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K-p-Nitrophenylphosphatase activity, Na and K content, Na permeability and membrane lipid composition in rabbit myocardium after cholesterol rich diet

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Summary. The aim of the present study was to investigate the effects of a cholesterol-rich diet on membrane function and lipid composition in rabbit myocardium. The activity and the ouabain sensitivity of the K-p-nitrophenylphosphatase (K-pNPPase), a partial reaction of the Na,K-ATPase, were diminished after a cholesterol/oil or pure cholesterol diet. The content of cholesterol, cholesterol esters and of several classes of phospholipids was enhanced in microsomes. A causal relationship is assumed between cholesterol accumulation and a decrease in membrane fluidity as well as in Na,K-ATPase activity. The intracellular Na content and the Na-Li-exchange rate were higher after the cholesterol diet. The increase in the Na content is supposed to be induced by a lower Na transport and a higher Na permeability. An enhanced Ca flux via the sarcolemma could be the consequence.

The biochemical and physiological functions as well as the physicochemical properties of membranes strongly depend on their lipid and cholesterol content. Variations in the cholesterol content or in the phospholipid composition of the lipid bilayer, caused e.g. by genetic factors, nutritional changes and the aging process, lead to alterations in membrane fluidity and lipid-protein interactions[2,3]. Thereby the permeability of ion channels and the activity of enzymes could also be influenced[4-7]. In this connection the contraction and relaxation of the isolated rabbit papillary muscle are influenced as a consequence of an altered Ca regulation in the heart after a cholesterol rich diet[8].

Na and Ca fluxes through the sarcolemma are functionally coupled[9]. Intracellular Na enrichment is also accompanied by Ca accumulation favoring the development of myocardial necrosis[10]. Therefore we investigated whether a cholesterol rich diet can modify mechanisms which are involved in cellular Na regulation. The K-p-nitrophenylphosphatase activity (K-pNPPase), the Na and K content and the Na-Li-exchange rate of the myocardium were measured after the diet. In microsomes and mitochondria the content of phospholipids and cholesterol was investigated.

Materials and methods. Hearts were obtained from rabbits fed for 12 weeks with a diet of standard pellets supplemented with either 2 g cholesterol or 2 g cholesterol mixed with 20 ml oil. In each test series data obtained from hearts of rabbits fed with standard pellets were used as control. After sacrifice by a blow on the head the heart was quickly removed, rinsed and freed of connective tissue and fat. About 2 g of the ventricular myocardium were frozen rapidly in liquid nitrogen and kept at −20 °C. The homogenization and the determination of the K-pNPPase activity were performed as described by Lamers et al.[11]. On the day of homogenization, the ventricle was weighed, dissected into small pieces and homogenized with 5 volumes of 0.25 M sucrose, 5 mM EGTA, 5 mM Tris-HCl (pH 7.0) in a Virtis 45 homogenizer at half-maximal speed. Homogenization was carried out for 4 periods of 5 sec alternating with 120 sec of cooling. After this, the homogenate was passed through a nylon filter under light pressure. All procedures were done on ice.

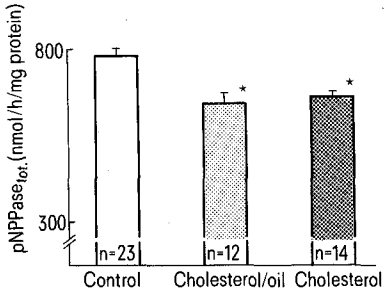

Figure 1. The total K-p-Nitrophenylphosphatase activity (pNPPase$_{tot}$) after the cholesterol and the cholesterol/oil diets; 2 $p \leq 0.05$.

Table 1. The Na and K content and the Na-Li-exchange rate (D) of the myocardium after cholesterol-rich diets

	Cholesterol/ diet (n = 10)	Control (n = 12)	Cholesterol/ oil diet (n = 15)	Control (n = 13)
Na (mmol/kg wet wt)	43.8 ± 4.5	33.5 ± 3.3	35.4 ± 0.1*	32.0 ± 0.4
K (mmol/kg wet wt)	66.7 ± 3.3	68.7 ± 2.8	61.8 ± 1.6	65.0 ± 2.2
K/Na	1.4 ± 0.05*	2.0 ± 0.10	1.8 ± 0.06*	2.1 ± 0.05
D	0.09 ± 0.02*	0.01 ± 0.02	–	–

* $2p \leq 0.05$.

