Cardiac Endothelial Cells Modulate Contractility of Rat Heart in Response to Oxygen Tension and Coronary Flow

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The aim of this study was to determine if endothelial cells in the heart release substances into the coronary perfusion medium that modify the contractility of myocardial cells. To assay the effects on the contractility of cardiac muscle of fluid that has passed through the coronary vasculature, a new method has been developed based on the cascade principle used to study vascular smooth muscle function. The coronary venous effluent from an isolated perfused working heart was collected periodically, and after reoxygenation it was used as the bathing medium for trabeculae isolated from the endocardial surface of another heart. The coronary venous effluent changed the contraction of the isolated trabeculae. The amplitude and the direction of the change depended on the degree of oxygen saturation of the coronary effluent before it was reoxygenated and the rate of coronary flow at the time the effluent was collected. The response of the trabecula to the coronary effluent was substantially altered by damaging the endocardial endothelium with a 1-second exposure to 0.5% Triton X-100 in Krebs’ solution. It was completely eliminated by damaging endothelial cells in both the perfused heart producing the effluent and the trabecula on which the effluent was assayed. Therefore, endothelial cells are required for the presence of cardioactive substances in the coronary effluent. The production of a labile endothelium-derived upregulating (positively inotropic) factor and a more stable endothelium-derived downregulating (negatively inotropic) factor has been demonstrated and appears to account for all of the changes in myocardial contractility produced by the coronary effluent. Neither of the endothelium-derived substances demonstrated in the isolated perfused heart is nitric oxide or endothelin. The concentration of the endothelium-derived upregulating factor is sensitive to oxygen tension, whereas the concentration of the endothelium-derived downregulating factor is sensitive to the rate of coronary flow but not oxygen tension. The coronary effluent appears to contain substances that stimulate secretion by the endothelial cells (preendothelial factors) as well as substances that have been produced by the endothelial cells (endothelial factors). The results indicate that during the passage of perfusion medium through the coronary vasculature upregulating and downregulating factors are added to the perfusate in relative concentrations that depend at least in part on local tissue PO2 and the rate of coronary flow. In the intact heart, this mechanism could operate to maintain balance between energy supply and work performed. (Circulation Research 1993;72:1044-1064)

KEY WORDS • endothelial cells • cardiac contractility

Over the last decade, a large body of data has been generated to show that endothelial cells are important intermediaries in the processing of information and transmission of signals within an organism. The classical observations of Furchgott and Zawadski1 showed that the vasodilatation in blood vessels produced by acetylcholine as well as other transmitters and humoral agents required the presence of endothelial cells. The substances that actually produced the relaxation were secreted by endothelial cells.

At least six biologically active substances are secreted by endothelial cells, including molecules that modulate the contraction of smooth muscle. Among these are nitric oxide, a potent relaxing agent,2 and endothelin, a powerful vasoconstrictor.3 Under normal conditions, both constricting and relaxing agents are present in the coronary blood simultaneously, with the net effect depending on the balance between the two.4 In addition to chemical transmitters, oxygen tension5 and shear forces6 also modulate the production by the vascular endothelium of agents that regulate smooth muscle contraction.

The contractility of cardiac muscle also responds to chemical transmitters, oxygen tension, and physical factors by modifying transmembrane calcium currents, intracellular calcium storage, and the contractile proteins themselves. The result is the establishment of new contractile states. It has not been possible to account for all of the differences in the contractility of isolated cardiac muscle by known mechanisms involving neurotransmitters, resting length, and electrolyte content of...
the bathing medium. An as yet unidentified factor or group of factors besides neurotransmitters that modify cardiac contractility appears to exist and may originate in the endothelial cells of the coronary blood vessels.\textsuperscript{7,8} In studies with isolated bundles of cardiac tissue, endothelial cells have been implicated in the process of cardiac adaptation.\textsuperscript{9-12} In these preparations, in which most of the endothelial cells are present in the endocardium, a decrease in the peak tension and in the time until the onset of relaxation can be produced by damaging the endocardium. These changes in contraction can be reversed by bathing the tissue in a medium conditioned by cultured cardiac endothelial cells.\textsuperscript{11,12} The identity of the substance producing the reversal has not yet been established. Although endothelin, a peptide produced by endothelial cells, has been shown to enhance cardiac contractility in vitro at low concentrations and receptors for endothelin have been found on the surface of myocardial cells, there is a difference of opinion about whether the peptide is involved in the reversal of the effect of endocardial damage.\textsuperscript{11,13}

To investigate the possibility that endothelin-derived factors are important regulators of cardiac contractility, the effect of fluid collected from the coronary vasculature of an isolated heart has been assayed on a cardiac trabecula isolated from another heart. The results indicate that cardioactive substances are added to the coronary perfusate. These factors can raise or lower cardiac contractility, and the rate of their production is sensitive to tissue oxygen tension and coronary flow. The effects of these factors are influenced by the integrity of the endothelial cells in the isolated trabecula and the perfused heart.

**Materials and Methods**

The primary aim of the study was to assay the effect of reoxygenated fluid from the coronary venous outflow on the contractility of isolated ventricular trabeculae. To accomplish this goal, two preparations were used simultaneously: one was an isolated trabecula from the endocardial surface of the right ventricle of a rat heart, and the second was an isolated perfused working heart. Because of the difference in the stabilization time of the two preparations, the trabecula was removed from the heart of the first rat approximately 90 minutes before the isolated heart was prepared from a second rat.

**Preparations**

Male Wistar rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing between 200 and 350 g were killed by rapid decapitation. Thin trabeculae or papillary muscles were isolated from the endocardial surface of the right ventricle of hearts as previously described.\textsuperscript{7} Great care was taken during the dissection to avoid touching the surface of the tissue bundle with dissection instruments or allowing the tissue bundle to shorten excessively, because each of these can cause significant damage to endothelial cells in the preparation. The tissue bundle was suspended between a force transducer (Grass Instrument Co., Quincy, Mass.) and a mechanical ground at approximately 2.2-μm sarcomere length based on the highly reproducible sharp increase in slope of the passive length–tension curve that occurs at this point. The tissue was bathed in approximately 30 ml Krebs' solution containing (mM) NaCl 118, KCl 4.8, KH\textsubscript{2}PO\textsubscript{4} 1.0, MgSO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25, CaCl\textsubscript{2} 2.5, and glucose 11.1, pH 7.4, in which 95% O\textsubscript{2}–5% CO\textsubscript{2} was continuously bubbled. Resting tension equaled 5–10 mN/mm\textsuperscript{2} cross-sectional area. Since the tissue was less than 1 mg, the bathing volume was 3×10\textsuperscript{-4} times that of the tissue. Temperature was maintained at 30±0.1°C by using a constant temperature circulating bath and continuously flowing water through a jacket. The tissue was stimulated to contract with electric shocks delivered through platinum field electrodes at 0.2 Hz. The stimuli were 5 msec in duration with a voltage 20% above the level necessary to produce maximum force response. Tension was continuously monitored with a strip-chart recorder, and periodically, the contractile waveform was captured by a computer through an analog-to-digital converter. Each waveform shown in the figures is a computer-averaged value of five consecutive contractions. The trabecula was maintained in standard Krebs' solution for approximately 120 minutes until the waveform of the contraction stabilized. The details of the process of stabilization have already been reported.\textsuperscript{8}

At the end of the experiment, the trabecula was quickly frozen in isopentane cooled by liquid N\textsubscript{2}, with its length fixed as previously described.\textsuperscript{14} Four-micron-thick transverse sections were cut with a cryostat maintained at −20°C or lower, and the cross-sectional area was measured through a Zeiss photomicroscope using a ×6.3 objective and a ×1.25 optovar.

