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RESPONSE OF AORTA CONNECTIVE TISSUE MATRIX TO INJURY CAUSED BY VASSOPRESSIN-INDUCED HYPERTENSION OR HYPERCHOLESTEROLEMIA

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The aim of the study was to investigate the effect of two various atherogenic stimuli (vasopressin-induced hypertension or hypercholesterolemia) on the collagen and glycosaminoglycan (GAG) content in the internal or external part of both thoracic and abdominal aorta, which are differently susceptible to atherosclerosis. Experimental rabbits were divided into four groups: controls, animals injected with physiological saline or vasopressin at the dose of 1 IU/kg from the 1st to the 25th day of experiment, respectively. The animals from group 4 were maintained on food, containing 0.25% cholesterol. Only in the vasopressin-treated group, the systolic blood pressure was elevated from 110 mmHg at the beginning, to 166 mmHg at the end of the study. After 14 weeks the aorta was dissected into internal and external parts. GAG fractions were separated and estimated as uronic acids. Collagen was evaluated as the hydroxyproline content in the tissue. Augmented total GAG and heparan sulphate (HS) level, plus no changes in the collagen content were seen in the internal part of the thoracic aorta in rabbits with hypercholesterolemia or hypertension. In the hypertensive animals, the changes were extended to the external part of the aorta and, additionally, comprised the elevation of the chondroitin-4 sulphate (C-4S) content. The two atherogenic stimuli increased the collagen level with no elevation of the GAG content in the abdominal aorta. A convergent effect of the injury, caused by hypertension or hypercholesterolemia on the collagen, total GAG and HS content was shown in the respective parts of the rabbit aortas. The common GAG, increased in the thoracic aorta, stand for the HS, in both hypertensive and hypercholesterolemic rabbits. As the sensitivity to atherosclerosis development in different segments of the aorta varies, they express various responses of the connective tissue matrix to injuries, caused by hypertension or hypercholesterolemia.

Key words: *atherosclerosis, connective tissue, collagen, glycosaminoglycans, heparan sulphate.*

INTRODUCTION

A hemodynamic stress and hypercholesterolemia, are known to be the main mechanisms, leading to vascular injuries, followed by reparative processes. The repair may promote early lesions with a subsequent advanced plaque

formation (1, 2, 3). Collagen accumulation is elevated in early atherosclerotic changes (1), comprising from 30 to 60% of the dry weight of advanced plaques (2). An increase in the collagen level was seen in reparative processes, following injury (4) and the progession of atherosclerosis (1, 2). The enhancement of glycosaminoglycans (GAG) and alteration of their pattern and properties in an artery play a great role in atherosclerosis developmet (5—7). GAG traps lipoproteins and forms with them complexes which are accumulated in the arterial wall. This process increases the intracellular ammassion of lipids. On the contrary, heparin (H) or heparan sulphate (HS) show an anticoagulant activity, inhibit growth of the smooth muscle cells and trigger the release of lipoprotein lipase (8).

The early progression-prone atherosclerotic lesions with a lipid accumulation in the intima can develop into advanced plaques, in which damages of the arterial wall structure, with a connective tissue accumulation are observed. Early progression-resistant lesions, which are morphologically similar with the progression-prone changes, do not progress or progress rather slowly. The mechanisms, involved in the transformation (or in the absence of transformation) of any early lesion into an advanced one, have not been explained so far (1). Early lesions appear first in the thoracic segment of the aorta (progression resistant location). However, in the abdominal part, early atherosclerotic changes are seen rather late but advanced plaques develop fairly quickly (1). The discrepancy between the predominant localisation of early atherosclerotic changes (thoracic aorta) and advanced arterosclerotic plaques (abdominal aorta) suggest a different susceptibility of the aorta to the development of advanced atherosclerotic plaques (1).

The concept of a non-specific mezenchymal reaction assumes a uniform response of extracellular matrix components, evoked by various irritants acting on the tissue. Kittlick suggested that, in different models of connective tissue development (skin wounds, cellulose sponge induced granuloma, damaged aortic wall), similar mezenchymal reactions were provoked (9). However, some experiments have shown that the response of the arterial wall to an injury depends on the involved vessel, the kind of stimulus and the species of experimental animals (10). The different experimental conditions and contradictory results, met in the literature (2, 11—15), do not allow to asses if the connective tissue response to injury, in both abdominal and thoracic segments, is uniform. In consequence the study was performed to define of the connective tissue response to injury caused by hypertension or hypercholesterolemia in the abdominal and thoracic aorta. We wanted also to verify working hypothesis that expected differences in repair process followed atherogenic injuries in various aortal segments are linked with their different sensitivity to atherosclerotic lesions development. Moreover, the study is aimed at explanation of the question, whether, irritants of different physiological

nature could cause the same reparative response of the corresponding aortal segments. This means assessing, content of collagen and glycosaminoglycans in the external and internal parts in the both thoracic and abdominal aorta.

