acyltransferase), PLA<sub>2</sub> (phospholipase A<sub>2</sub>), LPC (lysophosphatidylcholine), FA (fatty acid). **C.** LPLx (lysophospholipid x), PLx (phospholipid x), PLY (phospholipid y), LPLY (lysophospholipid y), TA (Acyl-CoA independent transacylase).

metabolism of FA. The second period includes nutritional transition from suckling of mother's milk to solid food intake and initiation of circadian rhythms (Babický *et al.* 1973, Smolich 1995).

Analysis of individual PL species demonstrates that mainly PC and PE are responsible for this uneven rise of total PL concentration during early postnatal development, since these two major PL account for almost 80 % of total PL in the rat heart (Novák *et al.* 2006). Other studies (Gudmundsdottir and Gudbjarnason 1983, Kumar and Chaudhuri 1993) also reported an increase in the amount of PC and PE in the developing rat heart but, due to different developmental stages analyzed, they did not observe these critical periods. PE is thought to play a crucial role during cardiomyocyte proliferation; there is evidence that this amino-PL plays a pivotal role in the cytokinetic process (Emoto and Umeda 2000). PE resides in the inner leaflet of the majority of eukaryotic plasma membranes (Zachowski 1993) including rat cardiomyocytes (Post *et al.* 1988). It is probably localized in PE-rich domains (Emoto and Umeda 2000) that tend to form a non-bilayer hexagonal structure, which has been shown to regulate various membrane-bound enzymes, such as phospholipase D (Nakamura *et al.* 1996) and protein kinase C (PKC) (Bazzi *et al.* 1992). On the other hand, PC is more disposed to maintain the lamellar

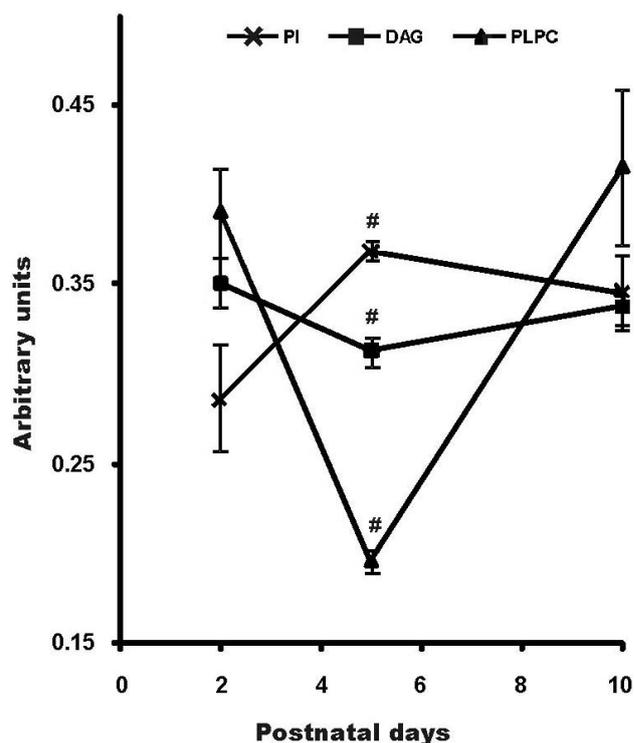
organization of membranes due to its large polar head group, which provides lateral pressure equilibrium between the headgroup and acyl chain level (Goni and Alonso 1999). Besides, PC plays an important role in PKC signaling (Lamers *et al.* 1992, Slater *et al.* 1996).

PC and PE occur not only as diacylglycerophospholipids but their *sn*-1 vinyl-ether-linked analogues, plasmalogens, are present in relatively high amounts in the rat myocardium as well (Novák *et al.* 2006, Post *et al.* 1988). Plasmalogens are less polar than their ester analogues because of the absence of the ester carbonyl dipole. This fact results in the changes of molecular conformation and consequently in different physicochemical properties of the lipid bilayer (Han and Gross 1990). Plasmalogen content in cardiac membranes differs among mammalian species. For example, the amount of choline plasmalogen (PLPC) is high in human, rabbit, dog and guinea pig myocardium (up to 20-40 % of PC), whereas in rat, mouse and hamster it comprises only 3-8 % of PC (van der Vusse *et al.* 1992). Ethanolamine plasmalogen (PLPE) concentration is approximately 5-fold higher than that of PLPC in the rat heart (Hack and Helmy 1988, Novák *et al.* 2006). In the course of postnatal development, the PLPC proportion drops transiently to a minimum by d5, grows again by d10, and then does not change till adulthood (Fig. 3). PLPE



**Fig. 2. A.** Weights of rat left ventricle (mg wet weight). **B.** Concentration of total phospholipids in rat left ventricle on postnatal days 2, 5, 10, 20, 40, 60, 80 and 100. Values are means  $\pm$  S.E.M. from 5 experiments in each group. # $p < 0.05$ , significant difference vs. previous stage (adapted from Novák *et al.* 2006).

proportion falls within the suckling-to-weaning transition (Novák *et al.* 2006). The dramatic changes in cardiac PLPC and PLPE proportion during early rat ontogeny might be related to the postnatal changes in intracellular  $Ca^{2+}$  transients that also take place in the first postnatal weeks (Escobar *et al.* 2004). This view is supported by a preferential distribution of plasmalogens in the inner leaflet of the sarcolemma (Gross 1984, Post *et al.* 1988) and the sarcoplasmic reticulum and by their propensity for inverted hexagonal phase formation (Glaser and Gross 1994), which are indications of their participation in ion transport (Chen and Gross 1994). In line with this, Ford and Hale (1996) showed that plasmalogens provide a critical lipid environment for the regulation of the trans-



**Fig. 3.** Developmental changes in the concentration of PI (phosphatidylinositol), DAG (diacylglycerol) and PLPC (choline plasmalogen). Values are expressed as arbitrary units (sum of concentrations measured on postnatal days 2, 5 and 10 is equal to 1). Values are means  $\pm$  S.E.M. from 5 experiments in each group. # $p < 0.05$ , significant difference vs. 2-day-old rats (adapted from Novák *et al.* 2006).

sarcolemmal sodium-calcium exchanger. Plasmalogens also play an important role as a source of second messengers: ether-linked diglycerides as effective activators of PKC (Ford and Gross 1990), and arachidonic acid (20:4n-6) (Glaser and Gross 1994), the precursor of eicosanoids and a potent signaling molecule (Lokuta *et al.* 1994).

