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EFFECTS OF HIGH FAT-, CHOLESTEROL-ENRICHED DIET ON THE ANTIOXIDANT DEFENCE MECHANISMS IN THE RABBIT HEART

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In 7 rabbits fed on hyperlipidic diet (0.5% cholesterol, 5% peanut oil and 5% lard) for 4 weeks, the ventricular myocardium was tested for antioxidant defences and thiobarbituric acid reactive substances. Seven age-matched rabbits served as controls. The hearts were previously subjected to 45 min Langendorff perfusion to study coronary flow, developed tension and resting tension; coronary effluent values of CPK activity, pH and UV absorbance at 250 nm (i.e., low molecular weight ATP catabolites) were also investigated. After 4 weeks of diet, a significant rise of plasma cholesterol ($P < 0.0001$) and tryglycerides ($P < 0.0001$) was observed. Total superoxide dismutase, catalase and glutathione transferase activities underwent a significant increase ($P < 0.05$) in the hyperlipidemic animals. On the contrary, a depression of glutathione reductase ($P < 0.01$) and selenium-dependent glutathione peroxidase ($P < 0.01$) activities, associated with decreased levels of non proteic thiol compounds ($P < 0.01$), was assessed. The selenium-independent glutathione peroxidase activity was not detectable in both groups. Thiobarbituric acid reactive substances levels were significantly increased in the hyperlipidemic rabbit myocardium ($P < 0.01$). Even though heart hemodynamics, CPK release and perfusate pH did not differ in control and experimental animals, higher 250 nm absorbance values ($P < 0.05$) were detected in the myocardial effluent of hyperlipidemic rabbits. In conclusion, high fat-, cholesterol-enriched diet induces an imbalance in the rabbit heart antioxidant defences, some of which are increased, whereas others are depressed, eventually resulting in enhanced myocardial lipid peroxidation. These biochemical changes are associated with higher perfusate values of UV absorbance at 250 nm, but not with significant CPK leakage or myocardial hemodynamics derangement.

KEY WORDS: Hyperlipidic diet, rabbit heart, superoxide dismutase, catalase, non proteic thiol compounds, glutathione-related enzymes, free radicals, lipid peroxidation, oxidative stress.

INTRODUCTION

Increased dietary intake of fat and cholesterol has been commonly accepted as favouring the occurrence of myocardial injury through the involvement of coronary arteries by atherosclerotic processes.

However, experimental evidence seems to suggests that high fat diets and hypercholesterolemia may also exert negative heart metabolic effects unrelated to

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atherogenic mechanisms, by altering the myocardial oxidants/antioxidants balance with enhanced susceptibility to oxidative stress.[1] In such a context, cholesterol may induce lipid peroxidation *in vivo*[2,3] and increase the activity of free radical generating cells, such as neutrophils[4,5] and platelets.[6] On the other hand, high fat diets may influence intracellular free radical generation, via increased microsomal P-450 activity,[7] peroxisomes stimulation[8,9] and enhanced activity of the prostaglandin pathway.[10] Moreover, increased availability and oxidation of fatty acids seem to greatly stimulate the mitochondrial oxygen radical production.[11] In this regard, enhanced cell lipid peroxides content has recently been shown after short-term hyperlipidic diets in the rat,[12] and increased breath pentane excretion (an *in vivo* peroxidation index) has been reported after intravenous infusion of a lipid emulsion in the human.[13]

Several lines of antioxidant defences are devoted to protect the cell against free radical attack,[14] which may induce lipid peroxidation and permeability alterations of the biomembranes.[14]

Even though 8 weeks fat-enriched diets have been shown to decrease the selenium-dependent glutathione peroxidase activity in the rat heart,[15] little is known concerning the wide spectrum of enzymatic antioxidant defences and the entity of lipid peroxidation in the mammalian myocardium during short-term hyperlipidic diets, which do not result in significant coronary atherosclerotic narrowing and secondary myocardial ischemia.[16–18]

To experimentally address this issue, rabbits were fed on hyperlipidic, cholesterol-enriched diet for 4 weeks and antioxidant defence mechanisms and thiobarbituric acid reactive substances were tested in the ventricular tissue. Heart hemodynamics and coronary effluent values of CPK activity, pH and UV absorbance at 250 nm (which is related to low molecular weight ATP metabolites) were also studied, in order to establish potential relationships between "oxidant" status and functional aspects of the heart.