Isolated perfused working hearts were prepared by a small modification of the preparation of Neely et al\textsuperscript{15} using hearts taken from male rats weighing between 250 and 350 g.\textsuperscript{16} The aorta, pulmonary artery, and left atrium were cannulated to control left atrial filling pressure and to measure cardiac output, coronary sinus flow, total coronary blood flow, and systolic and diastolic pressure. The rate of contraction was maintained at approximately 265 beats per minute. To avoid any possible effects of electrical stimulation of the isolated heart and to allow the detection of any tendency to arrhythmias, the hearts were not artificially paced. Any heart in which there was any arrhythmia or in which the spontaneous rate did not fall within the range of 250–280 beats per minute was rejected. The perfusate was Krebs' solution (same as used for isolated trabeculae) continuously equilibrated with 95% O\textsubscript{2}–5% CO\textsubscript{2} and maintained at 37°C. In the initial studies, the total volume of the perfusion system was 400 ml, and at the average rate of coronary flow, the medium was recirculated approximately four times per hour. In later experiments and in all of the experiments in which the vascular endothelium of the perfused heart was disrupted, the total volume of the perfusion system was reduced to 100 ml. Arterial and venous oxygen tension were continuously monitored with oxygen electrodes in the left atria and pulmonary artery. The electrodes were calibrated for each experiment, and O\textsubscript{2} tension was periodically checked by direct measurement of the dissolved gas. Cardiac power was calculated as mean pressure times cardiac output. At appropriate times after the contractile activity of the isolated trabecula had stabilized, the coronary sinus effluent was collected from the pulmonary artery for 1.5 minutes and reoxygenated with 95% O\textsubscript{2}–5% CO\textsubscript{2}. The effluent was brought to 30°C and used to replace the Krebs' solution bathing the trabecula, which was also maintained at 30°C. The change in the shape of the waveform of contraction...
was monitored continuously. The pH of the coronary venous effluent after reoxygenation was never more than 0.02 pH units below the original pH of the original perfusion medium measured at the same temperature.

**Method for Damaging Endothelial Cells**

In some experiments, the trabecula was exposed to a solution of 0.5% Triton X-100 in Kreb's solution for 1 second and then rinsed several times in normal Kreb's solution. The procedure has been shown to damage or remove the endothelial endothelium without altering the ultrastructural properties of the underlying myocardial cells in isolated mammalian cardiac papillary muscles\(^6\) and thin trabeculae.\(^7\) In measurements made on transverse sections of the trabecula, 80–95% of the total endothelial cell population (exclusive of capillary endothelium) in trabeculae of the thickness used in these studies was found in the endocardium.

To disrupt the endothelium of the isolated perfused working heart, the basic procedure described by Griffith et al.\(^7\) was used. The working heart was initially converted to a Langendorff-type perfusion using a constant 12–13 ml/min flow of normal Kreb's solution. After a 5-minute stabilization period, the perfusion fluid was switched to high-K\(^+\) Kreb's solution, and perfusion was continued at the same rate of flow. The K\(^+\)-rich solution was made by replacing 40 mM NaCl in the normal Kreb's solution with 40 mM KCl to give concentrations of 103 mM NaCl and 45.8 mM KCl. At the end of 10 minutes of the high-K\(^+\) perfusion, the heart was switched back to the normal Kreb's solution for 5 minutes to wash out the potassium before the working heart was reestablished. In general, the heart went into contracture within 15 seconds of initiating the high-K\(^+\) perfusion, and the mean perfusion pressure rose to approximately 180 mm Hg. During the first minute after restoration of normal Kreb's solution, the heart began beating slowly, and the mean perfusion pressure fell to approximately 125 mm Hg. Normal sinus rhythm was restored over the next 1–2 minutes. The perfusion solution used before the disruption of the endothelium was not mixed with the perfusion solution used after the disruption.

**Ultrastructure**

For transmission electron microscopy, perfused hearts were cut from the apparatus and fixed by perfusion with 2% glutaraldehyde in Kreb's solution. The hearts were then minced, fixed in fresh glutaraldehyde for 1 hour, and washed in Kreb's solution overnight. The tissue was further fixed in 1% osmium tetroxide in Kreb's solution for 1 hour and washed in Kreb's solution overnight. Dehydration was produced by two changes of 50%, 70%, and 90% ethanol for 15 minutes each. The final dehydration was produced by three changes of 100% ethanol and two changes of 100% propylene oxide for 15 minutes each. The samples were immersed in a 1:1 solution of propylene oxide and EM-Bed 812 for 1 hour, embedded in EM-Bed 812, and cured at 60°C for 36 hours. Sections were cut on a Sorvall Porter-Blum MT2-B ultramicrotome and stained first with 40% uranyl acetate in methanol for 25 minutes and then 1% lead citrate in water for 5 minutes. Photographs were taken on a Zeiss 109 or a Hitachi transmission electron microscope. For controls, hearts that had been removed from animals were fixed immediately by the same fixation protocol as used in the perfused hearts.

**Analytical Measurement**

The concentration of endothelin was measured by radioimmunoassay using a commercial kit from Peninsula Laboratories, Inc., Belmont, Calif.

**Statistics**

Differences in the mean values were evaluated by unpaired and, where appropriate, paired Students’ \(t\) test. For evaluating the relation between the percent \(O_2\) in the coronary venous effluent before reoxygenation and the relative change in peak isometric force developed, linear regression analysis was used. For evaluation of the significance of change in the pattern of response as a function of the rate of coronary flow and of exposure to 0.5% Triton X-100 for 1 second, Fisher’s exact test and Spearman’s correlation were used. Significance was assumed at \(p<0.05\).

**Results**

**Preparations**

*Cascade of perfused heart and isolated trabecula.* The coronary venous effluent of an isolated perfused working heart was collected from the right ventricle and reoxygenated with the same gaseous mixture (95% \(O_2\), 5% \(CO_2\)) that had been used to oxygenate the coronary perfusate before it entered the coronary arteries. The fluid was then used to bathe an isolated ventricular trabecula that had been removed from another heart. The contribution of endocardial endothelial cells in the trabecula to changes produced in the contraction of the trabecula by the coronary venous effluent was estimated by using a 1-second exposure to 0.5% Triton X-100 Krebs' solution to damage the endocardial endothelium between periods of exposure to the coronary venous effluent. The influence of the hemodynamic and metabolic state of the perfused heart on the effect of the coronary venous effluent could be evaluated inasmuch as preload and afterload on the heart, coronary flow, cardiac output, and oxygen tension were measured and/or controlled.

The isolated perfused working hearts achieved a stable level of function within 30–40 minutes after the establishment of coronary perfusion with oxygenated Krebs’ solution. Preload was set at 10 cm H\(_2\)O except for selected brief periods at 15 cm H\(_2\)O, and afterload was controlled by the height of the left ventricular outflow column, which remained constant throughout the experiments. Within 10–20 minutes, mean aortic pressure and systolic and diastolic pressure had reached a constant level, but other parameters of function required somewhat longer to stabilize. Coronary flow, cardiac work, and venous \(P_O_2\) all increased from the first measurement until they became constant between 20 and 40 minutes after cannulation of the isolated heart (Figure 1). A majority of the hearts achieved a constant efficiency by 20 minutes, but for those hearts with the lowest values, efficiency increased over an additional 20 minutes. The variability in efficiency among different preparations was reflected primarily in the amount of \(O_2\) extracted per unit volume of coronary perfusate and...
in cardiac output, but not in the rate of coronary flow or aortic pressure. The percent saturation of oxygen in the coronary venous effluent varied from 15% to 70%.