The concentration of CL, a marker of the inner mitochondrial membrane, increases markedly by d40 (Novák *et al.* 2006). This almost 3-fold increase suggests that mitochondrial membranes are predominant structures accounting for the growth of total PL concentration during the first five weeks of rat postnatal life. It has been shown that the interval between d1 and d4 is associated with the rapid and large accumulation of mitochondria and myofibrils in rabbit left ventricular (LV) myocardium. Mitochondria are packed more densely with cristae and the area of the mitochondrial inner membrane per unit of myofibrillar volume increases progressively throughout the perinatal period (Smith and Page 1977). In adult rat heart, the area of the mitochondrial inner membrane per unit cell volume exceeds that of the

sarcoplasmic reticulum by 16-fold and that of the sarcolemma by about 41-fold in LV myocytes (Page 1978). The rapid increase in CL amount in the neonatal rat heart has been confirmed by incorporation studies using radioactive precursors. The rate of CL biosynthesis is 4.5-fold higher in mitochondria isolated from neonatal rat hearts than in those from adults (Stuhne-Sekalec *et al.* 1990). CL is known to be associated with several inner mitochondrial membrane proteins (Hoch 1992), the best characterized being its interaction with cytochrome *c* oxidase. Paradies *et al.* (1997) demonstrated the close linkage between the content of CL in the mitochondrial membrane and cytochrome *c* oxidase activity in adult rat myocardium. In agreement with the rise of CL during early postnatal ontogeny (Novák *et al.* 2006), a 2-fold increase in the content of cytochrome *c* oxidase was found between birth and d30 in mitochondria of the neonatal rat heart (Škárka *et al.* 2003).

Although PI constitutes about 4-6 % of total myocardial PL, this quantitatively minor PL plays an important role in signal transduction as a precursor of phosphatidylinositol-4,5-bisphosphate, which is split by phospholipase C (PLC) to the second messengers inositoltrisphosphate and diacylglycerol (DAG) after G-protein-coupled receptor stimulation (Nishizuka 1992). We observed maximum PI concentration on d5 in rat LV (Novák *et al.* 2006). Together with the increase in PI concentration on postnatal d5, a significant drop occurs in the concentration of myocardial DAG (Fig. 3), PKC activator (Hamplová *et al.* 2005). Thus, these results suggest an inhibition of G-protein/PLC/PKC signaling between d2 and d5, i.e. within the narrow developmental window of the hyperplasia-to-hypertrophy switch of ventricular myocytes (Li *et al.* 1996).

Concerning developmental changes in PS, its concentration tends to rise by d5, then it falls by d10 and does not change thereafter (Novák *et al.* 2006). This minor amino-PL is distributed primarily in the inner leaflet of the plasma membrane (Post *et al.* 1988), where it serves as the cofactor of PKC. Besides this, exposure of PS on the plasma membrane surface in the outer leaflet is one of the earliest events in apoptosis (van den Hoff *et al.* 2000).

The content of sphingomyelin (SM) is about 4 % in newborns and declines to a half in adulthood (Novák *et al.* 2006). This decrease can be explained by the relative increase of mitochondrial membranes rare in SM, which is distributed mainly in the outer monolayer of the sarcolemma (Post *et al.* 1988) and can be concentrated in

lipid membrane domains, rafts and caveolae. Moreover, SM metabolites (ceramides and sphingosine-1-phosphate) play an important role as second messengers in the regulation of cell proliferation, cell-cycle arrest, apoptosis and angiogenesis (Chatterjee *et al.* 2006).

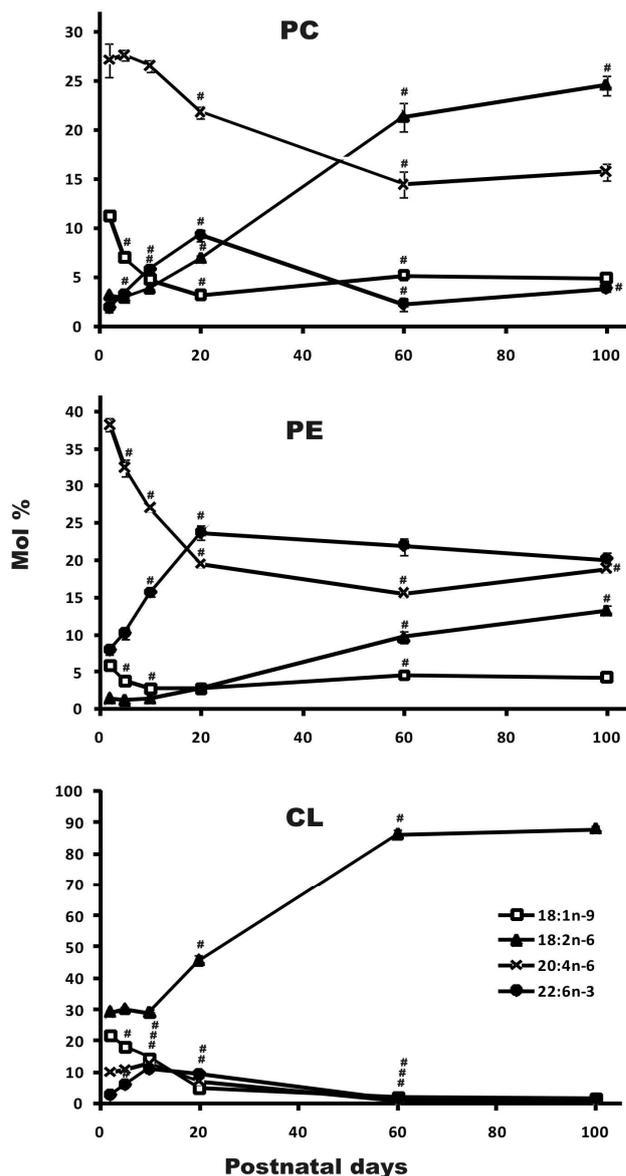
#### *Phospholipid FA composition*

As demonstrated in our developmental study (Novák *et al.* 2006), each of the individual myocardial PL species has a characteristic proportion of FA acyl chains from birth till adulthood that might be related to its specific role in membrane function. On the other hand, FA composition in all PL undergoes similar changes during ontogeny, though their magnitude and time course differ in individual PL. As an example, Fig. 4 shows the time course of changes in the proportion of four main unsaturated FA acyl chains in PC, PE and CL from postnatal d2 till adulthood. Briefly, the proportion of oleic acid (18:1n-9) and arachidonic acid (20:4n-6) is relatively high while linoleic acid (18:2n-6) and docosahexaenoic acid (22:6n-3) is low in all PL shortly after birth as compared with adults. The time course in FA composition indicates that the most dynamic developmental changes in PL occur up to the suckling-to-weaning transition.