The basic medium for the determination of the enzyme activity contained 100 mM Tris-HCl (pH 7.5 at 37 °C), 4 mM $MgCl_2$, 1 mM Tris-EGTA, 15 mM KCl and 3 mM of p-nitrophenylphosphate (sodium salt). The increase of the p-nitrophenol concentration was measured by spectrophotometry at 400 nm. The ouabain-sensitive part of the activity was calculated by subtracting the activity in the presence of 10^{-3} M ouabain, 4 mM Na^+ and 0.25 mM ATP. The inhibition of the ouabain-sensitive K-pNPPase by 10^{-6} M ouabain was measured, and the percentage of inhibition was calculated. Protein was determined with the Biuret method[12].

The remaining part of the tissue was dissected, weighed, separated into 2 portions, and incubated in 2 different ways with the 5-fold volume of a Na substituted Li-containing buffered solution (140 mM LiCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.3). In the first sample after a 10 min incubation at 4 °C the intracellular Na and K contents were analyzed, because at this temperature only the extracellular ions are washed out[13]. The second sample was incubated for 60 min at 37 °C. The Na-Li-exchange rate was calculated on the basis of an exponential exchange kinetic, from the Na concentrations in the tissue and the supernatant[13]. After drying and treating the tissue with 1 ml of 1 N HNO_3 per g dry weight the Na and K contents in the extract were measured by flame photometry.

The cell organelles were prepared in Tris-sucrose medium. The preparation of the mitochondria was performed as described in[14]. The 100,000 g pellet was regarded as being the microsomal fraction. After lipid extraction[15] the total cholesterol was estimated directly according to[16], modified for thin layer chromatography. The same extract was used for 2-dimensional thin layer chromatography of the phospholipids according to Folch et al.[17]. Phospholipid spots were visualized in I_2 vapor, treated with $HClO_4$, and inorganic phosphorus was determined[18]. Protein was determined by the method of Lowry[19]. The results are given as mean values ± SE compared by the Wilcoxon test and regarded as significantly different when $2p \leq 0.05$.

Results. 1. K-pNPPase activity and ouabain sensitivity. After combined cholesterol/oil and pure cholesterol diet the total K-pNPPase activity was 675.3 and 692.9 nmol/h/mg protein respectively, and was diminished significantly in comparison with the control value of 787.4 nmol/h/mg protein (fig. 1). The lower values of the total activity were the consequence of a decrease of the ouabain-sensitive part, whereas the ouabain-resistent part was unchanged. The ouabain-sensitive K-pNPPase activity amounted to 118.3 and 137.7 nmol/h/mg protein after cholesterol/oil and cholesterol diets respectively, and 202.7 nmol/h/mg protein in the control group (fig. 2). Calculating the percentage of ouabain-sensitive enzyme activity from the total one, possible changes of the total protein content of the homogenate after the diet are excluded. This percentage amounted to 19.7 and 21.0% after cholesterol/oil and cholesterol diets respectively, and was significantly lower than the control value of 26.0%. The percentage of inhibition of the ouabain-sensitive K-pNPPase by 10^{-6} M ouabain was determined as a measure of the ouabain sensitivity of the enzyme (fig. 3). After cholesterol feeding this percentage of inhibition was lower than that of the control, reaching 69.4 and 77.7% respectively. The smaller value of 70.6% after the cholesterol/oil diet did not differ significantly from the control one.

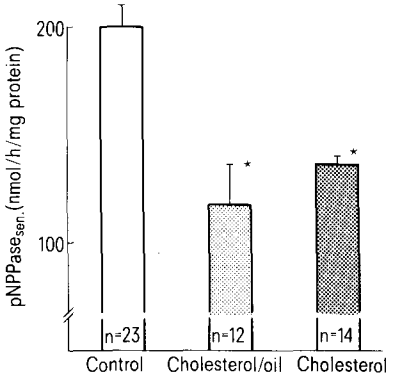

Figure 2. The ouabain-sensitive activity of K-p-nitrophenylphosphatase (pNPPase$_{sen}$) after the cholesterol and the cholesterol/oil diets; $2p \leq 0.05$.