Once the hearts achieved a constant level of function, they remained stable for the 2-hour period over which the assays of the coronary venous effluent were conducted. Cardiac work increased when the left atrial filling pressure was increased from 10 to 15 cm H$_2$O and then returned to the lower values as the preload was decreased. The increase in cardiac work was the same when the preload was again increased to 15 cm H$_2$O 80 minutes later. The increase in efficiency at the higher preload was attributed to the well-described property of the heart to increase work more efficiently by raising flow than by raising pressure. The variability of cardiac efficiency and coronary venous oxygen tension among the isolated perfused hearts was useful because it allowed the examination of the relation between the metabolic state of the perfused heart and the effect of its coronary venous effluent on the contractile behavior of the isolated trabeculae.

Transmission electron microscopy of thin sections of isolated perfused hearts revealed no sign of damage to endothelial cells as a result of the perfusion. No significant difference was observed in the ultrastructure of endothelial cells lining the small arteries between unperfused hearts fixed after removal from the animal and hearts perfused as long as 120 minutes (Figure 2). As a further check on the viability of coronary endothelial cells in the perfused hearts, the response of coronary flow to a bolus of methacholine (0.4 ml of $10^{-3}$ M) added to the coronary perfusate over 5 seconds was determined. In all six hearts in which this was done, coronary flow increased by $13.1 \pm 1.8\%$. After the methacholine passed through the coronary vessels, coronary flow returned to its previous level.
After their removal from hearts, trabeculae required up to 120 minutes for their contractile behavior to stabilize. The characteristics of the stabilization have already been described in detail.\textsuperscript{14} In 26 of 28 preparations in which this was carefully monitored, the peak tension and the time to half relaxation declined during this period, but the rate of rise of tension did not change. In the other two trabeculae, peak tension increased, and the time to half relaxation became longer without any change in the rate of rise of tension. Among
the 28 preparations, the mean decline in peak force was 14±4%, and the mean decrease in the time to 50% relaxation was 21±4%. After the period of stabilization, the peak tension developed by trabeculae stimulated at 0.2 Hz was related to the cross-sectional area in a manner that has already been described for the maximum calcium-activated force in detergent-skinned preparations.7 In very thin trabeculae, normalized force was generally greater than 100 mN/mm², but it declined as cross-sectional area increased. In tissues with cross-sectional areas greater than 0.2 mm², developed tension was 10–15 mN/mm². For this reason, only trabeculae with cross-sectional areas of ≤0.2 mm² were included in the data analysis. The maximum rate of rise of tension was 202±9 mN/mm² per second. The time to peak tension was 0.21±0.02 seconds.

Cascade with selectively damaged endothelial cells. The coronary vascular endothelium was damaged in the perfused hearts by a brief period of perfusion with a modified Krebs' solution containing 40 mM K⁺. This procedure has been reported to damage coronary endothelial cells and to eliminate the decrease in coronary resistance produced by cholinergic drugs.17 After the exposure of the heart to the modified Krebs' solution containing an additional 40 mM K⁺ and the reinstitution of normal Krebs' solution as the perfusion medium, there were some changes in the hemodynamic performance of the heart. Mean aortic pressure decreased from 68±3 to 57±1 mm Hg, a decrease of 16% (n=10, p<0.02). Mean aortic flow was reduced from 13.0±1.0 to 8.9±0.6 ml/min (p=0.01), and coronary blood flow declined from 9.2±0.9 to 7.2±1.0 ml/min (p=0.01). The relative degree of oxygen saturation in coronary venous blood dropped from 29±3 to 24±4 (p=0.04).

The decrease in mean aortic pressure developed by the perfused heart after damage to the vascular endothelial cells was very similar in amplitude to the decrease in peak contractile force developed by isolated trabeculae after damage to their endothelial cells by a 1-second exposure to Triton X-100. The very good correlation between the declines in aortic flow and mean pressure (r=0.94) was not unexpected, since there was no change in the resistance to flow from the left ventricle. The decline in coronary flow was proportionally less than the decline in aortic flow because of an apparent decrease in the resistance to coronary flow that was due to a change in the vascular smooth muscle from the high-K⁺ Krebs' solution (see below). Although the heart was operating as a Langendorff preparation with a constant flow, the brief exposure to the high-K⁺ Krebs' solution caused a 49±11% (p<0.01) increase in the pressure necessary to maintain the same rate of flow (from 77±7 to 115±7 mm Hg). After the exposure to the high-K⁺ Krebs' solution, there was no change in coronary resistance when methacholine was added to the perfusate either as a bolus of 0.4–0.8 ml of 1.0–100 μM solution or 10 μg/ml in perfusion medium for 30 seconds, amounts equal to 0.1–20 times the amount of drug that produced a decrease in coronary resistance in the heart before the vascular endothelium was damaged. There was a loss of response to cholinergic drugs. The decrease in coronary flow and the increase in coronary resistance were less than the values reported when the basal secretion of nitric oxide was inhibited by N⁴-monomethyl-L-argi-

nine.18 This is probably due to the fact that all endothelial function has been affected, not merely the secretion of nitric oxide. This smaller decline in coronary flow prevents the large decline in cardiac output seen with specific inhibition of secretion of nitric oxide.18

The ultrastructure of the cells in the heart perfused with high-K⁺ solution was examined with standard transmission electron microscopy of thin sections of hearts that had been histologically fixed after exposure to high-K⁺ solution and subsequent washout by standard Krebs' solution (Figures 3 and 4). The myocardial cells had normal-appearing sarcolemmas, myofilaments, mitochondria, and internal membrane systems except for some dilatation of the transverse tubular system, a not uncommon finding in perfused hearts. Within the arterial vasculature, there were very few endothelial cells. Those present had complete or near complete absence of the surface membrane on their lumen side and membrane damage of a lesser degree on the nonlumen side. In the three hearts in which ultrastructure was carefully examined, no normal endothelial cells were seen within the arterial vasculature. The capillaries and the venules had a normal appearance. The endothelial cells in the endocardium of the left ventricle, which would not have been subjected to high pressure during the period of Langendorff-type perfusion, also had a normal appearance. The smooth muscle cells in the arteries and arterioles had clearly been damaged by the perfusion with high-K⁺ solution. Most of the smooth muscle cells had lost part or all of their surface membranes. The density of the contractile filaments was greater than normal, and they appeared to be less organized than normal. The mitochondria were very dense, and the normal structure of the cristae was often obscured. There were numerous intracellular vesicles containing electron-dense material.

The damage to the vascular endothelial cells and the vascular smooth muscle cells was almost certainly the cause of the changes in hemodynamic performance and the smaller relative decline in coronary compared with aortic flow. Damage to the vascular smooth muscle as well as the vascular endothelial cells probably prevented the marked vasoconstriction that follows selective vascular endothelial cell damage and the consequent loss of nitric oxide production.18–20 When damage to only the vascular endothelial cells was produced by perfusion of the heart with the detergent CHAPS added to the Krebs' solution (20 mg to 12 ml of Krebs' solution19), there was a very large drop in aortic and coronary flows of approximately 50–60%. (A detailed report of a comparison of the effect of damaging endothelial cells by high-K⁺ solution or by a brief exposure to detergent will be presented in another manuscript in preparation.) Coronary venous oxygen saturation generally fell to under 10%, and hemodynamic performance of the working heart deteriorated rapidly (data not shown). This response to selective damage to the coronary endothelium has been reported by others.19 To maintain good performance of the heart after damage to the vascular endothelial cells, it was necessary to prevent the marked increase in coronary resistance and decline in venous oxygen tension. Therefore, the protocol that produced damage to vascular smooth muscle was used because it damaged the vascular endothelial cells and
maintained normal ultrastructure and good function of the cardiac myocytes.

To evaluate the inference from ultrastructure that high-K⁺ Krebs' solution damaged only endothelial cells in the arterial vasculature, the effect of the high-K⁺ Krebs' solution on the contraction of isolated trabeculae with undamaged endocardial trabeculae was measured. After exposure to the high-K⁺ solution, contractions by the trabeculae had the same peak tension and time course as before exposure. Therefore, it appears that the high perfusion pressure must accompany the high concentration of K⁺ for damage to the endothelial cells to occur.