MATERIALS AND METHODS

Study design

Forty male New Zealand rabbits, weighing 2.5 kg each were housed with a free access to commercial food pellets, as well as tap water ad libitum, being kept in light (L)-dark (D) conditions L:D = 12:12. The animals were divided into four groups: Group 1, intact control; group 2, injected with 0.9% solution of NaCl; group 3, vasopressin-administered animals; group 4 maintained on food containing 0.25% cholesterol.

On the 100th day of the experiment, the animals were sacrificed and the aortal wall was removed and cleaned from the connective tissue of the adventitia. Both the thoracic and abdominal aortas were separated 1 cm above the coeliac trunk. The two parts were then dissected into internal (intima and adjacent media) and external (media) layers of the aorta (15). All the oartas were microscopically controlled.

Systolic blood pressure was measured in conscious rabbits on the 1st, 50th and 100th day of the experiment (16). The hair of the right posterior limb was cut off in the internal surface and a 2 cm wide cuff connected with a manometer was installed on the upper part of the limb. Lower, a photocell was installed over the artery, as a pulse detector.

Drugs applied

The rabbits of the groups 2 and 3 were applied 25 injections (once daily) from the 1st to 25th day of experiment. In group 2, a 0.9% NaCl solution was injected into the margin vein of the rabbit ear in the volume of 0.1 ml/kg of body weight. Arginine vasopressin was dissolved in physiological saline and intravenously administered at the dose of 1 IU in 0.1 ml physiological saline/kg once daily (group 3).

Microscopic study

Slices, 8 μm thick, obtained from both the thoracic and abdominal aortas, were stained with hematoxylin and eosin and microscopically investigated under magnification of 100×.

Cholesterol estimation

In order to estimate total cholesterol 8 ml blood samples from the rabbit hearts were taken on the 100th day of the experiment for a biochemical analysis, according to the enzymatic method. The blood samples were collected after overnight fast being allowed to clot at room temperature. Serum was recovered by a low-speed centrifugation and kept frozen at −20°C until the examination. Serum total cholesterol was measured with the classic enzymatic methods (bio Merieux kits).

Determination of collagen

The total collagen was estimated, according to the Woessner's method as described earlier (17). An macerated tissue was extracted with ether-aceton and vacuum dried at 90°C. Samples of the total collagen were assayed for hydroxyproline by hydrolysis with 6 N HCl (3 ml/10 mg of dry tissue) at 110°C for 24 h. After hydrolysis, all hydrolizates were evaporated to dryness in a water bath and the precipitates were dissolved with 3 ml of redestilled water. The samples, neutralised by 1 N NaOH , were diluted to 10 ml with redestilled water. From the tubes, 0.2 millilitre samples were taken for a further analysis and diluted with redestilled water to 2 ml of the final volume. Hydroxyproline was oxidised to pyrrole by 1.25 ml of chloramine T in a citrate buffer (pH = 6.0) then shaken for 5 min and incubated for 20 minutes at 20°C. In order to remove the excess of chloramin T, 1.25 ml of 3.15 M perchloric acid was added. After 5 minutes, the samples were treated with 1 ml of 20% p-dimethylaminobenzaldehyde and incubated in a 60°C bath water for 20 minutes. The optical density was measured at 560 μm on a spectrophotometer.

