Experimental calcification of the aorta in rabbits: Effects of chelating agents and glucagon

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Summary

Experimental calcification of the aorta, induced in rabbits by a single dose of 8 mg of dihydro-tachysterol (AT 10), represents an optimal model for studying vascular diseases caused by lipid and calcium deposition on the artery wall. This lesion may be treated with chelating agents and/or with glucagon.

The effect of chelating agents and/or of glucagon on lipid and calcium deposition involving the aortic wall was studied in 12 adult white Flemish rabbits. The animals were divided into four groups and in each of them experimental calcification of the aorta was induced by AT 10. The first group did not receive any treatment and served as control. The second group received only ethylenediamine tetraacetic acid (EDTA) (120 mg daily). The third group received only glucagon (0.1 mg subcutaneously). In the fourth group, the glucagon (0.1 mg subcutaneously) was injected with EDTA (120 mg daily). The animals were killed 30 days after suspension of therapy. The aorta was rapidly harvested in its thoracic tract.

The calcium content of the sclerosed aorta was determined by flame photometer, while the cytochemical detection of calcium was performed using N,N-Naphthaloyl-hydroxyl-amine. Lipid deposition was studied by Sudan black B staining. Usual laboratory staining (hematoxyline - eosine and Toluidin blue) were used for detection of structural details. All morphological results were submitted to the quantitative analysis of images and statistical analysis.

The results of the present study suggest that glucagon may play a role in the biochemical and morphological modifications usually found in atherosclerosis. In fact, macroscopic and ultrastructural evaluation of our morphological results suggest that the pathological changes observed may be restored by administration of glucagons and/or by chelating therapy.

Introduction

Over the last few years, chelating therapy (for some cardiovascular diseases) has come back in to fashion with contrasting opinions *(Lamas & Ackermann 2000; Laslett 2002; Villarruz et al. 2002; Anderson et al., 2003)*.

The best-known chelating agent is ethylenediamine tetraacetic acid (EDTA). This drug has various biochemical effects: inhibition of lipid peroxidation, cell membrane stabilization, calcium channel blockade and free radical scavenging *(Chappel et al, 1995)*.

The general decalcifying mechanism of EDTA may derive from its affinity for divalent ions, especially calcium. After calcium binding it produces readily soluble non-ionized salts which are quickly and completely excreted by the kidney. EDTA exerts a distinct inhibitory and decalcifying effect on calcium deposition in peripheral vascular disease *(Olszewer et al. 1990)*. Glucagon, a product of pancreatic cells, is a 29-aminoacid, single-chain

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polypeptide. It plays a role in human lipid metabolism *(Malinovska et al., 1998)*. The secretion of glucagon is regulated by dietary glucose, insulin, amino-acids and fatty acids *(Davis & Granner, 1996)*. Secretion is also regulated by the autonomic innervation of the pancreatic islet. Glucagon interacts with glycoprotein receptor on the plasma membrane of target cells *(Sheetz & Jager, 1988)* and its effects are mediated by cAMP. Glucagon administration produces a decrease of calcium level, which was initially explained by an increased secretion of calcium by the kidneys *(Zamir et al, 1993)*. Elimination of calcium ions by means of EDTA strongly increases glucagon activity while Ca++ ions have an inhibiting effect. These observations indicate the possibility of an elevated transmission of the glucagon signal from receptor to the catalytic enzymatic unit by means of calcium blockers and, at the same time, the favourable action of calcium blockers on calcium and lipid deposition in vessels. These considerations led us to combine the favourable effects of EDTA and glucagon on lipid and calcium deposition in the aortic wall.

Materials and Methods

In our experiments, 12 male adult white Flemish rabbits (age: 1.5 year, weight: 3 kg) were used. All the animals were treated in agreement with the Helsinki Convention on the use of animals in Medical Research and with the Council Directive of the European Communities on the Protection of Animals used for Experimental and other Scientific Purposes (L358/1, November 24, 1986). Food and water were provided “ad libitum” and the animals were caged at room temperature. The rabbits were divided into four groups, of three rabbits each. In all groups, experimental calcification of the aorta was induced by a single dose of 8mg dihydrotachysterol AT10 introduced via stomach-gavage.

EDTA, as chelating agent, and glucagons, as lipid catabolising agent, were used to treat this vascular disease (experimental calcification of the aorta in rabbits).The induction of experimental calcification of the aorta was verified "in vivo" by thoracic radiography and post mortem by autopsy.

The rabbits were divided into four groups.The first group served as control and did not receive any therapy. The second group was injected i.v. with EDTA (120 mg every day for ten days). The third group received only glucagon (0.1mg subcutaneously). The fourth group received glucagon (0.1mg subcutaneously) plus 120 mg i.v. of chelating agent EDTA.

Previous experiments have shown that extensive calcification of the aorta begins on the third day after the administration of AT 10 and that it persists during the following 60 days. After 30 days, all the rabbits were killed and the arterial wall was harvested for macroscopic and microscopic examination: the calcium contained in the aortic wall was cytochemically detected according to the method of Zechmeister (1979) while the calcium contained in the sclerosed aorta was examined by means of a Zeiss flame photometer.

Quantitative analysis of images:

Quantitative analysis of the intensity of the staining was performed by means of a Quantimet Analyser (Leica™). The control values from samples incubated without substrate were considered as “zero”. Quantitative analysis of images (QAI) may provide incorrect results, because the main choices (i.e. the instructions for software) are made by each operator, according to his/her personal preferences. Therefore, it is mandatory to follow careful rules: the counts must be repeated at least 3 times using the double-blind technique and should be performed by different people, on different analysers and with samples identified by a number or a letter. The final results are obtained by another scientist, who identifies each sample and attributes specific values that then undergo statistical analysis. The values obtained in our experiments represent the intensity of staining for each type of tissue and are expressed as Conventional Units (C.U.) ± standard error of the mean; further details on QAI, including the definition of C.U., can be found in the Manual

of the Quantimet Leica 500 image analyser (1997).