Effect of Coronary Venous Effluent on Contractility

Patterns of change in contractility. Superfusion with coronary venous effluent from 13 of 20 isolated hearts caused a change in the peak tension developed by the trabeculae and in the time to half relaxation. Examples of the changes in the tension waveform are shown in Figure 5, and the change in peak tension for each of the hearts is shown in Figure 6. The rate of rise of tension was not altered. Within 2 minutes of exposure to the venous effluent, the change in contractility could be clearly distinguished from any effects that were simply due to the interruption of the stimulation during the change in solution. A steady state was reached by 15 minutes, and it remained for at least another 5 minutes, the longest period of exposure to the venous effluent in this series of experiments having been 20 minutes. No change occurred in the contractility of the trabeculae if the standard Krebs' solution was replaced by another aliquot of the same solution.

Four different patterns of change in the contraction of the trabeculae in response to superfusion with coronary venous effluent were observed. In some experiments, force increased; in others, force decreased. In eight of the 20 experiments, the contractility returned to control levels each time coronary venous effluent was replaced by standard Krebs' solution (Figure 5). In the other 12 experiments, there was a decrease in contractility to a level below the control value measured immediately before the exposure to the coronary effluent. This decrease occurred even in some cases in which the contractility had been elevated throughout the entire period of exposure to the coronary effluent. When a decrease below the control value in Krebs' solution was observed, it did not always occur after the first exposure to coronary venous effluent. In seven of the 12 experiments in which developed force was lower after an exposure to coronary venous effluent than before, the decline was not observed after the first period in coronary effluent but only after subsequent exposures of the trabeculae to coronary venous effluent.

Regardless of whether the developed force was reduced after the period in the effluent, a second exposure to effluent collected under the same conditions for the perfused heart produced a change in developed force in the same direction as the original exposure. In 11 experiments in which exposures of the same trabecula to effluent from the same isolated heart were compared, the differences between the two responses within each experiment were not significant (p>0.7). On 11 occa-

sions, two isolated trabeculae of similar thickness taken from the same heart were set up simultaneously so that the effects of the same coronary venous effluent on two different trabeculae could be determined at the same time. In each case, there was no significant difference in the response of the two trabeculae to the effluent.

The concentration of endothelin in aliquots of three collections of coronary effluent was less than $10^{-12}$ M, which is the lower limit of detection of the radioimmunoassay. This value is similar to that already reported for perfused isolated hearts.21

Effect of oxygen tension. Coronary venous oxygen saturation varied from 15% to 70% as a result of the difference in the efficiency of the perfused hearts. The change in the contraction of the trabecula produced by the venous effluent was related to the percent saturation of the venous effluent before it was reoxygenated with 95% O$_2$–5% CO$_2$ (Figure 6). With the higher levels of O$_2$ in the effluent, the force developed by the trabecula was increased, and with the lower levels, developed force decreased. The coefficient of determination of the relation between oxygen saturation and change in force changed (Figure 6, bottom panel). Modification of the contraction of the trabecula still occurred. However, there was a loss of the positive effect of coronary venous effluent collected at high oxygen tensions.

The pattern of recovery from exposure to the coronary venous effluent was also related to the oxygen tension of the effluent before it was reoxygenated (Figure 7). When the effects of the effluent were completely reversible with restoration of the control solution, the percent saturation was relatively high. In trabecula bathed with effluent with intermediate levels of oxygen saturation, the effect of first exposure on contractility was completely reversible, but subsequent exposures produced a residual decline in developed force. There was a residual decrease in developed force even after the first exposure when the degree of oxygen saturation was low.

Effect of coronary flow. When the coronary venous effluent was assayed on trabeculae in which the endocardial endothelium had been damaged or destroyed by Triton X-100, there was a significant correlation between the amplitude of the change in the contraction of the trabecula produced by coronary venous effluent and the rate of coronary flow (Figure 8). As coronary flow increased, the range of amplitudes of the change in contraction of the trabecula progressively decreased, and at coronary flow above 28 ml/min, no change in the contractility of the trabecula was observed regardless of the oxygen saturation of the venous effluent. Both Fisher's exact test and Spearman's correlation were
used to determine if the decrease in the range of amplitudes of the change in peak force as coronary flow increased was significant. Both tests indicated a level of significance at *p*<0.02. When trabeculae with intact endocardial endothelial cells were used to assay the coronary venous effluent, there was no significant effect of the rate of coronary flow on the amplitude of the change in the peak force developed by the trabeculae during a contraction. This last observation argues against simple dilution as the basis for the effect of the rate of coronary flow.

**Influence of thickness of the trabeculae.** A change in the contraction of the trabecula from superfusion with reoxygenated coronary venous effluent occurred in trabeculae that were less than 0.2 mm² in cross section. Thicker tissues showed little or no change (Figure 9). The amplitude of the response increased progressively as the thickness of the trabeculae decreased. The correlation between the oxygen tension of the venous effluent and the change in contractility was greater when the data were restricted to only thin trabeculae (Figure 10), indicating that a significant factor in the variability of the response of the trabeculae to the coronary effluent was the thickness of the trabeculae or something related to the thickness. Treatment of these trabeculae with 0.5% Triton X-100 in standard Krebs' solution for 1 second to damage the endocardial endothelium appeared to lower the thickness of the trabeculae to the point at which an effect on contraction was no longer observed, from approximately 0.2 mm² to approximately 0.08 mm² (Figure 9). Because of the limited number of experiments, the significance of the difference was borderline (*p*=0.09).

**Role of Endothelial Cells**

As Brutsaert et al⁹ have shown, exposure of an isolated papillary muscle to 0.1% Triton X-100 in Krebs' solution for 1 second damaged the endocardial endothelium, lowered the peak developed force, and produced an earlier onset of relaxation (Figure 11). If, however, the detergent was applied to trabeculae with residual reduced contractile force from an earlier 15-
FIGURE 5. Graphs showing the change in the shape of the contraction of trabeculae when coronary venous effluent temporarily replaces standard Krebs' solution. Waveforms before, during, and after superfusion with coronary venous effluent are given. Four different patterns of response are shown (see text). Upper panel: Force is reversibly decreased (coronary flow, percent O₂ saturation, and efficiency for the perfused heart producing the efferent are 12 ml/min, 37%, and 0.21, respectively). Lower panel: Force is increased, but there is a residual decrease (values as in upper panel are 10 ml/min, 42%, and 0.20, respectively).
FIGURE 5. Continued. Upper panel: Force is decreased, and there is a residual decrease (values as in upper panel [preceding page] are 17 ml/min, 20%, and 0.18, respectively). Lower panel: Force is reversibly increased (values as in upper panel [preceding page] are 20 ml/min, 58%, and 0.23, respectively).
minute exposure to coronary venous effluent that had oxygen saturation below 28% before reoxygenation, the result was different. The detergent increased peak developed tension to approximately the level that existed before the reduction of force from exposure to the coronary venous effluent. The results suggest that endothelial cells are involved in the changes in contractility produced by the coronary effluent. Coupled with the different patterns of response of trabecula to coronary effluent, they suggest that endothelial cells in the heart can produce both upregulating and downregulating cardioactive factors and that the mixture is sensitive to oxygen tension in the tissue. To evaluate these interpretations more rigorously, studies in which endothelial cells in the perfused heart and the isolated trabeculae were selectively damaged were carried out.