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MATERIALS AND METHODS

Male, New Zealand White rabbits, weighing 1.8–2 kg, were divided at random in 2 groups, with 7 rabbits in each group. The experimental group received for 4 weeks a hyperlipidic diet (100 g/day for each rabbit) composed by 89.5% standard rabbit pellets, 5% lard, 5% peanut oil and 0.5% cholesterol.[19] The percentage content of palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) was 24.5%, 44% and 13% in the lard and 10%, 46% and 33% in the peanut oil, respectively. The control group was given for the same period an equal amount of a standard rabbit diet, containing in the lipidic fraction 18% palmitic acid, 27% oleic acid and 40% linoleic acid. The detailed composition of these diets (supplied by S.A.Ge.M. Mangimifici, Roseto degli Abruzzi, Italy) has previously been reported.[19] Body weights were also weekly determined.

Plasma lipid analysis

Blood samples were obtained by ear bleeds before initiation of the assigned diet and just before sacrifice, as previously reported.[19] Total cholesterol and triglycerides were determined using enzymatic commercial procedures (Boehringer Biochemia Robin, Milano, Italy).

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Heart perfusion

After sacrifice by decapitation and midline thoracotomy, the hearts were rapidly mounted on the aortic cannula of a "tandem" Langerdorff perfusion apparatus (perfusion pressure: 80 mmHg). The perfusion buffer, consisting of 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$ and 11 mM glucose (pH: 7.4), was filtered through 5 μm Millipore filters, kept at 37°C and gassed with 95% O_2 and 5% CO_2. In order to avoid heart rate-dependent variability of hemodynamic parameters, the cardiac frequency was kept constant at 180 beats/min by right ventricular pacing (duration 1 ms; strength, 0.5 volts).[20] Resting tension and developed tension were recorded by a force transducer (model 7004, Basile, Italy) tied to the apex of the left ventricle and connected to a multichannel Sensormedics Dynograph R 611 recorder. Hearts were preloaded with a resting tension of 1.5 g.[20] Coronary flow was measured by one minute perfusate collections.[20,21] After a preliminary stabilization period of 20 min, the normothermic perfusion was performed for other 45 min to study hemodynamic parameters and myocardial effluent. The hearts were then perfused with ice-cold buffer, to obtain a rapid hypothermic arrest. Wet ventricular weights were determined after removing atria and great vessels. The organs were then stored in liquid nitrogen until analyses.

CPK activity, pH values and UV absorbance at 250 nm (A_{250}) of the perfusate

The myocardial effluent was collected during 45 min perfusion to determine CPK activity spectrophotometrically (340 nm, 25°C) with a commercial kit (CK NAC-activated, Boehringer Mannheim GmbH, Germany), pH values[21] and UV absorbance at 250 nm,[20,21] which reflects the release of low molecular weight ATP metabolites, mainly hypoxanthine and inosine.[20] CPK release was expressed as mU/min/g wet tissue or mU/ml/g wet tissue, whereas absorbance values at 250 nm were calculated as $A_{250} \times 10^3$/g wet tissue.

Homogenate and supernatant preparation

The ventricular myocardium was homogenized (1:6 w/v) in ice-cold 0.05 M potassium phosphate buffer (pH 7.4), containing 1 mM EDTA, with four 30 s bursts of a commercial homogenator (Ultra-Turrax, Tecmar Company, Cincinnati, OH), allowing 30 s rests between bursts.

