Suppression of Experimental Atherosclerosis by the Ca^{++}-Antagonist Lanthanum

POSSIBLE ROLE OF CALCIUM IN ATHEROGENESIS

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A B S T R A C T Agents inhibiting calcium deposition into arteries are known to suppress atherosclerosis in animals. However, the precise role of calcium in atherogenesis is unknown. In this study, the specific Ca^{2+}-antagonist lanthanum was used to attempt suppression of experimental atherosclerosis and to gain more insight into the possible effects of calcium on atherogenesis. Rabbits were fed an atherogenic diet with and without increasing doses of LaCl$_3$. All cholesterol-fed rabbits showed marked increases in serum cholesterol and Ca^{2+}. Untreated atherogenic animals revealed pronounced gross and microscopic atherosclerosis and striking increases in the aortic content of cholesterol, collagen, "elastin," and calcium as well as of elastin calcium, polar amino acids, and cholesterol. With increasing LaCl$_3$ dosage these abnormalities progressively decreased and were completely abolished at the highest dose. The ingested La^{3+} was absorbed only in small quantities and had no discernible effect on the calcium and connective tissue content of bone, skin, lung, heart, and skeletal muscle nor on myocardial function (left ventricle pressure and left ventricle dp/dt) or myocardial and muscle content in ATP and creatine phosphate. The data suggest that shifts in arterial Ca^{2+}-distribution may play a decisive part in atherogenesis, and provision of arterial calcium homeostasis by La^{3+} a pivotal role in its prevention, despite hypercholesteremia. Other inhibitors of calcium deposition into arteries may exert their protective effect by similar mechanisms. However, a direct inhibition of atherogenesis by La^{3+} cannot entirely be ruled out in this study, although no direct effects of La^{3+} on tissue metabolism have as yet been reported.

INTRODUCTION

As recently summarized by Ross and Glomset (1) the main processes leading to the formation of atherosclerosis plaques appear to be: (*a*) increased permeability of arterial endothelium to macromolecules such as lipoproteins; (*b*) migration of smooth muscle cells (SMCs)[1] from the media into the intima; (*c*) proliferation of these cells by mitosis; (*d*) secretion by the increased SMCs of excessive amounts of collagen, elastin, and glycosaminoglycans; as well as (*e*) endocytosis (phagocytosis) of lipids and/or lipoproteins by intimal cells. Calcium mineralization, especially of arterial elastin, also appears to be a frequent integral part of atherogenesis (2).

Previous studies in rabbits (3–7) and pilot studies[2] in monkeys (5, 6) have indicated that most of these atherosclerotic processes can be inhibited or completely suppressed by agents which prevent excessive deposition of calcium into arteries. These agents included ethane-1-hydroxy-1,1-diphosphonate, 3-amino-1-hydroxy-propane-1,1-diphosphonate, azacycloheptane-2,2-diphosphonate as well as 2-thiophenecarboxylic acid and its methylated or bromated derivatives. These compounds exerted their antiatherosclerotic effect despite unmitigated high serum cholesterol levels when given in large enough amounts. Similar protective actions of diphosphonic acids also have been reported by others (8–11) in animal models of atheroarteriosclerosis. In addition, other drugs that inhibit arterial deposition of calcium or its use also have been shown to be antiatherogenic. These drugs include Mg-EDTA (12), pyridinol carbamate (13), chondroitin sulfate A (14), reserpine, guanethedine, propanolol (15, 16) (thyro)calcitonin (17), sodium *p*-hexadecylaminobenzoate (18, 19) and, as discussed by Numano et al. (20) and Numano (21), manganese,

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[1] *Abbreviation used in this paper*: SMCs, smooth muscle cells.

[2] Report on the completed study is in preparation.

estrogen, and the phosphodiesterase inhibitor EG 626. On the other hand, agents that induce arterial calcium deposition such as vitamin D_3 have been known for a long time to promote atherosclerotic lesions (14).

These studies have led several investigators, including ourselves, to speculate that calcium ions and their local arterial tissue distribution as well as early focal deposition of calcium minerals on intercellular structural tissue components may play a decisive part in atherogenesis far beyond the well-known later gross calcification of plaques (2–7, 11, 19–21). However, in spite of mounting evidence for such a role, the precise action of calcium in the pathogenesis of the disease has not been established. A major reason for this may be that the concentration and activity of ionized calcium in tissues are difficult to measure directly. Because of this difficulty, investigators (22, 23) have measured in vitro the tissue activities of Ca^{2+} and the calcium-mediated cellular functions indirectly, including in arteries, by using the specific calcium ion antagonist, lanthanum. Lanthanum is known to be a potent competitor for cellular Ca^{2+} binding sites to which it adheres in a less reversible manner than does calcium.

In this investigation we therefore attempted to study the possible effect of calcium on the atherosclerotic processes and their graded control in vivo in rabbits by means of increasing doses of oral lanthanum.

METHODS

48 male New Zealand White rabbits weighing 2.0–2.5 kg were divided randomly into six groups of 8 rabbits each. Two groups received the control diet of Purina Rabbit Chow (Ralston Purina Co., Inc., St. Louis, Mo.); one group was fed only the chow (normal controls) while the other was given chow containing 40 mg lanthanum trichloride (ultra pure; Alfa Div., Ventron Corp., Danvers, Mass.) per kilogram body weight per day, with the $LaCl_3$ dissolved in deionized distilled water and adsorbed to the chow pellets. The other four groups received the slightly modified fibrogenic atherogenic diet described by Kritchevsky et al. (24), consisting of the basic chow with 8% peanut oil and 2% cholesterol (by weight) mixed into the chow. One of these groups was fed only the atherogenic diet (atherosclerotic control group), whereas the remaining three groups received this diet with $LaCl_3$ in the amount of (per kilogram body weight per day): 20, 30, and 40 mg, respectively. At the end of the 8-wk study period all animals were killed by an intravenous overdose of pentobarbital.

In vivo studies

Blood samples were drawn after an 18-h fast, once before starting the experiment and then in 2-wk intervals. The blood samples were analyzed as described (3) for total cholesterol (25), total calcium by atomic absorption spectroscopy (26), ionized calcium (27) by using a calcium ion analyzer (spacestat 20, Orion Research, Inc., Cambridge, Mass.), inorganic phosphorus (28), as well as for hemoglobin and complete blood count by standard methods at the Clinical Service Laboratories of University Hospital, Boston, Mass.

Systolic blood pressures were recorded in the untranquilized waking state by the method of Kramsch et al. (29) at the beginning of the experiment and then in 4-wk intervals. Briefly, a IITC-rat tail cuff was placed over the shaved tail of the rabbits, with the tail artery pressure being recorded on a Narco Physiograph (Narco Bio-Systems, Inc., Houston, Tex.) equipped with an IITC modified 59 preamplifier, after the rabbits had been resting for 30 min in a protective box. Simultaneous intra-arterial blood pressures were taken with a Sanborn 279 transducer (Hewlett-Packard Co., Palo Alto, Calif.) at the level of the left ventricle via a short catheter from a femoral artery in selected rabbits (three from each group), immobilized by intramuscular injection of the analgesic tranquilizer Innovar. The intra-arterial pressures were recorded on a Hewlett-Packard 7702 B recorder (Hewlett-Packard Co., Palo Alto, Calif.), and revealed agreement of the systolic blood pressures taken by the two techniques within 5–8 mm Hg. Before the blood pressure recording in the waking state, the heart rate of the rabbits was recorded with a stethoscope.

Postmortum studies

Morphological studies. At autopsy, the entire aorta, the proximal portions of the pulmonary arteries, and the heart, lungs, kidneys, adrenals, liver, spleen, and femurs as well as portions of the abdominal skin, and skeletal muscle (left musculus erector trunci) were removed. The aortas were opened longitudinally along the midthoracic line and cleaned of grossly adherent adventitial tissue. All organs were weighed after they had been cleaned of blood by brief immersion in normal saline and rapid blotting with dry gauze. Their wet weight was expressed as wet weight per kilogram body weight of the rabbit at the time at which it was killed. From the aortas of each group representing the most common gross intimal characteristics of that group (i.e., at least five aortas of comparable appearance) the aortic surface area involved with lesions was estimated. The opened aortas were covered with clear plastic, their contours traced, and the grossly visible lesions filled in with black ink. The total aortic surface area as well as the lesion areas then were measured by planimetry from the tracings. Small representative crosssections from plaque areas and adjacent normal areas of thoracic and abdominal aorta, as well as from comparable normal aortic areas of control rabbits, were taken for histological analysis. Similar horizontal sections were taken from the pulmonary arteries. Cross-sections of whole hearts about midway between the arteriovenous groove and apex, as well as tissue blocks of lung, kidney, adrenals, liver, spleen, skin, and skeletal muscle also were taken for histological studies. All tissue samples removed were processed for histological analysis as described (3).