Requirements for the presence of endothelial cells for cardioactive factors to be present in the coronary effluent. To determine if endothelial cells were essential for the effect of the coronary effluent on contractility to occur, it was necessary to show that the cardioregulatory activity in the coronary effluent disappeared when endothelial cells had been damaged in both the isolated perfused heart providing the coronary effluent and the trabecula used for the assay. Pairs of trabeculae were isolated from the same heart and exposed to aliquots of the same coronary effluent from a perfused heart. The

| Figure 6. | Plots showing the relation between the percent oxygen saturation in coronary venous effluent before it has been reoxygenated and the percent change in the peak force of an isolated trabecula bathed in the effluent. Each point represents the results from a different trabecula and perfused heart. Left atrial filling pressure was 10 cm H2O. Top panel: Before detergent. Correlation coefficient was 0.70; coefficient of determination was 0.49. Bottom panel: After detergent. Correlation coefficient was 0.09.

| Figure 7. | Bar graph showing the pattern of recovery of the contractile properties of the coronary venous effluent after a coronary effluent is replaced by standard Krebs’ solution as a function of the percent O2 saturation of the effluent. The average coronary venous percent O2 saturation (mean ± SEM) is given for each of the three patterns denoted by residual, residual delayed, and residual absent, which indicate, respectively, the following: 1) decrease in force after all exposures to coronary effluent (n=5), 2) return to normal control only after the first exposure to effluent (after subsequent exposures, there was a reduction from the level of force developed by the trabecula before the exposure) (n=10), and 3) return to normal control contraction after each of at least three different exposures to effluent (n=13). Differences among the categories are significant at p<0.05; in the case of residual compared with residual absent, differences are significant at p<0.01.

pairs consistently had the same response to the same coronary effluent. In pairs of trabeculae, the ratio of change in peak tension of the two tissues exposed to the same coronary effluent was 1.01±0.04 (p=0.99).

After the demonstration of near identical responses of the two trabeculae, two different types of experiments were conducted. In the first, endocardial endothelial cells in one trabecula were damaged, and then both trabeculae were exposed to aliquots of the same coronary effluent from a perfused heart in which the vascular endothelial cells had been damaged (protocols B and C of Figure 12). There was an increase of 30±7% in the peak force of the trabeculae with intact endothelial cells but only a 5±3% increase in the trabeculae with damaged endothelial cells (n=7) (Figure 13, Table 1). In four of the seven experiments, the coronary effluent from hearts with damaged endothelium had no effect on trabeculae with damaged endothelium. The difference in the response between the control and Triton X-100-treated trabeculae was significant at p=0.008. The response of the Triton X-100–treated trabeculae to the coronary effluent was not significantly different from zero (p>0.05). All or almost all of the effect of the coronary venous effluent on the contractility of a trabecula was lost when endothelial cells had been damaged in both the isolated perfused heart and the trabecula. The small response that may have remained in a minority of Triton X-100–treated trabeculae could have been due to the small residual amount of endothelial cells remaining in the blood vessels in the tissue that were not accessible to the detergent during its brief exposure to the tissue.

In the second type of experiment, two different samples of coronary effluent collected from the same
perfused heart (one before and one after damaging the vascular endothelium) were assayed on the same Triton X-100--treated trabecula (protocols B and D of Figure 12, Table 1). The coronary effluent collected before damage to the vascular endothelium of the perfused heart produced a significant change in contraction (−11±3%, p=0.01), whereas the coronary effluent collected after the damage to the vascular endothelium did not produce a significant change. Therefore, whether tested by using the same effluent on two different trabeculae or different effluent on the same trabecula, the conclusion was the same: no cardioactive effect was present in effluent when vascular endothelial cells in the perfused heart and endocardial endothelial cells in the trabecula had both been damaged.

Endothelial cells release a relatively stable downregulating (negatively inotropic) factor. The coronary effluent from perfused hearts with intact vascular endothelial cells was assayed on trabeculae in which endocardial endothelial cells had been damaged with Triton X-100 (protocol D in Figure 12). The time lag between the collection of the effluent and its application to the test trabeculae was between 4 and 5 minutes. In all nine experiments, the effluent caused a decline in peak tension. The mean decrease was 11±3% (p=0.01). In two of the nine experiments, the peak tension had increased by 13% and 27% after 1 minute but then began to fall, so that a net decrease in peak contractile force was present by 2 and 3 minutes, respectively. Force reached an approximately steady value by 5 minutes. The time constants for the decline in force after the coronary effluent was applied and for the recovery after the coronary effluent was replaced by

- **Figure 8.** Plots showing the relation between the amplitude of the change in peak force developed by trabeculae during exposure to coronary effluent and the coronary flow at the time the effluent was collected. In the top panel, the coronary effluent was assayed on trabeculae that had not been treated with 0.5% Triton X-100, and in the bottom panel, the trabeculae had been treated with Triton X-100 for 1 second. As the coronary flow increases, the amplitude of the change in force declines with Triton X-100--treated trabeculae but not with untreated trabeculae. The difference is significant (p=0.02) according to Fisher's exact test and Spearman's correlation.

- **Figure 9.** Plots showing the absolute value for the percent change in the force developed by trabeculae and the cross-sectional area in the same trabeculae before (top panel) and after (bottom panel) the endocardial endothelium had been damaged by a 1-second exposure to 0.5% Triton X-100. The change in the pattern of response produced by the exposure to Triton X-100 was significant at p=0.09.

- **Figure 10.** Plot showing the relation between percent oxygen saturation in coronary venous effluent before it was reoxygenated and the percent change in peak force in trabeculae with cross-sectional area of ≤0.06 mm² (correlation coefficient, 0.82; coefficient of determination, 0.67).
normal Krebs' solution were approximately 3 minutes. The amplitude of the decrease produced by the coronary effluent was not related to the oxygen tension in the coronary effluent as shown in studies above (Figure 6, bottom panel). However, downregulation produced on the trabeculae with damaged endothelial cells was related to the rate of coronary flow (as shown in studies above in Figure 8, bottom panel).

Endothelial cells release a labile upregulating (positively inotropic) factor. To minimize the time delay between the release of an endothelial factor and its action on cardiac myocytes, the coronary effluent from perfused hearts with damaged endothelial cells was assayed on trabeculae with undamaged endothelial cells (protocol C in Figure 12). In this experiment, only factors that stimulate endothelial cells to release cardioactive factors would be present in the coronary effluent; no endothelial factors themselves would be present. These endothelial cell-stimulating or preendothelial factors could act on endothelial cells immediately adjacent to cardiac myocytes in the trabeculae, and the effect of that interaction on contraction could be detected. In six of eight experiments, in which the percent $O_2$ saturation of the coronary effluent before reoxygenation was 20% or greater, there was an increase in the peak tension developed by the trabeculae of 30±7% (Figure 14) ($p<0.01$). In two additional experiments, the perfused hearts were subjected to a 4-minute period of ischemia that was concluded 10 minutes before the collection of the coronary effluent for application to the trabecula. The $O_2$ saturations of the coronary effluent were 8% and 19%. In these experiments, the coronary effluent caused declines in peak tension of 16% and 19% (Figure 14). The amount of change in force developed by the trabeculae with undamaged endothelial cells was related to the oxygen tension in the effluent before it was reoxygenated (Figure 14) (correlation coefficient, 0.86). The relation between $O_2$ change and change in force from coronary effluent is shifted to the left when the vascular endothelial cells in the perfused heart are damaged as if a downregulating factor has been reduced (compare the bottom panel of Figure 6 with Figure 14). This is the opposite of the effect of damaging the endocardial endothelium in the trabecula, which results in a loss of the upregulation (Figure 6, bottom panel).

The rate of onset and the reversal of the increase in peak tension caused by the coronary effluent were much faster than the appearance and disappearance of the decline in contractility described above. The maximum change occurred in both directions within 1 minute, the shortest period of time in which the measurement was made, since five twitches were signal-averaged to give the contour of the contractions used for data analysis.