Non proteic thiol compounds (NP-SH) were determined in the supernatant obtained after centrifugation at 800 × g for 3 min at 4°C, to remove fibrous material.

Thiobarbituric acid reactive substances (TBARS), a well known index of lipid peroxidation, were determined on the same supernatant, taken to a final concentration of 1:2 (v/v) with 2.3% KCl.[19,23]

Superoxide dismutase (SOD; EC 1.15.1.1.) and Catalase (EC 1.11.1.6.) activities were assayed on the supernatant obtained from a second centrifugation at 2000 × g for 10 min at 4°C. Glutathione reductase (GS-SG Red; EC 1.6.4.2.), Glutathione peroxidase (GSH-Px; EC 1.11.1.9.) and Glutathione transferase (GST; EC 2.5.1.18.) activities were measured on cytosol harvested after further centrifugation at 105000 × g for 60 min in a Beckman ultracentrifuge (model L3-50).

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Biochemical analyses

The total SOD activity was determined at 25°C, using the xanthine oxidase-nitroblue tetrazolium system, as described by Oberley and Spitz.[24] Standard curves were obtained with increasing amounts of bovine erythrocyte SOD (Sigma Chemical Co.). SOD activity was measured by its ability to inhibit 50% of the rate of nitroblue tetrazolium reduction at 560 nm in the assay condition. Each Unit of specific activity (U) represents 1 μg equivalents of bovine erythrocyte SOD.

Catalase activity was assayed on the appropriate supernatant (preincubated with 1% Triton X-100 for 30 min) by following at 25°C the decrease in absorbance at 240 nm due to specific H_2O_2 decomposition by catalase.[25] The reaction mixture (3 ml in quartz cuvettes) contained 50 mM KH_2PO_4 and 50 mM Na_2HPO_4, pH 7.0, 14 mM H_2O_2 (perhydrol, Merck, Germany) and 0.025–0.1 ml of the appropriate supernatant sample. Specific activity was expressed as U/mg supernatant protein. One Unit (U) is the amount of enzyme which decomposes 1 μmole of H_2O_2 for 1 min at 25°C.

NP-SH (of which GSH represents the main biochemical pool) were determined after extraction with 4% sulfosalicylic acid according to the 5,5'-dithiobis-2-nitrobenzoic acid-dependent Ellman's method,[26] as previously described.[19,23] After recording absorbance values at 412 nm with a double beam Varian DMS 200 spectrophotometer, NP-SH values were calculated as nmol NP-SH/g wet tissue (molar extinction coefficient: $13.6 \times 10^3\ M^{-1}\ cm^{-1}$).

For GS-SG Red activity measurement, an appropriate amount of cytosolic solution (0.05–0.1 ml) was added to the assay mixture (1 ml) containing 0.1 M potassium phosphate buffer, pH 7.4, 1 mM GS-SG, 1 mM EDTA, and 0.16 mM NADPH.[19,23] The enzymatic activity was performed measuring the disappearance of NADPH at 340 nm and expressed as U/mg protein. One unit (U) represents 1 nmole of NADPH oxidized/min. A molar extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ was used.

GSH-Px activity was assayed by the Paglia and Valentine's procedure.[27] The assay solution (2 ml) contained 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1.5 mM NaN_3, 1 mM GSH, 0.16 mM NADPH (Sigma Chemical Co.), 4 μg of glutathione reductase (Sigma Chemical Co.) and a suitable sample of enzyme solution (0.025–0.1 ml). After 5 min preincubation, the reaction was started with the addition of 0.25 mM H_2O_2. The value for a blank reaction with the enzyme source replaced by buffer was subtracted for each assay. The rate of reaction was recorded at 37°C, following spectrophotometrically the decrease in absorbance of NADPH at 340 nm. Specific activity was expressed as U/mg protein, each unit (U) representing 1 nmole NADPH oxidized/min.