Several factors may play a role in developmental changes in the FA profile of cardiac membrane PL, including nutrition, metabolic transition, hormonal changes and increasing workload. Results of dietary studies demonstrate that the FA composition of rat heart and other organs is highly responsive to the intake of n-3 and n-6 long-chain PUFA during postnatal life (Charnock *et al.* 1984, Suarez *et al.* 1996). Additionally, it was shown that reduced protein intake in pregnant rats alters the FA composition of membrane PL in the offspring after weaning (Burdge *et al.* 2003, Tappia *et al.* 2005). At the beginning of postnatal life, rats are fed exclusively by their mother's milk; hence, the FA profile of heart PL in sucklings is substantially influenced by the quality and quantity of milk fat (Berger *et al.* 1992, Huang *et al.* 1992). The composition of milk changes during lactation (Bitman *et al.* 1983, Bitman and Wood 1990) and the FA profile of milk triacylglycerols (TAG) shows marked interspecies differences in the chain length and SFA/PUFA ratio: whereas human milk contains a large proportion of long-chain PUFA (Bitman *et al.* 1983), in other species (cows, rats) the amount of SFA, MUFA and 18:2n-6 predominates (Bitman and Wood 1990, Gudmundsdottir and Gudbjarnason 1983).

However, in our developmental study (Novák *et al.* 2006) as well as in that of others (Gudmundsdottir and Gudbjarnason 1983), a high amount of PUFA incorporated into myocardial PC and PE of suckling rats was observed which is not in line with the FA composition of rat milk. The high content of PUFA in PL suggests the involvement of endogenous sources of FA in myocardial PL turnover during early ontogeny. Considering the limited ability for FA synthesis (Ghosal *et al.* 1969) and desaturation (Brenner 1971) in the myocardium, PUFA in PL must be derived from plasma TAG and/or nonesterified FA originating from shorter chain precursors by the desaturation-elongation process in the liver (Cunnane and Chen 1992b). In line with this view, a marked quantitative increase in long-chain FA in TAG was observed in rat liver during the first postnatal week (Chen and Cunnane 1992, Cunnane and Chen 1992a). During the suckling-weaning transition (Fig. 4), 18:2n-6 starts to increase, probably due to its high content in solid food. In contrast, 20:4n-6 declines gradually in all PL after weaning (Gudmundsdottir and Gudbjarnason 1983, Novák *et al.* 2006). The high proportion of 20:4n-6 in PL of rat myocardium after birth is in line with the results of Decrok *et al.* (2002) who show that the main PUFA in myocardial PL is 20:4n-6 on d2 post-hatching in king penguin, although there is a preponderance of n-3 PUFA in the yolk. The increase in 20:4n-6 and 22:6n-3 proportion in myocardial PL observed just after birth copy closely the alterations in FA composition in cardiac membranes after the administration of catecholamines (Gudbjarnason 1989, Gudbjarnason and Benediktsdottir 1996). We suppose that this membrane modification reflects an early response of the heart to stress connected with the transition from fetal to neonatal life. During adulthood and aging, from the 3<sup>rd</sup> till the 23<sup>rd</sup> month, the level of 18:2n-6 decreases in PC and that of 20:4n-6 increases in PC and PE of the rat heart which coincides with the down-regulation of  $\beta$ -adrenoceptors (Benediktsdottir *et al.* 1995, Gudbjarnason and Benediktsdottir 1996).

The gradual elevation of the 22:6n-3 content in all PL during the suckling period observed in our study (Novák *et al.* 2006) and by others (Ghebremeskel *et al.* 1999, Gudmundsdottir and Gudbjarnason 1983), cannot be explained by nutritional intake alone. The content of this PUFA in mother's milk even decreases during the development of rats (Guesnet *et al.* 1997) and human neonates (Gibson and Kneebone 1981). This process of hormonal maturation during postnatal development offers



**Fig. 4.** Time course (postnatal days 2, 5, 10, 20, 60 and 100) of changes in the proportion of 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid) and 22:6n-3 (docosahexaenoic acid) in PC (phosphatidylcholine), PE (phosphatidylethanolamine), CL (cardiolipin) (adapted from Novák *et al.* 2006). Values are means  $\pm$  S.E.M. from 5 experiments in each group. # $p < 0.05$ , significant difference vs. previous stage.

a more acceptable explanation for the 22:6n-3 increase. The plasma level of thyroid hormones in the rat increases gradually until the third postnatal week (Vigouroux 1976), resembling the time course of the increase in 22:6n-3 in cardiac PL (Fig. 4). Moreover, the increase in 22:6n-3 in the neonatal heart can even accelerate in hyperthyroidism (see further) (Hamplová *et al.* 2003). The developmental rise of 22:6n-3 can be also related to increasing adrenergic responsiveness of the developing

rat heart (Novotný *et al.* 1999). Stimulation of the heart by catecholamines leads to an increase of 22:6n-3 in myocardial PL of adult rats (Benediktsdottir *et al.* 1995, Gudbjarnason and Benediktsdottir 1996). Similar remodeling of myocardial FA composition was observed in response to different stress conditions, such as exposure of immature (Novák *et al.* 2004) or adult rats to chronic hypoxia (Ježková *et al.* 2002) or to systemic pressure overload (Nováková *et al.* 2002) (see further text). Gudbjarnason *et al.* (1978) described the positive correlation between heart rate of mammals, ranging from mice to whales, and 22:6n-3 content in their myocardial PL. Similarly, Hulbert *et al.* (2002) reported a substantial allometric decline in 22:6n-3 content in PL of the heart, skeletal muscle, liver and kidney with increasing body mass. The relationship mentioned above supports the hypothesis that the relative amount of 22:6n-3 can act as a membrane pacemaker for metabolic activity. Although its mode of action is not quite clear, the fundamental role of 22:6n-3 in membranes of many tissues is evident. This unique PUFA is known to readily incorporate into PL and thus significantly alter the basic properties of membranes, including fluidity and permeability. Recently, 22:6n-3 has been proposed to play an important role in the formation of lipid rafts (Shaikh *et al.* 2003) and thereby in a modification of function of proteins for which these regions provide a platform (Wassall *et al.* 2004). Many dietary studies suggest that the replacement of n-6 by n-3 PUFA in membrane PL might have beneficial effects on the heart and reduce the risk of sudden death (Durot *et al.* 1997).