Detemination of glycosaminoglycans (GAG)

The GAG were determined as uronic acids, according to Antonopoulos' (18) method, with Mier and Wood's modification (19). The samples were homogenised, deffated with an ether-acetone mixture (3 : 1) and the residue dried to constant weight at 90°C. The tissue was digested at 37°C in a solution, containing 100 mg of DL-cysteine and 186 mg of EDTA in 50 ml of 0.2 CH_3COONa. Then pH was adjusted to 6.8 with 1 N of NaOH. The material was then digested at 65°C (for 16 h) with papain (20 mg per sample). 0.5 ml of 50% TCA was added to each sample, and after centrifugation (4000/min during 30 min), the supernatant was dialysed against redestilled water for 12 h. A 5% solution of kalium acetate in ethanol was added to each dialysate and incubated for 24 h, at 4°C. After precipitation and centrifugation, the sediment was dissolved in 0.5 ml of 0.075 M NaCl. The GAG fractions were separated by column chromatography with cellulose powder CF-11. Each sample (0.2 ml) was put into the column (16 mm high and with diameter of 3 mm). The columns were successively eluted with 1% w/v aqueous CPC; 0.3 M NaCl and then with $MgCl_2$ solution, starting at 0.28 M and gradually increasing to 1.2 M. All the salt solutions contained 0.05% CPC. Uronic acids were determined by the carbasole method of Bitter's and Muir's (20): For further analysis, 1 ml samples of each GAG fractions: hialuronic acid (HA), heparan sulphate (HS), dermatan sulphate (DS), chondroitin-4sulphate (C-4S), chondroitin--sulphate (C-6S), heparin (H) were taken. 5 ml reagents were prepared from 100 ml H_2SO_4, (d = 1.84) and 9.53 g natrium tetraborate was added to each tube and cooled on ice. The samples were placed in a bath with boiling water for 15 min and, after cooling, 0.2 ml of carbasol was added to each sample. After mixing, the tubes were heated in a boiling water bath for 10 min. The optical density was read at 530 μm on a specol spectrophotometer.

Statistical analysis

The U Mann-Whitne's test was used for statistical analysis. Statistical differences between the groups were evaluated by Kruskal-Wallis's test.

RESULTS

Systolic blood pressure

On the first day of the experiment the systolic blood pressure was similar in all the investigated groups and ranged from 108.3 to 112 mmHg. However, in the vasopressin-applied animals, a marked enhancement of the systolic blood pressure up to 206 mmHg ($p < 0.001$), was seen on the 50 th day of the study. Hypertension in the vasopressin group (166 mmHg; $p < 0.01$) was still present on the last day of the experiment, in spite of the fact that vasopressin injections were stopped on the 25 th day. The systolic blood pressure in other groups was almost the same during the whole period of the experiment (*Fig. 1*) and no statistical differences were noted between the groups.

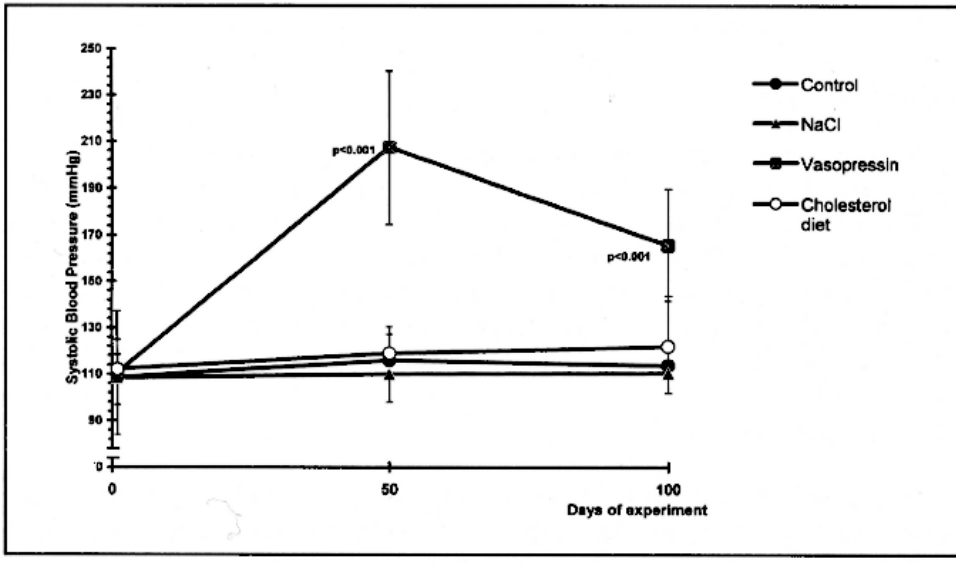

Fig. 1. Systolic blood pressure of the intact rabbits (Control), injected with NaCl (NaCl) or vasopressin (Vasopressin) and in the rabbits mantained on food containing 0.25% cholesterol (Cholesterol diet). Each bar represents the mean ± SD of eight samples.

