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ORIGINAL CONTRIBUTION

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Dietary oxysterols induce *in vivo* toxicity of coronary endothelial and smooth muscle cells

Summary Dietary cholesterol oxidation products (COPs) were reported to exhibit *in vitro* toxicity toward vascular cells. The aim of this study was to determine whether dietary COPs induce *in vivo* toxicity toward coronary arteries and to evaluate their effect on the coronary reactivity. Golden Syrian hamsters were fed either a normolipidic diet or a hyperlipidic diet with or without a mixture of COPs (1.4 mg/kg/day). At the end of the feeding periods, cardiac mitochondria and cytosol were prepared to determine the subcellular distribution of cytochrome c. Oxidative phosphorylation was evaluated with glutamate, pyruvate or palmitoylcarnitine as a substrate. The main coronary artery was examined all along its length by transmission electron microscopy (TEM). Plasma sterol concentrations were determined. Furthermore, at the end of the 3-month feeding period, the hearts were perfused at constant pressure by the Langendorff method. The endothelium-dependent reactivity to acetylcholine was evaluated. The myocardial sterol concentration was also estimated. After a 15-day diet with dietary COPs, a release of cytochrome c into the cytosolic fraction of the whole heart occurred, which indicated apoptosis of one or several types of cardiac cells probably induced by excess circulating cholestanetriol. The morphological data obtained by TEM after three months of diet suggested that mainly vascular cells (endothelial and smooth muscle cells) were damaged by dietary COPs, whereas cardiomyocytes appeared healthy. Furthermore, the mitochondrial oxidation of palmitoylcarnitine was reduced and that of pyruvate was increased, suggesting some maintenance of energy metabolism. This strengthens the hypothesis of apoptosis. Several changes in coronary reactivity suggesting an increased NO production were observed. In conclusion, dietary COPs triggered *in vivo* apoptosis of coronary cells through the release of cytochrome c in the cytosol. This toxicity was counterbalanced by an increased endothelium-dependent dilation.

Key words dietary oxysterols – coronary cells – toxicity – apoptosis

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Introduction

Cholesterol oxidation products (COPs) are either produced endogenously through oxidative stress and enzymatic reactions [1], or supplied by foods [2]. They are present in all diets containing cholesterol, but their amount is generally small [3]. However, when cholesterol-rich diets are subjected to different technological processes (heating, irradiation and storage), the level of COPs can be considerably higher. COPs are present in noticeable amounts in several food products such as egg powder and dry milk.

Once absorbed by the intestinal tract [4], they are transferred to the organs via low density lipoproteins (LDL) [5]. Thereafter, they are either incorporated into the organs or rapidly eliminated through bile acid synthesis [6]. In the organism, COPs are involved in diffe-

rent physiological processes including the regulation of cholesterol homeostasis [7], but also in different pathological events such as atherosclerosis [8]. Numerous investigations have focused on the atherogenous effect of COPs [9–11]. Compared to the oxysterol-free diets, COP-rich diets favor the development of atherosclerosis. However, this matter has been debated since several studies [12, 13] have indicated that cholesterol is more atherogenous than COPs. The differences might be due to the type of COPs added to the diet. Lyons et al. [14] have shown that dietary 7-ketocholesterol (7K) cannot accumulate in the aorta and is thus probably not atherogenic. If 7K is a common dietary COP, food products contain other more toxic COPs, such as α-epoxycholesterol (αE) and β-epoxycholesterol (βE) that are transformed into cholestanetriol (CT) in the body [15]. CT is probably the most cytotoxic COP to endothelial cells [16]. Instead of favoring atherosclerosis, COPs could only be toxic toward vascular cells.

In vitro, COPs are toxic toward different types of vascular cells (endothelial cells [16], smooth muscle cells [17], fibroblasts [18] and macrophages [19]). They induce apoptosis [20] through a mechanism involving excess intracellular calcium [21], since blockade of L-type calcium channels concurs to decrease COP-induced cellular death [22]. *In vivo*, their toxic effect on the heart has never been precisely described. Several points have not been elucidated yet such as the cellular type the most sensitive to the toxic effect of dietary oxysterols and the mechanism of action of dietary oxysterols. Moreover, COP-induced toxicity could alter the endothelium-dependent reactivity of the coronary network. It has been shown that oxidized LDL or COPs impair acetylcholine-induced dilation in coronary arterioles [23, 24] through a NO- and superoxide radical-related mechanism [25, 26]. This effect has been observed when COPs are incubated with the artery for a short duration. However, the influence of dietary COPs consumed for a long duration is still unknown.

The aim of this study was to evaluate the *in vivo* effect of a long-term consumption of oxysterols on the different cells participating in cardiac architecture and on coronary reactivity in the golden Syrian hamster previously fed four diets differing in their cholesterol and oxysterol contents. Thus, the toxicity of dietary COPs was estimated by evaluating the subcellular distribution of cytochrome c and by visualizing the main coronary artery and surrounding myocardium by TEM. Furthermore, the oxidative phosphorylation was evaluated in the whole myocardium. The coronary reactivity to acetylcholine (Ach) was also measured in the isolated perfused heart of hamsters. The accompanying changes in plasma and cardiac sterol concentrations as well as in myocardial calcium level were quantified.

Materials and methods

Preparation of dietary COPs

The mixture of oxysterols was prepared by heating cholesterol dissolved in a lipid matrix (lard) at 135 °C for seventy-two hours as previously described [27]. As evaluated by gas chromatography-mass spectrometry [27], the final extract contained 7α-hydroxycholesterol (9.2 %), 7β-hydroxycholesterol (14.3 %), 5α, 6α-epoxycholesterol (20.5 %), 5β, 6β-epoxycholesterol (20.5 %), a mixture of 6-ketocholesterol plus 25-hydroxycholesterol (5.9 %) and 7-ketocholesterol (24.3 %). The sum of the detected sterols represented 94.7 % of the total products separated by gas chromatography, the remaining being too low to be correctly identified and quantified. The amount of hydroperoxycholesterol was lower than 3 %.

Animals and diets

The present study was carried out in accordance with the French legislation describing the care and use of laboratory animals. One hundred weaning male golden Syrian hamsters (Janvier's breeding) were used to evaluate the effect of dietary oxysterols on cardiac tissue and fourty-eight animals were used to determine the influence of dietary COPs on coronary vessels. Golden Syrian hamsters were chosen for their high plasma level of LDL-cholesterol that is close to that of humans. To evaluate the effect of dietary oxysterols, we used weaning animals since they adapt more easily to semi-synthetic diets. The animals were housed in individual cages in an animal housing without specific pathogen microorganisms. The temperature (21 ± 1 °C), humidity (60 ± 10 %) and light/darkness cycle (12 h/12 h) were controlled.

For the evaluation of COPs toxicity, two sets of experiments with different diet durations (fifteen days or three months) were conducted, but the age of the animals at the end of the feeding period was kept constant by adjusting the age at the beginning of the diet. In the first set of experiments, fifty-six weanling hamsters were randomly allotted to four groups of fourteen animals after fourteen weeks of feeding with a normolipidic semi-synthetic diet. Each group was then fed a specific diet *ad libitum* for two weeks (15-day diet). The first group was fed a low-lipid diet without oxysterol (Low L group). The composition of the non-lipidic fraction of this diet has already been described elsewhere [27]. The low-lipid diet contained 2.5 % of a mixture of corn oil and fish oil (4:1 w/w). It was chosen as the control diet preventing the development of atherosclerosis. Its lipid content was low and similar to that found in commercial chows. Furthermore, the supply of polyunsaturated fatty acids was high and equilibrated, since these lipids, mainly those of the n-3 family, have been reported to exhibit anti-