It should be stressed that the FA profile in CL shifts most remarkably among membrane PL during heart ontogeny. Whereas in CL of newborns, besides 18:2n-6 also 18:1n-9 and 20:4n-6 acyl chains are present (Fig. 4), (Novák *et al.* 2006), an 18:2n-6-enriched CL is found in the adult heart, where 18:2n-6 constitutes 80-90 % of CL acyl chains and other chains are quite minor (Hoch 1992). Fig. 4 shows that 18:2n-6 and 20:4n-6 levels do not change during the suckling period and 18:2n-6 steeply raises later, followed by a decline of 20:4n-6. Both acyl chains maintain the constant level in the heart of 2- and 3-month-old rats. Lee *et al.* (2006) demonstrated that the FA composition of CL alters dramatically again with aging: the amount of 18:2n-6 decreases in favor of 20:4n-6 and 22:6n-3 in 24-month-old rats as compared with 4-month-old ones. It is believed that the acyl chain specificity of CL can be attained *via* remodeling, as the enzymes responsible for its *de novo* synthesis are not

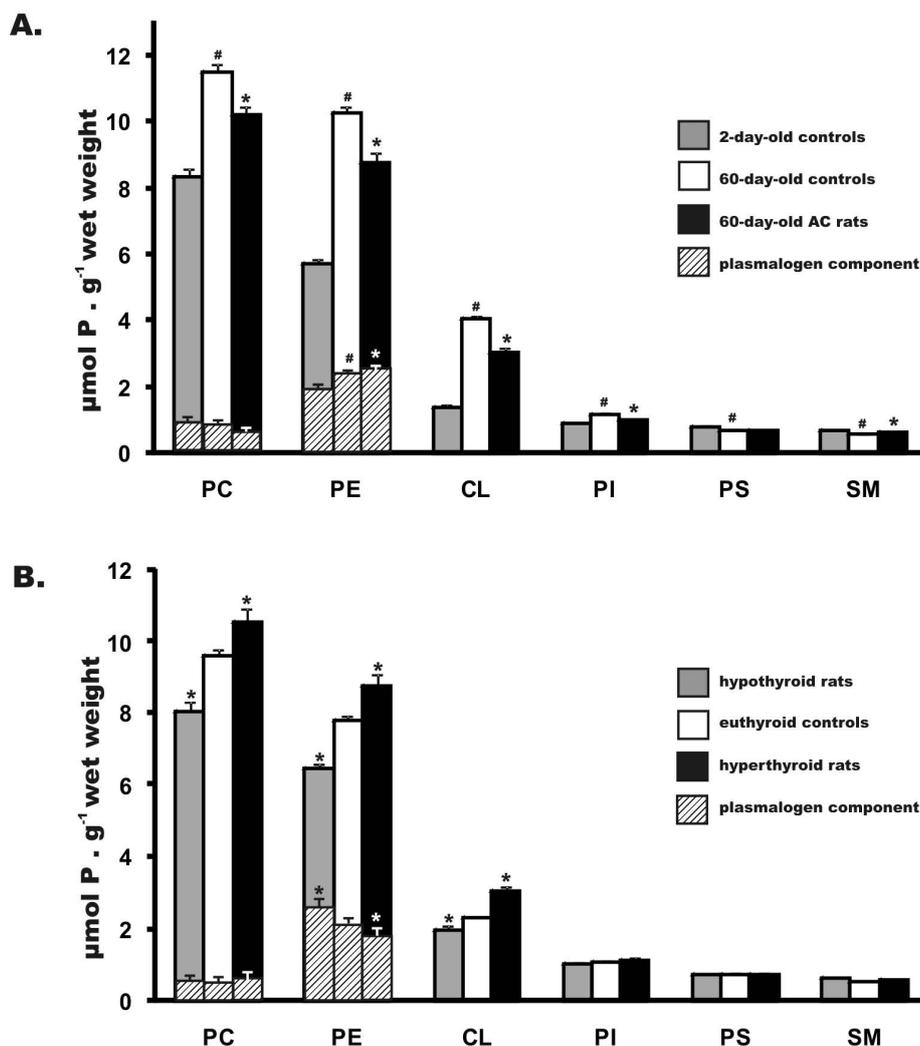
acyl-group specific (Rustow *et al.* 1989). The remodeling is achieved through coordinated biosynthetic and remodeling pathways (Fig. 1) (Hauff and Hatch 2006, Li *et al.* 2007, Schlame and Ren 2006). Tetralinoleoyl-CL is the most abundant of CL species in mammalian heart, its maximum molecular symmetry being crucial for tight interactions of CL with mitochondrial proteins (Schlame *et al.* 2005). In line with this assumption, cytochrome *c* oxidase was stimulated most effectively by CL rich in 18:2n-6 in reconstituted vesicles (Yamaoka-Koseki *et al.* 1991). In agreement with our developmental study on the rat heart, Cheng *et al.* (2008) observed a similar fatty acyl pattern in CL of the rat brain during the first few days after birth. Surprisingly, the low level of 18:2n-6 (about 10 mol%) persisted in the brain CL of adult rats in contrast with the high content of 18:2n-6 in CL of most mammalian tissues including the heart (Novák *et al.* 2006).

### **Effect of excessive workload on postnatal phospholipid remodeling**

#### *Systemic pressure overload*

In various forms of systemic hypertension, LV hypertrophy is considered as an adaptive response aiming to compensate for increased afterload and maintain normal hemodynamic functions. Hypertrophy involves specific qualitative alterations in gene expression as well as in cardiac cell phenotype (Dambrin *et al.* 1994, Ritter and Neyses 2003). Although the initial outcome is a compensatory growth of the heart, the excessive or long-lasting stimulus may lead to congestive heart failure. Myocardial adaptation to increased workload includes structural and functional alterations of cell membranes which is well documented for both protein (Moalic *et al.* 1993, Zheng *et al.* 1996) and lipid components (Mrnka *et al.* 1996, Reibel *et al.* 1986). Numerous results, obtained mostly with isolated cardiomyocytes, indicate that hydrolytic products derived from membrane PL by the action of phospholipases play an important role as second messengers in signal transduction involved in the stimulation of cell growth and proliferation (Dorn and Force 2005, Tappia 2007). In view of these observations, the remodeling of membrane PL induced by pressure overload during early ontogeny may influence the postnatal growth and other physiological characteristics of the developing heart.

In our experiments, we used a model of pressure overload induced in neonatal rats (d2 or d6) by the