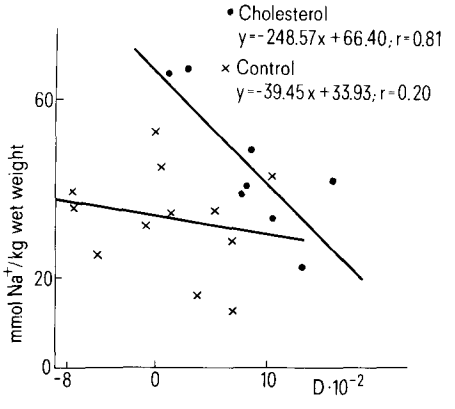

Figure 4. The correlation between the Na-Li-exchange rate (D) and the Na content of the myocardium after the cholesterol diet.

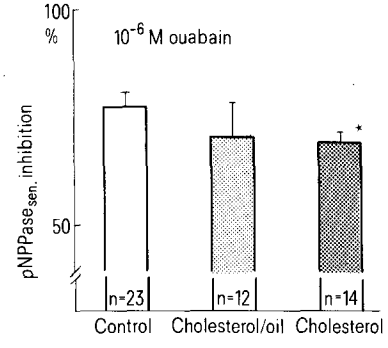

Figure 3. The percentage of inhibition of the ouabain-sensitive K-p-nitrophenylphosphatase activity (pNPPase$_{sen}$) by 10^{-6} M ouabain after the cholesterol and the cholesterol/oil diets; $2p \leq 0.05$.

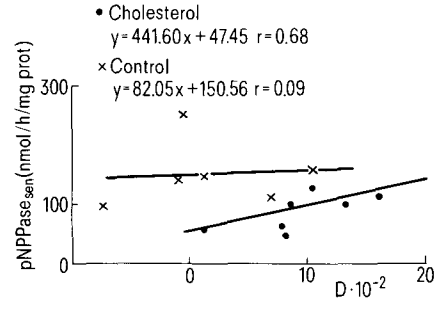

Figure 5. The correlation between the Na-Li-exchange rate (D) and the ouabain-sensitive activity of K-p-nitrophenylphosphatase (pNPPase$_{sen}$) after the cholesterol diet.

2. Na and K content of the myocardium. The cholesterol/oil diet caused a significant intracellular Na enrichment while no change was detected in the K content (table 1). After the pure cholesterol diet the enhanced Na content was not significant, and the K content was similar to the control value. The quotients of intracellular K and Na concentrations were decreased significantly in both cases as a consequence of the higher Na content. The greater Na-Li-exchange rate (D), calculated after the cholesterol diet, means that the Na efflux into the Na-free medium was increased (table 1). A functional relationship can be expected between changes in the Na content and the D after cholesterol diet. A smaller Na content was correlated with a greater D after the diet, while no significant correlation was found in the control (fig. 4). The calculated D includes the passive Na permeability as well as the active Na transport. Therefore the functional relationship between the ouabain-sensitive K-pNPPase activity and D was of interest. After a cholesterol diet these parameters show a significant correlation (fig. 5).

3. Phospholipid and cholesterol composition of heart microsomes and mitochondria. The contents of phospholipid classes, free cholesterol and cholesterol esters are represented in tables 2 and 3. In both fractions the content of free and esterified cholesterol was higher. After the diet phosphatidylcholine, lysophosphatidylcholine and sphingomyelin were enhanced in microsomes, whereas phosphatidylserine was diminished in mitochondria.

