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Developmental profiles of protective mechanisms of heart against peroxidative injury

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Summary: The developmental profiles of the protective mechanisms of heart against peroxidative injury during neonatal growth was examined in the pigs of three different age groups. Lipid peroxidation expressed in terms of malonaldehyde formation was considerably higher in the pig hearts of the 8–10 day age group compared to that either by newborn or adult age groups. The four principal antioxidative enzymes, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase (G6PD), were enhanced during early neonatal growth and, with the exception of G6PD, all other enzymes were further enhanced during further growth to adulthood. G6PD activity dropped significantly in adult heart. The phospholipid contents of myocardial membrane between newborn and week-old pigs did not vary significantly. Total phospholipids and phosphatidylcholine contents were significantly higher in adult heart compared to those in neonatal heart. The enzymes of phospholipid synthesis and degradation, fatty acyl CoA synthetase (FACS), phospholipase A_2 (PLA_2), lysophospholipase (LPL), and lysophosphatidylcholine acyltransferase (LPCAT) increased during early neonatal growth. During further growth to adulthood, FACS decreased, PLA_2 did not change, whereas both LPL and LPCAT increased significantly. Analysis of free fatty acids showed that palmitic and stearic acids decreased during the first week of growth, but increased during further growth to adulthood. Oleic acid did not change with aging, but arachidonic acid dropped in adult heart compared to that in neonatal heart. Linoleic, palmitoleic and free fatty acids increased dramatically during the first week of neonatal growth, but dropped thereafter. These results suggest that the unusual peroxidative status of the week-old pig heart is related to the presence of high concentrations of polyunsaturated fatty acids in the membrane phospholipids and not with the antioxidative defense system.

Key words: lipid peroxidation, phospholipids, deacylation-reacylation pathway, free fatty acids, unsaturated fatty acids, antioxidative enzymes

Introduction

Recent studies have demonstrated that hearts of adult animals are more susceptible to ischemic and reperfusion injury compared to those of neonates (17, 26). The greater vulnerability of adult hearts to injury has been shown to be due to their inability to maintain anaerobic glycolysis and adenosine triphosphate (ATP) levels during hypoxia compared to neonates (18). For similar heart rates and anoxic insults, the degree of myocardial contracture in the neonates was found to be less than in the adult (17). The severity of contracture in adult heart was shown to be due to the lower ATP and higher calcium concentrations. This observation was also supported by a recent study which demonstrated that myocardial Ca^{++} uptake during reperfusion is greater in adults than in neonates (26).

The influx of Ca^{++} during ischemia and reperfusion is known to cause lipase activation resulting in the breakdown of membrane phospholipids (3, 6, 10). In addition, reperfusion

of ischemic myocardium is associated with the generation of oxygen-derived free radicals (19, 29) which attack polyunsaturated fatty acids and phospholipids, causing lipid peroxidation (9, 24). The loss of membrane phospholipids during ischemia and reperfusion has been shown to be due to the defective reacylation of lysophosphatidylcholine, a deacylated product of the major phospholipid component of myocardial membrane, phosphatidylcholine (3, 10, 34). It seems plausible, therefore, that the developmental changes in the enzymes of deacylation-reacylation pathway of phospholipid synthesis may offer a promising insight into the mechanism of greater vulnerability of adult hearts to reperfusion injury.

In this study, we compared the activities of the enzymes of phosphatidylcholine-lysophosphatidylcholine cycle pathway in the hearts of newborn, one-week-old, and adult pigs. The activities of these enzymes were evaluated in conjunction with the contents of various phospholipids and free fatty acids and antioxidative enzymes of myocardial membrane.

Materials and Methods

Yorkshire pigs of both sexes were used in our experiment. The pigs were divided into three groups. The first group consisted of newborn pigs born within 48 hours, the second group consisted of neonates between 8 and 10 days of postnatal age, and the third group consisted of full-grown two-month-old adult pigs. The animals were tranquilized with ketamine (Ketaject 50 mg/kg, i.m.) and anesthetized with pentobarbital (Nembutal 25 mg/kg, i.v.). They were placed on positive pressure artificial respiration using room air, and the chest was opened with a midline incision through the sternum. Several biopsies from the heart were taken, washed of adhering blood, and weighed immediately before processing of the tissues (wet weight). The tissue biopsies were divided into several groups. The first group was dried to a constant weight to obtain the dry weight of the heart. Another group was homogenized in ice-cold Tris-Sucrose buffer containing 10 mM Tris-HCl, pH 7.4 and 0.32 M Sucrose (1 g tissue/5 ml buffer), pH 7.4 using a Teflon-coated, hand-held tissue homogenizer.

Membrane fractionation was then carried out by differential centrifugation according to the method described by Mukherjee et al. (25) with some modification. The homogenate was centrifuged at 450 g to remove cell debris. The supernatant fluid was then centrifuged at 10,000 g for 20 min to precipitate mitochondria. The precipitate was dissolved in the buffer and recentrifuged. The final precipitate was resuspended in assay buffer. The supernatant from the above step was further centrifuged at 100,000 g for 1 h to settle microsomes. The precipitate was resuspended in buffer and centrifuged between 10,000 g and 100,000 g to obtain a purer form of microsomes. The final microsomal pellet was suspended in the assay buffer. Appropriate marker enzyme monoamine oxidase was assayed as described previously, to judge the purity of the preparation (13).

Assay of enzymes

Phospholipase A_2 activity was determined in the microsomal fraction of heart by incubating 0.3 μmol of phosphatidylcholine (made up to a specific radioactivity of about 10^5 cpm/μmol with phosphatidyl-[^{14}C-choline]) with appropriate amounts of enzyme (50 μg of microsomal protein) in the presence of 10 mM glycylglycine, pH 8.0, 4 mM $CaCl_2$, and 0.5 % cholate in a total volume of 0.5 ml (41). Control experiments performed without microsomal enzyme showed negligible amounts of labeled PC hydrolysis. After incubation at 37 °C for 60 min with shaking, the reaction was terminated by adding 30 μl of 0.2 M EDTA and 2.15 ml of chloroform-methanol (5:8, v/v). Lipids were extracted and phospholipid separated as described below. The spots were visualized under iodine vapor, scraped off into counting vials, and counted for radioactivity incorporated into lysophosphatidylcholine after adding 5 ml of methanol and 10 ml of scintillation fluid. The percentage of phosphatidyl [^{14}C] choline degraded to lysophosphatidyl [^{14}C] choline was calculated, and the phospholipase activity was expressed as nmol of phosphatidylcholine hydrolyzed per min per mg protein.