Cholesterol concentration

Total cholesterol in the serum of the rabbits, maintained on food containing cholesterol, increased 5-times, comparing to the control and other groups ($p < 0.001$). No alterations of the cholesterol level were observed in the control, NaCl or vasopressin treated animals (*Fig. 2*).

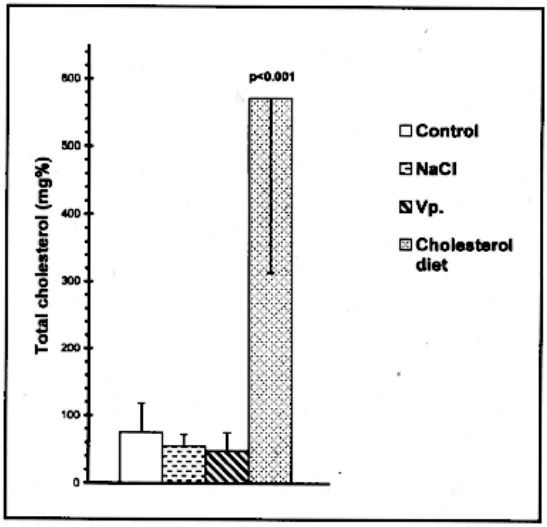

Fig. 2. Concentration of total cholesterol in plasma of the intact rabbits (Control), injected with NaCl (NaCl) or vasopressin (Vasopressin) and in the rabbits mantained on food, containing 0.25% cholesterol (Cholesterol diet). Each bar represents the mean ± SD of eight samples.

Collagen content

Vasopressin-induced hypertension does not exert any effect on the collagen level in the thoracic aorta. However, in the thoracic segment collagen content tends to decrease in hypercholesterolemic rabbits ($p = 0.053$). Both hypertension ($p < 0.05$) or hypercholesterolemia ($p < 0.025$) augmented the collagen content in the internal part of the abdominal aorta. Moreover, the collagen level elevation was visible even in the external part of the abdominal segment in the vasopressin group ($p < 0.025$) (*Fig. 3*).

Glycosaminoglycans (GAG) content

Hypertension and hypercholesterolemia increased GAG content in the internal part of the thoracic aorta ($p < 0.05$). This phenomenon was also found in the thoracic segment of the vessel in hypertensive rabbits ($p = 0.02$). GAG level in the abdominal aorta was not altered in the two investigated groups comparing with the intact and NaCl injected controls (*Fig. 4*). Heparan sulphate (HS) level was increased in the internal part of the thoracic aorta in hypertensive and hypercholesterolemic ($p < 0.05$) rabbits, as well as in the external part of the vasopressin-treated animals ($p = 0.005$). Chondroitin-4sulphate (C-4S) content was found to be elevated by hypertension in both the internal ($p < 0.02$) vnd external ($p < 0.025$) parts of the thoracic aorta. In the abdominal aorta, however, content of HS, C-4S was increated, comparing to the thoracic segment ($p < 0.001$) in all investigated groups (*Table 1*). The other fraction of GAG were not influenced by experimental conditions (data not shown).