Table 2. The lipid composition of the mitochondria of the myocardium after the cholesterol/oil diet

	Control	Cholesterol/ oil diet
Phosphatidylcholine	1.36 ± 0.11 (n = 5)	1.29 ± 0.13 (n = 11)
Phosphatidylethanolamine	0.78 ± 0.09 (n = 5)	0.68 ± 0.07 (n = 11)
Sphingomyelin	0.22 ± 0.01 (n = 5)	0.21 ± 0.02 (n = 11)
Phosphatidylserine	0.09 ± 0.01 (n = 5)	0.05 ± 0.01* (n = 11)
Lysophosphatidylcholine	0.15 ± 0.01 (n = 5)	0.14 ± 0.02 (n = 11)
Lysophosphatidylethanolamine	0.08 ± 0.01 (n = 5)	0.06 ± 0.01 (n = 11)
Cholesterol	9.8 ± 0.9 (n = 12)	17.1 ± 1.2* (n = 6)
Cholesterol esters	1.4 ± 0.2 (n = 11)	10.2 ± 0.9* (n = 4)

Phospholipids: µg/phosphorus/mg protein, cholesterol and cholesterol esters: µg/mg protein; * 2 $p \leq 0.05$.

Table 3. The lipid composition of the microsomes of the myocardium after the cholesterol/oil diet

	Control	Cholesterol/ oil diet
Phosphatidylcholine	2.82 ± 0.08 (n = 5)	3.43 ± 0.21* (n = 10)
Phosphatidylethanolamine	2.08 ± 0.05 (n = 5)	2.25 ± 0.16 (n = 9)
Sphingomyelin	0.34 ± 0.03 (n = 5)	0.44 ± 0.03** (n = 10)
Phosphatidylserine	0.14 ± 0.01 (n = 5)	0.14 ± 0.02 (n = 10)
Lysophosphatidylcholine	0.18 ± 0.01 (n = 5)	0.28 ± 0.03** (n = 10)
Lysophosphatidylethanolamine	0.12 ± 0.02 (n = 5)	0.14 ± 0.02 (n = 10)
Cholesterol	31.9 ± 1.9 (n = 12)	58.8 ± 8.1 (n = 6)
Cholesterol esters	7.2 ± 1.5 (n = 11)	23.6 ± 3.1 (n = 6)

Phospholipids: µg phosphorus/mg protein, cholesterol and cholesterol esters: µg/mg protein; * 2$p \leq 0.10$; ** 2$p \leq 0.05$.

Discussion. The feeding of lipid and cholesterol diets led to a large increase in the plasma content of cholesterol-rich lipoprotein fractions. The plasma level of cholesterol, equilibrating during cholesterol/oil and pure cholesterol feeding, is 51.7–103.4 mM and 22.2 mM respectively, compared with about 1 mM for the control[20]. Considerable attention has been paid to the functional consequence of diet-induced modifications of the membrane lipid composition[2–5,7,8,21–23]. Because of its lipid requirement[6,24] and functional coupling to the Ca regulation in the myocardial cell[25] the Na,K-ATPase activity was investigated. The ouabain-sensitive part of the K-pNPPase represents the rate of dephosphorylation of the Na,K-ATPase[11,26] and is correlated also with the Na transport[27]. To preserve the lipid surroundings of the enzyme protein the activity was measured in the whole homogenate. Only left ventricular tissue was used, since a difference in K-pNPPase activity between right and left ventricle has been described[11]. After both cholesterol/oil and pure cholesterol diets the decrease of ouabain-sensitive K-pNPPase activity points to a dominating effect of the cholesterol component of the diet on the enzyme activity. The decreased sensitivity of the enzyme to 10^{-6} M ouabain after the diet is in agreement with the possible influence of membrane lipids on the inhibitory effect of glycosides[28].

The assumption of an altered heart muscle membrane composition was confirmed by the results concerning the cholesterol and the phospholipid content in microsomes and mitochondria. The striking cholesterol enrichment of the microsomal fraction is expected to be responsible for the measured functional alterations. Different mechanisms for the transfer of cholesterol into the cell and its incorporation into membranes have been discussed[29]. As demonstrated on myocardial and endothelial cells, not only the content, but also the distribution of cholesterol within the plasma membrane are specific[30]. Accumulation of the sterol in a protein-rich region of the membrane causes a decrease in its fluidity. Cholesterol esters, if incorporated into the cytoplasmic membrane, make the membrane more rigid than does free cholesterol[31]. Cholesterol incorporation into an artificial membrane, decreasing its fluidity, inhibits Na,K-ATPase activity[32]. A direct inhibitory effect of the sterol on the enzyme protein must also be considered[33].