Lysophosphatidylcholine acyltransferase activity was assayed according to the method described by Okuyama et al. (27) as modified by Chien et al. (3). The assay mixture contained in a final volume of 0.4 ml, 50 nmol of 1-palmitylglycerophosphocholine, 0.05 μCi of [^{14}C]-lysophosphatidylcholine, 40 nmol of palmitoyl CoA, 10 μmol of $MgCl_2$, 50 μmol of Tris-HCl buffer (pH 7.2) and 100 μg of microsomal

protein. After a 10 min incubation period at 37 °C in a shaking water bath, the reaction was stopped with 3 ml of methanol and the lipids extracted as described below. After separation of the phospholipids, phosphatidylcholine and lysophosphatidylcholine fractions were counted for radioactivity.

Fatty acyl CoA synthetase activity was measured by the formation of palmitoyl CoA from isotopic palmitate as described elsewhere (8). The incubation mixture contained in a total volume of 1 ml, 0.2 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mg Triton X-100, 50 mM $MgCl_2$, 20 mM ATP, 200 mM potassium (1-^{14}C) palmitate, 500 µM CoASH and 50 µg of microsomal enzyme protein. Incubations were performed for 10 min at 37 °C and terminated by the addition of 1 ml of Dole's Reagent (isopropanol: n-heptane: 0.5 M H_2SO_4 40:10:1, by vol). Unreacted palmitate was removed by washing with heptane. The lower phase was assayed for radioactivity.

Lysophospholipase was assayed in the microsomal fraction of heart by incubating 50 µg of microsomal protein with 200 nmol of 1-(1-^{14}C) palmitoyl-S_n-glycero-3-phosphocholine (sp. act. 100 dpm/nmol) in a total volume of 0.5 ml with 100 mM phosphate buffer, pH 6.5, at 37 °C for 10 min (37). The reaction was stopped by adding 2.5 ml of a solution containing isopropanol:heptane:1N H_2SO_4 (400:100:10, by vol). The remaining lysophosphatidylcholine was removed by adding 100 mg of silicic acid, and the released fatty acids were extracted with 3 ml of a solution containing heptane and water (1:1, v/v). The heptane layer was counted for radioactivity to estimate released fatty acids.

Antioxidative enzymes were assayed from the heart homogenates. Superoxide dismutase (SOD) was determined by its inhibitory action on the superoxide-dependent reduction of ferricytochrome by xanthine-xanthine oxidase (35). The reaction mixture contained in a total volume of 1.6 ml, 0.02 M phosphate buffer, pH 7.0, 0.1 mM xanthine, 2 µM cytochrome C, and 20 µl homogenate. The reaction was initiated by adding xanthine oxidase, and the rate of cytochrome C reduction was monitored at 417 nm by a spectrophotometer. The assay of GSH-peroxidase was based on the coupling of the enzyme to NADPH via GSH-reductase, and the rate of NADPH oxidation was measured spectrophotometrically at 340 nm (35). The assay mixture contained in a total volume of 1 ml, 0.25 mM reduced glutathione, 0.2 mM cumene hydroperoxide, 0.12 mM NADPH, 1 unit of GSH-reductase, 0.091 mM EDTA, 50 mM Tris-HCl buffer (pH 7.6), and 20 µl homogenate. The specific activity was expressed as µM NADPH oxidized per min per mg protein. GSH-reductase was assayed enzymatically using an assay mixture containing 87.7 mM Tris-HCl buffer, pH 8.0, 0.94 mM EDTA, 4.6 mM oxidized glutathione (GSSG), 0.16 mM NADPH, and 20 µl of homogenate in a total volume of 2.87 ml (31). The change in absorbance was followed at 340 nm at 25 °C. G-6-P-dehydrogenase activity was measured by incubating 20 µl of homogenate in 2.92 ml reaction mixture containing 86 mM triethanolamine buffer, pH 7.6, 0.5 mM NADPH solution, and 0.67 mM G-6-P solution (11). The reaction was monitored at 340 nm at 25 °C.

Assay of fatty acids and phospholipids

The third group of tissue biopsies was used to estimate phospholipids. Lipids were extracted according to the method of Folch et al. (14), as modified by Shaikh and Downar (34). Approximately 100 mg of myocardial tissue was homogenized in a 5 ml solution of ice-cold chloroform-methanol mixture (2:1, vol/vol) containing 0.005 % butylated hydroxy toluene using a Polytron homogenizer (Brinkman, NY). Appropriate amounts of labeled fatty acid and phospholipid were added as internal standards to monitor the recoveries of fatty acids and phospholipids during extraction. Separate standards were provided for each phospholipid class in order to account for the selective loss of different phospholipid classes. The recovery of phospholipids was also verified by extracting the heart tissue in the presence of labeled phospholipids of known radioactivity.

The extracted lipids were separated into neutral and phospholipids by silicic acid column chromatography as described previously (16). The phospholipid fractions were subjected to a one-dimensional thin layer chromatography (TLC) technique as described by Shaikh and Downar (34). TLC was performed on silica gel H plates which were activated by heating at 120 °C for 60 min prior to use. Lipid extracts were quantitatively transferred to the TLC plates, and the chromatograms were developed at room temperature using chloroform-methanol-acetic acid-water (75:25:3:4 v/v/v/v) as developing solvent. Phospholipid fractions were localized by iodine vapor.

Silica gel areas corresponding to various phospholipids were scraped and quantitated by phosphorus estimation as described by Bartlett (1). Free fatty acid (FFA) contents of myocardium were assayed by high pressure liquid chromatography (HPLC) after converting the FFAs into their corresponding

phenacyl ester derivatives using a modification of the method of Wood and Lee (38). A lower concentration of acetonitrile was used initially in order to separate palmitoleic and arachidonic acids (2). 25 µl of phenacyl esters of fatty acids were injected onto an IBM C18 column (250 × 4.5 mm). An initial mobile phase of 58 % acetonitrile was run for 48 min, followed by 66 % acetonitrile for an additional 74 min. A linear gradient raising the acetonitrile concentration from 66 % to 88 % was run over 54 min, followed by 24 min at 88 % acetonitrile. The phenacyl derivatives of fatty acids were identified by comparing with the retention times of known standards.