GST activity was determined by following at 340 nm the rate of conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB, Aldrich Europe, Belgium), according to the Habig's method,[28] with slight modifications as previously reported.[19,23] One specific activity unit (U) was defined as the amount of the enzyme conjugating 1 nmole of substrate/min/mg protein.

The glutathione peroxidase activity of GST (namely, selenium-independent glutathione peroxidase: GST-Px) was investigated by Lawrence and Burk's method,[29] using 1.2 mM cumene hydroperoxide (CHP, Sigma Chemical Co.) as substrate in the same assay system of GSH-Px, but replacing CHP for H_2O_2.[19,23]

TBARS were measured by the modified method of Ohkawa *et al.*[30] An aliquot of the homogenate was added to 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution

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(pH 3.5), 1.5 ml aqueous solution of 0.8% 2-thio-barbituric acid (TBA: Sigma Chemical Co.) and 1 μmole of butylated hydroxytoluene in ethanol as antioxidant. After heating at 95°C for 60 min and cooling, the chromogen was extracted with n-butanol plus pyridine (15:1, v/v). After a brief centrifugation to separate the phases, the absorbance of the organic layer was read at 532 and 520 nm against an appropriate blank. For calculations, the difference between absorbance values at 532 nm and those recorded at 520 nm,[31] and a molar extinction coefficient of 1.54×10^5 M^{-1} cm^{-1} (Ref. 14) were used, expressing the values as nmol TBARS/g wet tissue.

Protein concentrations were determined by Bradford's method,[32] using gamma-globulin as standard.

Statistical analysis

Results were calculated as means ± SD. Unpaired Student's t test was used to compare hemodynamic and biochemical data between the two groups. Plasma lipids values were analysed by the Student's t test for paired data. $P < 0.05$ was regarded as statistically significant.

RESULTS

Plasma lipids

After 4 weeks of diet, plasma cholesterol and triglycerides values rose from 49 ± 10 and 63.8 ± 14.6 mg/dl to 568.3 ± 134 ($P < 0.0001$) and 365.8 ± 157.2 mg/ml ($P < 0.0001$), respectively.

Body and wet ventricular weight

At the end of the experimental time, body weight and wet ventricular weight did not differ in control and fat-fed rabbits (2.3 ± 0.21 vs 2.53 ± 0.24 kg, and 4.69 ± 0.31 vs 4.98 ± 0.43 g, respectively; P = NS).

Hemodynamic parameters

After 45 min perfusion, developed tension values were 4.15 ± 0.45 and 4.05 ± 0.3 g in control and fat-fed rabbits, respectively (P = NS), whereas resting tension remained at "zero" value in both groups throughout the experiment. Although coronary flow levels were higher in hyperlipidemic animals at the end of perfusion, the values did not reach the limit of statistical significance (4.65 ± 0.7 vs 4 ± 0.6 ml/min/g wet tissue, $P = 0.087$).

CPK activity, pH values and UV absorbance at 250 nm of the perfusate

After 45 min perfusion, CPK release was similar in control and hyperlipidemic animals (44.9 ± 11.1 vs 48.1 ± 13.4 mU/min/g wet tissue, P = NS). Perfusate pH value (which has been shown to decrease in hypoxic hearts[21]) was not different from that of the freshly prepared Krebs–Henseleit buffer in both groups of rabbits. At the end of the experimental time, absorbance values at 250 nm were 2.8 ± 0.9 vs

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TABLE I
Myocardial antioxidants and thiobarbituric acid reactive substances in control and fat-fed rabbits

	Control	Fat-fed
Total SOD	7.15 ± 1.3	9 ± 1.6^a
Catalase	19.05 ± 6.8	27.2 ± 6^a
NP-SH	868 ± 102	701 ± 91^b
GS-SG Red	91 ± 24	56 ± 18^b
$GSH\text{-}P_x$	226 ± 28	176 ± 21^b
GST	324 ± 37	391 ± 54^a
GST-Px	ND	ND
TBARS	8.5 ± 2.1	15 ± 3.8^b

Total SOD: Total superoxide dismutase activity (U/mg protein); Catalase: Catalase activity (U/mg protein); NP-SH: Non proteic thiol compounds (nmol NP-SH/g wet tissue); GS-SG Red: Glutathione reductase activity (U/mg protein); GSH-Px: Selenium-dependent glutathione peroxidase activity (U/mg protein); GST: Glutathione transferase activity (U/mg protein); GST-Px: Selenium-independent glutathione peroxidase activity (U/mg protein).