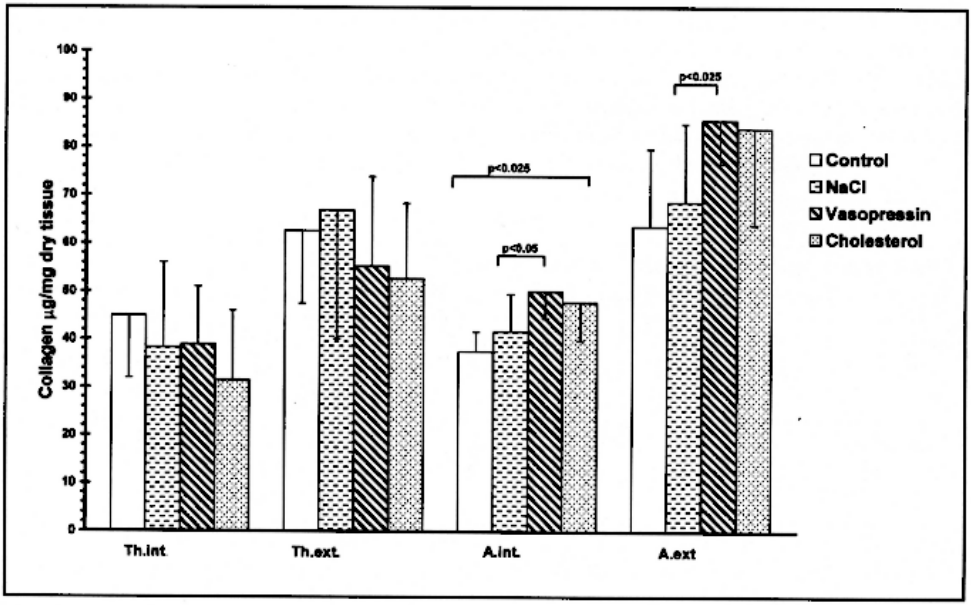

Fig. 3. Collagen content in internal (Th.int.) or external (Th.ext.) parts of the thoracic aorta and internal (A.int.) or external (A.ext.) parts of the abdominal aorta in the intact rabbits (Control), injected with NaCl (NaCl) or vasopressin (Vasopressin) and in rabbits mantained on food containing 0.25% cholesterol (Cholesterol). Each bar represents the mean ± SD of eight samples.

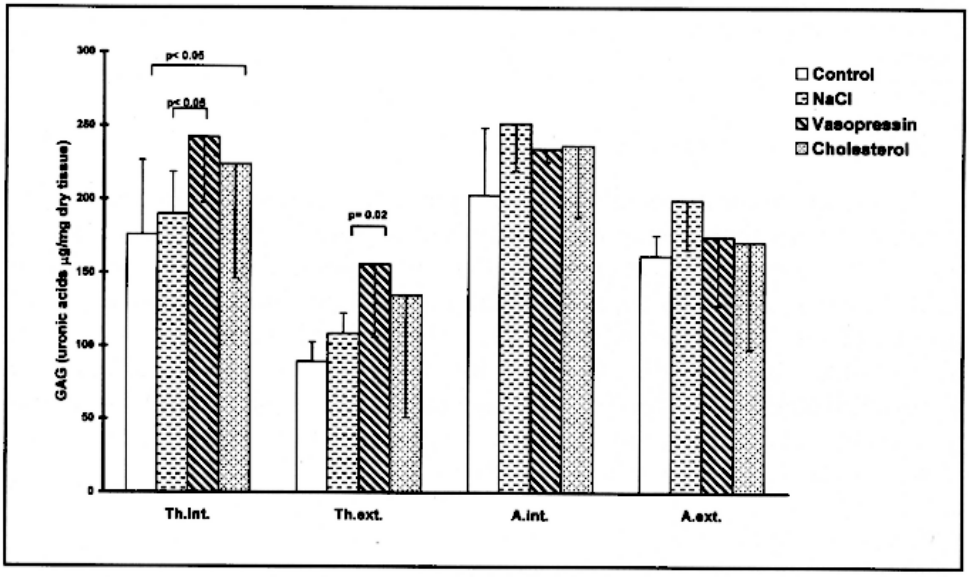

Fig. 4. Glycosoaminoglycans (GAG) content in internal (Th.int.) or external (Th.ext.) parts of the thoracic aorta and internal (A. int.) or external (A. ext.) parts of the abdominal aorta in the intact rabbits (Control), injected with NaCl (NaCl) or vasopressin (Vasopressin) and in the rabbits mantained on food, containing 0.25% cholesterol (Cholesterol). Each bar represents the mean ± SD of eight samples.

Table 1. Content of Heparan Sulphate (HS), Chondroitin-4Sulphate (C-4S) in the internal and external parts of the thoracic (Th) or abdominal (A) aorta of controls, NaCl or vasopressin-injected rabbits, as well as the animals maintained on cholesterol containing diet. Each value is expressed as the mean of 8 rabbits ± SD. For evaluation of the statistically significant differences of HS and C-4S level between the respective parts of the aorta the vasopressin treated rabbits were compared with NaCl applied animals, but cholesterol fed group with controls.