Assay for lipid peroxidation

The fourth group of biopsies was carefully homogenized in ice-cold Krebs Ringer bicarbonate buffer, pH 7.4, using a Teflon-coated, hand-held homogenizer so that there was no excessive cell damage, and it was then suspended in a suitable amount of the same buffer to give a final protein concentration of 5 mg/ml. Lipid peroxidation was stimulated by incubating the suspending tissue with vigorous shaking at 37 °C for 3 h as described previously (39). At varying time intervals (30 min, 60 min, 120 min, and 180 min), buffer samples were withdrawn to estimate the extent of lipid peroxidation. The lipid peroxidation was measured by monitoring malonaldehyde formation with the thiobarbituric acid (TBA) reaction as described previously (12). Two milliliters of the buffer sample was mixed with 0.25 ml of cold 5N HCl, 0.5 ml of 40 % trichloroacetic acid, and 0.5 ml of 2-thiobarbituric acid (TBA); the mixture was boiled at 100 °C for 10 min and rapidly cooled. The mixture was subjected to centrifugation at 3,000 rpm for 5 min, and the absorbance at 535 nm of the supernatant was spectrophotometrically determined to measure the amount of malonaldehyde formed. Protein was estimated according to Lowry et al., using fat-free bovine serum albumin as standard (22). DNA was estimated as described by Richards (33). Analysis of data was performed by paired t-test or two-sample t-test. All measurements are expressed as the mean values plus or minus the standard error of the mean (mean ± SEM). Results were considered significant when $p < 0.05$.

Results

Tissue water, protein and DNA contents of heart

The results for heart weight, tissue water, and protein and DNA contents as a function of age are shown in Table 1. Heart weights of one-week-old pigs almost doubled compared to those of newborns. The hearts further grew seven times in weight in two months. Water content of heart did not vary significantly between newborn and week-old hearts, but it increased slightly during maturation to adulthood.

Table 1. Comparision of weight, water, protein and DNA contents of heart.

	Neonatal		Adult
	Newborn (0–2 days) (n = 6)	(8–10 days) (n = 6)	2 months (n = 6)
Heart weight (g)	10.63 ± 1.014	21.94 ± 1.057*	145 ± 5.03**
Water content (%) of heart	81.3 ± 0.2	81.8 ± 0.2	82.6 ± 0.3
Heart protein/animal (g)	1.03 ± 0.039	3.05 ± 0.024*	19.22 ± 0.853**
Heart DNA/animal (mg)	25.7 ± 1.165	59.8 ± 4.096*	256 ± 5.112**
Protein/DNA ratio	40 ± 1.3	51 ± 3.8*	75 ± 3.7**

Results are expressed as Mean ± SE of six different animals in each group.
* $p < 0.001$ compared to (0–2) days age group.
** $p < 0.001$ compared to either (0–2) days age group or (8–10) days age group.

Myocardial protein and DNA contents tripled and doubled, respectively, within 8–10 days of development, and enhanced by 6 times and 4 times, respectively, during the next two months of age.

Activities of fatty acyl CoA synthetase, phospholipase A_2, lysophospholipase and lysophosphatidylcholine acyl transferase

All the enzyme activities were measured in the microsomal fraction of the heart. The purity of the microsomal fraction was confirmed by the absence of monoamine oxidase as described in the Methods section.

Hearts of the newborn pigs showed the least amount of enzyme activities amongst the three different age groups. Significant enhancement of fatty acyl CoA synthetase, phospholipase A_2 and lysophospholipase activities was noticed within the first week of neonatal growth (Fig. 1). Lysophosphatidylcholine acyltransferase activity was also increased appreciably after one week of birth, but the difference was not statistically significant. During further development to adulthood, fatty acyl CoA synthetase dropped by 10% and phospholipase A_2 did not increase any further, but both lysophospholipase and lysophosphatidylcholine acyltransferase increased significantly compared to those of neonatal animals.

In order to ascertain the extent of loss of enzyme activities during the course of subcellular fractionations, the relative specific activities of the enzymes vs. percentage of total recovered protein in microsomal fractions were calculated. Enzymatic activities in the total homogenate and total recoveries thereof in microsomal fraction, expressed as percent of homogenate activity, were as follows: Fatty acyl CoA synthetase 42.67, recovery 85%; phospholipase A_2 35.35, recovery 88%; lysophosphatidylcholine acyltransferase 31.67, recovery 91%; lysophospholipase 28.28, recovery 84%.

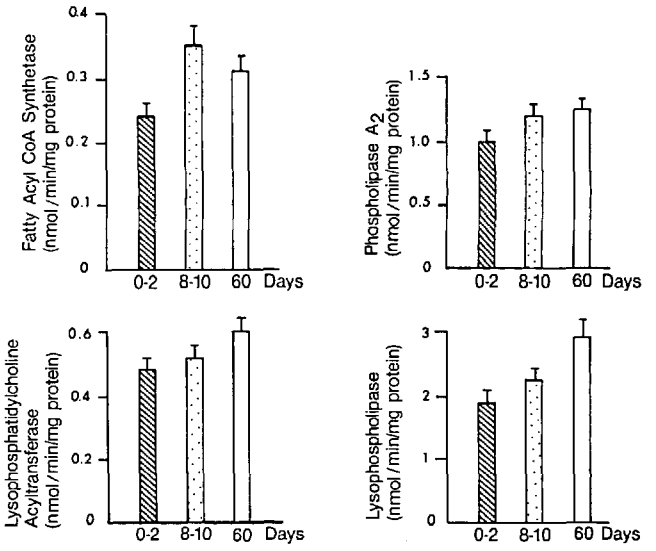

Fig. 1. Activities of fatty acyl CoA synthetase, phospholipase A_2, lysophospholipase, and lysophosphatidylcholine acyl transferase as a function of age. Each bar graph represents Mean ± SE of 6 different pigs in each group.

