PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 157–162 (1986)

Alterations in Canine Coronary Arteries Produced by Chronic Cigarette Smoking (42238)

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Abstract. Segments of left circumflex coronary artery were obtained from beagles that had been exposed to cigarette smoke for 2 years and from nonsmoking controls. A ring sample from each was used to determine passive (0-Ca^{2+} and 2 m*M* EGTA) and active (75 m*M* K^+) force–length relations. Other contiguous samples were used to determine collagen and elastin content, water content and distribution, and total electrolyte content. No significant differences were found in passive mechanical properties or the collagen and elastin content of arteries from the two groups of animals. Maximum values of active force development were found to be increased in arteries from smoking dogs as were values on the ascending part of the active force–length curve. Total water content and the distribution space of an extracellular marker (^{60}Co-EDTA) were not significantly different among arteries from the two groups. This along with the similarity of total electrolyte contents suggests that values of cell volume were not different between the two groups and that the above differences in active force development represent alterations in smooth muscle cells at the subcellular level. These results suggest that the contribution of chronic smoking as one of a group of cardiac risk factors may be due to an effect of augmenting coronary artery smooth muscle contractility.

A considerable body of evidence exists that links chronic cigarette smoking to coronary heart disease and a variety of other cardiovascular disorders (1). Most of the studies upon which this conclusion is based have been derived from epidemiological as well as autopsy-type studies. Using results from patients undergoing selective coronary arteriography, Herbert showed a direct correlation between coronary artery disease and cigarette consumption (2). His study suggested that a correlation exists between the number of cigarettes consumed and the severity of coronary artery disease as well as an accelerating effect of cigarette smoking on the development of coronary artery disease.

Auerbach *et al.* (3), in an exhaustive study of autopsy material from male patients, found that cigarette smoking had a greater association with atheroma in coronary arteries and intramyocardial arteries and arterioles. They found moderate and advanced fibrotic intimal thickening of the coronary arteries more frequently in smokers than in nonsmokers, which was correlated with the amount of cigarette smoking. Advanced hyaline thickening was found in over 90% of those smoking two or more packs of cigarettes per day. In a similar type of study, Strong and Richards (4) also found a close association between chronic cigarette smoking and atherosclerosis in coronary arteries of autopsied males from 25 to 64 years of age. Also, Naeye and Truong (5) observed that proliferative lesions which contained collagen and elastin fibers and abnormally oriented muscle cells were more prevalent in intramyocardial arteries of smokers than nonsmokers.

In contrast, the physiological and pathophysiological mechanisms responsible for this increased incidence of coronary artery disease have never been clearly established. Accordingly, it was the objective of the experiments described herein to evaluate the effects of chronic cigarette smoking on coronary arteries of beagle dogs. The results of these experiments document an increased contractile response in coronary arteries from smokers.

Materials and Methods. *Animals.* These experiments were performed using coronary arteries from 19 male beagle dogs (3.5 years old). The animals were studied under the direction of Dr. Oscar Auerbach and Dr. Julio

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0037-9727/86 $1.50

Frasca at the Veterans Administration Hospital in East Orange, New Jersey. All 19 animals were prepared for the cigarette smoking studies with airway tracheostomies performed under sterile conditions. A tracheostomy collar containing a polyethylene tube with a one-way inhale/exhale valve was fitted to the animals. The tubing was attached to a piston-type smoking device (A.D. Little Co.). Cigarettes calibrated to contain 1.5 mg nicotine and 30 to 35 mg tar were inserted into the smoking pump. Cigarettes of the test group (9 dogs) were lit allowing the smoke to be inhaled, while the cigarettes of the control group (10 dogs) remained unlit. The test dogs smoked 12 cigarettes a day, 7 days a week for 2 years. The animals smoked continuously, up to the day before they were sacrificed, and tissues were removed for study.