Biochemical studies. The remaining unfixed tissue of aorta, heart, lung, skin, liver, bone, and skeletal muscle was used to determine lipids, collagen, "elastin," calcium, and nonlipid phosphorus. The intima-media of aorta was stripped from the luminal side (30), minced finely into pieces of about $2 \times 2 \times 2$ mm. Heart, lung, and skeletal muscle were minced in a similar manner; skin by using a Polytron model PT 20 ST homogenizer (Brinkman Instruments, Inc., Westbury, N. Y.). The minced tissues were lyophilized for the determination of their dry weight.

One portion of each lyophilized tissue then was delipidated with chloroform-methanol 2:1 (vol/vol), and their total and ester cholesterol contents were determined from the lipid extracts as described (31); the dry defatted weight of the tissues was determined from the residues after lipid extraction. The delipidated tissues then were divided into two

samples each, one for the determination of collagen content, the other for the determination of calcium and nonlipid phosphorus by the methods described (3, 7). Briefly, for collagen determinations, aortic and heart tissue and skeletal muscle were extracted with boiling 0.1 N NaOH by the method of Lansing et al. (32); skin and lung tissue by a modification of the Lansing method described by Keeley et al. (33). After centrifugation of the samples, the supernatant fluids (containing the alkali hydrolysates of collagen) were hydrolyzed further with 6 N HCl and the hydroxyproline content of the acid hydrolysates was determined (34), using a factor of 7.46 for the conversion of the hydroxyproline values to collagen. For the determination of calcium and nonlipid phosphorus, the delipidated tissue samples were ashed in alumina crucibles overnight at 600°C and portions of the ashes were dissolved either in concentrated HCl (calcium determination) or concentrated H_2SO_4 (phosphorus determination). From the respective acid digests of the ashes the calcium content was determined by atomic absorption spectroscopy (35); that of phosphorus by colorimetric measurement (28). In addition, the right femur of each rabbit was divided into epiphysial ends and shaft (dyaphysis) which were prepared separately for the determination of their calcium mineral content as described (7). The content of calcium and nonlipid phosphorus of the delipidated epiphyses and diaphyses were determined as described above for other tissues.

The nondelipidated portions of each lyophilized aortic intima-media, skin, and lung were taken for analyzing the content and composition of their elastin fractions as described for aorta (3). The elastin fractions were isolated by extraction of the tissues with boiling 0.1 N NaOH with the same methods as described above for extracting collagen from delipidated aorta (32) or lung and skin (33). The isolated elastin fractions then were delipidated, desiccated, and their dry weight determined. Their contents of total and ester cholesterol as well as of triglycerides and free fatty acids were determined from the lipid solutions by thin-layer chromatography according to a modification of the method of Downing (36) as described (7). The calcium and phosphorus contents were determined from small samples of the dry elastin preparations with the same methods as those described above for whole tissue. In addition, the amino acid composition of the elastin fractions was determined as described (31) by hydrolyzing the defatted elastin preparations with 6 N HCl at 110° for 24 h and analyzing the hydrolyzed amino acids with a Technicon amino acid autoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.) according to the method of Hamilton (37).

A 1-g specimen from each liver also was analyzed for its content in total and ester cholesterol as well as in triglycerides and free fatty acids. The methods used for the preparation of liver tissue were the same as those described above for other tissues; the methods for the determination of its lipid content and composition were those described above for elastin preparations.

Urine samples were obtained from the bladder at autopsy in all animals and analyzed for their hydroxyproline content by the method of Kivirikko (38). Urinary hydroxyproline content was expressed per milligram urinary creatinine that remained constant at different concentrations of the excreted urine (39). Creatinine clearance over 24 h was comparable in all groups and within the normal range for rabbits (40), indicating normal glomerular filtration rates.

Special studies. In selected untreated and lanthanum-treated rabbits, cardiac function was assessed under pentobarbital anesthesia and assist respiration by intubation via a tracheostomy. The thorax was opened by midline sternotomy and left ventricular pressures were measured by direct catheterization through apical puncture with a wide bore

needle attached via short (2–3 cm) polyethylene cannula to a Statham P 23 transducer (Statham Instruments, Inc., Oxnard, Calif.) at the level of the left ventricle. Pressures were recorded on an Electronics for Medicine, Inc. (Pleasantville, N. Y.) DR 8 recorder before and during increasing both preload and afterload by abdominal compression and transient (3 s) cross-clamping of the aortic root, respectively. In almost all animals, at least one ventricular premature beat occurred during the aortic cross-clamping; the following beat demonstrated postextrasystolic potentiation against the clamped aorta.

In other selected animals from the normal control group, the lanthanum-fed control group and the group fed only the atherogenic diet, skeletal muscle (right musculus erector trunci) and hearts were removed in vivo and analyzed for their content in adenosine triphosphate and creatine phosphate by a modification of the methods of Lamprecht and Trautschold (41) and Adam (42), with the same Sigma ATP-kit (Sigma Chemical Co., St. Louis, Mo.) for both analyses. Thorax and abdomen of the rabbits were opened in midline under anesthesia and assist respiration as outlined above, and the normally perfused skeletal muscle as well as the whole beating heart were instantly freeze-clamped by thongs precooled in liquid nitrogen. The tissues then were stored in a liquid nitrogen tank until processed for biochemical analysis.

Since direct determinations of lanthanum in biological fluids and tissues as yet have not been reported, preliminary studies were conducted in selected rabbits and cynomolgus monkeys (Macaca fascicularis) given control diets containing 40 mg $LaCl_3$/kg body wt per d (two animals per dietary group of each species). Blood samples (drawn 4 and 12 h after meal ingestion) and 24-h urines and feces of monkey as well as liver and bone (left femur) of rabbits (autopsy material) were lyophilized, ashed, dissolved in concentrated HCl, and brought to a constant volume with deionized distilled water as described above for atomic absorption spectroscopy. The samples then were analyzed (43) for lanthanum content in a plasma emission echelle spectrometer (Spectraspan model III A: courtesy of Spectrametrics, Inc., Andover, Mass.). Appropriate La^{3+} standard solutions of various concentrations were used to calibrate the instrument, which was found to detect accurately this rare earth element at a level as low as 2 ppb.

With the exception of the lanthanum determinations, where the data were averaged, the significance of all data was determined by statistical analysis of unpaired data.

RESULTS

In vivo studies

The animals consumed their respective diets well throughout the experiment, except for one rabbit on the atherogenic diet without lanthanum that died prematurely. All rabbits gained weight, with the average weight gain in the groups varying between 592 and 654 g. The weight increases among the groups did not differ significantly. Waking state systolic blood pressures (range: 91–112 Hg) and heart rate (202–215 beats/min) as well as hemoglobin (9.8–14.3 ml/100 ml blood), erythrocyte count 4.4–6.0 × $10^6/\mu$l), leukocyte count (7.1–13.4 × $10^3/\mu$l), and leukocyte differential were comparable between all groups and within the normal limits for rabbits (40). The platelet count for the group receiving only the atherogenic diet (range:

322–448 × $10^3/\mu l$) was somewhat but not significantly lower ($P > 0.05$) than the normal values (40) in the normal control groups and the other groups (range: 385–512/μl).