Preendothelial and endothelial factors. As shown above, the coronary venous effluent contains both cardioactive factors released by endothelial cells and sub-

**FIGURE 11.** Plots showing the effect of a 1-second treatment with 0.5% Triton X-100 in Krebs' solution on the peak isometric force (expressed in relative terms) of a series of isolated trabeculae that have been superfused only with Krebs' solution (top panel) and that have been exposed to coronary venous effluent with $O_2$ saturation of less than 28% before reoxygenation (bottom panel) (at these $O_2$ levels, the decrease in force remained after the removal of the coronary effluent). Asterisk indicates significant difference at $p<0.05$.

**FIGURE 12.** Diagrams illustrating the protocols for experiments described in "Role of Endothelial Cells." MYO, myocaridium; ENDO, endothelial cells.

**FIGURE 13.** Plot obtained from recordings of two trabeculae, one treated with Triton X-100 to damage endothelial cells (○) and the other with undamaged endothelial cells (●), exposed between the arrows to aliquots of the same coronary venous effluent collected from a perfused heart with damaged vascular endothelium.
Table 1. Effect of the State of the Endothelial Cells on the Cardioactivity of Coronary Effluent

<table>
<thead>
<tr>
<th>State of endothelial cells</th>
<th>Change in peak tension</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfused heart Intact</td>
<td>30±7%</td>
<td>7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Damaged*</td>
<td>-11±3%</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>Damaged†</td>
<td>5±3%</td>
<td>8</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM. The same perfused hearts were used for all three sets of conditions.

The same perfusate was assayed on two different trabeculae from the same heart. There was no significant difference in the response of the two trabeculae to coronary effluent with both sets of endothelial cells intact.

Perfusate collected from the same perfused heart at two different times was assayed on the same trabecula.

stances that stimulate endothelial cells to release the cardioactive factors. The activity of preendothelial and endothelial factors can be partially separated by examining the effects of selective damage to endothelial cells in the perfused heart or the trabeculae (Figure 15).

In the top panel of Figure 15, the effect of damaging the vascular endothelial cells in the perfused heart on the change in the force of contraction of trabeculae produced by coronary effluent has been plotted (protocols A and C in Figure 12). The results show that damaging the vascular endothelial cells has little effect on the change in contractility of trabeculae when the original effect of the effluent was to decrease peak force. The effect of damaging the vascular endothelial cells is quite different when the effluent enhanced the contractility of trabeculae: the size of the increase in force becomes greater. These data indicate that coronary effluent contains a preendothelial upregulating factor and a preendothelial downregulating factor. There is no evidence for an endothelial upregulating factor and no need to postulate a downregulating endothelial factor. In addition, there must be some antagonistic or inhibitory effect produced by the endothelial cells in the perfused heart against an upregulating factor formed by the endothelial cells in the trabeculae.

When the endocardial endothelial cells in the trabeculae are damaged and the vascular endothelial cells in the perfused heart remain intact (protocols A and D in Figure 12), all positive effects disappear, and there is a small increase in the negative effect of the effluent (Figure 15, bottom panel). These data show that the endocardial endothelial cells in the trabeculae are the source of the endothelial upregulating factor and that the coronary effluent contains no endothelial upregulating factor. All of the downregulating effect can be accounted for by the endothelial downregulating factor, although a preendothelial downregulating factor may also be present.

In these studies, the downregulating effect of the coronary effluent is not significantly altered by selectively eliminating the effects of either the preendothelial or endothelial downregulating factor individually. The level of activity of the two downregulating factors is the same, suggesting that they are in equilibrium. This would be expected if the factors were relatively stable, since the coronary perfusion medium had been recirculated for a period of time before the collection of the coronary effluent. The absence of any endothelial upregulating factor in the presence of a preendothelial upregulating factor in the coronary effluent supports the notion that the endothelial-derived upregulating factor is relatively labile. Although probably originally present in the coronary fluid, the factor has rapidly decayed. The fact that the collective effect of the more stable preendothelial factors is upregulation is com-

Figure 15. Plots showing the relation between the effect of coronary effluent before and after damage to endothelial cells. Top panel: Damage to endothelial cells in the perfused heart. Bottom panel: Damage to endothelial cells in the trabeculae.
pletely consistent with the decrease in mean developed pressure and cardiac output by perfused hearts after damage to the vascular endothelium.

Discussion

The existence of cardioregulatory factors in the coronary effluent from isolated perfused working hearts has already been shown in a study suggesting that their effect required the presence of normally functioning endothelial cells. This work also suggested that both upregulating (positively inotropic) and downregulating (negatively inotropic) effects were produced. These inferences have been tested in studies in which there has been selective damage to vascular endothelial cells in perfused hearts and endocardial endothelial cells in isolated ventricular trabeculae. Changes in contractility as a result of endothelial factors have been called upregulation and downregulation in preference to changes in inotropy because of the pathological connotation usually attached to the term negative inotropy.

To investigate the possibility that coronary endothelial cells produce substances that modify the contractility of the myocardial cells, the effect of the coronary venous effluent from an isolated perfused working heart has been assayed on the development of force of an isolated cardiac trabecula. When a bathing medium identical to the fluid entering the coronary artery system of the perfused heart was replaced by reoxygenated coronary venous effluent, the contraction of the trabecula changed in most cases. The alterations in the contractility were not uniform. In some experiments, developed force increased; in others, it decreased. After damage to endothelial cells in both the perfused heart producing the coronary effluent and the trabecula on which the coronary effluent was assayed, no change in the contraction of the trabecula was produced by the coronary effluent. The direction and the amplitude of the change seemed to depend on the state of endothelial cells in the perfused heart and the trabecula as well as tissue oxygen tension and rate of coronary flow in the perfused heart.

Procedure for Damaging Endothelial Cells in the Perfused Heart

Four different methods have been described in the literature for damaging the vascular endothelium in an organ or a tissue. These are perfusion with a low concentration of a detergent, a hypotonic solution, a selection of enzymes, or a standard electrolyte medium that has been modified to contain 40 mM K+. In all of the published applications of these methods, the aim of the research was to compare the behavior of the blood vessels with and without normal endothelium. The function of the organ itself was not a major consideration.

In the study reported here, the goal was to study changes in function of the heart in the presence and absence of normally functioning endothelial cells. When either enzymes, detergent, or hypotonic solution is used to damage vascular endothelium, two undesirable consequences occur. The capillaries are disrupted, allowing the specialized perfusion medium to pass into the extracellular space in an uncontrolled fashion, and the damage to the endothelium inhibits the normally high basal level of nitric oxide production in isolated perfused vessels. The result of the latter in the heart is a marked increase in coronary resistance and sharply decreased coronary flow. As a result of the markedly reduced coronary venous oxygen tension, the hemodynamic performance deteriorates rapidly, resulting in much lower cardiac output (data not shown).

With the technique of perfusion with a medium containing an elevated concentration of potassium ions, it is possible to exercise greater control over the effects of the damage. The part of the vascular system within which endothelial damage occurs can be limited to prevent disruption of the capillaries, and the large decline in mean pressure and output produced by the heart can be prevented. For these reasons, perfusion with a high-K+ Krebs’ solution was used to damage the endothelium.

The damage to the endothelial cells in the blood vessels is almost certainly due to the increase in the perfusion pressure required to maintain flow during the period in which the heart operates as a Langendorff preparation with constant flow. The high concentration of K+ causes strong contraction of the smooth muscle in the arteries and a sharp decline in pressure in the capillaries and veins. As a result, the endothelial cells in the arteries and arterioles are stripped from the walls of the blood vessels, and the endothelial cells in the capillaries and veins remain intact. During the process of stripping off the endothelial cells, the surface membranes of the smooth muscles are damaged, producing calcium loading, intense contraction, and disruption of the intracellular organization. After the washout of the high-K+ solution, the smooth muscle cells are left with intracellular disorganization including heavy calcium loading of mitochondria and sarcoplasmic reticulum. Apparently, this damage prevents the smooth muscle cells in the arteries and arterioles from producing the large increase in coronary resistance that normally would have occurred after the loss of the basal secretion of nitric oxide. This interpretation is supported by the observations that the short exposure to the high-K+ solution does not alter the contractile function of the trabeculae.