atherogenic properties [28]. The second group of animals was fed a hyperlipidic diet (High L group) composed of the low-lipid diet supplemented with 15 % lard and 3 % cholesterol. We chose this diet to determine whether excess dietary saturated fatty acids and cholesterol induce coronary atherosclerosis in the hamster. According to Sima et al. [29], this hyperlipidic diet induces coronary atherosclerosis in the hamster. However, these authors did not verify the purity of the dietary cholesterol used. In the present study, the presence of oxysterols in the high-lipid diet was checked by gas chromatography. This diet was totally devoid of any trace of oxysterols. The third group of animals (High L + COPs) was fed the hyperlipidic diet in which 0.4 % cholesterol was replaced by a mixture of oxysterols. This proportion was similar to that found in human nutrition. Compared to the whole diet, the proportion of oxysterols was small, since it corresponded to 0.01 %. The dietary supply of oxysterols corresponded to 1.4 mg/kg of body weight/day. The fourth group (Low L + COPs) also received the mixture of oxysterols, but added to the normolipidic diet at a dose that supplied the same amount of dietary oxysterols as in the High L + OS group (1.4 mg/kg of body weight/day). In the four diets, the antioxidant vitamin E was omitted in order to facilitate the development of atherosclerosis. To avoid peroxidation in the different diets, the chows were prepared and totally replaced every two days. As shown by gas chromatography, cold storage of the lipid fraction did not promote the formation of oxysterols during the experiment. Similarly, storing diets in cages at room temperature for two days did not lead to the oxidation of cholesterol.

In the second set of experiments, forty-four weaning hamsters were allotted to four groups of eleven animals after four weeks of feeding with a normolipidic semi-synthetic diet. The animals were then fed the four specific diets mentioned above for twelve weeks (3-month diet).

For the evaluation of the effect of dietary COPs on coronary artery reactivity, after one month of feeding with a normolipidic semi-synthetic diet, the hamsters were randomly allotted to four groups of twelve animals. Each group was fed one of the four specific diet *ad libitum* for three months.

Mitochondria extraction

At the end of each diet, cardiac mitochondria from the animals dedicated to the study of COPs toxicity were prepared. Nine and six hamsters per dietary group were used for the 15-day and 3-month diets, respectively. The animals were anesthetized with intraperitoneal injection of sodium pentobarbital (54 mg/kg). Blood samples were collected from the abdominal aorta of the 3-month fed animals. They were centrifuged (1850 g, 20 min, 4 °C) and the plasma was collected. A total of 2,6-di-tert-butyl-4-methylphenol (0.1 %) in ethanol was added to plasma samples to avoid peroxidation. After a rapid thoracotomy, the heart was collected and the mitochondria were extracted according to Palmer et al. [30]. After the first low-speed and high speed centrifugations, the supernatant contained cytosol and microsomal membranes. It was ultra centrifuged (105,000 g, 1 h, 4 °C) to purify the cytosolic fraction. Immediately after preparation, the cytosolic fraction was frozen and stored at –80 °C to limit the action of cellular proteases. At the end of the purification procedure, the mitochondrial pellet was suspended at the approximate concentration of 20 mg/mL. The concentrations of mitochondrial proteins were determined according to Lowry et al. [31].

Mitochondrial respiration

Mitochondrial respiration was evaluated at 37 °C with a Clarke electrode, an oxymeter (YSI 5300) and a recorder. The respiration medium was composed of sucrose 230 mM, Tris-HCl 10 mM, KH_2PO_4 2.5 mM, EDTA 2 mM, lipid-free serum albumin 0.1 %, pH 7.4. The measurement chamber was totally filled with respiration medium and the mitochondria were added (0.25 mg/mL). The chamber was immediately closed with a pierced glass cap fitted onto a Hamilton-syringe needle. After a 10-s interval, the oxidative substrate was added. Three oxidative substrates were tested for each mitochondrial preparation: palmitoylcarnitine (24 µM) + malate-Na (1 mM), glutamate (10 mM) + malate-Na (1 mM) and pyruvate (10 mM) + malate-Na (1 mM). After another 10-s interval, a mixture of ADP-K and magnesium acetate (ADP-Mg complex) was added to the medium (final concentration of 360 µM). The state III and IV respiration rates were determined according to Chance and Williams [32] and the ADP:O ratio according to Estabrook [33].

Assay of respiratory complexes

NADH cytochrome c reductase (complex I + III), succinate cytochrome c reductase (complex II + III) and cytochrome c oxidase (complex IV) were determined in isolated mitochondria according to Pitkänen et al. [34], Merante et al. [35] and Glerum et al. [36], respectively. The activity of complexes I + III and II + III was determined in frozen mitochondria whereas that of complex IV was measured in fresh preparations.

Transmission electron microscopy

For each diet duration, the five remaining hamsters of every dietary group of the COPs toxicity study were used to study the morphology of cardiac tissue and coronary

artery by transmission electron microscopy (TEM). After anesthesia and heparinization, the hearts were immediately perfused according to the Langendorff method at a pressure of 60 mmHg with sodium cacodylate buffer (100 mM, pH 7.4) containing glutaraldehyde (2.5 %). Pieces of myocardium including the total length of the main coronary artery were dissected in a fixative solution (2.5 % glutaraldehyde in 100 mM cacodylate buffer, pH 7.4) and the samples were fixed for four hours at 4 °C. After rinsing in the same buffer, samples were post-fixed in 1 % osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) for one hour at 4 °C. They were then dehydrated using a series of increasing ethanol concentrations (up to 100 %) followed by propylene oxide and were embedded in Epon. Sections were cut using a Reichert Ultracut E ultramicrotome. Thin sections were collected on copper grids and subsequently stained with 2.5 % uranyl acetate in ethanol for 20 min followed by lead citrate in water for 10 min. Sections were examined with a Hitachi H600 electron microscope operating at 75 kV. In each heart, fifteen sections harvested all along the main coronary artery and surrounding muscle tissue at regular intervals (of approximately 500 μm length) were observed by TEM. Thus, the total length of the main coronary artery was visualized, which allowed the appreciation of the morphology from the origin of the artery to the apex of the heart.

Western blotting analysis of cytochrome c

The assay of cytochrome c was conducted on the mitochondrial and cytosolic fractions issued from each heart [37]. It was carried out by SDS-PAGE using a 15 % polyacrylamide gel. After electrophoresis, transfer onto a nitrocellulose membrane and blockade of the non-specific sites, the membrane was incubated with mouse anti-cytochrome c antibody (Chemicon International Inc.) at the concentration of 1 μg/mL for one hour. The ECL analysis system (Amersham Bioscience) with peroxidase-linked anti-mouse antibody was used to reveal the spot of cytochrome c. For mitochondrial determination, 15 mg of protein was used, whereas 190 mg was necessary for the cytosolic fraction. All the data were expressed as a percentage of the same cytochrome c-rich mitochondrial sample and normalized for 15 mg of protein. The reproducibility of the method was good since the cytochrome c-rich mitochondrial sample used as a reference was repeated six times with a mean of 23562 ± 290 density units. Moreover, as little as 2 ng of cytochrome c can be detected by this technique.