The mean values for serum constituents are summarized in Table I. The mean serum cholesterol levels rose in all rabbits on the atherogenic diet with or without lanthanum to levels above 2,000 mg/100 ml within 2 wk and remained at that level through the study with the cholesterol values among all cholesterol-fed groups being not significantly different. The mean ionized serum calcium but not the total calcium rose significantly in rabbits on the atherogenic diet without LaCl$_3$. Serum Ca^{2+} levels remained significantly elevated—along with significant elevations of total calcium—in atherogenic animals treated with lanthanum regardless of dosage, with the rises in total calcium being mainly due to increased ionized calcium. The serum inorganic phosphorus remained unchanged in all groups. Lanthanum treatment alone had no effect on these serum constituents.

Postmortem studies

MORPHOLOGICAL RESULTS

Macroscopic findings. The average weight per kilogram body weight of kidneys and hearts was comparable in all groups, with all kidneys being grossly normal. However, the heart of all untreated rabbits on the atherogenic diet showed marked gross coronary atherosclerosis. In the atherogenic group treated with the lowest dose of LaCl$_3$, coronary atherosclerosis consisted only of a few small dots of lipid infiltration

and was absent in the atherogenic groups treated with the two higher dosage levels and the lanthanum-treated control rabbits. The dry weight of the intima-media of the aortas was comparable within each group if expressed per kilogram body weight. However, there was a highly significant increase ($P < 0.01$) in mean aortic weight from 34.8 mg/kg body wt in the normal control group to 54.0 mg/kg in the untreated group on the atherogenic diet. The increases in mean aortic weight in atherogenic animals became progressively smaller with increasing dosage of lanthanum: 46.4 mg for the group receiving daily 20 mg LaCl$_3$/kg body wt ($P < 0.01$ compared to normal); 38.5 mg for the 30 mg LaCl$_3$ group ($P < 0.05$ compared to normal); and 35.5 mg for the 40 mg LaCl$_3$ group, which was no longer significantly different from that of the normal control group. No significant change in mean aortic weight occurred in the normal group of rabbits treated daily with 40 mg LaCl$_3$/kg body wt. Fig. 1 shows the outlines of representative aortic surface area involvement with lesions for each atherogenic group. There was a progressive reduction in aortic surface area involvement with increasing dosage of lanthanum from 64% (untreated atherogenic) to 16% (20 mg LaCl$_3$), and <3% (40 mg LaCl$_3$); aortas of control animals with and without lanthanum treatment revealed no lesion involvement.

Microscopic findings. Fig. 2 shows the micrograph of a cross-section of thoracic aorta from a normal control rabbit. The intima consisted of one layer of cells on an intact internal elastica, which was followed in an orderly fashion by layer upon layer of intact medial elastic laminas alternating with layers of unaltered medial smooth muscle cells. Cross-sections through

TABLE I
Content of Serum Components in Control Rabbits and Rabbits on the Atherogenic Diet
with and without Lanthanum

| Experimental groups | Total cholesterol | Calcium | | | Inorganic phosphorus |
		Total	Ionized	% Ca^{++}	
		mg/100 ml; Mean ±SD			
Control diet	67±52	13.2±1.1	5.2±0.4	38±4	7.1±0.8
Atherogenic diet without LaCl$_3$	2573±568	13.3±1.2	6.1±0.5*	45±3*	6.4±0.7
Atherogenic diet + 20 mg LaCl$_3$/kg body wt	2431±498	14.8±1.0‡	6.2±0.7*	46±5*	6.6±0.6
Atherogenic diet + 30 mg LaCl$_3$/kg body wt	2035±921	15.3±1.3*	6.4±0.7*	47±6*	6.1±0.8
Atherogenic diet + 40 mg LaCl$_3$/kg body wt	2537±672	16.0±1.2*	6.7±0.6*	47±5*	6.1±0.8
Control diet + 40 mg LaCl$_3$/kg body wt	92±43	13.7±0.8	5.1±0.9	37±3	5.9±1.0

* $P < 0.01$ compared to untreated control.
‡ $P < 0.05$ compared to untreated control.

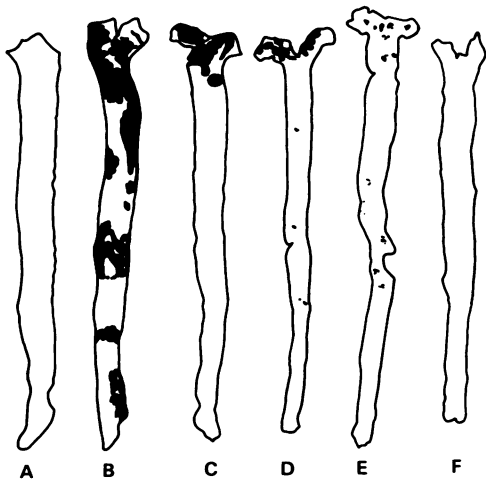*D. M. Kramsch, A. J. Aspen, and C. S. Apstein*

FIGURE 1 Tracings of representative aortic surface area involvement with lesions in percent for each of the experimental groups. (A) Control diet without LaCl₃, 0%; (B) Atherogenic diet without LaCl₃, 64%; (C) Atherogenic diet plus 20 mg LaCl₃/kg body wt, 16%; (D) Atherogenic diet plus 30 mg LaCl₃/kg body wt, 7%; (E) Atherogenic diet plus 40 mg LaCl₃/kg body wt, <3%; (F) Control diet plus 40 mg LaCl₃/kg body wt, 0%.
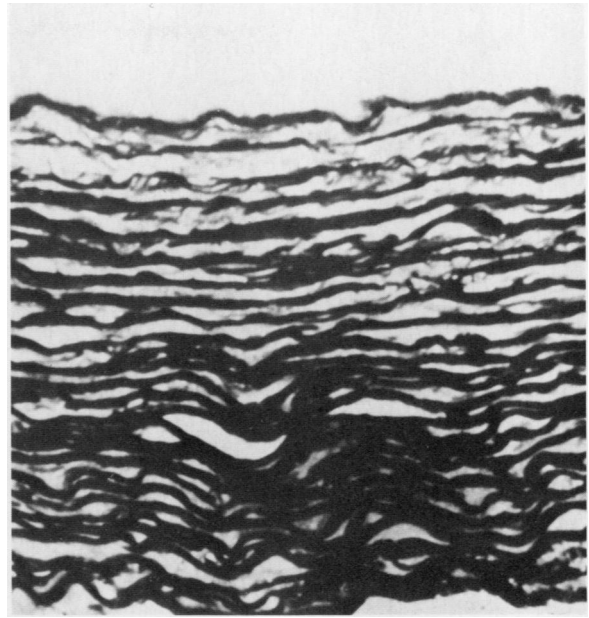

FIGURE 2 Histological cross-section of normal thoracic aorta of a rabbit on the control diet without lanthanum, showing a thin intima including an intact internal elastica (top black line) overlying an orderly structural normal media. Verhoeffs-Van Gieson; ×102.

aorta of lanthanum-treated control animals had a similar appearance.

Fig. 3, A–D, shows serial microscopic sections through one of the characteristic fibrous-fatty aortic plaques elicited in rabbits fed the atherogenic diet without lanthanum. The intima was raised considerably by a marked increase in cellularity, and there was a striking accumulation of collagen in the intima and subintimal media; the internal elastica and many of the subintimal medial elastic laminas were severely deranged and fragmented (Fig. 3A). There was heavy deposition of calcium (Fig. 3B), of lipid (Fig. 3C), and of glycosaminoglycans (Fig. 3D) on the damaged elastica. The increased intimal cells also contained large deposits of Oil Red O-stainable lipids (Fig. 3C). However, only minor deposits of stainable calcium were seen between calcified elastic laminas, including in overlying cells (Fig. 3B). Moderate accumulations of glycosaminoglycans also occurred over accumulated collagen (Fig. 3D).

Fig. 4A and B shows serial sections through one of the larger lesions still present in rabbits receiving the atherogenic diet and treated with 20 mg LaCl₃/kg body wt. The cellularity of the intima was only moderately increased, with the intimal cells present being mainly of the foam cell type (Fig. 4A) and containing Oil Red O-stainable lipid. There was no accumulation of collagen and glycosaminoglycans, but the internal and subintimal elastic laminas still were slightly deranged and often stained mildly for lipids. However, there was no detectable calcification of the elastica

(Fig. 4B). Increase of the dosage of LaCl₃ to 30 mg/kg resulted in further reduction of the cellularity of the intima, absence of collagen and glycosaminoglycan accumulations, normal appearance of the arterial elastica, and absence of calcification.