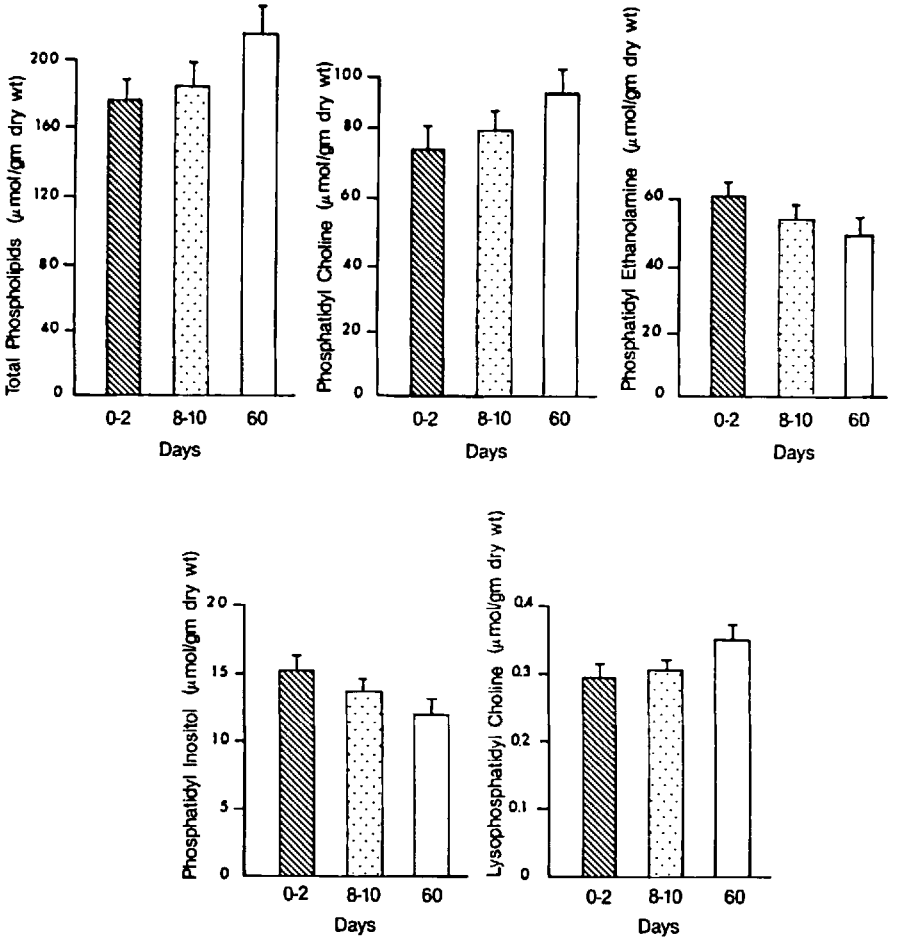

Fig. 2. Membrane phospholipid content as a function of age. Each point represents the mean ± SE of 6 different pigs in each group.

Membrane phospholipids as a function of age

Total as well as major individual phospholipid classes were measured in the heart biopsies obtained from newborn, one-week-old and two-month-old pigs. As mentioned in the Methods section, recoveries of phospholipid fractions were tested with exogenously added labeled phospholipids of known radioactivity. The recoveries of the total phospholipid were 99.0 ± 1.4 %. The recoveries of phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and lysophosphatidylcholine (LPC) were 98.9 ± 1.2 %, 99.2 ± 1.0 %, 97.5 ± 1.5 %, and 96.2 ± 1.7 %, respectively.

Neither the total phospholipids nor the individual phospholipids, PC, PE, PI, and LPC, varied statistically in quantities between newborn and one-week-old age groups (Fig. 2). Significant enhancement in the contents of phospholipids PC and LPC were, however, achieved during further growth to adulthood. Thus, total phospholipids PC and LPC

Table 2. Fatty acid composition of myocardial lipids.

Fatty acid		0–2 days	8–10 days (n mol/g dry heart wt.)	60 days
Laurate	12:0	30.4 ± 2.5	34.5 ± 5.1	28.9 ± 1.9
Myristate	14:0	43.6 ± 8.1	78.7 ± 9.0*	53.0 ± 6.7
Palmitate	16:0	307 ± 16.7	299 ± 23.4	295 ± 25.2
Palmitoleate	16:1	37.5 ± 4.1	52 ± 10.7*	11.5 ± 2.3
Stearate	18:0	331 ± 30.9	346 ± 37.2	395 ± 54.1
Oleate	18:1	160 ± 14.0	183 ± 15.4*	159 ± 16.4
Linoleate	18:2	115 ± 13.2	186 ± 19.6*	131 ± 14.9
Arachidonate	20:4	117 ± 10.2	144 ± 15.7*	64.1 ± 7.6
Total FFA		1310 ± 109	1510 ± 96	1181 ± 121

* $p < 0.05$ compared to 0–2 days age group. Results are expressed as Mean ± SE of 6–7 different animals in each group.

contents of adult heart membrane were about 29 %, 17 %, and 10 %, respectively, higher, whereas PE and PI contents were about 17 % and 15 % lower, respectively, compared to newborn animals.

Free fatty acids as a function of age

Fatty acid analyses were conducted on the total lipid extract from newborn, week-old, and adult pig hearts. Amongst saturated fatty acids identified in the heart, palmitic acid and stearic acid were present in the highest quantity (Table 2). Palmitic acid concentration was decreased during the first week of growth, but increased during further development, such that its concentration in two-month-old pig heart was the same as that in the newborn heart. The stearic acid concentration followed a similar pattern, but its content in the adult heart was significantly higher compared to any other age groups.

Of all the polyunsaturated fatty acids identified in our study, arachidonic and oleic acid were present in the greatest quantity. Oleic acid concentration did not change with aging, but arachidonic acid content dropped significantly in the hearts of adult animals compared to those of newborn and week-old pigs. Linoleic acid increased dramatically during the first week of growth and development and dropped slightly during further growth to adulthood. Palmitoleic acid concentration also increased during the first week of aging, but dropped significantly in the hearts of adult animals. Total free fatty acid (FFA) concentration in the week-old pig heart was higher compared to newborn hearts, but the FFA concentration in adult heart was significantly lower compared to any other age groups.

In vitro lipid peroxidation

Lipid peroxidation in heart homogenates of pigs of varying ages was followed for 3 hours by *in vitro* incubation, and the amounts of thiobarbituric acid (TBA) reactive materials formed were measured as a function of incubation time (Fig. 3). Two-month-old pig hearts showed minimal production of TBA-reactive materials, newborn pigs showed a relatively greater, but not appreciable, amount of lipid peroxidation, whereas 8–10-day-old pig hearts showed generation of massive amounts of TBA-reactive materials. For two-month-old pigs, lipid peroxidation did not change with incubation time, but progressive increase in lipid peroxidation occurred for both groups of neonatal animals.