The animals were killed with an anesthetic overdose (pentobarbital). A 1.5-cm segment of left circumflex coronary artery was obtained, placed in iced physiological salt solution (PSS), and transported within 2 hr to our laboratory. The PSS had the following composition (mM): 116.5 NaCl, 22.5 $NaHCO_3$, 1.2 NaH_2PO_4, 2.4 Na_2SO_4, 4.5 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 5.6 dextrose at pH 7.4. Remaining tissues in these animals were used by Dr. Auerbach's group for studies of morphological changes and were unavailable to us.

Upon arriving at the laboratory, the arteries were trimmed of loose adherent tissue, cut into rings approximately 3–5 mm wide, and incubated in normal PSS for 3 hr at 37°C. They were then placed overnight in PSS at 0°C. The PSS was continuously aerated with a 95% O_2–5% CO_2 mixture. The next morning, the segments were placed into a metabolic shaker in PSS and maintained for 3 hr at 37°C, aerated with 95% O_2–5% CO_2. This procedure has been employed frequently in the past in our as well as other laboratories and shown to produce the most consistent results with regard to achievement of normal ion gradients across smooth muscle cell membranes (6–9).

Mechanics. One group of rings was used to determine force–length relationships under conditions of active and passive smooth muscle using an apparatus which has been previously described in detail (10). The apparatus consists of a modified Brush penmotor used as an electronically driven lever. The rings were attached to an isometric force transducer on one side, and to a modified arm in the penmotor on the other side. By applying an electrical signal to the penmotor input, changes in ring length could be produced and measured through an internally provided signal in the penmotor. Continuous measurements of force were obtained from the force transducer and the two signals (force and length) recorded on an X–Y plotter. The rings were immersed in PSS at 37°C which was continuously aerated with 95% O_2–5% CO_2. The ring was allowed to equilibrate for an additional 1-hr period after placement in the apparatus. At this point, the ring was stretched to a stable preload of 1 g. The normal PSS was then exchanged for one which contained 75 mM K^+ substituted on an equal molar basis for Na^+. This produced a maximal response to this form of activation. The high K^+-PSS also contained 1 μM phenoxybenzamine and 1 μM propranolol to block adrenergic responses of neuronally released norepinephrine. The isometric response to K^+ was monitored until it reached a steady state. At this point, the length was decreased until force was zero. The subsequent length–force response to the slow increase in length was recorded until a maximum force of 50 g was obtained (total time to peak force = 10 min). Ring force was then again returned to zero. The bath was drained, rinsed, and refilled with a Ca^{2+}-free PSS containing 2 mM EGTA to inactivate the muscle. Ring length was then cycled between the same force limits (i.e., 0 and 50 g) at a rate of 10^{-2} Hz until a steady-state curve was produced (approximately 30 to 45 min). The passive length–force curve as well as the length–force curve under activated conditions were both recorded on the X–Y plotter. These procedures are summarized schematically in Fig. 1.

When these responses were complete, the distance between the pins was measured in order to calibrate the length scale using a precision reticule in one eyepiece of a stereo dissection microscope. The ring was removed from the apparatus, cut open, and the total length (circumference) measured. The width of the ring was measured at six equally spaced distances along the length using a precision tool microscope.

The plotter length–force graphs were converted to digital form using a digitizer tablet (Talos Model 620) and transferred to a digital