Regulating Factors Added to the Coronary Venous Effluent

Since Krebs’ solution contains only ions and glucose, an alteration in contractility from a loss of material from the medium is unlikely. The change in contractility is almost certainly due to the addition of some substance or substances, as the perfusate passes through the coronary blood vessels. The classical work of Furchgott and Zawadski and subsequent studies have shown that endothelial cells lining the lumen of blood vessels produce substances that can modify the tone of the vascular smooth muscle by releasing at least six different substances with vasoactive properties, including nitric oxide, a potent vasorelaxant, and endothelin, a powerful vasoconstrictor. Changes in oxygen tension and shear force on the endothelial cells are also effective stimuli for endothelial secretion.

A role for endothelial cells in the modulation of cardiac contractility has been suggested by several different kinds of studies. Removal or damage of the endocardial endothelium lining in isolated trabecula decreases peak tension and shortens the time to the
onset of relaxation in isolated ventricular trabeculae.9 This change can be reversed by exposing the tissue bundle to the fluid incubating a culture of cardiac endothelial cells.11,12 Under certain conditions, one can observe a higher level of activity of actomyosin ATPase in cardiac cells in the immediate vicinity of coronary arteries even when those arteries are not being perfused.8 Of the substances elaborated by endothelial cells, at least one, endothelin, is known to have a powerful inotropic action on cardiac tissue.23

The importance of the endothelial cells in the production of cardioregulatory factors in the coronary effluent is shown conclusively by the studies in which the vascular endothelial cells in the perfused heart and endocardial endothelial cells in the test trabecula had been damaged. Under these conditions, there was at most a very small effect of coronary effluent on the contraction of the trabecula. A small effect could have been due to residual endothelial cells in the blood vessels or to a small effect of factors released by myocytes acting directly on other myocytes. The data do not allow a distinction between these two possibilities, but they do affirm that endothelial cells are required at least for almost all of the effect on contractility that can be transmitted through a medium perfusing the vascular system.

Two different types of factors that are involved in the endothelial regulation of cardiac contraction are present in the coronary venous effluent: factors liberated by the endothelial cells (endothelial factors) and factors that act on the endothelial cells to regulate their release of cardioactive factors (preendothelial factors). The effluent from a perfused heart with damaged endothelial cells can still alter the contraction of a trabecula even though it does not contain endothelium-derived factors. The effluent contains signals to endothelial cells in the trabecula that result in a release of factors from these endothelial cells. Damaging the endocardial endothelial cells in the trabecula removes essentially all effect on contractility of the trabecula by the coronary effluent collected from a heart with damaged endothelium.

Conversely, damaging the endocardial endothelial cells in the trabecula removes the possibility of producing endothelial factors in the trabecula in response to preendothelial factors. Therefore, the change in contractility of the trabecula with damaged endothelial cells in response to coronary effluent must be due to endothelial factors produced by the cells in the perfused heart. This interpretation is confirmed by the disappearance of the cardioregulatory activity when the vascular endothelial cells in the perfused heart as well as endocardial endothelium, which constitutes approximately 90% of the total endothelium in the trabecula, have been damaged.

Separate Endothelium-Derived Upregulating and Downregulating Factors

By using different combinations of damaged versus undamaged endothelial cells in the perfused hearts and the trabeculae, it is possible to isolate the effects of individual cardioactive components of the coronary effluent. Two specific individual factors have been demonstrated, an upregulating factor whose effect disappears very quickly when it has been removed and a downregulating factor with an effect that has a substantially longer time constant for decay. The upregulating factor becomes very prominent when the endothelial cells in the perfused heart have been damaged but the endothelial cells in the trabecula are undamaged. Under these conditions, the diffusion distance and time for material released by the endothelial cells to reach the cardiac myocytes are very short. An upregulating effect is present at Po2 levels lower than those required when no endothelial cells have been damaged. This can be explained if the downregulating effect produced by endothelial cells in the perfused heart partially obscures the more labile upregulating effects. Such an interpretation would explain why damaging the endothelial cells in the trabeculae virtually eliminates any upregulating effect.

The endothelium-derived downregulating factor is more stable, and its effect can be seen even after a delay of a few minutes before its application to the trabecula. Its effect has a slower onset and a slower decay than the upregulating factor. The differences in the kinetics and stability of the two factors can explain several observations reported in “Results,” but more direct experiments are necessary to support the interpretation. The difference in decay rates could be why the residual effect after removal of the coronary effluent is a decrease in contractile force and why a net downregulating effect is sometimes preceded by a rapidly appearing but transient increase in contractile force. The influence of oxygen tension on the relative concentrations of the upregulating and downregulating factors could explain the decreasing likelihood of a residual downregulating effect as the Po2 increases.

The different patterns of recovery from the exposure to coronary effluent could be due to the different kinetics of the upregulating and downregulating endothelial factors and the differences in relative amounts according to tissue oxygen tension. In many ways, this type of regulation of contractility, determined by the balance of two opposing effects with different kinetics, resembles what has already been described for the response of vascular smooth muscle to endothelium-derived factors. Endothelial cells produce both upregulating and downregulating substances that can be present at the same time, the net effect on smooth muscle contraction being determined by the balance between the opposing actions.24

It is possible to explain all of the observations of the effects of coronary venous effluent on myocardial contractility as the sum of the individual effects of the endothelium-derived upregulating and downregulating factors. The general shape of the top panel of Figure 6 is very similar to the sum of the bottom panel of Figure 6 and Figure 14. The upregulating factor appears to be closely related to the oxygen tension, whereas the downregulating factor appears to be relatively insensitive to oxygen tension but relatively sensitive to coronary flow. The combined effect of the two factors produces a regulatory mechanism that responds to changes in oxygen tension and coronary flow, the two key parameters most concerned with energy supply and energy utilization.

It is not possible at this time to identify the two endothelial factors. The stability of the downregulating factor, as shown by the existence of the effect after
minutes, a period of time equal to approximately 20 half times for decay of the nitric oxide, indicates that it cannot be that substance. The absence or very low concentration of endothelin in the coronary effluent argues against it as a significant factor in these studies, although endothelin, normally considered to be relatively stable, may be degraded in this preparation. However, it is important to note that the lack of a role for either nitric oxide or endothelin in the isolated perfused heart does not mean that these substances are unimportant in the regulation of contractility of the heart in the intact organism.

Mechanisms of Regulation of Contractility

One important mechanism by which endothelial cells modify contractility is through modulation of a cAMP-dependent mechanism that regulates the ATPase of actomyosin by raising or lowering enzymatic activity.25,26 Integrity of endothelial cells is required for the function of the cAMP-dependent regulatory mechanism,27 and when the endothelial cells are damaged, this type of regulation disappears.

It is likely that contractile force as well as ATPase activity of actomyosin is regulated by this cAMP-dependent mechanism. In earlier studies, it was shown that maximum calcium-activated force could be increased in permeabilized rat heart by a sequence of two successive reactions, the first initiated by cAMP and the second by Triton X-100 or another nonionic detergent.28 In light of the findings reported here, it is not unreasonable to conclude that the second reaction involved the release of factors from the endothelial cells by the Triton X-100. The ability of Kentish and Jewell29 to see only a small effect in their efforts to reproduce the results is probably a consequence of their failure to ensure the integrity of the endothelial cells in their preparations.