Heart perfusion

After the 3-month diet, the hamsters dedicated to the study of COPs influence on coronary artery reactivity were anesthetized with sodium pentobarbital (60 mg/kg of body mass) and heparinized (850 IU). After rapid thoracotomy, the hearts were removed and immediately placed in a cold Krebs-Heinselett buffer until cessation of beating. They were then perfused isovolumetrically at constant pressure (60 mmHg) according to the Langendorff mode with a Krebs-Heinselett buffer containing NaCl (118 mM), KCl (5.6 mM), $CaCl_2$ (1.9 mM), $MgCl_2$ (1.2 mM), $NaHCO_3$ (20 mM), KH_2PO_4 (2 mM) and glucose (11 mM). The perfusion fluid was continuously oxygenated with carbogen. A latex balloon was introduced into the left ventricle. It was connected to a pressure gauge and amplifier (Hugo Sachs Elektronik) to evaluate the left ventricular pressure during the cardiac cycle. The balloon was filled until the diastolic left ventricular pressure reached 10 mmHg. A flow probe related to a flow meter (Transonic Systems, Inc.) allowed to continuously evaluate the coronary flow. The flow probe, located just upstream of the aortic cannula, allowed the evaluation of the coronary flow in the whole perfused heart. All the parameters of cardiac functioning were transmitted to a computer and analyzed using an appropriate data acquisition system (HSE Isoheart, Hugo Sachs Elektronik). The heart was perfused under basal conditions for 15 min, which allowed the stabilization of cardiac functional parameters (left-ventricular developed pressure, heart rate and coronary flow). Thereafter, acetylcholine was infused to evaluate the coronary reactivity. The infusion flow was regulated so as to correspond to 1 % of the coronary flow. Since the hearts were perfused under constant pressure, the changes in coronary flow reflected the changes in vascular tone in the whole myocardium. Several concentrations of acetylcholine were tested (1, 5 and 10 μM). The pharmacological solutions were prepared extemporaneously before each infusion. Between each infusion, a re-equilibration period was observed to enable the re-establishment of cardiac physiological parameters to the values measured before infusion. The different parameters of cardiac functioning collected or calculated by the data acquisition system were the following: diastolic and systolic pressures of the left ventricle, left ventricular developed pressure (LVDP), dP/dt min, dP/dt max, coronary flow and heart rate. These parameters were measured continuously throughout the perfusion procedure. To evaluate the effect of acetylcholine on the coronary flow, left ventricular developed pressure, dP/dt max, dP/dt min and heart rate, the value of every parameter was collected just before the infusion of acetylcholine (pre-infusion value) and at the top of the coronary flow peak. The difference between these two values was then divided by the pre-infusion value. Twelve hearts were perfused in each group. At the end of the perfusion, the hearts were frozen at the temperature of liquid nitrogen and stored at –80 °C until biochemical analysis.

Biochemical analysis

Each perfused heart was reduced to powder in liquid nitrogen. A first aliquot (100 mg) made it possible to determine myocardial dry weight. This determination was carried out on each heart. The remaining powder was used to measure myocardial calcium and oxysterols. As a large amount of myocardial powder was required and the heart weight was low (approximately 500 mg), the myocardial powder was randomly pooled by two in each dietary group. This reduced the number of experiments to six per group. Determination of myocardial oxysterols was carried out after extraction of myocardial lipids according to Folch et al. [38]. Myocardial and plasma oxysterols were assayed as previously described [27].

Total myocardial calcium was extracted with perchloric acid (final concentration = 0.6 M). Calcium crystals were dissolved by heating the homogenate at 80 °C for one hour in hermetically sealed tubes. After cooling, the homogenate was centrifuged (500 g, 20 min, 4 °C). Lanthane chloride (10 g/L) was added to the supernatant to avoid the artefact due to phosphate ions and calcium was evaluated by atomic absorption spectrometry, using a wavelength of 422.7 nm.

Statistical analysis

The results are expressed as mean ± S. E. M. The data were submitted to a 2-way analysis of variance [39] describing the effect of hyperlipidemia (Hef), that of dietary oxysterols (Oef) and the cross-interaction (C. I.) between these two factors. All the calculations were carried out using the NCSS 2001 software. When necessary, the comparison of means was done using a Newman-Keul's test. A p value lower than 0.05 was considered as significantly different.

Results

After the 15-day or the 3-month period on the four diets, the animals were healthy as evidenced by the coat aspect and their interest in the environment. In hamsters fed the low-lipid diet devoid of dietary oxysterols for three months, the plasma cholesterol concentration was 13.41 ± 0.48 mg/dL (Table 1). It was not modified by the addition of oxysterols to the low-lipid diet. Conversely, the high-lipid diets greatly increased the plasma cholesterol concentration (+ 161 % compared to the low-lipid diets). As evidenced by the significant cross-interaction, this increase was modulated by the addition of oxysterols to the diet. The presence of dietary COPs in the high-lipid diet significantly increased plasma cholesterol concentration (+ 28 %) compared to the oxysterol-free high-lipid diet. The total COPs to cholesterol ratio (sum of each COP concentration divided by the plasma concentration of cholesterol) was 0.46 ± 0.06 % in the plasma of rats fed the low-lipid diet devoid of exogenous oxysterols. Total COP concentration was not altered by excess dietary oxidized or not oxidized lipids. Whatever the dietary conditions, 4βOH was the most abundant plasma COP. In the Low L group, its concentration was more than ten-fold as high as that of the most abundant (βE) of other oxysterols. The other oxysterols displayed lower concentrations, with αE being the most concentrated, 7βOH the less abundant and 7K, 7αOH, CT and 27OH intermediate. The plasma concentration of certain oxysterols was modulated by the diet. Hyperlipidemia increased the concentration of βE and 7αOH (+ 50 and + 126 %, respectively), but reduced that of 4βOH (–33 %) and of the mixture of 25OH + 6K (–60 %). Addition of oxysterols to the diet contributed to increasing 7K and CT (+ 125 and + 194 %, respectively) and to decreasing 4βOH (–38 %) and the mixture of 25OH + 6K (–48 %). As evidenced by the significant cross-interactions, the increase in 7K and CT was modulated by the amount of dietary lipids. It was higher with the low-lipid diet (+ 343 and + 343 % for 7K and CT) than with the high-lipid diet (+ 23 and + 90 % for 7K and CT, respectively). For 4βOH, the cross-interaction between the effect of excess dietary lipids and oxysterols was not significant.

The influence of the diet on myocardial sterol levels was determined after three months on the different diets (Table 2). Myocardial cholesterol (5.6 ± 0.3 mg/g dry weight in the Low L group) was not altered by hyperlipidemia and dietary oxysterols (data not shown). This was similar for total myocardial COPs (approximately 1 % of total cholesterol). As in plasma, 4β-hydroxycholesterol was the most abundant oxysterol (62 % of total cardiac oxysterols in the Low L group), but the amount of β-epoxycholesterol was also high (23 % of total cardiac COPs in the Low L group). Thereafter, the oxysterols detected by decreasing order of abundance were (in percent of total myocardial oxysterols in the Low L group): α-epoxycholesterol [7], 7-ketocholesterol [3], cholestanetriol [2], 7β-hydroxycholesterol [1], 7α-hydroxycholesterol [1] and the mixture of 25-hydroxycholesterol + 6-ketocholesterol [1]. The level of cholestanetriol was modulated by the diet. It was highly increased by the presence of COPs in the low-lipid diet (+ 670 %), but it was not modified by dietary oxysterols under hyperlipidic conditions. The 7-ketocholesterol and mixture of 25-hydroxycholesterol + 6-ketocholesterol varied similarly. Under normolipidic conditions, their concentration was increased by dietary COPs (+ 171 and + 380 % for 7-ketocholesterol and 25-hydroxycholesterol + 6-ketocholesterol, respectively). Conversely, under hyperlipidic conditions, their concentration was decreased by the presence of oxysterols in the diet (–36 and –65 % for

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Table 1 Effects of the diet on plasma sterol levels