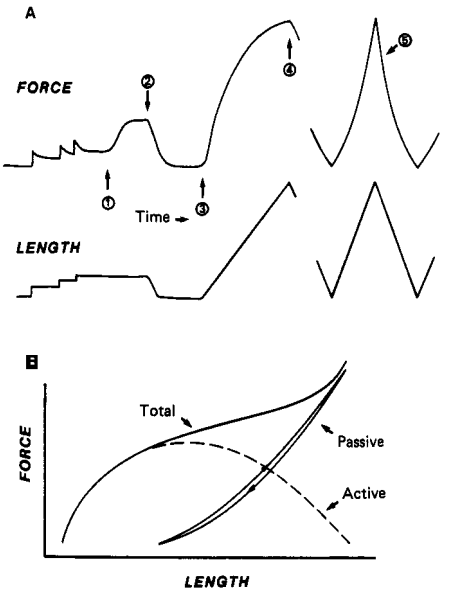

FIG. 1. Diagrammatic representation of methods used to determine active responses. Activation (75 mM K^+) was initiated from a 1-g preload (1) to a steady-state response (2), then lowered to a tension of zero (3). The length was slowly increased until tension reached a maximum value of 50 g (4) then reversed. Following relaxation of smooth muscle, the length–tension response to a slow stretch-release cycle was determined (5). Differences in values of tension under active and passive conditions at a particular value of length were used to construct the active force–length curve shown in the lower half of the figure.

computer (PDP 11/23) for analysis. The active curve was divided into 50 equal force steps and at each step the value of ring length was determined by three-point interpolation. Values of passive length–force were obtained from the Ca^{2+}-free PSS plus 2 mM EGTA curve at equivalent values of ring length. Differences in force under active and passive conditions between the two curves were obtained and divided by a cross-sectional area of the ring to determine active tangential wall stress. The passive length–force data were also divided by wall cross-sectional area to obtain a measure of passive mechanical properties (10).

The active length–force curve was normalized in the following manner. The value of ring length at which active force was zero was set to a value of zero. The value of ring length at which the active force was maximum was set to a value of 1. Values of active force were obtained between 0 and 1 in steps of 0.1 using three-point interpolation. Values of active force were obtained above a normalized length of 1 in steps of 0.05 by three-point interpolation. Values of active force were averaged among the various experiments for the arteries within the two groups at specific values of normalized diameter. This type of normalization procedure was found to produce the smallest dispersion in experimental data. The passive length–force curves were also divided into 50 increments of equal length from zero force up to maximum value. Data were averaged for all arteries in the two groups at each step producing average data with force and length standard errors.

Chemistry. One ring was used for the determination of total collagen and elastin content. Collagen and elastin were separated by repetitive application of heat and pressure (11). Collagen is solubilized by this procedure while elastin remains in the residue. Soluble and insoluble components were separated and their hydroxyproline content was determined separately. From this information and standard factors, values of collagen and elastin content were determined for tissues from the two groups of animals.

Other contiguous segments of coronary arteries were employed for the determination of extracellular water space using ^{60}Co chelated to EDTA as a marker (12). Following their initial incubation in PSS, the samples were transferred to a similar solution containing the radioactive marker for 15 min. They were removed from the incubation medium and lightly blotted to remove surface water. Tissue weights were determined before and after oven drying at 90°C for 20 hr. Wet and dry weights were used to determine total water content. The arterial samples and known amounts of radioactive loading solutions were counted in a gamma counter. From this information, values of extracellular water space were computed. Values of cell water space were estimated as the difference between total water content and the distribution space for the marker. The samples were ashed with hydrogen peroxide (30% v/v) containing excess $AgNO_3$ to precipitate tissue chloride. The ash was dissolved in 0.1 N HNO_3 to which 10 mM LiCl was added. Electrolyte content was determined by atomic absorption spectroscopy (Perkin, Elmer Model 2380) and expressed on the basis of tissue dry weight.

Statistical significance between various data

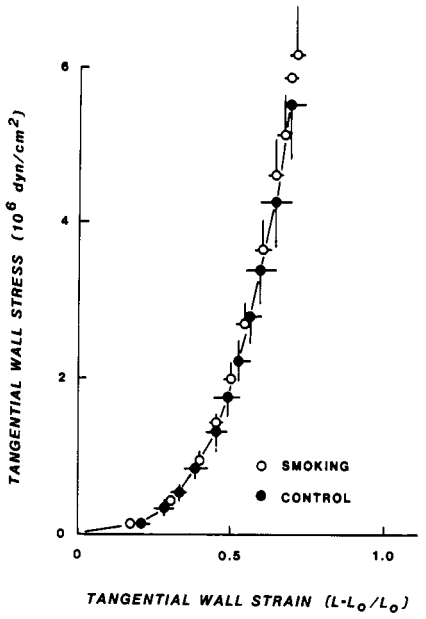

FIG. 2. Relation between tangential wall stress and strain for coronary arteries from control (●) and smoking dogs (○) under conditions of passive smooth muscle. Symbols are means and bars represent means ± 1 SEM. No significant differences were found between the two groups. N = 10 for controls and N = 9 for smokers.