Fig. 5A and B shows a representative section through one of the few small aortic lesions seen in rabbits receiving the atherogenic diet and treated with 40 mg LaCl₃/kg body wt. The structure of the vessel was essentially normal with the exception of a small layer of foam cells overlying an unbroken internal elastia (Fig. 5A). Deposition of Oil Red O-stainable lipids occurred mainly in the small number of cells present in this superficial intimal layer (Fig. 5B), indicating that lipid infiltration of cells may be inevitable once they have migrated to the intima.

In untreated rabbits on the atherogenic diet, the common pulmonary and the major coronary arteries showed regularly severe microscopic lesions of about the same type as in the aorta; the branch arteries of these organs and the arteries of other organs (liver, kidneys, adrenals, spleen, skin, and skeletal muscle) less regularly. Treatment of atherogenic animals with 20 mg LaCl₃/kg resulted in complete suppression of these lesions in the pulmonary arteries but not in the arteries of other organs; this was only achieved by treatment with 30 and 40 mg LaCl₃/kg body wt. All arteries of control animals with and without lanthanum treatment were free of disease.
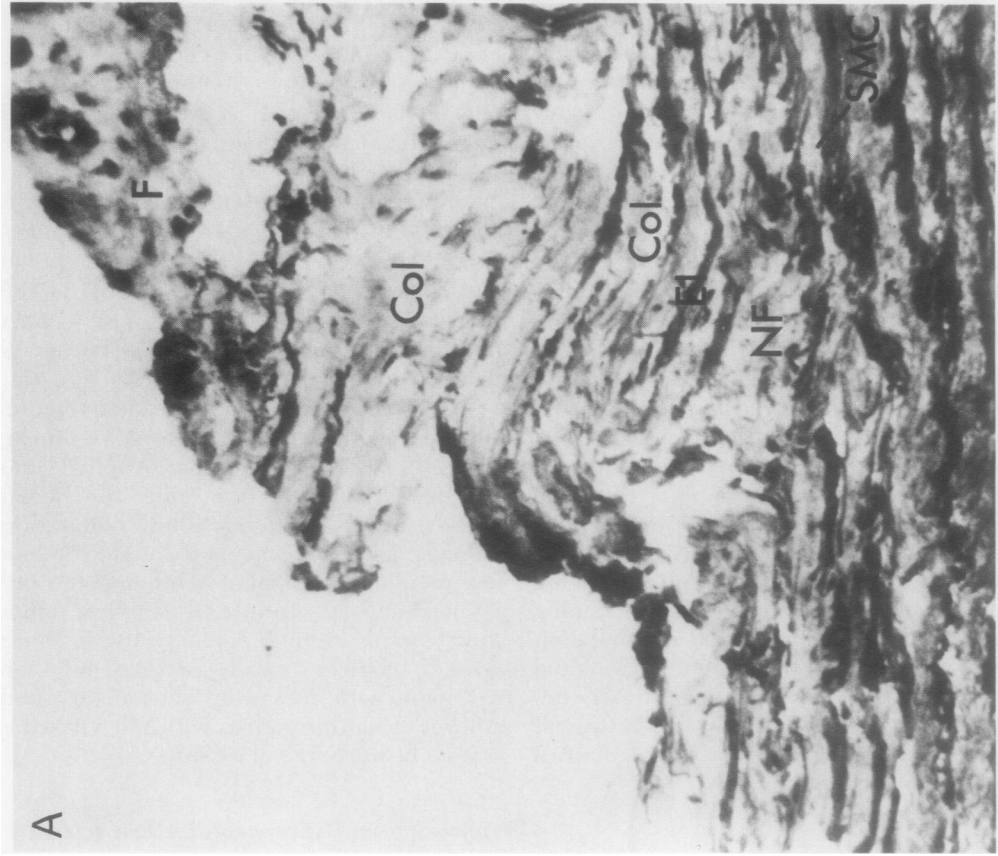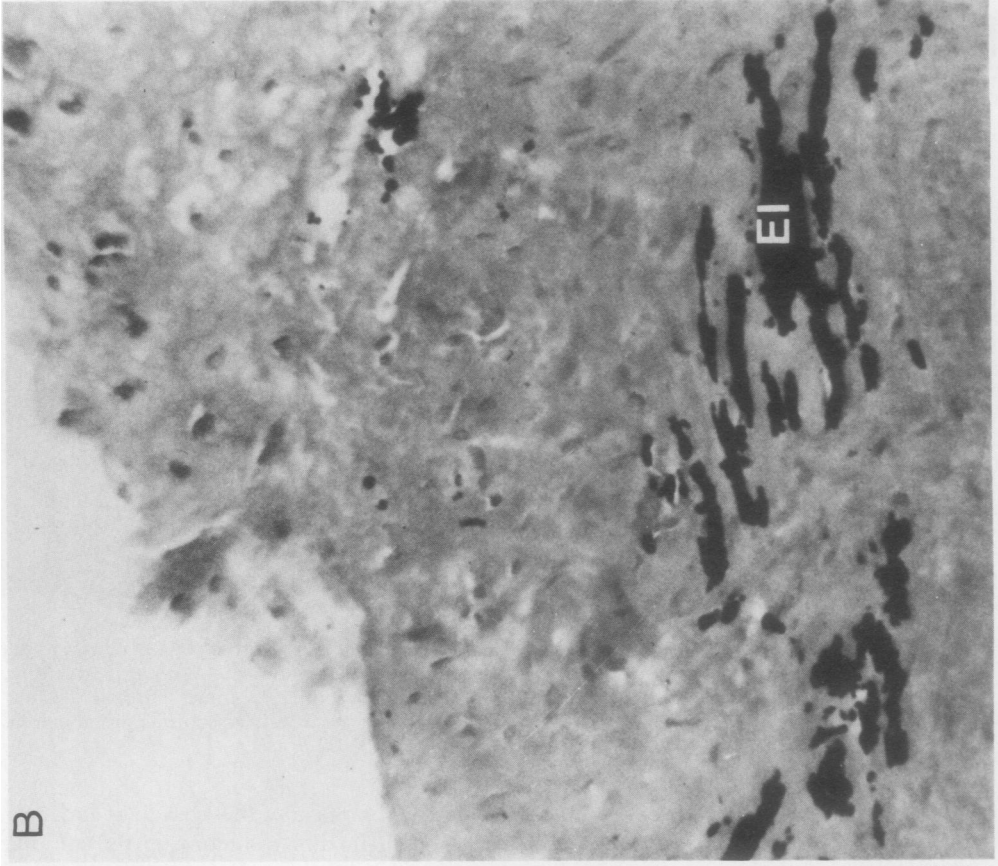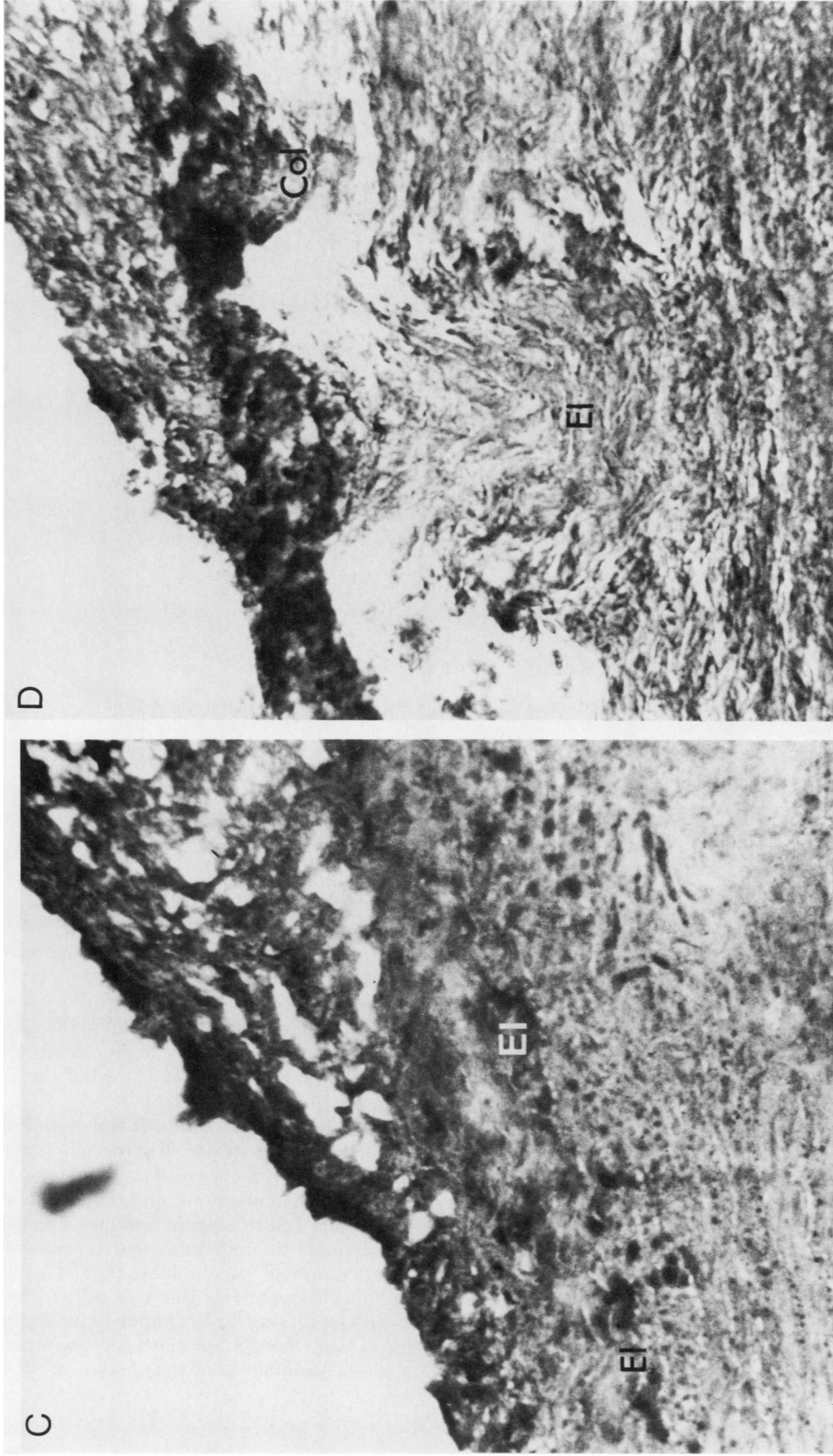
FIGURE 3 (A–D) Serial histological sections of a characteristic plaque of a rabbit fed the atherogenic diet without lanthanum treatment. Approximately the top half of the micrographs represents the markedly raised intima; the bottom half, the subintimal media. (A) Gomori trichrome-aniline blue, showing that the raising of the intima was due to an increased cellularity and accumulation of collagen (light grey, Col); the increased intimal cells appeared to be smooth muscle type cells (SMC) as well as "foam cells" (F) of unidentifiable origin. In the center of the necrotic subintimal foam cells (left center, NF). (B) Yasue's calcium stain-light green, showing dense deposition of calcium (black) mainly on the fragmented intimo-medial plaque elastica (EL). (C) Hematoxylin-Oil Red O, showing lipid deposition (black) in intimal and subintimal cells and on the deranged plaque elastica (EL). (D) PAS-alcian blue, showing depositions of glycosaminoglycans (dark grey) over areas of intimal and subintimal collagen (Col) accumulations as well as on the deranged plaque elastica (EL). ×102.
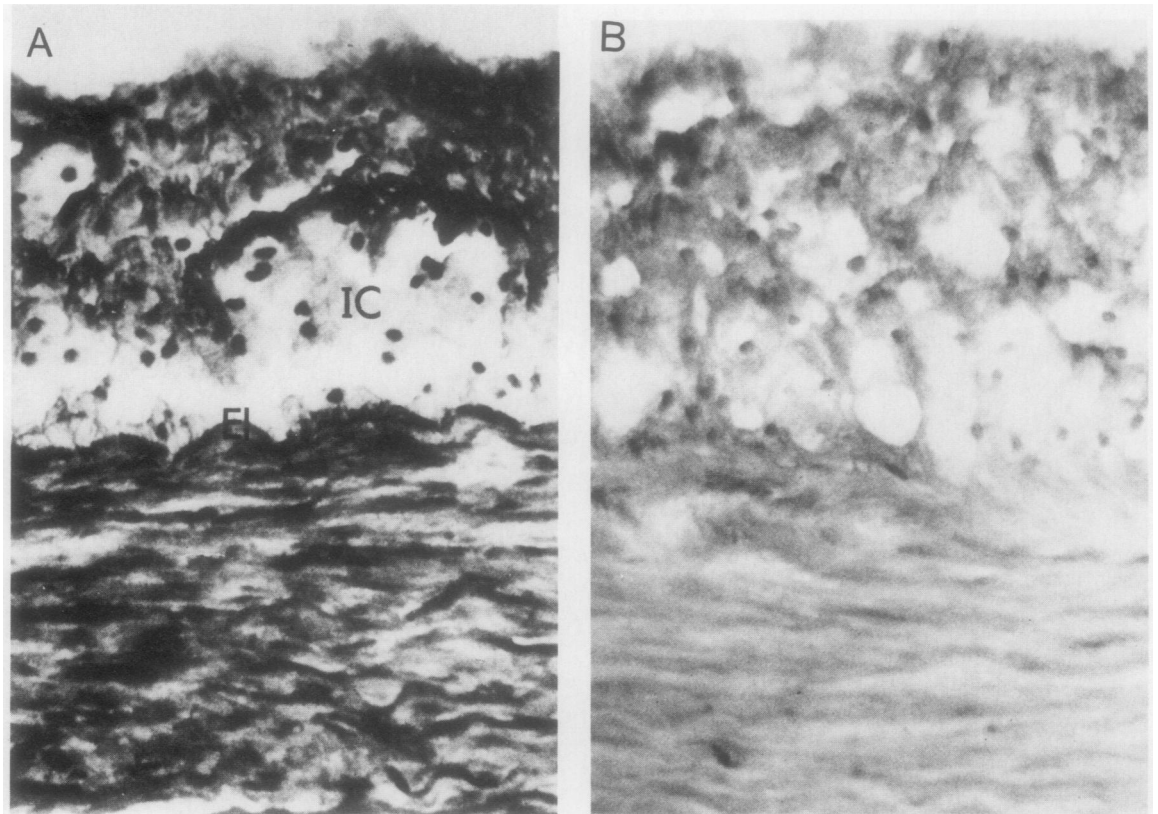
FIGURE 4 (A and B) Serial sections through a typical aortic plaque of a rabbit fed the atherogenic diet and treated simultaneously with 20 mg LaCl₃/kg body wt. (A) Gomori trichrome-aniline blue, showing lack of collagen accumulation, marked inhibition in the increase of intimal cellularity (IC) and of elastica (El) derangement. B: Yasue's-light green, showing absence of calcification. ×102.