TBARS: Thiobarbituric acid reactive substances (nmol TBARS/g wet tissue).

Biochemical data are expressed as means $\pm$ SD of 7 rabbits in each group.

$^a p < 0.05$ vs control group.

$^b p < 0.01$ vs control group.

ND, not detectable.

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3.9 ± 0.8 $A_{250} \times 10^3$/g wet tissue in control and fat-fed rabbits ($P < 0.05$). When expressed as the sum of the four values detected during 45 min perfusion, CPK and A_{250} values were 9.9 ± 2.4 vs 9.4 ± 2.05 mU/ml/g wet tissue ($P = NS$), and 12.6 ± 3.3 vs 17.8 ± 4 $A_{250} \times 10^3$/g wet tissue ($P < 0.05$) in control and hyperlipidemic animals, respectively.

Biochemical data

The enzymatic activities of the antioxidant defence system, NP-SH and TBARS levels in the two groups of animals are summarized in Table I. Total SOD and catalase activities increased significantly ($P < 0.05$) in the experimental group, as well as GST activity ($P < 0.05$). On the contrary, a marked depression of GS-SG Red and GSH-Px activities ($P < 0.01$), associated with NP-SH decrease ($P < 0.01$), was assessed in the hyperlipidemic rabbit myocardium. GST-Px was not detectable neither in controls nor in fat-fed rabbits. Finally, ventricular TBARS values were significantly higher in fat-fed than in control animals ($P < 0.01$).

DISCUSSION

The present study demonstrates that myocardial antioxidant defences and lipid peroxidation are significantly modified in rabbits fed on high fat-, cholesterol-enriched

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diet. Particularly, total SOD and catalase activities appear significantly increased. It is known that linoleate-rich fats may induce the microsomal cytochrome P-450 system,[7] which could favour increased superoxide anion generation and SOD activation.[14] The SOD-mediated superoxide dismutation may increase the cellular pool of H_2O_2, with synergic activation of catalase.[33] In such a context, it is worth noting that both SOD and catalase activities have been reported to increase in the liver of rats fed linoleate-enriched diets.[7,34] Moreover, catalase activation could partly be related to the peroxisomal metabolism of dietary lipids,[8,9] with higher H_2O_2 generation essentially by fatty acyl CoA oxidase.[8] This enzyme may also be stimulated by hypoinsulinemia,[35] which has been reported in cholesterol-fed rodents.[36]

Our results confirm in the rabbit heart the not yet fully explained dysfunction of the GSH-redox cycle, previously shown in the liver of cholesterol-fed rats.[3] Increased oxidants levels and tissue metabolic alterations induced by hyperlipidic diet could potentially explain the depressed activity of GSH-Px. In fact, the enzyme seems to be inhibited by hydroxyl radicals and peroxides, especially if low GSH levels occur.[37] On the other hand, palmitoyl CoA[38] (which increases in the heart after fat loading[39]), hypoinsulinemia[40] and linoleate,[40–42] besides aldehydic peroxidation products,[42] inhibit glucose-6-phosphate dehydrogenase, so critically influencing NADPH availability for GS-SG Red and GSH-Px. Thus, the rate of NADPH generation limits that of peroxide reduction, allowing tissue peroxide accumulation.[33] The reduced GS-SG Red activity (which even under physiological conditions is low in the heart[43]) may result in a significant depression of myocardial NP-SH content in the hyperlipidemic animals, though a specific radical-induced NP-SH decrease may also be involved.[44] Moreover, GS-SG Red could adversely be influenced by the hyperactivity of GST, since glutathione S-conjugates have been shown to inhibit GS-SG-Red activity.[45] Even though a GST isoenzymatic study was not performed, the undetectable GST-Px activity suggests the absence of a 2-2-like "basic" isoenzyme in the rabbit heart.[46] Thus, the potential antioxidant properties of GST in the hyperlipidemic rabbit myocardium could be due to hydroxyalkenals-scavenging "near neutral" isoenzymes, which represent more than 50% of GST activity against CDNB in the rat heart[47] and may specifically be activated by oxygen radicals.[48]