Oxysterol	Low L	Low L + OS	High L	High L + OS	Anova
7βOH	$0.03^{a} \pm 0.01$	$0.10^{b} \pm 0.01$	$0.09^{b} \pm 0.02$	$0.04^{a} \pm 0.01$	Hef: NS Oef: NS CI: $p < 0.001$
27OH	0.05 ± 0.01	0.08 ± 0.03	0.03 ± 0.01	0.04 ± 0.01	Hef: NS Oef: NS CI: NS
CT	$0.07^{a} \pm 0.01$	$0.31^{b} \pm 0.04$	$0.10^{a, c} \pm 0.02$	$0.19^{d} \pm 0.02$	Hef: NS Oef: $p < 0.001$ CI: $p < 0.01$
7αOH	$0.09^{a} \pm 0.02$	$0.10^{a} \pm 0.02$	$0.23^{b} \pm 0.02$	$0.20^{b} \pm 0.01$	Hef: $p < 0.001$ Oef: NS CI: NS
7K	$0.14^{a} \pm 0.01$	$0.62^{b} \pm 0.17$	$0.30^{a} \pm 0.03$	$0.37^{a, b} \pm 0.04$	Hef: NS Oef: $p < 0.01$ CI: $p < 0.05$
αE	0.20 ± 0.06	0.21 ± 0.02	0.27 ± 0.02	0.22 ± 0.03	Hef: NS Oef: NS CI: NS
25OH + 6K	$0.35^{a} \pm 0.11$	$0.15^{b} \pm 0.02$	$0.11^{b} \pm 0.01$	$0.09^{b} \pm 0.01$	Hef: $p < 0.01$ Oef: $p < 0.05$ CI: NS
βE	$0.37^{a} \pm 0.05$	$0.67^{b} \pm 0.07$	$0.80^{b} \pm 0.12$	$0.76^{b} \pm 0.10$	Hef: $p < 0.01$ Oef: NS CI: NS
4βOH	$3.92^{a} \pm 0.66$	$2.25^{b} \pm 0.30$	$2.42^{b} \pm 0.36$	$1.72^{b} \pm 0.34$	Hef: $p < 0.05$ Oef: $p < 0.01$ CI: NS
TOS	5.31 ± 0.61	4.55 ± 0.42	4.61 ± 0.58	3.76 ± 0.40	Hef: NS Oef: NS CI: NS
Chol	$13.41^{a} \pm 0.48$	$13.26^{a} \pm 0.59$	$30.59^{b} \pm 1.76$	$39.14^{c} \pm 2.76$	Hef: $p < 0.001$ Oef: $p < 0.05$ CI: $p < 0.05$

The results are expressed in µg/mL, except for cholesterol (mg/dL). The number of experiments was eleven per group. *Low L* animals fed the low-lipid diet; *Low L + OS* animals fed the low-lipid diet supplemented with oxysterols; *High L* animals fed the high-lipid diet; *High L + OS* animals fed the high-lipid diet supplemented with oxysterols; *7βOH* 7β-hydroxycholesterol; *7K* 7-ketocholesterol; *βE* β-epoxycholesterol; *αE* α-epoxycholesterol; *CT* cholestanetriol; *7αOH* 7α-hydroxycholesterol; *27OH* 27-hydroxycholesterol; *25OH + 6K* mixture of 25-hydroxycholesterol and 6-ketocholestanol; *4βOH* 4β-hydroxycholesterol; *TOS* total oxysterols (sum of each COP concentration); *Chol* cholesterol; *Hef* effect of the hyperlipidic diet; *Oef* effect of dietary oxysterols; *CI* cross-interaction between these two factors; *NS* not significant; a,b,c,d: significantly different by Newman Keul's test

Table 2 Effect of the 3-month diet on myocardial sterol levels

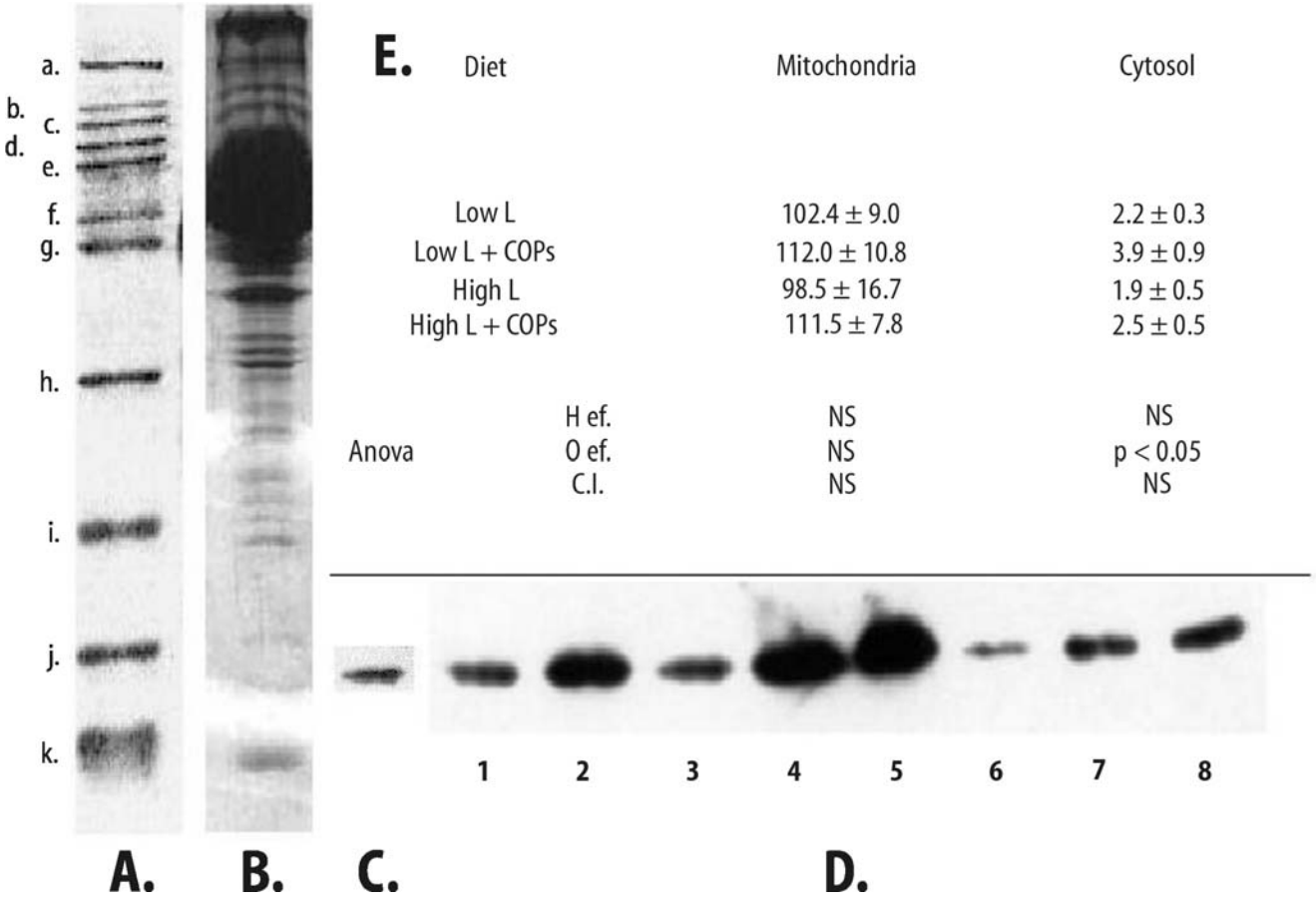
Oxysterol	Low L	Low L + COPs	High L	High L + COPs	Anova
7K	1.7 ± 0.6^{a}	4.6 ± 1.2^{b}	$3.6 \pm 0.7^{a, b}$	$2.3 \pm 0.7^{a, b}$	Hef: NS Oef: NS CI: $p < 0.05$
CT	$1.0^{a} \pm 0.3$	$7.7^{b} \pm 1.9$	$1.2^{a} \pm 0.4$	$1.2^{a} \pm 0.2$	Hef: $p < 0.01$ Oef: $p < 0.01$ CI: $p < 0.01$
25OH + 6K	0.5 ± 0.2^{a}	2.4 ± 1.1^{b}	$2.0 \pm 0.6^{a, b}$	$0.7 \pm 0.2^{a, b}$	Hef: NS Oef: NS CI: $p < 0.05$

The results are expressed in µg/g dry weight. The number of experiments was six per group. *Low L* animals fed the low-lipid diet; *Low L + COPs* animals fed the low-lipid diet supplemented with oxysterols; *High L* animals fed the high-lipid diet; *High L + COPs* animals fed the high-lipid diet supplemented with oxysterols; *7K* 7-ketocholesterol; *CT* cholestanetriol; *25OH + 6K* mixture of 25-hydroxycholesterol and 6-ketocholestanol; *Anova* 2-way analysis of variance; *Hef* effect of the hyperlipidic diet; *Oef* effect of dietary oxysterols; *CI* cross-interaction between these two factors; *NS* not significant; a, b: significantly different by Newman Keul's test

7-ketocholesterol and 25-hydroxycholesterol + 6-ketocholesterol, respectively). The level of other myocardial COPs was not modified by the diet.