BIOCHEMICAL RESULTS

Aorta. The biochemical changes observed in intima-media from whole aorta were in agreement with the structural alterations in arteries. Table II summarizes the changes in the content of aortic components for each group. As compared to normal control animals, animals given the atherogenic diet without lanthanum showed large increases in aortic collagen, elastin, free and ester cholesterol, calcium and nonlipid phosphorus. In contrast, animals on the atherogenic diet treated with lanthanum revealed a progressive reduction of these aortic components with increasing dosage of LaCl₃. Lanthanum in the amount of 40 mg LaCl₃/kg body wt abolished completely the effect of the atherogenic diet, with the exception of the aortic cholesterol content, which still was slightly but significantly above normal. The content in aortic components of lanthanum-treated control rabbits was not significantly different from that of normal controls. Changes in the concentration of these aortic components (milligram per gram dry intima-media) followed a similar pattern. As compared

to normal control animals, untreated atherogenic animals revealed highly significant increases ($P < 0.01$) in the concentration of collagen, cholesterol, calcium, and phosphorus and significant increases ($P < 0.05$) in the concentration of elastin. With increasing LaCl₃ dosage these atherogenic changes became progressively smaller, revealing no significant difference to normal controls values for all components at the 40-mg dosage level except for a slightly higher cholesterol concentration. The values for LaCl₃ treated controls also remained normal.