Statistical analysis of data:
The statistical analysis performed included basic statistical methods such as mean values, maximum and minimum limits, variations, Standard Deviation (SD), Standard Error of Mean (SEM) and correlation coefficients (1985). Correlation coefficients denote a significant level when $P<0.001$.

Results
Our results are summarized in Tables 1 and 2. Table 1 deals with morphological evaluation of calcification in the aortic intima of treated and untreated rabbits. The intimal surface of the aorta shows the macroscopic and microscopic changes clearly reported in the tables.

Table 2 shows the lipid deposition and the calcium evaluation in the aortic intima of treated and untreated rabbits. As can be seen, chelant therapy strongly reduces calcium deposition, while glucagon therapy strongly reduces lipid deposition. Combined therapy, chelating agent plus glucagon, is able to reduce both these two pathological parameters.

Glucagon and EDTA: effect on the arterial wall.
Macroscopic evaluation of the intimal surface of the aortic wall showed that in the control group the intimal surface of the aorta was totally calcified. On the contrary, in the second group there were areas of calcification, and in the third group the intimal surface of the aorta did not show any macroscopically visible changes (Tables 1 and 2).We may

Table 1. Morphological evaluation of the experimental calcification in the aortic wall of the treated and the untreated rabbits.

Groups of rabbits	Morphological changes of the intimal surface of the calcified aorta in rabbits, as observed in a light microscope.
I Untreated	Total calcification and degenerative changes of the myocytes.
II Chelant	Strong decrease of the calcification (no visibile).
III Glucagon	Strong decrease in lipid deposition.
IV Chelant plus Glucagon	No visible calcification, no lipid deposition.

Table 2 Combined evaluation of lipid and calcium deposition in experimental calcification in the aortic wall of the treated and the untreated rabbits.

Groups	*Lipid deposition (Sudan Nero B)	**Ca (flame photometer)	**Ca (biochemical detection)
I Untreated	38.4 ± 1.3	410.8 ± 10.2	315.4 ± 8.7
II Chelant	34.1 ± 1.6	160.6 ± 9.8	96.4 ± 6. 3
III Glucagon	10.2 ± 0.8	406.6 ± 11.4	314.1 ± 9. 3
IV Chelant plus Glucagon	6.8 ± 1.1	157.8 ± 10.3	90.3 ± 5.8

* Results are expressed as C.U. (Conventional Units see Methods) ± SEM
** Results are expressed as μg /mg of tissue (fresh weight).

conclude that glucagon influenced lipid deposits, whereas it affected calcium deposition much less.

Lipid and calcium deposition in the aortic wall: role of glucagon.

Lipid deposition in myocytes is followed by invasion of blood capillaries into the arterial wall. Numerous calcium deposits, in a disperse form or in the form of microcristalline precipitates, were found. In some smooth muscle cells of the media, signs of repair process could be seen towards the end of the study period.

Slight morphological changes were found in the aortic wall of the treated animals. Only few isolated lipid deposits were observed (Table 1 and 2).

Discussion

Our results demonstrated that glucagon alone substantially reduces infiltration of lipids in the aortic wall, but its action is less effective on calcium deposits. EDTA substantially reduces the extent of calcium deposits, but is less influential in lipid infiltration.

Since EDTA also reduces the content of neutral lipids in the blood serum, it may enhance the effect of glucagons *(Olszewer et al., 1990; von Rij et al., 1994)*. On the other hand, apart from its major effect (prevention of lipid inclusions into the aortic wall), glucagon promotes the effects of EDTA by reducing calcium deposits *(Chappel 1993)*.

In our experience, the combination of glucagon and EDTA appears to be useful for clinical practice too. However, the secondary effect of long term administration of EDTA on the permeability of the walls of blood vessels with the potential risk of hemorrhage, should receive further attention. The findings obtained from the second experimental model (used to analyze the possible role of glucagon on lipid and calcium deposition in the aortic wall), brought to light another example of a close relationship between lipid accumulation (during weaning) and calcium deposition in the arterial wall. Several mechanisms conducive to lipidosis of the arterial wall have been described: 1) increased infiltration of blood lipids; 2) increased synthesis of lipids; 3) decreased degradation of lipids; 4) disturbance of lipid flow across the vessel wall. Furthermore, free oxygen radicals may be implicated *(Cranton & Frackolton, 1984)*. It can be presumed that the fat droplets present in the myocytes of the aortic wall represent accumulated neutral lipids: cholesterol probably forms a part of these droplets. Glucagon administration played a role in the accumulation of lipids in the aortic wall as a lipolytic factor. It is probable that glucagon also influences the metabolic activity of myocytes in relation to lipids, since the lipolytic activity of myocytes was increased and as a result lipids in these cells decreased. This reduced damage to the structural elements of the arterial wall. Glucagon may even play a role in the synthesis of lipids in the arterial wall. It has also been found that the reversible hypolipidic effect of glucagon consisted of the transfer of plasmatic lipids and cholesterol from plasma to thrombocytes *(Caren & Corbo, 1970)*. The decreased calcium deposition in the arterial wall observed after glucagon administration also may be the consequence of a hypocalcemy caused by the glucagon.

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