The concentration of cytochrome c in the mitochondrial fraction and its presence in the cytosolic fraction was estimated in the four dietary groups after fifteen days of feeding. The amount of cytochrome c in the mitochondrial fraction (Fig. 1) was not affected by the diet. This was not the case for the cytosolic fraction. Western blotting analysis showed that the amount of cytochrome c was low in the Low L (deposits 6, 7 and 8) and High L groups (deposits 1 and 3). Conversely, the presence of oxysterols in the diet noticeably increased the amount of cytochrome c in this fraction. This was true in normolipidic conditions (deposits 4 and 5), but also during hyperlipidemia (deposit 2). The Table included in Fig. 1 shows the statistical results for the cytosolic fraction. Dietary oxysterols increased significantly the release of cytochrome c in the cytosolic compartment.

To determine which changes in tissue morphology accompanied these pathological modifications, the main coronary artery and surrounding myocardium were observed by TEM after three months of diet. Whatever the dietary group, the cardiomyocytes exhibited a classical structure with healthy mitochondria. This was not always the case for the coronary vessel. In the Low L group (Fig. 2A), the luminal surface of endothelial cells was smooth and the basal lamina was thin and constant.



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Diet		Mitochondria	Cytosol
Low L		102.4 ± 9.0	2.2 ± 0.3
Low L + COPs		112.0 ± 10.8	3.9 ± 0.9
High L		98.5 ± 16.7	1.9 ± 0.5
High L + COPs		111.5 ± 7.8	2.5 ± 0.5
Anova	H ef.	NS	NS
	O ef.	NS	$p < 0.05$
	C.I.	NS	NS

Fig. 1 Effect of the diet on the subcellular distribution of cytochrome c. **A** Electrophoresis of molecular weight markers: a.) myosine (205 kD); b.) β-galactosidase (116 kD); c.) phosphorylase b (97 kD); d.) transferrine (80 kD); e.) bovine serum albumin (66 kD); f.) glutamate dehydrogenase (55 kD); g.) ovalbumin (45 kD); h.) carbonic anhydrase (30 kD); i.) trypsin inhibitor (21 kD); j.) lysozyme (14 kD); k.) aprotinin (6.5 kD). **B** Coloration of the gel with Coomassie blue after electrophoretic separation of cytosolic proteins. The homogeneity in the density of the different sample treated indicates that the same amount of proteins was deposited on the gel for western blotting analysis of cytosolic and mitochondrial cytochrome c. **C** Western blotting of bovine heart cytochrome c (12,327 KD) revealed with the mouse anti-cytochrome c antibody. **D** Example of cytochrome c detected in the cytosolic fraction of different samples randomly selected in the hyperlipidic and oxysterol-treated hearts: Low L group: deposits 6, 7 and 8; Low L + COPs group: deposits 4 and 5; High L group: deposits 1 and 3; High L + COPs group: deposit 2. **E** Digited data concerning the effect of the 15-day diets on the subcellular distribution of cytochrome c. The number of experiments was nine per group. The Western blotting analysis was performed with 15 µg of mitochondrial protein or 190 µg of cytosolic proteins. The results are always expressed as the percentage of the cytochrome c spot of the same mitochondrial sample. It was normalized to 15 µg of mitochondrial or cytosolic proteins. *Low L* animals fed the low-lipid diet; *Low L + OS* animals fed the low-lipid diet supplemented with oxysterols; *High L* animals fed the high-lipid diet; *High L + OS* animals fed the high-lipid diet supplemented with oxysterols; *Hef* effect of the hyperlipidic diet; *Oef* effect of dietary oxysterols; *CI* cross-interaction between these two factors; *NS* not significant

Moreover, several layers of smooth muscle cells were perfectly organized. The sub-endothelial space was devoid of any smooth muscle cells. Dead or dying cells were not observed. In the High L group (Fig. 2B), part of the coronary wall was similar to that observed in the Low L group. However, some zones (approximately 34 % of the total zones observed) were different. In these zones, the endothelial cells appeared healthy, whereas the smooth muscle cells were scarce and stretched out. Dead or dying cells were not observed. However, a proliferation of collagen fibers occurred in the vascular wall. Smooth muscle cells were never observed in the sub-endothelial space, which indicates that atherosclerosis did not develop. In the High L + COP group (Fig. 2D), this was very different. In about 23 % of the zones observed, the compactness of the vascular wall was much higher and constituted a swelling in the vessel lumen. The luminal surface of the endothelial cells was scalloped in numerous zones. Some of the smooth muscle cells migrated into the lamina. These cells and endothelial cells constituted a thin cap. The media was swollen and full of collagen and refringent materials. It constituted a gruel responsible for the thickening of the vascular wall. In the gruel, most of the smooth muscle cells were dead or dying. The gruel was separated from the vessel lumen only by a brittle layer of cellular material. These morphological changes suggested the occurrence of cytotoxicity. The Low L + COP group (Fig. 2C) resembled the High L + COP group. The endothelial cells were damaged. The sub-endothelial space contained smooth muscle cells. Collagen, refringent materials, cellular debris and damaged smooth muscle cells were present in the media, which suggested the occurrence of cytotoxicity. However, its severity was lower than in the High L + COP group. In the Low L + COP group, the endothelial cells were less damaged and the cellular cap seemed to be less damaged. A lower number of morphological abnormalities (12 % of the observed zones as compared with 23 % in the High L + COP group) was observed and their aspect was less severe.

After the 15-day diet, the toxic effect of dietary COPs on vascular cell morphology was not yet observed (photographs not shown). Polarographic measurements were however conducted (Table 3). As suggested by the