was analyzed by one-way analysis of variance (13). The F statistic was calculated to determine if significant differences existed among the groups. If they did exist, a modified t statistic was determined (14) and used to test for statistical significance ($P > 0.05$).

Results. Figure 2 gives a summary of passive coronary artery mechanics. Shown in this diagram are averaged tangential stress–strain relationships. As indicated in the diagram, no significant difference existed in values of passive mechanical properties for coronary artery samples obtained from control and cigarette smoking dogs.

There were no significant differences in collagen or elastin content or their ratio for arteries from the two groups. Collagen content averaged 34.4 ± 1.5% dry wt in control, and 36.6 ± 1.4% dry wt in smoker coronary arteries. Elastin content averaged 23.0 ± 7% dry wt in control, and 21.9 ± 1.0% dry wt in smoker coronary arteries. This finding is consistent with the lack of change in passive mechanical properties of the coronary arteries.

A summary of the variation of active stress (force/area) with normalized muscle length is given in Fig. 3. Values of active stress development were significantly higher for the smoking than the control dogs for values of normalized muscle length between 0.4 and 1.0. Values at smaller muscle length were larger in the case of smoking dogs, but did not quite reach the level of statistical significance. At values above 1, the increase in scatter among the experimental data prevented the identification of a significant difference.

Table I shows a comparison of average values of total water content and its distribution in these coronary arteries. There were no significant differences in total water content, extracellular (^{60}Co-EDTA space) or cell water, among arteries from the two groups. This finding, combined with the fact that there were no significant differences in total collagen and elastin content, indicates that the relative cell volume of the coronary artery wall in control and smoking dogs was not significantly different. This suggests that the increase in force development represented an increase in the ability of individual cells to develop contractile force.

A summary of arterial wall electrolyte content is given in Table II. There were no statistically significant differences in total content of K^+, Na^+, Ca^{2+}, Mg^{2+}, or Cl^-.

Discussion. The results of these experiments document for the first time an increase in

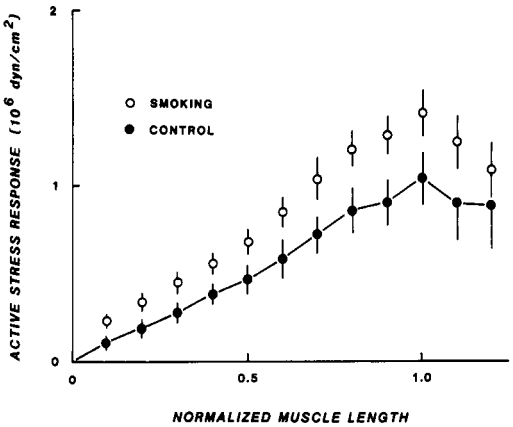

FIG. 3. Variation of active stress response to K^+ as a function of normalized muscle length for the two groups of arteries. Symbols are as defined in Fig. 2 and represent means ± 1 SEM. Significantly larger values of active stress response were found for coronaries from the smoking group. N = 10 for controls and N = 9 for smokers.