The reasons for the alterations in the content of several phospholipid classes induced by the cholesterol/oil diet are not clear. An oil diet modifies the distribution of the phospholipid classes in a way not understood yet[22]. The possibility of an effect of feeding cholesterol only on the distribution of the phospholipid classes was not proved. If the inhibition of the Na,K-ATPase reduces the Na pump capacity also, the cell accumulates Na and losses K. Only a distinct Na enrichment of the myocardial cells was measured after the cholesterol rich diet. This fact could be explained by a higher Na permeability coinciding with a decreased Na transport, which was measured as a higher Na-Li-exchange rate in the myocardium. Contradictionary to our results in isolated rat heart cells cholesterol depletion leads to an increased Na influx[5]. After cholesterol diet changes of the Na content, the Na-Li-exchange rate and the activity of the K-pNPPase correlate with one another. If the Na-Li-exchange rate and the K-pNPPase activity are high, the Na content is relatively low. Thus, the Na enrichment seems to be reduced by a compensatory activation of the Na,K-pump to different extents. This type of adaptive enzyme regulation after a long time of treatment with cardiac glycosides has been described[34]. It is assumed that the modification in intracellular Na is of great importance for the beat-to-beat regulation of Ca within the myocardial cell, furthermore an intracellular Na enrichment is followed by a Ca accumulation[8,25].

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Release of labile cyclo-oxygenase products of arachidonic acid from kidney by endotoxin[1]

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Summary. The possible release of prostaglandin (PG)-like substances was studied in isolated perfused kidneys from intact and from intrarenal endotoxin (Lipopolysaccharide-LPS)-injected rabbits, using the venous outflow superflow superfuse assay organ technique. Injection of LPS into the renal artery of an LPS-pretreated kidney caused a release of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2)-like materials into the venous effluent as verified by the responses of the specific assay organs. No detectable release of these substances was found in the venous outflow of LPS-injected intact kidney. The possible role of labile cyclo-oxygenase products of arachidonic acid in the Shwartzman reaction is discussed.

It has been shown that many hemodynamic events induced by LPS are accompanied by an increased release of PGs from the lung[2]. This observation led to the assumption that PGs might be involved in pathological derangements of the tissues by LPS. The results of a recent study indicate that LPS may cause the release of PGs from the lung in in vivo but not in vitro conditions[3]. It has been shown that kidney contains stable and unstable PGs and this organ can readily metabolize these lipids[4]. The present study was undertaken to investigate the possible action of LPS on the release of PGs from isolated perfused intact and LPS-pretreated rabbit kidney.

Material and methods. The experiments were carried out on adult rabbits of either sex weighing 2.0–3.0 kg. The animals were anesthetized with sodium pentobarbital (35 mg/kg i.v.) and the left kidneys were isolated, perfused with warmed (37 °C) and oxygenated (5% CO_2 in O_2) Krebs' solution as described previously[5]. A group of rabbits were prepared for LPS treatment. For this procedure the animals were anesthetized with sodium pentobarbital and after the anesthesia was established an incision was made on the left flank and the kidney was exposed. LPS (*E. coli*, lipopolysaccharide W.0111:B_4, Difco Laboratories) was injected intracortically in the upper pool of the kidney at the dose of 100 µg in 0.1 ml saline. In a group of animals (4 rabbits) only saline (0.1 ml) was injected intracortically. After that the flank was surgically sutured and the animals were kept in room temperature in separate rabbit cages. They were allowed food freely, and ordinary water. After 24 h the LPS-injected kidneys were isolated, perfused with Krebs' solution. The venous return was continously superfused over a series of assay organs prepared in cascade[6]. The contamination of the venous outflow by urine was eliminated by a polyethylene cannula inserted into the ureter.

Spirally cut rabbit aorta (RA)[7], rat stomach fundus (RSF)[8] and rabbit coeliac artery (CA)[9] were used as assay organ. These assay organs were selected for the separation of TXA_2 and PGI_2-like material in the kidney outflow. TXA_2 consistently produced a contractile response in 3 assay organs[4,9] while PGI_2 a definite relaxation in CA with extremely low concentrations[9]. The initial tension applied was 0.5–1.0 g, and the contractions were recorded on a Grass polygraph (Model 79