Table III shows the constituents of isolated elastin preparations of aorta. In rabbits fed the atherogenic diet without lanthanum there were large increases in elastin cholesterol, calcium, nonlipid phosphorus, and in the polar amino acids: aspartic and glutamic acid, lysine, and arginine; the remaining amino acids were comparable to those of elastin from normal control aorta. Simultaneous treatment of animals on the atherogenic diet with increasing doses of lanthanum progressively suppressed these alterations of elastin and prevented them completely at the dosage of 40 mg
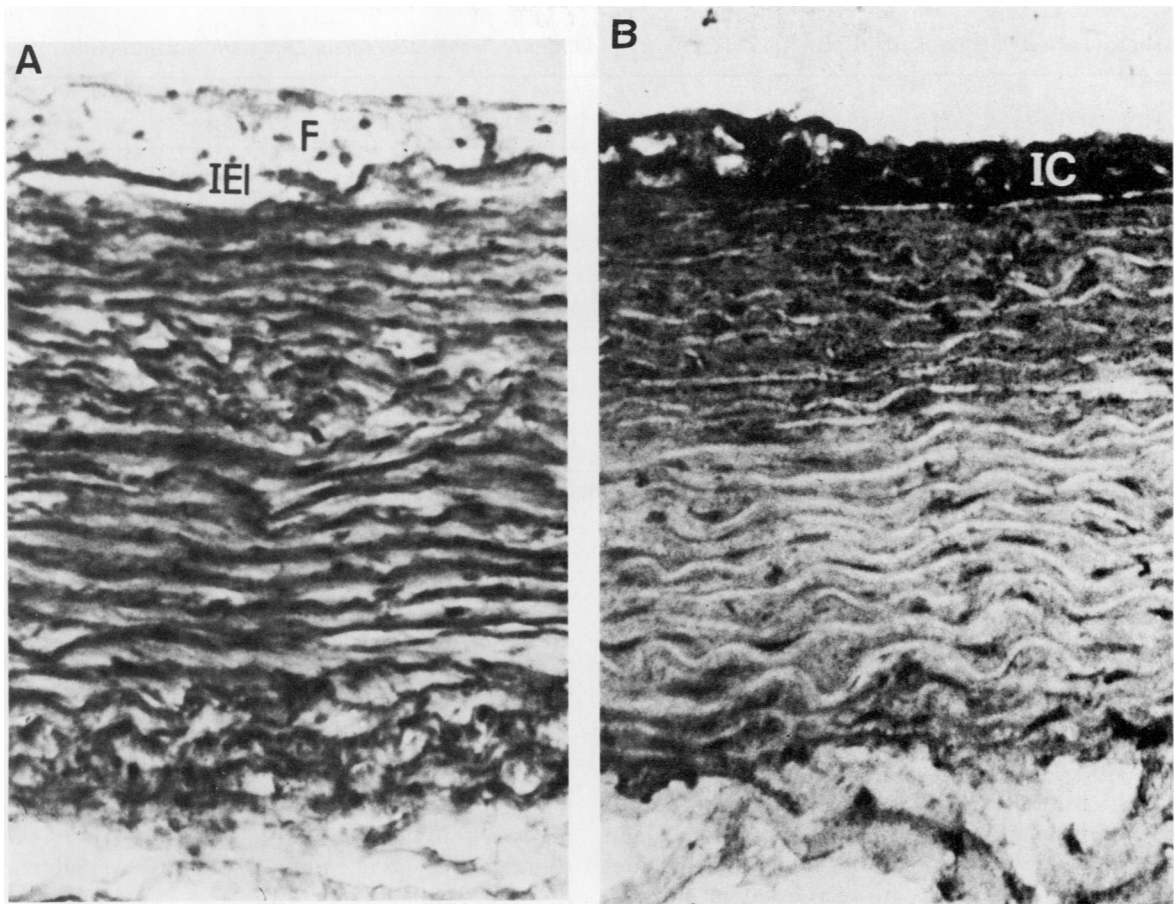
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FIGURE 5 (A and B) Serial sections through a representative plaque from rabbits fed the atherogenic diet and treated with 40 mg LaCl₃/kg body wt. (A) Gomori trichrome-aniline blue, showing an essentially normal aortic wall except for an intima which was minimally raised by foam cells (F) overlying an unbroken internal elastica (IEl) (cf. Fig. 2). (B) Hematoxylin-Oil Red O, showing lipid deposition (black) predominantly in the minimally increased intimal cells (IC). ×102.

LaCl₃/kg body wt. The aortic elastin constituents of lanthanum-treated control rabbits were comparable to those of normal aortic elastin from untreated controls.

Other organs. Table IV lists the content in collagen, elastin, and cholesterol in organs other than arteries. With the exception of the elevated cholesterol of lung and skin in rabbits on the atherogenic diets, there was no significant difference in the content of these constituents in lung, skin, heart, and skeletal muscle between the experimental groups. Likewise, the composition of elastin from lung and skin of untreated and lanthanum-treated control and atherogenic animals was comparable. The findings indicate that the collagen and elastin of these organs was neither affected by the atherogenic diet nor lanthanum. As shown in Fig. 6 the urinary excretion of hydroxyproline decreased significantly from normal levels in animals fed only the atherogenic diet. This decrease presumably represents decreased turnover as well as

increased deposition of body collagen, apparently to a large part into arteries. On the other hand, with increasing doses of LaCl₃ there was a further decrease in urinary hydroxyproline excretion which was about in the same order of magnitude in control animals fed 40 mg LaCl₃ as in atherogenic animals receiving that dose. However, this decrease may represent decreased collagen synthesis or secretion induced by La³⁺ in synthesizing cells, especially in arteries.

The mean content of lipids in liver of rabbits fed only the atherogenic diet increased markedly above normal (total cholesterol rose 771% with the main increases being ester cholesterol; triglycerides rose 285% and free fatty acids 256%). No significant changes occurred in lanthanum-fed control animals, indicating that lanthanum treatment at this dosage level did not cause fatty degeneration of liver. On the contrary, with increasing dosage of LaCl₃ the liver lipid content decreased progressively in atherogenic rabbits, with

TABLE II

Components of Aortic Intima-Media from Control Rabbits and Rabbits on the Atherogenic Diet with and without Lanthanum

Experimental groups	Collagen	Elastin	Total cholesterol	Percent ester cholesterol	Total calcium	Nonlipid phosphorus
Control diet	2.8±0.4	10.3±2.8	0.3±0.2	7±2	0.03±0.007	0.04±0.02
Atherogenic diet without LaCl₃	6.5±1.3*	22.1±1.7*	3.5±1.2*	50±6*	0.06±0.012*	0.09±0.03*
Atherogenic diet + 20 mg LaCl₃/kg body wt	4.2±0.6*	17.3±1.9*	1.9±1.0*	55±12*	0.04±0.010‡	0.06±0.01‡
Atherogenic diet + 30 mg LaCl₃/kg body wt	3.6±0.6‡	14.1±2.6	1.4±0.7*	42±5*	0.03±0.010	0.04±0.03
Atherogenic diet + 40 mg LaCl₃/kg body wt	2.9±0.8	13.6±4.7	0.6±0.1‡	19±6*	0.03±0.008	0.05±0.02
Control diet + 40 mg LaCl₃/kg body wt	2.4±0.9	10.4±2.0	0.1±0.1	N.D.§	0.02±0.011	0.03±0.03

Absolute amounts in milligram per whole aorta per kilogram body weight; mean±SD.
* $P < 0.01$ compared to untreated control.
‡ $P < 0.05$ compared to untreated control.
§ Not detectable.

triglycerides and free fatty acids being normal at the 40 mg/kg dose. However, the mean liver cholesterol content still was elevated 292% at this treatment level, indicating that lanthanum as given inhibited only moderately cholesterol deposition in susceptible and accessible cells such as hepatocytes and other cells.

No significant differences were detected between the experimental groups in the content of calcium and inorganic phosphorus in heart, skeletal muscle, skin and bone, indicating that the structural and functional calcium and phosphorus levels of these organs may have remained unchanged by the athero-genic diet and/or lanthanum treatment. These findings were corroborated in special studies.