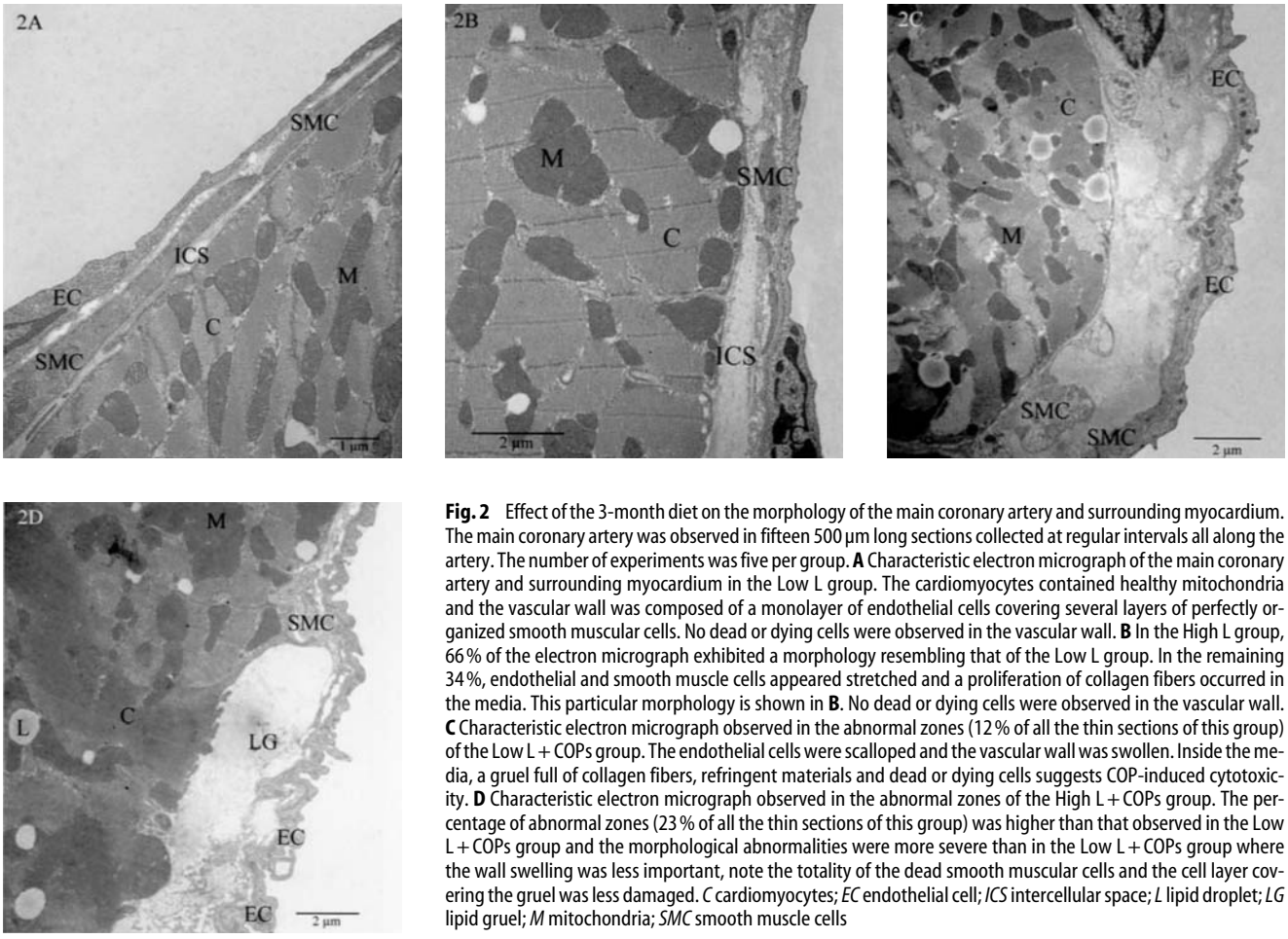

Fig. 2 Effect of the 3-month diet on the morphology of the main coronary artery and surrounding myocardium. The main coronary artery was observed in fifteen 500 µm long sections collected at regular intervals all along the artery. The number of experiments was five per group. **A** Characteristic electron micrograph of the main coronary artery and surrounding myocardium in the Low L group. The cardiomyocytes contained healthy mitochondria and the vascular wall was composed of a monolayer of endothelial cells covering several layers of perfectly organized smooth muscular cells. No dead or dying cells were observed in the vascular wall. **B** In the High L group, 66 % of the electron micrograph exhibited a morphology resembling that of the Low L group. In the remaining 34 %, endothelial and smooth muscle cells appeared stretched and a proliferation of collagen fibers occurred in the media. This particular morphology is shown in **B**. No dead or dying cells were observed in the vascular wall. **C** Characteristic electron micrograph observed in the abnormal zones (12 % of all the thin sections of this group) of the Low L + COPs group. The endothelial cells were scalloped and the vascular wall was swollen. Inside the media, a gruel full of collagen fibers, refringent materials and dead or dying cells suggests COP-induced cytotoxicity. **D** Characteristic electron micrograph observed in the abnormal zones of the High L + COPs group. The percentage of abnormal zones (23 % of all the thin sections of this group) was higher than that observed in the Low L + COPs group and the morphological abnormalities were more severe than in the Low L + COPs group where the wall swelling was less important, note the totality of the dead smooth muscular cells and the cell layer covering the gruel was less damaged. *C* cardiomyocytes; *EC* endothelial cell; *ICS* intercellular space; *L* lipid droplet; *LG* lipid gruel; *M* mitochondria; *SMC* smooth muscle cells

Table 3 Effect of the 15-day diets on mitochondrial oxydative phosphorylation

		State III	State IV	RCI	ADP:O
	Low L	336±15	41±3	8.4±0.3	2.65±0.02
	Low L + COPs	338±7	43±1	7.7±0.2	2.64±0.02
GLUTAMATE	High L	324±7	40±2	8.3±0.4	2.68±0.02
	High L + COPs	343±13	41±3	8.2±0.4	2.62±0.02
	H ef.	NS	NS	NS	NS
Anova	O ef.	NS	NS	NS	NS
	C. I.	NS	NS	NS	NS
	Low L	334±14	50±2	7.0±0.2	2.77±0.02
	Low L + COPs	365±11	52±2	7.1±0.3	2.74±0.04
PYRUVATE	High L	324±15	46±2	7.0±0.1	2.76±0.03
	High L + COPs	362±10	53±1	7.2±0.1	2.80±0.03
	H ef.	NS	NS	NS	NS
Anova	O ef.	$p < 0.01$	$p < 0.05$	NS	NS
	C. I.	NS	NS	NS	NS
	Low L	265±9	35±2	7.8±0.3	2.65±0.04
	Low L + COPs	229±9	37±2	6.0±0.4	2.61±0.01
PCARNITINE	High L	240±8	33±2	7.3±0.3	2.69±0.03
	High L + COPs	220±9	35±3	6.2±0.3	2.62±0.06
	H ef.	NS	NS	NS	NS
Anova	O ef.	$p < 0.01$	NS	$p < 0.001$	NS
	C. I.	NS	NS	NS	NS

The number of experiments was nine per group. State III and IV respiration rates were expressed as ng atoms of oxygen/min/mg and the ADP:O ratio as nmoles/ng atom of oxygen. *State III* state III respiration rate; *state IV* state IV respiration rate; *RCI* respiratory control index; *ADP:O* ADP:O ratio; *Low L* normolipidic diet without oxysterols; *Low L + COPs* normolipidic diet containing oxysterols; *High L* hyperlipidic diet without oxysterols; *High L + COPs* hyperlipidic diet containing oxysterols; *PCARNITINE* palmitoylcarnitine; *H ef.* effect of hyperlipidemia; *O ef.* effect of dietary oxysterols; *C. I.* cross interaction between these two factors; *NS* not significant

characteristics of glutamate oxidation (high state III respiration rates associated with low state IV respiration rates), the mitochondrial preparation was of good quality. The state III respiration rate, state IV respiration rate, RCI (Respiratory Control Index) and ADP:O ratio measured with this substrate were not modified by hyperlipidemia and dietary oxysterols. This was not the case for pyruvate. The state III and state IV respiration rates were increased by dietary oxysterols (+10 and +9%, respectively), but the RCI value and ADP:O ratio remained unchanged. Hyperlipidemia did not modify the characteristics of pyruvate oxidation. When palmitoylcarnitine was used as a substrate, the effect was different. Dietary oxysterols decreased the state III respiration rate (–11%) without affecting the state IV respiration rate and ADP:O ratio. As a consequence, the RCI value decreased (–19%). To further understand the decrease in state III respiration, the activity of respiratory complexes was evaluated (data not shown). Hyperlipidemia did not modify the activity of respiratory complexes. However, dietary oxysterols increased that of NADH cytochrome c reductase and that of succinate cytochrome c reductase (+25 and +27%, respectively, $p < 0.01$), without modifying that of cytochrome c oxidase.

The respiratory characteristics of cardiac mitochondria were also evaluated after three months of diet (data not shown). Using glutamate as a substrate, the state III respiration rate was high (292 ± 5 ng atoms of oxygen/min/mg of proteins) and the state IV respiration rate was low (38 ± 1 ng atoms of oxygen/min/mg of proteins) in the Low L group. This led to high RCI value (7.8 ± 0.6) and ADP:O ratio (2.46 ± 0.04 nmoles/ng atom of oxygen), which suggested the good quality of the mitochondrial preparation. Hyperlipidemia and dietary oxysterols did not modify these characteristics. With pyruvate as a fuel, the oxidative capacities were slightly lower (239 ± 9 and 35 ± 2 ng atoms of oxygen/min/mg of proteins for the state III and state IV respiration rates, respectively) than with glutamate, but the ADP:O ratio was higher (2.62 ± 0.05 nmoles/ng atoms of oxygen). The characteristics of pyruvate oxidation were modified neither by hyperlipidemia nor by dietary oxysterols. With palmitoylcarnitine as a substrate, this was different. The state III respiration rate was significantly decreased by dietary oxysterols (from 167 ± 10 and 153 ± 9 ng atoms of oxygen/min/mg of proteins in groups Low L and High L, respectively, to 136 ± 8 and 135 ± 8 ng atoms of oxygen/min/mg of proteins in groups Low L + COPs and High L + COPs, respectively), but the state IV respiration rate was not modified. This contributed to greatly decreasing the RCI value in the COP-fed animals (–18%). Hyperlipidemia did not change the characteristics of palmitoylcarnitine oxidation.