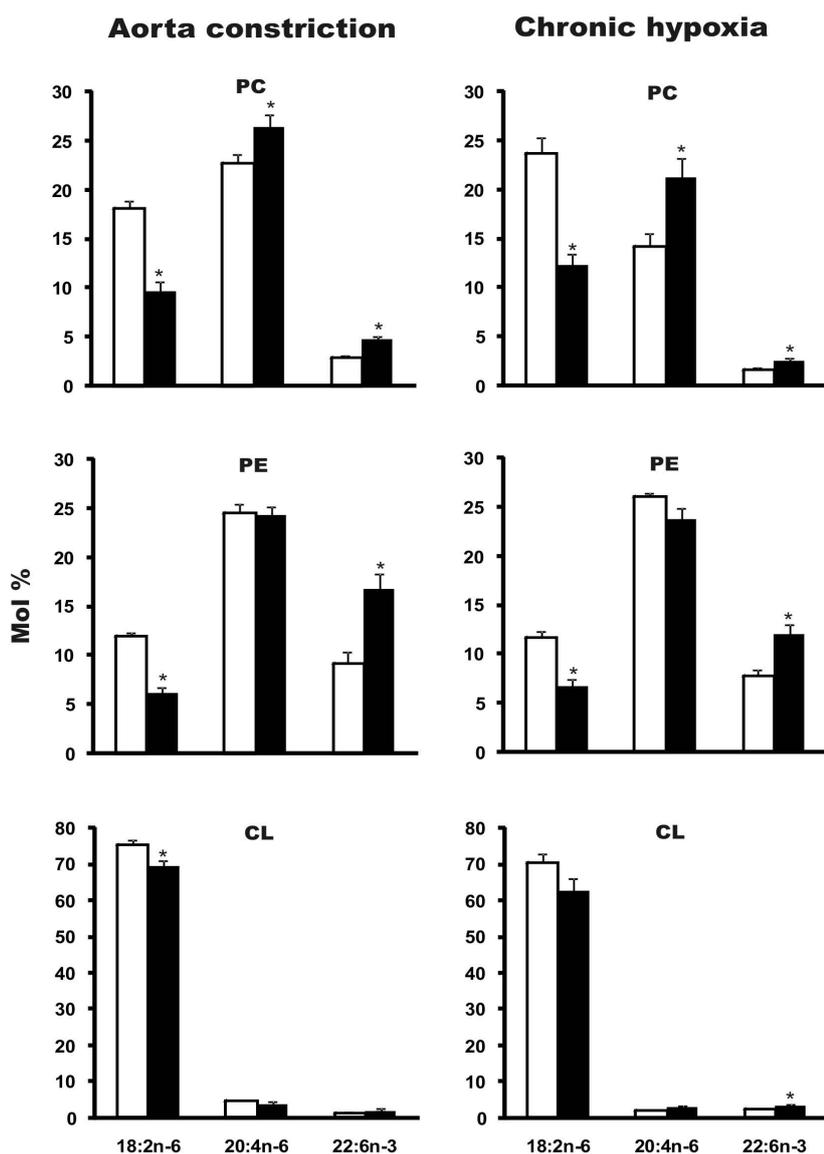


**Fig. 5. A.** Concentration of PC (choline phosphoglycerides), PE (ethanolamine phosphoglycerides), CL (cardiolipin), PI (phosphatidylinositol), PS (phosphatidylserine), SM (sphingomyelin) in left ventricles of 2-day- and 60-day-old controls and 60-day-old aorta-constricted (AC) rats (AC was induced on postnatal day 2). Values are means  $\pm$  S.E.M. from 5 experiments in each group. # $p < 0.05$ , 60-day-old vs. 2-day-old controls; \* $p < 0.05$ , AC vs. 60-day-old controls (adapted from Mrnka *et al.* 1996). **B.** Phospholipid concentration in left ventricles of 21-day-old rats in different thyroid states. Values are means  $\pm$  S.E.M. from 5 experiments in each group. \* $p < 0.05$ , hypothyroid or hyperthyroid rats vs. euthyroid controls (adapted from Hamplová *et al.* 2003).

abdominal aorta constriction (AC) as described earlier (Kolář *et al.* 1998). This model allows adaptive responses of the myocardium which is still in the proliferative phase of the ventricular myocyte growth (Clubb and Bishop 1984, Sedmera *et al.* 2003). AC in neonatal rats leads to a pronounced increase in the LV mass and to a moderate increase in the right ventricle (RV) mass during maturation (LV/BW by 60 %, RV/BW by 20 %), indicating the transition from the compensatory phase of hypertrophy to heart failure. Concentrations of PC, PE, CL and PI are lower in the LV (by 11 %, 14 %, 24 % and 15 %, respectively) of 60-d-old AC rats as compared with age-matched controls (Mrnka *et al.* 1996). The concentrations of SM and PLPE increase (by 10 % and 8 %, respectively) (Fig. 5A). The decrease in the concentration of main PL species (especially CL) reflects the impaired development of membrane structures (predominantly mitochondria) as documented by quantitative electron microscopy in AC hearts (Page and McCallister 1973).

Figure 6 shows changes in the main PUFA profile induced by pressure overload in PC, PE and CL of the LV. In PC, the proportion of 18:2n-6 decreases and it is compensated by the increase of 20:4n-6 and 22:6n-3 PUFA in LV of 60-d-old AC vs. age-matched controls. In PE of AC rats, the proportion of 18:2n-6 decreases, while the proportion of 22:6n-3 increases, compared with the controls. In CL of AC rats, the proportion of 16:0, 18:0 and 18:1n-7 increases (not shown) while 18:2n-6 decreases compared with the controls (Fig. 6). Alterations in PL concentration and FA acyl chain profile in the RV were similar but less pronounced than in the LV. The comparative analysis of 60-day-old intact and age-matched sham-operated controls did not show any difference in the concentration of PL and their FA profiles (not shown).

A similar PL remodeling in pressure overload hypertrophy induced by AC has been well documented in adult rats. The reduction in the proportion of 18:2n-6 and increased accumulation of 22:6n-3 in most of PL are



**Fig. 6.** Changes in proportions of 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid) and 22:6n-3 (docosahexaenoic acid) in PC (phosphatidylcholine), PE (phosphatidylethanolamine) and CL (cardiolipin) in the left ventricle of 60-day-old aorta-constricted rats (unpublished data) and in the right ventricle of 40-day-old chronically hypoxic rats (adapted from Novák *et al.* 2004); controls (white columns), load (black columns). Values are means  $\pm$  S.E.M. from 5 experiments in each group. \*  $p < 0.05$ , significant difference vs. corresponding controls.

nearly identical with those observed in our model of pressure overload induced just after birth. In contrast, the myocardial concentration of CL and proportion of 20:4n-6 in PC in adults are not influenced by AC (Reibel *et al.* 1986). Recently, considerable attention has been paid to CL remodeling in human and experimental heart failure. It has been proposed that pathological remodeling of CL (e.g. loss of tetralinoleoyl-CL and the rise in CL species containing 18:1n-9, 20:4n-6 and 22:6n-3) relates to impaired mitochondrial function and thereby may play a role in the initiation of heart failure (Heyen *et al.* 2002, Sparagna *et al.* 2007). The results presented in Fig. 6A point to two interesting observations: (i) loss of 18:2n-6 in CL is negligible compared to that of PC and PE, and (ii) the decline of 18:2n-6 in PC and PE is compensated by incorporation of 20:4n-6 in PC and 22:6n-3 in PC and PE. Moreover, our results suggest that this phenomenon

occurs both in pressure-overloaded hearts (AC) and in hearts of rats exposed to chronic hypoxia (Fig. 6). Xu *et al.* (2003) demonstrated a CL remodeling pathway in rat liver mitochondria that involves the CoA-independent transfer of 18:2n-6 acyl chains by the transacylation reaction directly from PC or PE to monolyso-CL. This energy-independent transacylation exhibits a clear specificity for the 18:2n-6 acyl chain and was shown to completely convert tetraoleoyl-CL to tetralinoleoyl-CL. These data suggest the possible way how to keep tetralinoleoyl-CL level and symmetry under conditions of decreased energy production in the overloaded heart. Thus, we suppose that PC and PE could serve as the storage of 18:2n-6 for regeneration of fully functional CL in the myocardium subjected to increased load from the early postnatal period. Yamashita *et al.* (1997) observed in the liver a CoA-independent transacylase, which

catalyzes the transfer of C20 and C22 PUFA from diacyl-PL to various lyso-PL. This observation could help to explain the increased accumulation of 20:4n-6 and 22:6n-3 into PC and PE observed under various stress conditions.