TABLE I. WATER CONTENT AND DISTRIBUTION (% Dry Wt)

Group	N	Total water	^{60}Co-EDTA Space	Cell water
Control	9	78.1 ± 0.7	62.4 ± 3.3	16.5 ± 2.8
Smoking	8	76.4 ± 1.2	59.1 ± 1.2	17.8 ± 1.9

Note. Values are means ± 1 SEM.

maximum force development (contractility) of coronary arteries from dogs exposed to chronic cigarette smoke for a period of 2 years. This increase in contractility was in response to a nonspecific activator, K^+. K^+ activates vascular smooth muscle by at least two mechanisms. The first mechanism involves a direct effect on the smooth muscle membrane producing depolarization and increasing calcium influx causing contraction. An intracellular Ca^{2+} pool is also released by K^+ activation which contributes to force development. The second mechanism involves an activation of adrenergic nerve endings in the walls of blood vessels leading to a release of norepinephrine and an α-adrenoreceptor-mediated contraction along with a β-adrenergic-mediated relaxation (15). Contributions from neuronal transmitter release were blocked in this study by phenoxybenzamine and propranolol treatment. This suggests that augmented calcium influx with potassium activation could account for the observed differences in maximum force development, possibly along with augmented release of intracellular Ca^{2+} stores.

The results of these experiments are supported indirectly by results of a recent study by Klein *et al.* (16). They evaluated the effects of chronic cigarette smoking on coronary vascular reserve. The latter was assessed from the hyperemic response to coronary artery injection of contrast media during diagnostic cardiac catheterization. The coronary vascular reserve was substantially smaller in chronic smokers than nonsmokers. Klein *et al.* suggest that this could be due to an action of nicotine inducing chronic ganglionic stimulation thereby limiting the capacity for maximal vasodilation due to chronically increased α-adrenergic tone (16). As an alternative possibility, they suggest a depressed vasodilatory mechanism, perhaps related to β-adrenergic receptors.

An interesting secondary finding of this study was the fact that no significant difference existed in the passive mechanical properties of coronary arteries. In addition, no significant changes were found to occur in the collagen or elastin composition of coronary arteries, the amount and distribution of water, or the total amount of arterial wall electrolytes. Since all these factors are ultimately related to arterial smooth muscle cells, this result suggests that cigarette smoking does not influence extracellular protein biosynthesis by coronary artery vascular smooth muscle cells or less likely affects synthesis and degradation in a parallel manner. This may add some additional support to the concept that another site of action is responsible for these changes in active force development, rather than directly the arterial smooth muscle cells.

These findings lead to two additional questions. What are the causes of these changes and what are their consequences? In terms of the initiating factors, several factors could be evolved. Carbon monoxide, nicotine, and oxidants present in cigarette smoke have all been implicated in causing alterations of arterial wall function and alterations in body functions associated with smoking (17, 18). In terms of consequences, an increase in contractility to vasoactive stimuli could contribute to the increase in coronary artery disease associated with cigarette smoking. A causal link between augmented contractility of coronary artery smooth muscle and coronary artery disease remains to be proven.

TABLE II. ARTERIAL WALL ELECTROLYTE CONTENTS (m*M*/kg Dry Wt)

Group	N	Ca^{2+}	Mg^{2+}	K^+	Na^+	Cl^-
Control	9	90.3 ± 14.6	25.5 ± 2.0	104 ± 11	659 ± 31	530 ± 35
Smoking	8	99.8 ± 30.7	25.3 ± 4.0	134 ± 15	638 ± 49	492 ± 38

Note. Values are means ± 1 SEM.

The work performed in this research project was supported in part by Grants HL 28476, HL 28459, and HL 24512 from DHEW, and a research grant from the Southeastern Chapter of the American Heart Association. Dr. Santamore is a recipient of a Career Development Award, HL 01026, from NIH. Dr. Tulenko is a recipient of a Young Investigators Award, HL 24512, from NIH. The authors wish to acknowledge the generous support of Dr. Oscar Auerbach and Dr. Julio Frasca (deceased) for allowing us access to tissues from their animals. The expert technical assistance of Ms. Susie Veit and the secretarial assistance of Ms. Maxine Blob are gratefully acknowledged.

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Received June 6, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted September 23, 1985.