Due to the toxic effect of COPs on artery wall, their impact on coronary reactivity to acetylcholine was measured after three months on the four diets. Basal coronary flow, LVDP, dP/dt max, dP/dt min and heart rate were 49 ± 4 mL/min/g dry weight, 56 ± 6 mmHg, 1962 ± 252 mmHg/s, –1218 ± 156 mmHg/s and 264 ± 18 beats/min in the Low L group, respectively. Their value was not modified by hyperlipidemia and dietary oxysterols (data not shown).

Ach induced a rapid dilation that only lasted a few seconds. Ach-induced dilation was calculated by determining the percent change between the value measured at the peak of dilation and that measured just before the beginning of dilation. To evaluate the modification of other cardiac physiological parameters, the same times were used. Ach-induced changes in coronary flow were measured at three concentrations (1, 5 and 10 μM). The endothelium-dependent dilation (Fig. 3) was low when Ach was infused at the concentration of 1 μM, since it constituted only 22 ± 2% of pre-infusion coronary flow. Between 1 and 5 μM, the dilation was increased (+106%) and it stabilized thereafter. The increase in coronary flow was not associated with noticeable modifications of the LVDP, dP/dt max, dP/dt min and heart rate.

Hyperlipidemia did not influence the physiological parameters of cardiac functioning (coronary flow, LVDP, dP/dt max, dP/dt min and heart rate) during Ach-in-

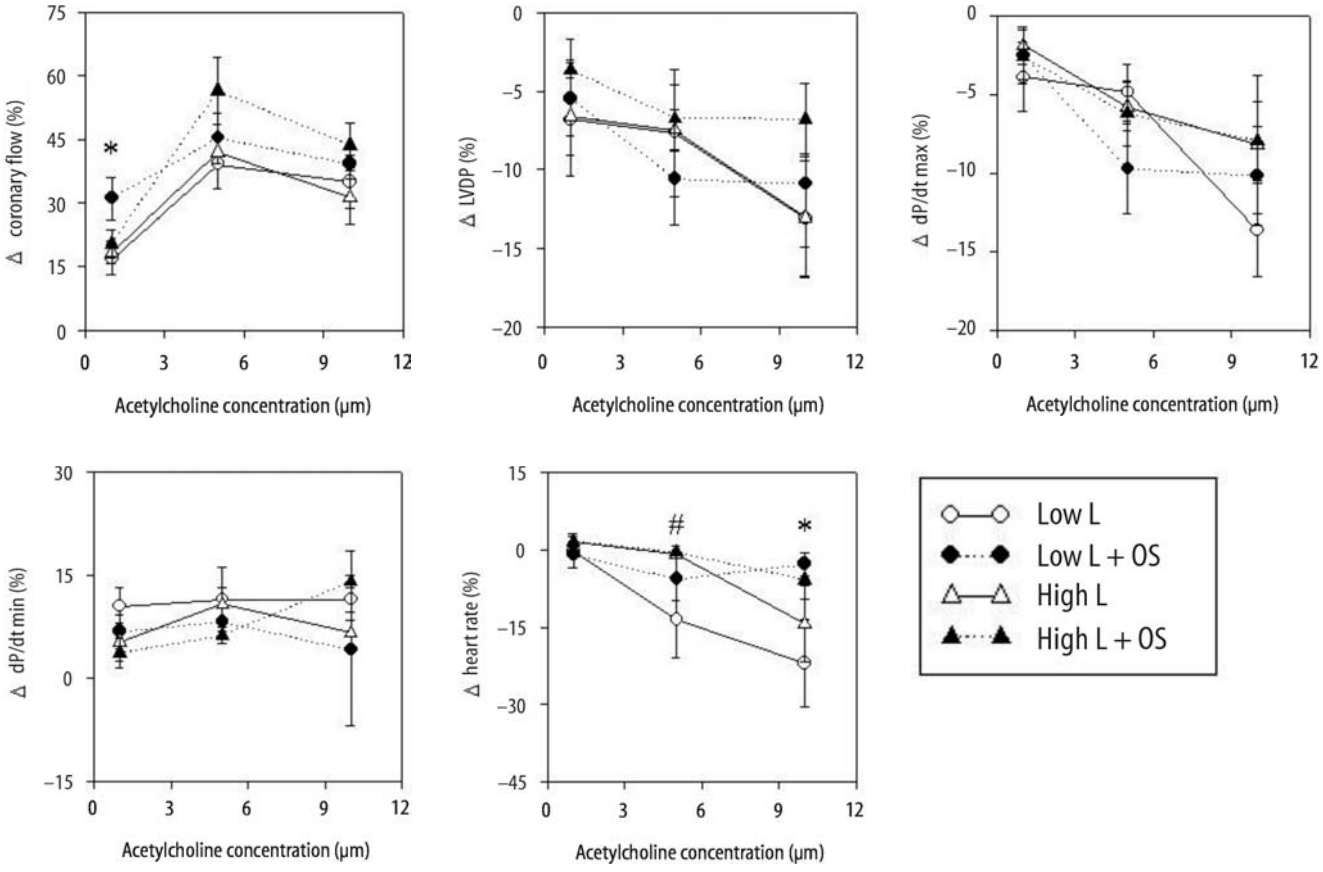

Fig. 3 Effects of the diet on cardiac functioning during Ach-induced dilation. The number of experiments was twelve per group. *Δ coronary flow* change in coronary flow; *Δ LVDP* change in LVDP; *Δ dP/dt max* change in dP/dt max; *Δ dP/dt min* change in dP/dt min; *Δ heart rate* change in heart rate; *Low L* low-lipid diet; *Low L + COPs* low-lipid diet supplemented with oxysterols; *High L* high-lipid diet; *High L + COPs* high-lipid diet supplemented with oxysterols; #: significant effect of hyperlipidemia ($p < 0.05$); *: significant effect of the dietary supply of oxysterols ($p < 0.05$); the interaction was never significant

duced dilation. Dietary COPs contributed to greatly modifying Ach-induced dilation, but this effect was observed mainly at the concentration of 1 μM, that is to say in the ascending phase of the curves plotted against the infused Ach concentration. When Ach concentration equals 1 μM, dietary COPs significantly increased Ach-induced dilation (+ 46 %) without altering the parameters of cardiac mechanical activity. Thereafter, at higher Ach concentrations (that is to say when the curves tended to stabilize), the effect of COPs was not significant.

Myocardial calcium accumulation was evaluated. In the Low L and High L groups, the myocardial calcium level was low (0.53 ± 0.04 and 0.56 ± 0.04 mg/g dry weight, data not shown). This parameter tended to be increased by dietary oxysterols (0.58 ± 0.05 and 0.71 ± 0.10 mg/g dry weight in groups Low L + COPs and High L + COPs, respectively; $p = 0.11$; NS). This increase appeared to be higher in the High L + COP group (+ 30 %) compared to the Low L + COP group (+ 7 %). However, no significant difference was observed, probably because of the low number of samples in each dietary group ($n = 6$).