Groups:	Control		NaCl		Vasopressin		Cholesterol	
Toracic aorta (Th.)	Internal part of Th.	External part of Th.	Internal part of Th.	External part of Th.	Internal part of Th.	External part of Th.	Internal part of Th.	External part of Th.
Heparan Sulphate (HS)	25.03 ±6.51	16.31 ±5.18	23.9 ±5.42	15.58 ±4.17	32.19 ±6.84 ($p < 0.05$)	23.02 ±3.52 ($p = 0.005$)	35.07 ±4.39 ($p < 0.05$)	19.24 ±5.23
Chondroitin-4Sulphate (C-4S)	9.13 ±4.58	14.1 ±5.62	13.62 ±6.25	11.3 ±4	20.43 ±5.25 ($p < 0.02$)	20 ±9.49 ($p < 0.025$)	13.36 ±2.18	12.93 ±3.31
Abdominal aorta (A)	Internal part of A.	External part of A.	Internal part of A.	External part of A.	Internal part of A.	External part of A.	Internal part of A.	External part of A.
Heparan Sulphate (HS)	55.2 ±4.67	41.8 ±7.12	69.25 ±32.3	49.35 ±11.47	71.88 ±13.78	43.38 ±11.04	73.24 ±16.93	47.13 ±8.39
Chondroitin-4Sulphate (C-4S)	40.08 ±20.13	26.76 ±6.64	53.39 ±24.92	34.01 ±10.19	53.76 ±29.44	29.59 ±5.67	43.08 ±22.76	30.13 ±11.71

Histological studies (data not shown)

In the cholesterol fed rabbits, the foam cells stratified in adjacent layers associated with thickenning of the intima were found mainly in the thoracic aorta. The intima is as thick as the media and shows a higher cell density than in the control group. In the abdominal aorta a focal intima thickening, with groups of the foam cells, is visible. The thickness of the intima amounts to about 10%—20% of the media width. Only in the abdominal aorta of hypertensive rabbits a hypertrophy of the media with disturbed cell orientation was visible. NaCl injections do not alter the structure and thickness of the aorta, which is identical with the controls.

DISCUSSION

The model of prolonged hypertension, caused by vasopressin injection, used for the induction of atherosclerotic changes in blood vessels is well known (21) but mechanism of elevated blood pressure can not be explained. In spite of fact

that vasopressin is known to contract all types of vessels in rabbits, rats dogs and man (22), antidiuretic effect of the peptide or it influence via central neural pathways are also considered to be responsible for the development of hypertension (23). In our study, the systolic blood pressure was continuously elevated during the whole period of the experiment, even when vasopressin application after 25 injections was finished (*Fig. 1*). Vasopressin itself does not change the connective tissue content. All the alterations, in the blood vessels of rabbits with vasopressin-induced prolonged hypertension, were correlated with the duration of hypertension (21). The collagen content and the tensile strength of gastric wounds in the rabbits were not influenced by vasopressin treatment (24). Any prolonged effect of vasopressin-induced hypertension on the total cholesterol concentration in the plasma was not observed in that study (*Fig. 2*).

The cholesterol diet increased the total cholesterol concentration in the plasma (*Fig. 2*); (25) but the systolic blood pressure was not affected by hypercholesterolemia within the whole period of the experiment (*Fig. 2*). The diet with cholesterol applied to the New Zealand rabbits within 7 months, did not influence either the systolic or the diastolic blood pressure, which supports our results (25).

Injury of a blood vessel is reported to increase the collagen synthesis in the vessel wall (12, 13, 26). The main concept of advanced plaque formation suggests enhanced fibrosis as a response to the lipid deposits in the vessel and leading to fibrotic cap development (1, 2, 26). In our study, in the internal part of the thoracic segment, a tendency towards an inhibition of collagen accumulation by cholesterol diet is shown (*Fig. 3*). This phenomenon consegregates with a much higher lipoprotein deposition in the thoracic aorta than in the abdominal part. The correlation between cholesterol content in the aortal wall and collagen accumulation is not clear. Controversial data were obtained in experiments reporting cholesterol diet effect on collagen synthesis. Hence, elevation, retrardation or no effect of hypercholesterolemic conditions on collagen synthesis were found. Moreover, increased collagen sythesis by injury was inhibited by cholesterol enriched diet in rabbits aorta (14). Barne's hypothesis suggests that the immediate effect of hyperlipidemic conditions may inhibit collagen synthesis (14). The inhibition of collagen synthesis, *in vivo* (27) and in vitro (28), by hyperlipidemic conditions has already been reported and explained to be due to the cytotoxic action of lipids (14). Earlier studies have suggested various response of different anatomical segments of aorta to atherogenic irritans. Thus, progressive decrease in collagen synthesis along the aorta and elevated it synthesis in the thoracic segment have been proved in rabbits (15). Contrary to that, a higher prolyl hydrolase activity, increased in hydroxyproline concentration and a higher incorporation of ^{14}C-proline was found in the abdominal aorta of hypertensive rats, while in the thoracic segment, all those parameters were not changed (12, 13, 26). Irregular