#### *Chronic hypoxia*

Chronic myocardial hypoxia is the major pathophysiological feature of various cardiopulmonary diseases, such as chronic obstructive pulmonary disease and cyanotic congenital heart defects. It is also naturally present in fetuses and in individuals living at high altitude (Ošťádal and Kolář 2007). It was shown that the adaptation to chronic hypoxia leads to a variety of morphological, biochemical and functional changes in order to maintain homeostasis with minimum energy expenditure (Ošťádal *et al.* 1999). Chronic hypoxia affects both ventricles that need to adapt to decreased oxygen availability but the RV must in addition cope with increased afterload due to pulmonary hypertension. Pulmonary hypertension, RV hypertrophy and myocardial remodeling are the characteristic features of chronic hypoxia. Besides the potentially adverse influence on the cardiopulmonary system, it is well established that the heart adapted to chronic hypoxia exhibits an increased tolerance to acute ischemic injury manifested as a reduction of myocardial infarct size, improvement of post-ischemic contractile dysfunction and limitation of life-threatening ventricular arrhythmias (Kolář and Ošťádal 2004, Ošťádal and Kolář 2007).

We have shown that, in addition to the remodeling of extracellular matrix, myofibrillar proteins and metabolic enzymes (Ošťádal *et al.* 1995, Pelouch *et al.* 1993, Bass *et al.* 1989), the remodeling of cardiac membrane PL also takes place in the heart of both neonatal (Novák *et al.* 2004, Oka *et al.* 2008) and adult hypoxic rats (Balková *et al.* 2009, Hlaváčková *et al.* 2007, Ježková *et al.* 2002). Rats exposed to intermittent hypobaric hypoxia (7000 m, 8 h/day) from postnatal d4 till d40 exhibit RV hypertrophy (36 %) while LV hypertrophy is less pronounced. The concentration of myocardial PL is unchanged except for a slight but significant decrease in mitochondrial CL in the LV (by 9 %). On the other hand, our study on adult rats adapted to the same hypoxic conditions demonstrates a greater effect on CL, namely in the RV (decrease by 19 %) (Ježková *et al.* 2002). The relative stability of membrane PL concentration in newborn hearts exposed to chronic hypoxia is in good agreement with other studies

indicating higher resistance of newborn rat heart to oxygen deprivation compared with adults (Ošťádal *et al.* 1999). The absence of a decrease in the concentration of major PL despite a significant rise in the heart mass, which was observed in young rats, suggests that hypertrophy induced by intermittent hypoxia is accompanied by a proportional increase in the synthesis of membrane PL. In contrast, the cardiomegaly induced by LV pressure overload in rats during the early postnatal period might be an example of disproportion between the synthesis of myocardial proteins and PL, resulting in a decrease in PL concentration (Mrnka *et al.* 1996).

The adaptation to chronic hypoxia leads to a substantial decrease in 18:2n-6 proportion, which is compensated by an increase in 20:4n-6 and 22:6n-3 acyl chains in both PC and PE. In CL, chronic hypoxia causes an increase in the 22:6n-3 acyl chain proportion (Fig. 6). No left-to-right ventricle difference in FA composition of PC and PE is observed in young rats, in agreement with the previous report on adult rats adapted to the same hypoxic conditions (Ježková *et al.* 2002). It may be suggested that these changes are due to hypoxia itself rather than due to increased RV afterload.

During exposure to hypoxia, numerous metabolic pathways can participate in the remodeling of FA composition in membrane PL; the deacylation-reacylation cycle where PLA<sub>2</sub> and acyltransferases cooperate, desaturation-elongation processes, and PL *de novo* synthesis belong to these pathways. The increase in the 20:4n-6/18:2n-6 ratio might be caused by activation of the desaturation-elongation pathway of 18:2n-6. The presence of both  $\Delta$ -6 desaturase and elongase in cardiac myocytes was reported (Lopez Jimenez *et al.* 1993). In our study (Novák *et al.* 2004), we have shown that the decreased content of 18:2n-6 was compensated by the elevation of 22:6n-3, which is a poorer substrate for PLA<sub>2</sub> than n-6 PUFA (Nalbone *et al.* 1990). Moreover, acyl-CoA synthetase with preferential affinity for 22:6n-3 was found in cardiac tissue (Bouroudian *et al.* 1990). Kawaguchi *et al.* (1991) reported that hypoxia leads to PL breakdown due to the activation of PLA<sub>2</sub>. CoA-independent transacylase with high specificity for C20 and C22 unsaturated acyl chains (mentioned in connection with LV pressure overload) should be also taken into consideration (Yamashita *et al.* 1997). We can speculate that increased oxidative stress could play a role in the process of membrane PL remodeling. Chronic hypoxia is associated with increased myocardial oxidative stress as evidenced by marked lipid

peroxidation (Kolář *et al.* 2007, Yoshikawa *et al.* 1982). It was shown that PLA<sub>2</sub> preferentially hydrolyzes damaged FA acyl chains from PL, thereby allowing their repair by the deacylation-reacylation process and the protection of membranes against further oxidative damage (De Windt *et al.* 1998).

#### Thyroid states

Thyroid hormones (TH) are important players in the control of ontogenetic development of cardiac membrane structures. TH appear in the plasma of rats on prenatal day 18 (Pic and Bouquin 1985) and reach their maximum levels in the third postnatal week (Vigouroux 1976). It has been well documented that hypothyroidism slows down the postnatal maturation of ventricular tissue whereas hyperthyroidism accelerates this process (Dieckman and Solaro 1990, Hoch 1988, Simonides and van Hardeveld 1987). In the immature rat myocardium among others, TH regulate the development of the coronary capillary network (Heron *et al.* 1997), early maturation of cardiac adrenergic signaling (Novotný *et al.* 1999, Whitsett *et al.* 1982), maturation of Ca<sup>2+</sup> handling (Černohorský *et al.* 1998, Kolář *et al.* 1992, Wibo *et al.* 1995) and also the PL remodeling of cardiac membranes (Hamplová *et al.* 2003, Kumar and Chaudhuri 1993, Vasdev *et al.* 1977).