Special studies

Fig. 7 shows the content of ATP and creatine phosphate in heart and skeletal muscle. There was no significant difference in the level of these high energy phosphates in either organ between normal and lanthanum-fed controls and animals on the atherogenic diet. Likewise, oral lanthanum had no detrimental effect on cardiac function as evidenced by the absence

TABLE III

Constituents of Elastin from Control Rabbits and Rabbits on the Atherogenic Diet with and without Lanthanum

Experimental groups	Total cholesterol	Total calcium	Nonlipid phosphorus	Aspartic acid*	Glutamic acid*	Lysine*	Arginine*
	mg/g elastin; mean±SD			Residues/1,000 residues; mean± SD			
Control diet	10.4±2.3	0.8±0.3	0.8±0.07	2.1±0.5	13.5±1.2	4.0±0.2	5.1±0.3
Atherogenic diet without LaCl₃	37.4±8.5‡	2.1±0.7‡	1.9±0.24‡	13.1±2.3‡	31.1±7.6‡	9.3±1.8‡	8.9±0.9‡
Atherogenic diet + 20 mg LaCl₃/kg body wt	24.6±9.7‡	1.5±0.3§	0.9±0.11§	4.1±0.9‡	17.0±1.8‡	3.8±0.3	5.8±0.4§
Atherogenic diet + 30 mg LaCl₃/kg body wt	11.5±1.6	1.2±0.3	0.8±0.13	2.2±0.4	14.6±0.8	3.6±0.6	5.4±0.2
Atherogenic diet + 40 mg LaCl₃/kg body wt	8.9±2.5	0.7±0.2	0.6±0.29	2.4‖	12.6‖	3.9‖	5.3‖
Control diet + 40 mg LaCl₃/kg body wt	8.5±3.7	0.9±0.1	0.5±0.30	2.4±0.3	13.9±0.7	4.2±0.4	5.5±0.3

* The remaining elastin amino acids were not significantly different in all groups.
‡ $P < 0.01$ compared to untreated control.
§ $P < 0.05$ compared to untreated control.
‖ Average of three elastin samples.

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TABLE IV
Content of Collagen, Elastin, and Cholesterol in other Organs of Control Rabbits and Rabbits on the Atherogenic Diet with and without Lanthanum

Experimental	Lung			Skin			Heart Collagen	Skeletal muscle Collagen
	Collagen	Elastin	Cholesterol	Collagen	Elastin	Cholesterol		
				mg/gddt*; mean±SD				
Control diet	33.0±8.5	0.02±0.009	21.7±9.7	10.6±2.1	2.1±0.5	53.8±11.5	35.5±11.8	13.7±2.1
Atherogenic diet without LaCl₃	29.5±6.7	0.03±0.012	99.8±8.7‡	11.7±0.5	2.0±0.6	256.2±106.8‡	36.7±8.9	11.3±4.1
Atherogenic diet + 20 mg LaCl₃/kg body wt	41.0±9.5	0.02±0.005	70.2±9.5‡	—	—	—	41.3±5.3	15.7±4.6
Atherogenic diet + 30 mg LaCl₃/kg body wt	43.5±9.8	0.03±0.013	49.0±6.5‡	—	—	—	38.4±10.7	14.8±3.7
Atherogenic diet + 40 mg LaCl₃/kg body wt	31.3±4.3	0.03±0.014	36.0±7.4§	—	—	—	35.3±11.8	—
Control diet + 40 mg LaCl₃/kg body wt	40.7±7.9	0.03±0.013	19.8±7.3	9.9±3.7	2.4±0.7	46.4±18.1	—	8.7±4.9

* Gram dry defatted tissue.
‡ $P < 0.01$ compared to untreated control.
§ $P < 0.05$ compared to untreated control.

of significant differences in heart rate, left ventricular systolic pressure and dp/dt with cross-clamping and with postextrasystolic potentiation. Further evidence, derived from current studies in our laboratory with exercising non-human primates,[3] showed that oral lanthanum, even in the highest doses given, does not appear to adversely affect heart and muscular funtion and coordination. Cynomolgus monkeys fed 40 mg LaCl₃/kg body wt per d have run to date, 1 h daily for over 10 mo, in a treadmill without detectable difference in performance to untreated controls.

The preliminary lanthanum determinations in monkey revealed that about 65% of the daily ingested lanthanum was excreted in the feces, much of which may not have been absorbed. Small amounts (average:
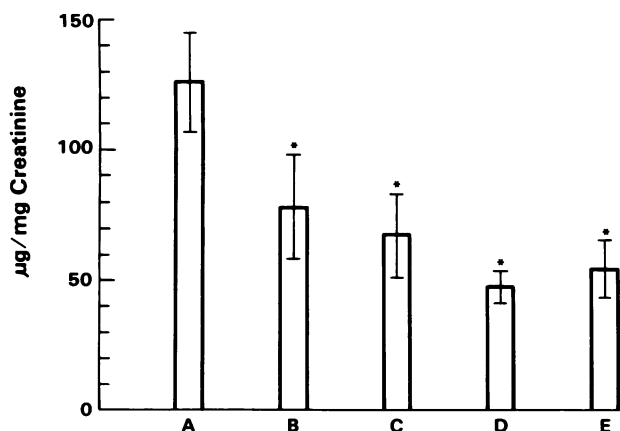
[3] Unpublished results.

FIGURE 6   Urinary hydroxyproline excretion of the experimental groups; in μg/mg creatinine, mean±SD. (A) Control diet, 126±19; (B) Atherogenic diet without LaCl₃, 78±20; (C) Atherogenic diet plus 20 mg LaCl₃/kg body wt, 67±16; (D) Atherogenic diet plus 40 mg LaCl₃/kg body wt, 47±6; (E) Control diet plus 40 mg LaCl₃/kg body wt, 54±11. (*) $P < 0.01$ compared to untreated control.

A: Control diet, no LaCl₃
B: Atherogenic diet + 40 mg LaCl₃
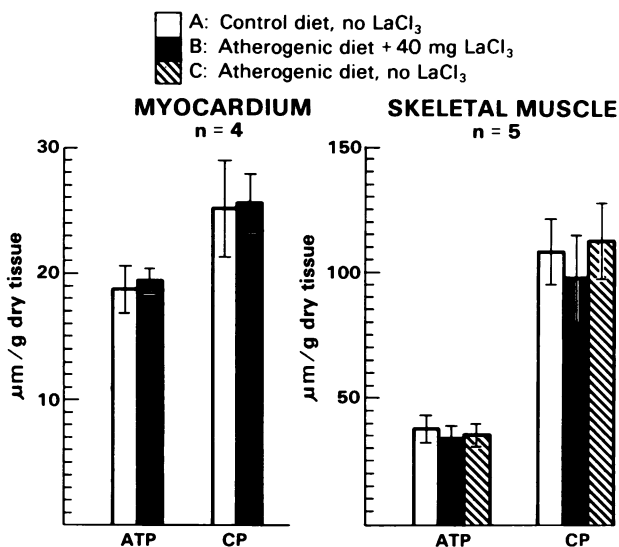C: Atherogenic diet, no LaCl₃

FIGURE 7   Content of ATP and creatine phosphate (CP) in myocardium and skeletal muscle of the experimental groups; in micromolar per gram dry tissue, mean±SD. (A) Control diet without LaCl₃; (B) Control diet plus 40 mg LaCl₃/kg body wt; (C) Atherogenic diet without LaCl₃. Myocardial ATP: A, 18.7±1.9; B, 19.4±1.0. Myocardial CP: A, 25.1±3.8; B, 25.5±2.3. Skeletal muscle ATP: A, 37.5±5.5; B, 33.5±5.3; C, 34.9±4.2. Skeletal muscle CP: A, 107.6±13.3; B, 97.1±17.5; C, 111.5±14.9.

64.5 μg) were contained in 24-h urines. 4 h after a test meal an average of 17.1 μg La³⁺/100 ml were found circulating in whole blood; none after 12 h. After 8 wk of treatment with 40 mg LaCl₃/kg body wt about 33 μg La³⁺ (average) were found in whole liver of rabbit; surprisingly, none was detected in bone. Liver and bone, body fluids, and feces of untreated animals contained no detectable lanthanum.