distribution of collagen in early atherosclerotic changes was confirmed by Kraty and coworkers in human. The elevated level of that protein was seen in the intercostal and renal arteries but renal changes contained higher content of collagen (3). The present data (*Fig. 3*) refers to early atherosclerotic lesions and reports that increase of the collagen accumulation was seen only in the abdominal aorta known as more sensitive for advanced plaques formation; atherosclerosis-prone location, (1, 2). Thus, higher tendency to accumulation of collagen in the abdominal aorta has been proved. The two different in their nature stimuli such as hypertension or hypercholesterolemia exerted similar acceleratory effect on collgen accumulation in the abdominal aorta but this action was not seen in the thoracic segment.

Enhancement of GAG in different models of atherosclerosis was reported. The high content of total GAG, HS, chondroitin sulphate (CS) and DS was found in the balloon catheter-induced atherosclerosis (11), centrifuged smooth muscle cells or rats with genetic or experimental hypertension (29). However, pattern of GAG was dependent on the maturity of changes. An elevation of HS (7), a higher expression of HS-containing proteoglycans (5) and a higher incorporation of sulphate into GAG (5) in fresh lesions have been reported, but in advanced plaques a decreased content of HS and a higher capacity of CS- and DS-containing proteoglycans have been shown (6). On the other hand, dramatic decrease of HS in the aorta is related to aneurysm development and is called the marker of the vessel destruction (30). Both hypercholesterolemia and hypertension enhanced the total GAG and HS content in the thoracic aorta (*Fig. 4*, *Table 1*), but the elevation of chontroitin-4sulphate (C-4S) level was seen only in the thoracic segment of the hypertensive rabbits. Our data shows, that the changes of GAG in the vessel wall were determined by anatomical localisation and were seen only in thoracic aorta known to be resistant to advanced lesions development (1, 2). Feeding with cholesterol also caused a sequential elevation of various fractions of GAG but the maximal content of CS in the aorta appeared very late (7). We suppose that no influence of hypercholesterolemia on the C-4S content in the rabbit thoracic aorta, observed in our experiment, could be due to the various dynamic of the connective tissue development induced by hypertension or high level of cholesterol in the plasma. The level of all the C-4S and HS (*Table 1*) in the abdominal aorta is 2—3 times higher than in the thoracic segment. This phenomenon was found in the all groups, the intact control and NaCl treated rabbits including. This means that diversity of the GAG pattern between two investigated parts of the aorta is not caused by atherogenic stimuli but is supposed to modify function of a cell (31) and it response to irritants. Differences in cell environment comprises the influence on cell phenotype, migration and gene expression and, manipulation of the extracellular matrix changed the response of cells to stimuli (31). Thus, not uniform changes in the

abdominal and thoracic aorta exerted by investigated stimuli are supposed to be due to disparities in composition of the extracellular matrix.

The models of hypertension and hypercholesterolemia, applied in that study, were sufficient to induce early changes in the aorta. No advanced lesions were found. The two, different in their nature stimuli: hypertension or hypercholesterolemia, induce a convergnt response of the connective tissue in the respective parts of rabbit's aorta but the response is dependent on the anatomical localisation of injury. As the sensitivity to atherosclerosis development in thoracic and abdominal segments of the aorta varies, they express various responses of the connective tissue matrix to injuries, Thus, in the abdominal aorta, the two stimuli increase in the collagen content, when the GAG level is constant. Contrary to that, in the thoracic segment an elevation of GAG was found but the collagen level was not increased in the two applied models. The content and pattern of GAG, in the extracellular space of the thoracic or abdominal aortas, are different, and these disparities are supposed to be responsible for modification of cell reaction to the injures in the two parts of the aorta. This study stresses also the importance of local factors (dependent on anatomical localisation), which may determine the connective tissue response to injury. In both hypertensive and hypercholesterolemic rabbits, the common GAG increased in the thoracic aorta (known to be resistant to advanced lesions development) stand for the HS. According to our data and taking into consideration the antiproliferative, anticoagulative properties of HS (8) and the inhibitory effects of GAG (structurally similar to HS) on collagen gene expression (32), HS could be potentially involved in the inhibition of collagen accumulation in the thoracic aorta and could be involved in the retardation of the atherosclerotic process development.

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