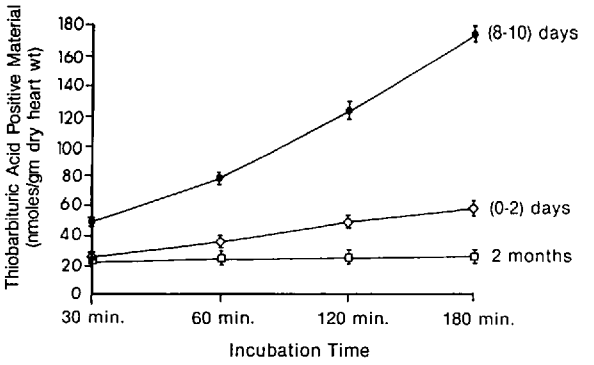

Fig. 3. Thiobarbituric acid reactive material production in pig heart homogenates during development. Each point represents the mean ± SE of 6 different experiments: (◊) – newborn, (●) – week-old, (□) – 2-month-old.

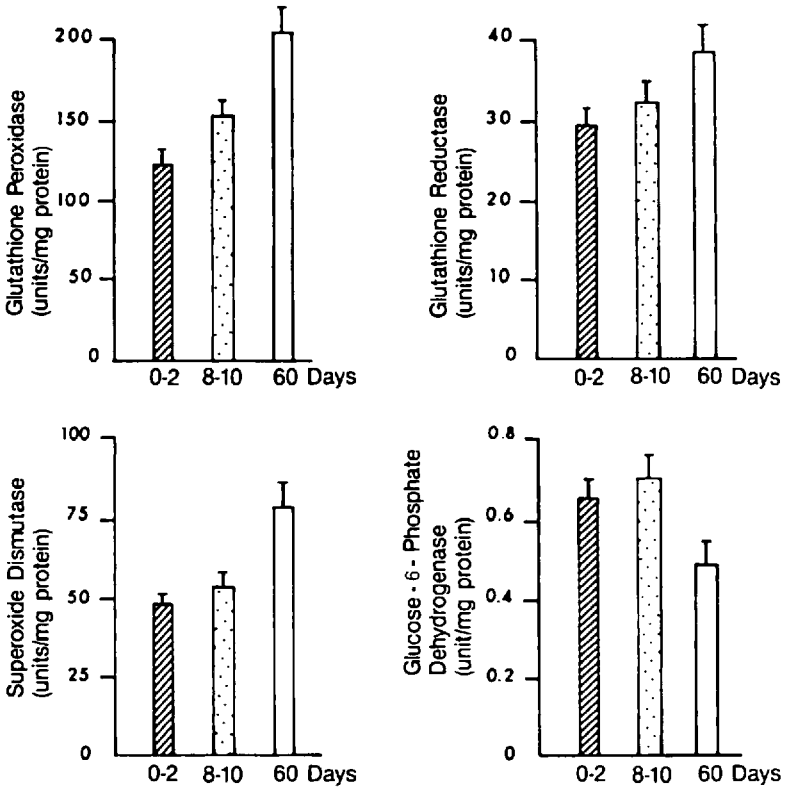

Fig. 4. The activities of glutathione peroxidase, glutathione reductase, superoxide dismutase, and glucose-6-phosphate dehydrogenase as a function of age. Each bar graph represents Mean ± SE of 6 different pigs in each group.

Antioxidative enzymes as a function of age

The activities of the enzymes are shown in Figure 4. All of the enzymes were present in significant amounts in the hearts of newborn pigs. Antioxidative enzymes changed with aging, but the development pattern varied for each enzyme. For example, myocardial SOD activity increased only slightly within one week after the birth, but enhanced by about 40 % during the next two months of growth to adulthood. GSH-peroxidase activity was enhanced by 12 % within the first 10 days of neonatal life, and increased further by 30 % in two-month-old animal heart. GSH-reductase also increased progressively, although by a small amount, within the first two months of growth and development. The activity was stimulated by 5 % within one week after birth, and then by 15 % during further aging to adulthood. G-6-P-dehydrogenase activity in the hearts of 8–10-day-old pigs was 5 % higher compared to those in the 0–2 day-old-animals (Fig. 4). This value dropped significantly to 40 % of the activity after two months of aging.

Discussion

There is increasing evidence to indicate with a high degree of probability that endogenous free radical reactions play a significant role in the degradation of biological systems. Peroxidative cleavage of polyunsaturated lipids studied in a variety of biological tissues, including lung (20, 39), liver (40) and kidney (20) has been associated with the oxidative destruction of membrane lipids. A similar study was never performed in the heart. In this report, we have demonstrated that the lipid peroxidative process in heart is associated with the neonatal rather than adult heart. Furthermore, a unique age-specific myocardial lipid peroxidation was found to occur after incubation of neonatal pig heart homogenates in the absence of any added factors. As measured by the formation of malondialdehyde, lipid peroxidation was minimum in two-month-old adult pig heart. Newborn pigs within 48 hours of birth also showed very little lipid peroxidation initially, but in contrast to adult heart, malondialdehyde formation increased with incubation time. Striking results were obtained with the hearts from week-old pigs. Not only was the initial lipid peroxidative product higher compared to newborn and adult animals, but it increased dramatically with the duration of incubation. After 3 hours of incubation, more than three times the amount of malondialdehyde was formed compared to any other age group. Similar results were previously reported for lungs (20, 39). Lipid peroxidation was not detected in rat lung homogenates prepared from animals immediately after birth, but appeared by the second day, reached a maximum at 5 days of age, and gradually disappeared by 20 to 21 days after birth. It has been hypothesized that a soluble inhibitor may be present in the lung, kidney and liver cells of older animals, inhibiting the lipid peroxidation process (20). Such a soluble inhibitor may also be present in the hearts of adult animals.