Other changes in the function of the cardiac myocytes in response to endothelial factors must also be considered. Changes in the calcium sensitivity of the contractile system and in the calcium currents through the sarcolemma and/or sarcoplasmic reticulum have been suggested.9,13

Role of Coronary Flow

As coronary flow in the perfused heart increased, the amplitude of the change produced by coronary venous effluent in the contractility of the trabeculae with damaged endocardial endothelial cells decreased. At the highest coronary flow rates observed, the venous effluent had no effect on the contraction of these trabeculae. A similar correlation did not exist, however, when the assay was performed on trabeculae with intact endocardial endothelium. In other words, when the assay was sensitive only to factors apparently acting directly on the myocardial cells, the response of the trabecula was sensitive to the rate of coronary flow in the isolated heart from which the coronary effluent had been collected. When factors that acted on the endocardial endothelial cells as well as those acting directly on the myocytes were assayed, the sensitivity to the rate of coronary flow was obscured. A simple explanation for this difference is that factors acting directly on the myocytes are released by vascular endothelial cells whose function is altered by the shear force produced by coronary flow. Factors acting on endothelial cells arise from other cells in the myocardium, presumably myocytes, whose function is not directly influenced by the physical forces associated with flow through the coronary vessels.

Rates of coronary flow in isolated perfused hearts in general are high. The lowest rates in the isolated hearts studied here were about equal to the highest rates in the in situ heart found during intense exercise, whereas the highest rates in the isolated heart were beyond the physiological range. A peak production of cardioactive factors by vascular endothelial cells may occur at coronary flow corresponding to the maximum values found in the organism. To demonstrate the accuracy of this speculation, it will be necessary to work with isolated perfused hearts having a lower rate of coronary flow. Indirect support for this notion can be found in previous work, in which quantitative histochemistry was used to detect spatial heterogeneity in actomyosin ATPase activity in cardiac muscle. There was evidence that, over a period of up to 2 hours after the termination of perfusion of coronary blood vessels, the production of an upregulating substance by cells in the blood vessels gradually ceased.8

The most likely basis for the effect of coronary flow is the shear force on the endothelial cells lining the blood vessels rather than dilution of material with increasing coronary flow. The insensitivity to the rate of coronary flow when the coronary effluent is assayed on trabeculae with intact endothelial cells argues against simple dilution. The rate of secretion of vasoactive material and the activity of certain ion channels in endothelial cells have already been shown to be sensitive to shear force.5,6,30 However, these data do not specifically demonstrate shear force as the important element. Other effects of variable coronary flow could be responsible for the modulation of the amplitude of the change in contractility. It is unlikely that coronary flow exerts its effect through oxygen tension in the tissue, because oxygen tension appears to be maintained by the variable coronary flow. The major determinant of venous oxygen tension under the conditions of controlled preload and afterload seems to be cardiac efficiency, a property of the cardiac cells that is set in most preparations either during the dissection or the initial perfusion of the isolated heart.

Endocardial Versus Vascular Endothelium

Since the regions of the perfused heart where endothelial cells were damaged by the high-K+ Krebs' solution were restricted to those subjected to a high perfusion pressure and since the ultrastructure of the endocardial endothelium appeared to be normal, it is likely that the actual cause of the damage is the perfusion pressure itself. The elevated K+ serves to increase the pressure and the velocity of the perfusion fluid by producing contraction of the vascular smooth muscle and the cardiac myocytes. There is no sign of damage to the endocardial endothelium of the perfused heart and no effect on the contraction of the trabecula from incubation in the high-K+ Krebs' solution, where pressure is not abnormally high.

In the absence of intact endocardial endothelial cells in the trabecula, no upregulating effect was seen from coronary effluent. Only a downregulating effect is seen, and that requires intact vascular endothelial
cells in the perfused heart. When the arterial endothelial cells in the perfused heart have also been damaged, the downregulating effect of the coronary effluent disappears as well, even though endothelial cells in the capillaries, veins, and endocardium are undamaged. These results indicate that the endothelial cells in the capillaries, veins, and endocardium are not producing downregulating factor in the isolated perfused heart, but it does not mean that they are totally nonproductive of cardioregulatory factors. In the isolated perfused heart they could be secrting the labile upregulating factor, which decays too quickly for detection under the conditions of the experiment. From other studies, it appears that capillaries and venules do not produce cardioregulatory factors, but in the isolated trabeculae it is clear that the endocardial endothelium does produce cardioregulatory factors. When the vascular endothelium of the perfused heart has been damaged, an effect of the coronary effluent on contractility of the trabeculae is still observed, although it is different. This effect can be eliminated by damage to the trabecular endocardial endothelium as well. The endocardial endothelial cells are not functioning merely as a diffusion barrier, inasmuch as damage to the endocardial endothelium on the surface of the trabecula decreases the extent to which the effect of the effluent penetrates into the trabeculae.

Physiological Function

On the basis of the data presented, it appears likely that endothelial cells play a significant role in modulating the contractility of the heart in situ. Local oxygen tension and the rate of coronary flow appear to be prime determinants of the nature of the function of this mechanism by influencing the relative concentrations of upregulating and downregulating endothelium-derived factors. Although it is clear that the regulatory system is sensitive to oxygen and coronary flow, it is not yet clear the extent to which oxygen tension acts directly on the endothelial cells and/or on other cells in the myocardium, in particular the cardiac myocytes, to release preendothelial factors.

One attractive model that explains the data would have both cardiac myocytes and endothelial cells interacting. The endothelial cells would release cardioactive substances in response to coronary flow and local O2, and the cardiac myocytes would influence endothelial function in accordance with the metabolic state of the myocytes. Under conditions of high work relative to energy supply, the loop would serve to downregulate contractility and decrease energy requirements. When energy supply is relatively high, upregulation of contractility could occur. This would constitute a form of autoregulation. Evidence in favor of the existence of such a mechanism has already been published.

Preendothelial factors either diffuse from their site of formation, presumably myocardial contractile cells, into the blood through the capillaries or through the wall of the artery to the endothelial lining. If they enter the bloodstream through the capillaries, they must remain stable through the circulation, including the lungs, before they reach the coronary arterial endothelium or, in the case of the left ventricle, the endocardium, where they can stimulate from endothelial cells the release of factors that actually act on the contractile cells. Endothelial factors entering the circulation in the arteries could diffuse through the capillaries to exert their effect without the need to ever enter the general systemic or the pulmonary circulation. Preendothelial factors would have to be relatively stable in the blood at least on a time scale of many seconds or a few minutes, whereas endothelial factors could be relatively unstable. A degree of instability of endothelial factors might be desirable to increase the responsiveness of the contractile cells to changing conditions. In the relatively incomplete data pertaining to the stability, endothelial factors do appear to be less stable than preendothelial factors.

A regulatory mechanism in which myocardial cells release factors according to the functional state of the cell and these factors in turn stimulate or modify the endothelial release of cardioactive factors modulating function of myocardial cells has the advantage of providing a site for information integration from multiple sources. It is quite possible that other organs, in particular the lungs and the kidney, may release substances into the blood that influence the production of cardio-regulatory factors by endothelial cells in the heart and thereby integrate the function of the heart with activity in other organs. In the regulation of vascular smooth muscle, many substances produced all over the body have effects of the vascular endothelial cells. In this comparison with the regulation of vascular smooth muscle, it is interesting to note the relative stability of endothelium-derived factors. For vascular smooth muscle, the relaxing factor is very labile, and the major contracting factor isolated so far (endothelin) is relatively stable. In blood vessels, the reserve is in the ability to dilate and lower the resistance to blood flow. In the heart, where the reserve is in the ability to increase contractility, upregulation is the more labile.

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C Ramaciotti, G McClellan, A Sharkey, D Rose, A Weisberg and S Winegrad

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