DISCUSSION

As reviewed by Weiss (22, 23), lanthanum has been shown to displace and replace Ca²⁺ on certain selected cell membrane loci, thereby blocking some—but not all—cellular influx and efflux of Ca²⁺, including arterial SMCs. Although its location in the tissues appears to be confined to the extracellular space and adjacent membrane surfaces, larger amounts of La³⁺ profoundly inhibit in vitro important cellular responses in which Ca²⁺ plays a significant role: the excitation-contraction coupling in muscle (skeletal, cardiac, and smooth muscle); the stimulation-secretion coupling of many secretory processes; the impulse discharge and conduction of nerve cells; and it also may inhibit the stimulus-division coupling of cell mitosis (44).

In this study, lanthanum treatment did not lower the elevated serum cholesterol and the Ca²⁺ levels in rabbits fed the atherogenic diet, but it prevented the rise in arterial calcium content and at the same time suppressed all major processes of atherosclerosis. Comparable antiatherosclerotic effects of similar doses of oral lanthanum recently have also been found in non-human primates on an atherogenic diet.[4] Although a direct effect of La³⁺ on arterial tissue cannot be entirely ruled out to explain the inhibition of atherogenesis in this study or the monkey studies,[4] there is no evidence available to support this possibility. No direct effects of La³⁺ on tissue metabolism have as yet been reported despite an abundance of in vitro studies (22, 23) with lanthanum as a tool in exploring the tissue activities of Ca²⁺.

On the other hand, because of the known importance of Ca²⁺ on many cellular functions and the known specific Ca²⁺-antagonistic effects of La³⁺ in vitro, the results of our in vivo studies are not surprising. Many of the atherogenic processes need calcium-dependent energy derived from high energy phosphates such as ATP and GTP. It is very likely that the marked increase in ionized serum calcium and total calcium in atherosclerotic aortas of cholesterol-fed animals not receiving lanthanum treatment, may have provided excessive amounts of free Ca²⁺ for an increased Ca- and Mg-dependent ATPase activity. In any cell containing

contractile proteins, including vascular SMCs, the relaxation-contraction cycle is dependent upon the myoplasmic concentration of Ca²⁺ (45), with increasing amounts of Ca²⁺ leading to increasing contractability. Moreover, a gradient of extracellular calcium ions appears to be instrumental in the directional movement of cells such as in chemotaxis (46). These cellular responses to focal increases in Ca²⁺ content may account for the migration of cells into the atherosclerotic intima. The contraction of vascular endothelial cells also appears to be influenced by Ca²⁺ (20), with increasing contraction presumably resulting in increased permeability of the endothelial barrier to blood-born elements such as lipoproteins as well as platelets and leucocytes. Substantial numbers of platelets and monocytes (circulating macrophages) have been found in experimental arterial lesions (1, 47). The adhesion of platelets to connective tissue, their aggregation and release reaction also appear to be Ca²⁺-dependent (48). These reactions of platelets exposed to arterial collagen are thought to be important factors in the initiation of atherogenesis (1) as well as in the thrombotic complications of late atherosclerosis (49).

Calcium ions have been implicated as intracellular messengers in the regulation of other important cellular functions such as secretion of proteins (50), the control of hormone-induced lipolysis and of several enzymes involved in lipomobilization (51) as well as of mitosis (52, 44). Ready availability of Ca²⁺ and its unrestricted influx into cells may facilitate excessive mitotic responses to invading mitogenic stimuli leading to intimal cell proliferation. Ca²⁺ also appears to mediate cellular functions operative in the synthesis and degradation of connective tissue. The anticalcific agent ethane-1-hydroxy-1, 1-diphosphate, which has been shown to inhibit calcium transport through cellular membranes (53), also inhibits proline hydroxylation in collagen (54), a necessary step for the secretion of procollagen from collagen synthesizing cells (55). Ca²⁺-dependent mechanisms, which may be responsible for the excessive secretion of elastin (56) and glycosaminoglycans in lesions are currently being studied by us in cultures of arterial SMCs. Monocyte-derived macrophages presumably are capable of degrading connective tissue proteins due to their high content in proteolytic enzymes, including collagenase and elastase (57). The elastolytic activity of macrophage elastase was inhibited by the calcium chelator EDTA (58).

There is abundant evidence that calcium also may play a direct role in many important extracellular events in atherosclerosis. Ca²⁺ has been suggested to be responsible for the binding of human plasma low density and very low density lipoproteins to sulfated glycosaminoglycans extracted from arteries, to form

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[4] Kramsch, D. M. and A. J. Aspen. Presented at the Fifth International Symposium on Atherosclerosis, Houston, Tex., November 1979.

insoluble complexes (59). Calcium also appears to alter other arterial matrix macromolecules. It has been demonstrated to cause configurational changes in arterial elastin in vitro, exposing hydrophobic sites and giving rise to increased absorption of other hydrophobic molecules such as cholesterol (60); at appropriate concentration and ionic strength it significantly increases elastolysis by pancreatic elastase;[5] and it appears to be instrumental in the binding of secondary, mostly polar proteins to lesion elastin either through Ca^{2+} bridges or simply by incrustation of whole elastin fibers with calcium minerals (61). Elastin preparations isolated from atherosclerotic lesions by hot alkali invariably have an increased content of polar amino acids (31), which appears to be the prerequisite for an increased binding of cholesterol esters to the abnormal elastin protein (62). Robert et al. (17) recently demonstrated that treatment with (thyro)-calcitonin can prevent the binding of other proteins to elastin as well as the development of entire immunogenic lesions in arteries.

Calcium also is prominently involved in the early mineralization of focal arterial lesions, including in atherosclerosis. The principal target tissue for beginning arterial calcification appears to be the elastica (2), which has been shown in vitro to calcify in preference over arterial collagen (63). Proposed nucleation sites for calcium mineralization include: the polar microfibrillar glycoprotein associated with the elastin (64), the likewise closely associated polar proteoglycans (65), or neutral peptide groups of the elastin itself (66). Calcium mineralization of collagen and other arterial constituents presumably are later events in the pathogenesis of atherosclerosis.

It is possible that the mechanisms involved in the graded arterial responses to the graded dosage increases of lanthanum in this study may be: (a) stepwise reduction toward normal in the available free Ca^{2+} concentration of arteries; (b) partial dislodging and replacement of Ca^{2+} by La^{3+} on selected superficial cell membranes sites; (c) restriction of excessive movements of calcium ions across cellular membranes into and out of pertinent cells; (d) and presumably inhibition of inordinate binding of calcium to macromolecules of the intercellular matrix (e.g., elastin, proteoglycans, and perhaps glycosaminoglycans). It is of interest, however, that even the largest dosage of lanthanum administered orally did not impair the general well-being, and the normal growth and development of the still growing animals under study. Specifically, all organ systems tested such as heart, arteries, skeletal muscle, lung, skin, bones, blood and, by inference, hemopoetic and nervous system, appeared to have retained their normal functional and

structural calcium and phosphorus. This may have been caused by the low absorption noted for oral lanthanum.

From the protective action displayed by the hormone thyrocalcitonin, which normalizes elevated calcium ion concentrations, Robert et al. (17) recently have postulated that homeostasis of calcium may be important in the prevention of immunogenic arterial injury. Likewise, Fassina (51) recently stressed the importance of calcium homeostasis in the normal regulation of certain pertinent aspects of lipid- and potentially lipoprotein metabolism. This study supports the concept that maintenance of calcium homeostasis in arterial and other tissues may be an essential factor in the prevention of atherogenesis. Similar homeostatic mechanisms may have been operating in the protective action of other anticalcium agents used by us and others to inhibit atherogenesis, including thiophene compounds and diphosphonates. In this context it should be noted that thiophenecarboxylic acids appear to have a thyrocalcitonin-like effect (67). The findings suggest that agents capable of regulating functional calcium levels in arteries may offer alternative methods in the treatment of atherosclerosis, even when elevated serum cholesterol levels and hyperlipoproteinemia cannot be effectively controlled.

ACKNOWLEDGMENTS

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