It is generally believed that a complex of inhibitors are normally present in the whole intact tissue. Homogenization of tissue apparently causes partial or total destruction of these inhibitors, thus initiating a chain of lipid peroxidation reactions. These inhibitors might include such products as the breakdown of the normal iron binding in tissue or the antioxidant complexes. Since the lipid peroxidizing activity occurred in such age-specific patterns and in association with immaturity, a deficiency of a factor or antioxidative enzyme normally providing protection against oxidative free radical injury in mature animals may result in the observed malondialdehyde formation. Previous results indicate that several antioxidative enzymes including superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase and their co-factors serve to protect the membrane phospholipids from free radical injury by breaking the autocatalytic

chain reactions of lipid peroxidation (4). We, therefore, measured the activities of these antioxidative enzymes in our study. Our results indicate that activities of these enzymes are lower in newborn pig hearts compared to those in week-old hearts. For example, SOD, best known as the scavenger of free radicals, is present in the newborn pig hearts in significant amounts. This enzyme activity is enhanced slightly within 8–10 days of neonatal age, and increased further by 40 % during the later phase of growth and development. A number of studies have confirmed the biologic importance of SOD in the prevention of oxygen-free radical cytotoxicity (32). When the presence of SOD in a tissue is not adequate to prevent the free radicals, the generated toxic radicals attack the unsaturated lipids in the cell causing lipid peroxidation. These toxic lipid peroxides are converted into hydroxy fatty acids, which are then metabolized by β-oxidation pathway (Fig. 5). The enzyme which is responsible for this conversion is glutathione peroxidase. Activity of this enzyme was enhanced by about 12 % during the first week of neonatal development, and increased by 30 % during further growth to adulthood.

Since glutathione (GSH) is essential for the normal function of many cells, it must be regenerated to ensure proper repair and proliferation of myocardial cells. The reducing

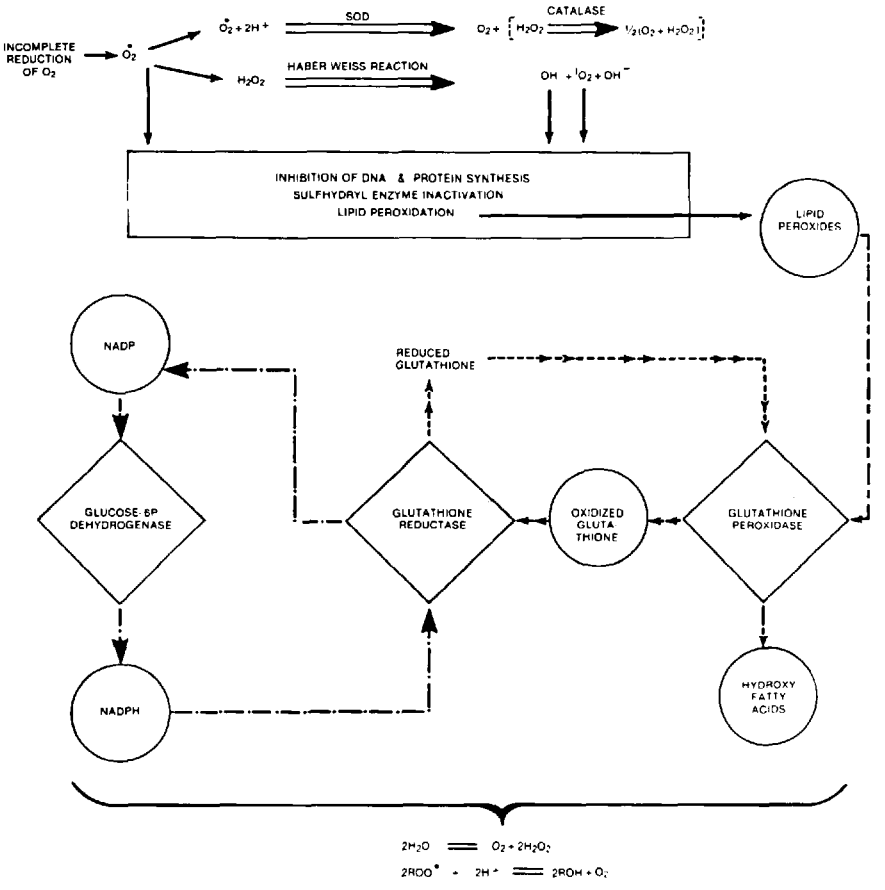

Fig. 5. Scheme of protective mechanism of heart against peroxidative injury.

equivalents used by GSH-peroxidase are regenerated by GSH-reductase, which transfers the reducing equivalents from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to produce intracellular supplies of reduced GSH. Our result indicates a 5 % increase in GSH-reductase activity during the first 10 days of neonatal heart maturation. About a 15 % increase of this enzyme activity was noticed during further development of the heart.

When the supply becomes low, NADPH can be regenerated through the action of pentose phosphate shunt enzyme, G-6-P-dehydrogenase which is abundantly present in heart tissue. It appears from our study that neonatal pigs are born with relatively higher activity of this enzyme. A very small increase in activity is noticed in the hearts of 8–10-day old-pigs compared to that of newborns. Less than 70 % of G-6-P-dehydrogenase activity (compared to newborns) is present in the hearts of adult pigs, suggesting that adult pigs do not depend upon the pentose phosphate shunt activities for the maintenance of NADPH levels in heart like the neonatal pigs. The presence of a relatively lower level of this enzyme activity also suggests that adult hearts may be less susceptible to the peroxidative injury since lipid peroxidation in biologic tissue is not only checked by G-6-P-dehydrogenase activity on lipid peroxides, but also lipid peroxidase may actually be prevented by this enzyme as a result of direct scavenging of the highly toxic $OH^{\cdot}$ radicals (23). Indeed, our study showed relatively lower amounts of the malonaldehyde generation when heart homogenates were incubated in buffer for prolonged time periods. In addition to providing the necessary supply of NADPH, G-6-P-dehydrogenase may be a critical ingredient for the biosynthesis of adenine nucleotides and fatty acids essential for the repair of free radical-induced damaged tissue (36). However, although we did not measure malic enzyme and 6-phosphogluconate dehydrogenase activities in heart, it may be equally interesting to know the development profiles of these two enzyme activities since they are also able to regenerate necessary NADPH when the supply becomes low (36).