We examined the effect of hypo- and hyperthyroidism on the PL composition in the developing rat heart. The hypothyroid state was induced by propylthiouracil (PTU) in drinking water given to nursing mothers from the postnatal d2 till d21. Hyperthyroidism was produced by daily injection of triiodo-L-thyronine (T<sub>3</sub>) to newborns in the same period of time (Hamplová *et al.* 2003). Hypothyroidism decreases the relative weights of the LV and RV by 22 % and 29 %, respectively, compared with euthyroid animals on postnatal d21. Fig. 5B shows the decrease in the concentration of major PL, suggesting that normal maturation of cardiac membranes is delayed in hypothyroidism (Hamplová *et al.* 2003). The reason for the slowing down of membrane PL maturation in hypothyroid rats may be either the nutrition deficit as hypothyroid mothers are hypolactating (Babický and Nováková 1985, Zeisel *et al.* 1986), or cardiac hypofunction with low heart rate, low velocity of contraction, and last but not least a lack of stimulatory effect of TH on the expression and activity of enzymes involved in PL metabolism (Hoch 1988, Taylor *et al.* 2002).

In contrast, we found an increase in the relative

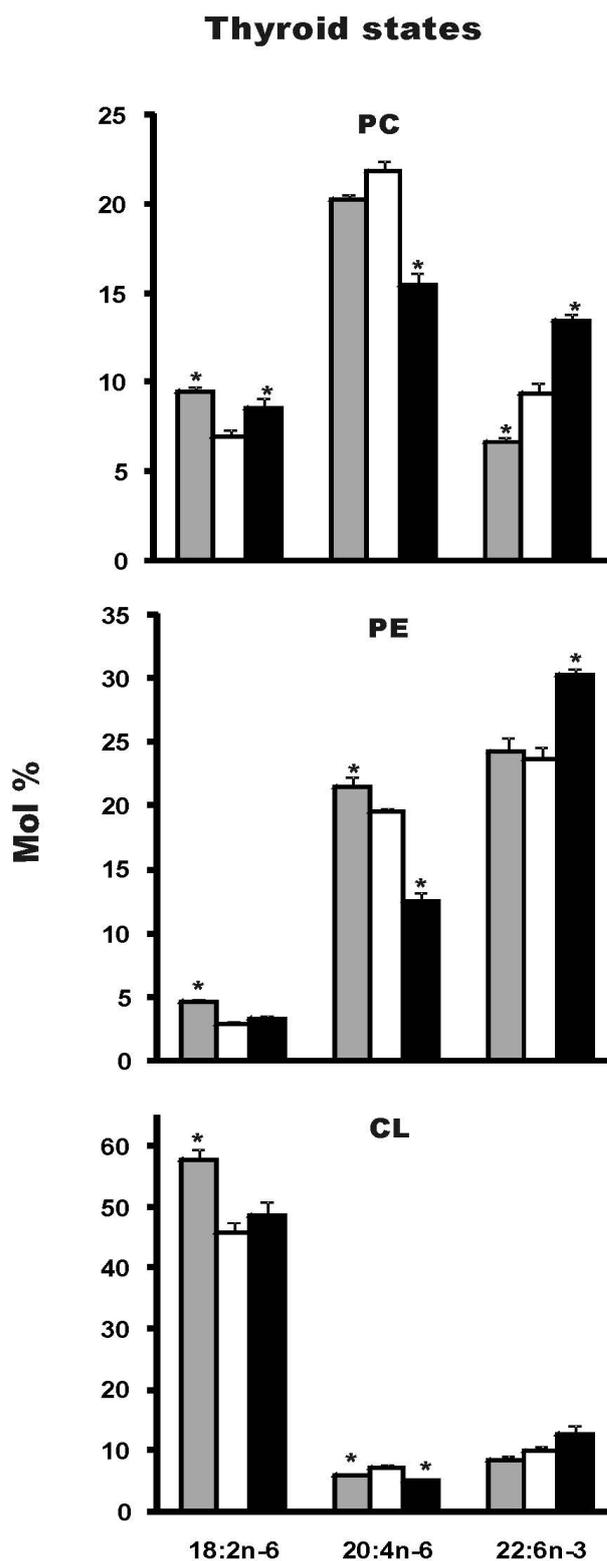
weights of the LV and RV (by 55 % and 80 %, respectively) and an increase in the concentration of major PL in the hyperthyroid rat myocardium on postnatal d21 (Fig. 5B). This is in line with the observation that hyperthyroidism also stimulates biosynthesis of CL and PC in the adult heart (Cao *et al.* 1995, Limas 1980). Moreover, the maturation of ventricular membrane structures is accelerated in hyperthyroid neonatal rats as demonstrated by Jarkovská *et al.* (1994). It has been shown that in hyperthyroid rats the mitochondrial contribution to cell volume increases and the volume of myofibrils remains constant (McCallister and Page 1973) while in euthyroid rats, the contribution of myofibrils and mitochondria to myocardial cell volume increases proportionally during postnatal development (Page *et al.* 1974). In addition, the elevated number and size of mitochondria in the cardiac muscle of hyperthyroid adults in comparison with euthyroid controls was reported (Page and McCallister 1973). Accordingly, we observed a markedly increased relative proportion of CL as a marker of preferential growth of mitochondrial structures in hyperthyroid immature myocardium (Hamplová *et al.* 2003). Both altered thyroid states also change the proportion of PLPE: hypothyroidism increases and hyperthyroidism decreases it, compared with euthyroid controls. Because plasmalogens are predominant PL of cardiac sarcolemma and their content is relatively low in mitochondria (Post *et al.* 1988), the observed differences may suggest that the proportion of mitochondrial to extramitochondrial membranes differs in the two altered thyroid states (Fig. 5B).

In our study (Hamplová *et al.* 2003), both hypo- and hyperthyroidism caused marked changes in FA acyl composition of individual PL in comparison with euthyroid controls (Fig. 7). Hypothyroidism maintains similar PL acyl chain composition in ventricles of 21-day-old rats as in euthyroid rats just after birth (Berger *et al.* 1992, Ghebremeskel *et al.* 1999, Gudmundsdottir and Gudbjarnason 1983), except for 18:2n-6, the content of which is higher in all PL compared with the euthyroid group. Similarly, the content of 18:2n-6 increases in cardiac mitochondria of hypothyroid adult rats (Hoch 1982). A possible explanation of the rise in 18:2n-6 and concomitant decrease in the 20:4n-6/18:2n-6 ratio is the inhibition of  $\Delta$ -6 desaturase activity by the lack of TH. The diminished  $\Delta$ -6 desaturase activity, the rate-limiting step in the conversion of 18:2n-6 to 20:4n-6, was observed in the liver of hypothyroid adult rats (Faas and

Carter 1982). The remodeling of FA composition after PTU treatment led to a decrease of the unsaturation index (UI) in PC. This decrease was caused by a redistribution of high unsaturated FA acyl chains in favor of the less unsaturated ones due to a marked decrease in the proportion of 22:6n-3 as compared with euthyroid controls (Hamplová *et al.* 2003) (Fig. 7). Pehowich (1995) reported a similar decrease of the 22:6n-3 content in cardiac mitochondria of hypothyroid rats. Decreased UI is also observed in cardiac mitochondria from hypothyroid adult rats (Hoch 1982) and in the sarcolemma from hypothyroid adult rabbits (Szymanska *et al.* 1991). In hyperthyroid hearts, a decreased n-6/n-3 PUFA ratio in PC and PE is caused by the decrease in 20:4n-6 and increase in 22:6n-3 contents. Enhanced conversion of 20:4n-6 to prostaglandin E2 may contribute to the lower 20:4n-6 level in PL of hyperthyroid hearts (Gudbjarnason 1975). The changes in the balance between n-6 and n-3 PUFA may have significant biological implications because eicosanoids derived from these two PUFA series exert adverse biological activities (Dimitrow and Jawien 2009).