Finally, the marked increase of TBARS in the hyperlipidemic rabbits seems to emphasize the occurrence of myocardial oxidative stress *in vivo*. In such a context, the dysfunction of the GSH-redox cycle may be crucial, in light of the key antioxidant role exerted by GSH-Px[49,50] and of the low SOD and, especially, catalase activities in the heart.[51] Moreover, the increased SOD/GSH-Px ratio could favour higher peroxides levels in the hyperlipidemic rabbits myocardium. On the other hand, enhanced oxidants generation, higher unsaturation index of cardiac phospholipids[15] and increased endoperoxides biosynthesis along the prostaglandin pathway[14] could also be involved in the rise of myocardial TBARS.

In spite of the biochemical changes induced by the hyperlipidic diet, significant hemodynamic alterations are not observed in the fat-fed animals, thus suggesting that greater levels of oxidative stress may be necessary to induce myocardial hemodynamic derangement. Our results agree with the findings of Tilton *et al.*[18] and Blumlein *et al.*,[22] who failed to demonstrate significant hemodynamic changes in the perfused hearts of cholesterol-fed rabbits. However, it is worth noting that higher values of A_{250} (which reflect the presence of low molecular weight ATP metabolites[20]) are detected in the myocardial effluent of the hyperlipidemic animals with respect to controls, though CPK release was similar in the two groups. These results could

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suggest that increased dietary intake of fats and cholesterol results in some permeability alterations of heart biomembranes, with coronary vascular cells involvement. In this regard, enhanced permeability of coronary endothelium has been shown in the perfused rabbit heart after short-term cholesterol feeding.[18] The absence of significant changes in CPK release, perfusate pH values and myocardial hemodynamics (sensitive indexes of ischemic-hypoxic injury[20-21]) furtherly argues against a coronary atherosclerosis-related hypoxic damage in hyperlipidemic animals. Accordingly, literature data have proved evidence that the entity and duration of fat supplementation must be greater than those used in the present study to induce significant coronary atherosclerosis in the rabbit.[16-18] In this context, a preliminary evaluation of hematoxylin-eosin stained sections of rabbit ventricular myocardium has revealed no discerneable narrowing of both epicardial and intramural arteries after feeding the present diet for 4 weeks (unpublished observations from our laboratory).

In conclusion, 4 weeks feeding high fat-, cholesterol-enriched diet involves an imbalance in the rabbit heart antioxidant defences, some of which are increased (total SOD, catalase and GST activities), but others depressed (NP-SH and the activity of GS-SG Red and GSH-Px), eventually resulting in enhanced myocardial oxidative stress. This phenomenon seems to be directly fat-related and is not associated with significant heart hemodynamics impairment or CPK leakage during normally oxygenated Langerdorff perfusion, though higher UV absorbance values at 250 nm are detectable in the myocardial effluent of hyperlipidemic animals. It could be hypothesized that the fat-related myocardial oxidative stress may enhance the heart damage associated with free radical-mediated disease entities, such as ischemia-reperfusion[14,52] and inflammatory or drug-induced cardiomyopathies.[14] In this context, increased susceptibility to ischemia-reperfusion injury has been shown in the rabbit heart after short-term (i.e., 3 weeks) dietary cholesterol supplementation.[18] However, further studies are advisable to more specifically address this issue in animal and human experimental models.

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