The results of our study indicate that lipid peroxidative process is significant only in week-old pig hearts. Comparison between newborn and week-old pigs shows that the heart of the week-old pig undergoes a greater degree of lipid peroxidation, even though it has relatively higher levels of antioxidative enzymes, suggesting that peroxidative status is not a function of antioxidative enzymes. Since it is well known that lipid peroxidation requires NADPH for the enzymatic reactions (15), and polyunsaturated fatty acids localized primarily in the microsomal phospholipids are the most likely substrates for the NADPH-catalyzed microsomal lipid peroxidation (30), we examined the enzymes of major pathways for myocardial phospholipid synthesis in the microsomal fraction because alteration in tissue enzyme activity is a more sensitive and specific indicator of tissue damage than any other known biochemical measurements. Our results indicate that the enzymes of deacylation-reacylation pathway of phospholipid metabolism and synthesis are relatively low in the hearts of neonatal animals. Significant enhancement of fatty acyl CoA synthetase, which activates fatty acids; phospholipase A_2, which deacylates phospholipids; and lysophospholipase, which hydrolyze lysophospholipids into free fatty acids are noticed within the first week of neonatal growth. Lysophosphatidylcholine acyltransferase, which reacylates lysophospholipids into phospholipids, is also increased appreciably after one week of birth, but the difference is not statistically significant; this suggests that compared to newborn pig heart, in week-old heart the degradation of membrane phospholipids may be greater than the de novo synthesis resulting in the presence of higher amounts of free fatty acids. However, neither the total phospholipids, nor the individual phospholipids such as PC, PE, PI and LPC varied in quantities between the hearts of newborn and week-old animals. Significant enhancement in the contents of total phospholipids and PC is achieved during further growth to adulthood. In concert, lysophosphatidylcholine acyltransferase, but not phos-

pholipase A_2, increased in the hearts of adult animals compared to neonates. Lysophospholipase activity is also increased in adult pig hearts, suggesting that not only the reacylation but the hydrolysis of lysophospholipids also is higher in adult animal hearts compared to those in neonatal hearts. These results also suggest that, similar to adult heart (3, 10, 34), a deacylation-reacylation cycle of phospholipid synthesis exists in neonatal hearts.

Since lipid peroxidation occurs by the oxidation of polyunsaturated fatty acids of microsomal phospholipids, we determined the composition of fatty acids isolated from the hearts of three different age groups. Compared to neonatal pig heart, week-old heart has more unsaturated fatty acids as indicated by the presence of significantly higher amounts of linoleic acid and palmitoleic acids. The total amount of FFA is also higher in the heart of this group of animals. Although it cannot be supported from our study, it is likely that the amount of linoleic and palmitoleic acids into the phospholipid or triglyceride pool of membrane lipids is relatively higher in the week-old pig heart than those in the newborn heart. In adult heart, FFA as well as unsaturated fatty acids, particularly arachidonic and palmitoleic acid, were present in significantly lower amounts compared to neonatal heart. The results suggest that increase in lipid peroxidation in the hearts of week-old animals may be due to an increase in polyunsaturated fatty acid content since the less unsaturated species are less susceptible to lipid peroxidation and will not decompose to form malondialdehyde (7). The presence of relatively lower amounts of FFA, especially arachidonic acid, may explain why negligible amounts of lipid peroxidation occur in the heart of adult animals, because arachidonic acid is the most preferred substrate for malondialdehyde formation via cyclo-oxygenase pathway (28).

Even though newborns are particularly susceptible to the lipid peroxidative process and this process is further stimulated during the initial phase of growth and development, the peroxidation process does not appear to have any toxic effect on the cells. Our study shows that deoxyribonucleic acid (DNA) content of normal pig heart increases after birth until the animal matures. Protein synthesis follows a similar pattern. Recent work suggested that superoxide anions alter the structure of DNA (21). More recently is has been shown that oxygen radicals actually inhibit DNA synthesis from thymidine due to the inhibition of thymidine kinase activity (5). These investigators also noticed stimulation of DNA-polymerase activity, an enzyme involved in DNA repair, further suggesting oxygen radical-mediated DNA injury. Our study, however, did not indicate any injury to DNA. Although superoxide anions can affect the DNA growth, the possibility of such interactions can only depend upon the probability of free radical generation in the nucleus near the DNA molecules and the absence of adequate SOD in the immediate vicinity. Studies characterizing myocardial DNA biosynthesis as a function of aging are extremely limited. To the authors' knowledge, no work has been performed regarding the effects of free radicals on protein and DNA content of heart during growth and development.

The findings of this study may be of great significance in those pathophysiological conditions of heart where reperfusion or reoxygenation of ischemic myocardium is essential. Recent studies demonstrated that adults are more susceptible to the ischemic injury than neonates (17, 18, 26). Our results indicate that some factor or factors other than antioxidative defense system or the peroxidative status of heart are responsible for greater reperfusion injury as observed by Jarmakani and his coworkers (17, 18). The antioxidative defense system against the peroxidative injury appears to be the strongest for adult heart and weaker for the week-old hearts. The results of this study suggest that the ability of the neonatal or adult pig heart to tolerate free-radical mediated myocardial injury is governed by a delicate balance between the peroxidative status, membrane integrity regulated by phospholipid and free fatty acids, and myocardial defense system controlled by various antioxidative enzymes.

The result further suggests that an *in vivo* enzymatic pathway exists in the heart for reducing lipid peroxides (Fig. 5). The ability of the heart to offset this challenge is determined not only by the total activities of myocardial antioxidative enzymes, but also by the capability of the heart to increase its antioxidant enzymes in response to an injury during reperfusion and the proximity of these protective systems to the site of generation of cytotoxic agents. Although the physiologic stimulus for the postnatal development profiles in antioxidant defense capacity is not established from our study, at least one explanation can be speculated. The marked increase in metabolic activity and increasing total oxygen consumption of growing neonates could provide sufficient increase in oxygen-derived free radical production to stimulate modulation of antioxidative status of the heart during growth and development. Our observations on the unique profile of the peroxidative status of the heart during growth and development support this hypothesis. Such modification in the peroxidative status of the heart during aging is likely to contribute to the modulation in the contents of membrane-bound phospholipids rich in polyunsaturated fatty acids, which could be of great importance in maintaining the integrity and function of myocardial membranes.