### Concluding remarks

Table 1 shows the changes in 20:4/18:2, n-6/n-3 ratios and UI in rat heart during postnatal development (d2, d21, d40 and d60) and effects of additional workload (hyperthyroidism, chronic hypoxia and aorta constriction). The gradual decline of 20:4/18:2 between d2 and d40 occurs in PC, PE and CL due to the rise in 18:2n-6 and fall in 20:4n-6 contents. The n-6/n-3 ratio in PC and PE decreases till d21 mainly due to the significant increase in n-3 PUFA proportion. In contrast, the remarkable rise in the n-6/n-3 ratio appears on d40 in all PL, mainly due to the increase in the 18:2n-6 proportion. The value of UI decreases on d40 and does not change thereafter. Hyperthyroidism lowers the 20:4/18:2 ratio because of the increase in 18:2n-6 in all PL. On the other hand, due to the pronounced drop in 18:2n-6, chronic hypoxia and LV pressure overload increase the 20:4/18:2 ratio in PC and PE. Concerning the influence of additional workload, UI increased only in PE of pressure-overloaded (AC) hearts. These results show that the period between postnatal d21 and d40 is characterized by metabolic conversion from suckling-weaning to the adolescence accompanied by nutritional transition from high-fat mother's milk to carbohydrate-rich solid food with a high 18:2n-6 content. In this period, the



**Fig. 7.** Proportion of 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid) and 22:6n-3 (docosahexaenoic acid) in PC (phosphatidylcholine), PE (phosphatidylethanolamine) and CL (cardiolipin) in left ventricles of hypothyroid (grey columns), euthyroid (white columns) and hyperthyroid (black columns) 21-day-old rats (adapted from Hamplová *et al.* 2003). Values are means  $\pm$  S.E.M. from 5 experiments in each group. \*  $p < 0.05$ , hypothyroid or hyperthyroid rats vs. euthyroid controls.

**Table 1.** Effect of increased workload on 20:4/18:2 and n-6/n-3 ratios and unsaturation index in myocardial phospholipids.

	d2	d21	d21-T <sub>3</sub>	d40	d40-CH	d60	d60-AC
<b>PC</b>							
20:4/18:2	8.55±0.37	3.19±0.19 <sup>#</sup>	1.82±0.09 <sup>*</sup>	0.63±0.09 <sup>#</sup>	1.83±0.24 <sup>*</sup>	1.15±0.09	2.75±0.21 <sup>*</sup>
n-6/n-3	10.69±1.30	2.17±0.13 <sup>#</sup>	1.43±0.11 <sup>*</sup>	15.9±2.67 <sup>#</sup>	7.38±1.21 <sup>*</sup>	10.40±0.67 <sup>#</sup>	5.27±0.20 <sup>*</sup>
UI	162.0±11.3	195.4±5.2 <sup>#</sup>	193.3±1.6	135.0±4.5 <sup>#</sup>	153.7±10.2	165.6±3.2	177.6±7.6
<b>PE</b>							
20:4/18:2	27.79±3.66	7.31±0.63 <sup>#</sup>	3.97±0.29 <sup>*</sup>	2.25±0.12 <sup>#</sup>	3.63±0.30 <sup>*</sup>	2.06±0.16	3.98±0.57 <sup>*</sup>
n-6/n-3	4.11±0.39	0.81±0.03 <sup>#</sup>	0.49±0.02 <sup>*</sup>	3.93±0.39 <sup>#</sup>	2.09±0.23 <sup>*</sup>	3.49±0.36	1.66±0.11 <sup>*</sup>
UI	268.3±5.9	268.9±6.2	267.8±5.2	197.5±3.5 <sup>#</sup>	207.1±4.9	200.5±9.2	235.3±9.4 <sup>*</sup>
<b>CL</b>							
20:4/18:2	0.35±0.03	0.16±0.01 <sup>#</sup>	0.10±0.01 <sup>*</sup>	0.03±0.01 <sup>#</sup>	0.05±0.01	0.06±0.01	0.05±0.03
n-6/n-3	7.36±0.35	5.16±0.60	4.25±0.71	25.83±2.76 <sup>#</sup>	16.78±1.72 <sup>*</sup>	48.60±7.05 <sup>#</sup>	31.53±5.96
UI	208.0±4.7	233.1±6.8	238.9±6.4	180.0±4.6 <sup>#</sup>	177.8±5.8	188.1±1.9	181.4±2.6

Values are means ± S.E.M. from 5 experiments in each group. PC (phosphatidylcholine), PE phosphatidylethanolamine), CL (cardiolipin), d – postnatal day, T<sub>3</sub> – hyperthyroid group (left ventricle, d2-d21, adapted from Hamplová et al. 2003), CH – chronic hypoxia (right ventricle, d4-d40, adapted from Novák et al. 2006), AC – aorta constriction (left ventricle, d2-d60, unpublished data), UI – unsaturation index calculated as mol% of individual unsaturated FA multiplied by the number of double bonds. <sup>#</sup> p<0.05 vs. previous stage, \* p<0.05 vs. corresponding age-matched controls.

unsaturation of the membrane lipid bilayer decreases as evidenced by the observed decrease in UI, caused by the fall of 20:4/18:2 and rise in n-6/n-3 ratios in all PL species. The considerably lower polyunsaturation of all PL on d40 may be explained by high proportion of mitochondrial membranes in the adult heart compared to the neonatal one. In agreement, Thesalouhidou *et al.* (2006) have shown that mitochondrial PL from skeletal muscle are significantly less polyunsaturated than those from the whole tissue. Lower UI in adulthood may contribute to higher selective resistance of mitochondrial PL toward oxidative stress.

Our comparative analysis shows that both the physiological increase of the load during normal postnatal

development and additional workload imposed by various interventions early after birth lead to a decrease in the n-6/n-3 ratio, i.e. an increase of n-3 PUFA at the expense of n-6 PUFA in all myocardial PL. This change can be considered as a general adaptive response of cardiac membranes to stress.

### Conflict of Interest

There is no conflict of interest.

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