Discussion

One of the aims of this study was to evaluate the effect of dietary lipids (excess saturated fatty acids plus cholesterol and mixture of COPs) on plasma concentrations of COPs and cholesterol. Our results indicate that excess dietary lipids (saturated fatty acids and cholesterol) and oxysterols modulate the composition of plasma COPs. Both dietary manipulations (excess dietary lipids and oxysterol supply) contributed to increasing plasma autoxidation products. This could be due to an increased production or a reduced elimination. With excess dietary lipids, plasma βE was increased. βE is mainly an autoxidation product [40] that results here from endogenous oxidation of cholesterol in the High L group. The supply of dietary oxysterols increased the level of other autoxidation products (7K and CT). They result from incorporation of dietary autoxidation products, 7K being directly provided by the food and CT resulting from the endogenous transformation of dietary αE and

βE. However, we are not sure that the degradation rate of 7K and CT was not modulated by dietary COPs. The increase in plasma autoxidation products observed in both dietary conditions was associated with a decrease in 4βOH. The *in vivo* formation of 4βOH has been mainly attributed to the activity of a cytochrome P450-related enzyme [41]. Circulating autoxidation products could inhibit the formation of 4βOH or accelerate its elimination. The role of this last COP is unknown. The COP-related decrease in 4βOH was associated with an increase in plasma cholesterol concentration under hyperlipidemic conditions. This was not observed when cholesterol was not present in the diet, which suggests that plasma 4βOH should contribute to reducing intestinal cholesterol absorption.

Another aim of this study was to determine whether dietary oxysterols trigger some toxicity toward the different cells participating in the architecture of cardiac tissue. Since certain dietary COPs induce toxicity toward cultured cardiac cells through a mechanism involving apoptosis [42, 43], we evaluated the subcellular distribution of cytochrome c in the cytosolic and mitochondrial compartments of the whole heart. Dietary COPs, but not hyperlipidemia, induced a release of cytochrome c in the cytosolic compartment. This release was characterized by an 85 % increase in cytochrome c in the cytosolic fraction. It could be explained by the occurrence of apoptosis in certain cells participating in the architecture of the cardiac tissue, but its low magnitude indicated that not all cell types were concerned. The damaged cells were not characterized with this technique. We thus visualized the main coronary artery and surrounding myocardium by TEM to know if cardiomyocytes and/or vascular cells were concerned. As observed all along the length of the main coronary artery, cardiomyocytes were healthy. However, endothelial and smooth muscle cells were noticeably damaged in numerous zones of the vascular wall. Some of the smooth muscle cells appeared dying or even dead after three months of diet. Endothelial and smooth muscle cells constitute a noticeable volume of the myocardium, since cardiomyocytes represent only 30 % of the cardiac tissue. However, they contain less mitochondria than the highly oxidative cardiomyocytes. Despite this, dietary COPs promoted a measurable release of cytochrome c in the cytosolic fraction of the whole myocardium as soon as fifteen days after the beginning of COP administration. This suggests that dietary COPs could induce *in vivo* apoptosis of vascular cells, although apoptosis has not been directly measured in the present study. The morphological abnormalities were associated with plasma accumulation of cholestanetriol. Cholestanetriol was not present in the diet. However, the epoxycholesterols contained in the COP mixture administered to the animals are known to be transformed into cholestanetriol *in vivo* [44]. Cholestanetriol is the most toxic COP [45] and its plasma accumulation in the Low L and High L groups of rats fed dietary COPs could explain the toxicity observed toward vascular cells. Plasma CT of the Low L + OS group was higher than that of the High L + OS group, although the most severe cellular damages appeared in the High L + OS group. The toxic effect of CT might be stimulated by the excess plasma cholesterol or the low 4βOH concentration observed in this last group. The toxic effect of cholestanetriol on vascular cells seemed to be mediated by apoptosis, as in cultured cells [42, 43].

The oxidative phosphorylation was evaluated in the mitochondria of the whole myocardium after 15 days and 3 months of feeding. Three substrates were used separately to evaluate the oxidative phosphorylation from every possible energy source: pyruvate, palmitoylcarnitine and glutamate as markers of carbohydrate, lipid and amino acid oxidation, respectively. As regards the 15-day diet, dietary COPs triggered a decrease in palmitoylcarnitine-related oxidation that was also observed after the 3-month diet. This was not related to a decrease in mitochondrial cytochrome c, since Western blotting analysis of this protein indicated that its intermembrane space concentration was not significantly altered by dietary COPs. This was not due either to a decrease in the respiratory complex activity, since the activity of complex I + III and complex II + III were increased by dietary COPs and that of complex IV tended to be increased. Another mechanism was thus responsible for this change in oxidative metabolism and other investigations of the mitochondrial functioning appear necessary to understand this effect. An increased matrix amount of calcium could be responsible for this phenomenon [46–48]. Although not significant, myocardial calcium tended to be increased by dietary COPs. Mitochondria are known to buffer excess cytosolic calcium. A slight and not significant increase in myocardial calcium could thus mask a large augmentation of the matrix calcium concentration. Whatever the causes of the changes in the oxidative phosphorylation, our results indicated that the mitochondria were affected by dietary COPs. This suggests alterations in the energy metabolism. A direct measurement of matrix calcium could allow a better understanding of the mechanism of action of dietary COPs, peculiarly since this cation is involved in the development of apoptosis.

In contrast to palmitoylcarnitine-related oxidation, pyruvate oxidation was increased after fifteen days of feeding with dietary COPs. One explanation might be the increased activity of complex IV. The resulting maintenance of the energy production strengthens the hypothesis of apoptosis. After cytochrome c release into the cytosol and binding to the apoptosome, activation of procaspase-9 necessitates energy in the form of dATP [49]. Apoptosis occurs only in energized cells. If cells are hypo-energized, they die through oncosis. The fact that pyruvate oxidation was maintained and even increased

by dietary oxysterols suggests that the energy status of the cells was good, which allowed apoptosome activation. After three months of feeding, the increase in pyruvate oxidation induced by COPs disappeared, which indicated that other metabolic regulations could be involved, such as changes in mitochondrial cytochrome c content or respiratory complex activity. However, we did not measure these parameters after such a duration of feeding.

The third aim of this study was to assess the effect of cholesterol- and/or COP-rich diets on the coronary reactivity to acetylcholine in perfused hearts to determine whether COP-induced toxicity related to long-term treatment with these lipids allowed the adaptation of coronary vessels. Under basal conditions, cardiac functioning was not modified by hyperlipidemia and dietary oxysterols. The persistence of coronary flow in groups exhibiting morphological abnormalities agrees with the data from TEM that indicate the occurrence of toxicity without obstructing lesions.

Ach triggered a transient dilation. It binds to muscarinic receptors that allow the production of dilating nitric oxide [50]. The hyperlipidic diets did not modify Ach-induced dilation. Dietary COPs affected Ach-induced changes in vascular tonus. When plotted against the concentration of acetylcholine, Ach-induced vasodilation seemed to obey Michaelis-Menten kinetics. Changes in vascular tone induced by dietary COPs were observed only at low Ach concentrations (1 μM). They were characterized by an increase in Ach-induced dilation. This was not associated with alterations of cardiac mechanical activity. Thereafter, the maximal dilation was not significantly altered by dietary COPs. Since dilation was modified by dietary COPs, this probably results from a better sensitivity to acetylcholine and an increased NO production by endothelial cells. Dietary COP-induced toxicity was evaluated at the level of big coronary vessels. The increase in acetylcholine-induced dilation suggests that the micro-vessels were not damaged and even sustained an adaptative response in order to compensate the evil functioning of the big coronary vessels.

In conclusion, the *in vivo* toxicity of dietary COPs toward vascular cells was demonstrated. It occurred through the release of cytochrome c in the cytosol and probably through apoptosis. The impact of dietary COPs on the plasma sterol concentrations suggests that vascular cell apoptosis was probably triggered by the accumulation of cholestanetriol. Moreover, oxysterol-induced changes in Ach-related coronary reactivity were compatible with increased NO production. This phenomenon corresponds to an adaptation mechanism. The COP-induced toxicity toward vascular cells is thus counterbalanced by this adaptation mechanism.

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