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References

1. Bartlett GR (1959) Phosphorus assay in column chromatography. J Biol Chem 234:466–468
2. Borch RF (1975) Separation of long chain fatty acids as phenacyl esters by high pressure liquid chromatography. Anal Chem 47:2437–2439
3. Chien KR, Han A, Sen A, Buja LM, Willerson JT (1984) Accumulation of unesterified arachidonic acid in ischemic canine myocardium. Circ Res 54:313–322
4. Chow CK, Tappel AL (1972) An enzymatic protective mechanism against lipid peroxidation damage to lungs of ozone-exposed rats. Lipids 7:518–524
5. Clement A, Hubscher U, Junod AF (1985) Effects of hyperoxia on DNA synthesis in cultured porcine aortic endothelial cells. J Appl Physiol 59:1110–1116
6. Corr PB, Gross RW, Sobel BE (1984) Amphiphatic metabolites and membrane dysfunction in ischemic myocardium. Circ Res 55:135–154
7. Dahle LK, Hill EG, Holman RT (1962) The thiobarbituric acid reaction and the autoxidation of polyunsaturated fatty acid methyl esters. Arch Biochem Biophys 98:253–261
8. Das DK (1982) Fatty acyl CoA synthetase in rat lung. Purification and properties. Lung 160:207–218
9. Das DK, Engelman RM, Rousou JA, Breyer RH, Otani H, Lemeshow S (1986) Pathophysiology of superoxide radical as potential mediator of reperfusion injury in pig heart. Basic Res Cardiol 81:155–166
10. Das DK, Engelman RM, Rousou JA, Breyer RH, Otani H, Lemeshow S (1986) Role of membrane phospholipids in myocardial injury induced by ischemia and reperfusion. Am J Physiol 251:H71–H79
11. Das DK, Ganguely M (1982) Mechanism of the control of pulmonary and hepatic fatty acid synthesis by the thyroid hormones. Arch Bisochem Biophys 218:142–155
12. Das DK, Neogi A (1984) Effects of superoxide anions on the (Na+K) ATPase system in rat lung. Clin Physiol Biochem 2:32–38
13. Das DK, Steinberg H (1985) Specific binding of serotonin in rat lung. Am J Physiol 248:E58–E63
14. Folch J, Lees M, Sloan-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

15. Girotti AW (1985) Mechanisms of lipid peroxidation. J Free Radical Biol Med 1:87–95
16. Hamilton JG, Comai K (1984) Separation of neutral lipids and free fatty acids by high performance liquid chromatography using low wavelength ultraviolet detection. J Lipid Res 25:1142–1148
17. Jarmakani JM, Nagatomo T, Langer GA (1978) The effect of calcium and high energy phosphate compounds on myocardial contracture in the newborn and adult rabbit. J Mol Cell Cardiol 10:1017–1029
18. Jarmakani JM, Nagatomo T, Nakazawa M, Langer GA (1978) Effect of hypoxia on myocardial high-energy phosphates in the neonatal mammalian heart. Am J Physiol 235:H475–H481
19. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Luechesi BR (1984) Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 54:277–285
20. Kehrer JP, Autor AP (1977) Age dependent lipid peroxidation in neonatal rat lung tissue. Arch Biochem Biophys 181:73–81
21. Little JB, Williams JR (1977) Reactions to Environmental Agents. Handbook of Physiology. Am Physiol Soc, Sect 9, Chapter 8 (Bethesda, MD)
22. Lowry DH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 1983:265–270
23. McCay PB, Gibson DD, Fong KL, Hornbrook KR (1976) Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. Biochem Biophys Acta 431:459–468
24. Meerson FZ, Kagan VE, Kozlov YP, Belkina LM, Arkhipenko YV (1982) The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. Basic Res Cardiol 77:465–485
25. Mukherjee A, Bush LR, McCoy KE, Duke RJ, Hagler H, Buja LM, Willerson JT (1982) Relationship between α-adrenergic receptor numbers and physiological responses during experimental canine myocardial ischemia. Circ Res 50:735–741
26. Nakanashi T, Yound HH, Shimizu T, Nishioka D, Jarmakani JM (1984) The relationship between myocardial enzyme release and Ca^{++} uptake during hypoxia and reoxygenation in the newborn and adult heart. J Mol Cell Cardiol 16:519–532
27. Okuyama H, Yamada K, Ikezawa H (1975) Acceptor concentration effect in the selectivity of acyl Coenzyme A: 1-acylglycerophosphonylcholine acyltransferase system in rat liver. J Biol Chem 250:1710–1713
28. Otani H, Engelman RM, Rousou JA, Breyer RH, Das DK (1986) Enhanced prostaglandin synthesis due to phospholipase breakdown in ischemic-reperfused myocardium. Control of its production by a phospholipase inhibitor or free radical scavengers. J Mol Cell Cardiol 18:953–961
29. Otani H, Engelman RM, Rousou JA, Breyer RH, Lemeshow S, Das DK (1986) Cardiac performance during reperfusion improved by pretreatment with oxygen free-radical scavengers. J Thoracic Cardiovasc Surg 91:290–295
30. Porter NA (1984) Chemistry of lipid peroxidation. Methods Enzymol 105:273–282
31. Ray LE, Prescott JM (1975) Isolation and some characteristics of glutathione reductase from rabbit erythrocytes. Proc Soc Exp Biol Med 148:402–409
32. Reiss U, Dovrat A, Gershon D (1980) Biological and clinical aspects of superoxide and superoxide dismutase. Elsevier/North Holland, New York
33. Richards GM (1964) Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal Biochem 57:369–376
34. Shaikh NA, Downar E (1981) Time course of changes in porcine myocardial phospholipid levels during ischemia. Circ Res 49:316–325
35. Steinberg H, Greenwald RA, Moak SA, Das DK (1983) The effect of oxygen adaptation on oxyradical injury to pulmonary endothelium. Am Rev Resp Dis 128:94–97
36. Tierney DF, Ayers L, Herzog S, Yang J (1973) Pentose pathway and production of reduced nicotinamide adenine dinucleotide phosphate. Am Rev Resp Dis 108:1348–1351
37. Van Den Bosch H, DeJong JGN (1975) Studies on lysophospholipases. iv. The subcellular distribution of two lysolecithin hydrolyzing enzymes in beef liver. Biochim Biophys Acta 398:244–257
38. Wood R, Lee T (1983) High-performance liquid chromatography of fatty acids: quantitative analysis of saturated, monoenoic, polyenoic and geometrical isomers. J Chromatogr 254:237–246

39. Yoshioka T, Shimada T, Sekiba K (1980) Lipid peroxidation and antioxidants in rat lung during development. Biol Neonata 38:161–168
40. Yoshioka T, Utsumi K, Sekiba K (1977) Superoxide dismutase activity and lipid peroxidation of the rat liver during development. Biol Neonata 32:147–153
41. Zahler P, Kramer R (1981) Methods in Enzymology. Academic Press, New York, vol 71, pp 690–698

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