

EXPERIMENTAL STUDIES

Reactivity-Based Coronary Vasospasm Independent of Atherosclerosis in Rhesus Monkeys

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Objectives. We studied the hypothesis that in the absence of vascular pathology, coronary artery vasospasm occurs as a result of local regions of vascular muscle hyperreactivity. We aimed to explore the basis for a functional etiology of those vasospasms not explained on a structural basis.

Background. Ovariectomized rhesus monkeys (*Macaca mulatta*) without injury or significant vascular disease were stimulated with platelet release products, and angiograms were compared with those from vasospasms induced in human patients.

Methods. We used intracoronary (IC) injections of serotonin, thromboxane A₂ (U46619), endothelin 1 or angiotensin II in concentrations 3 to 10 times that which reduced coronary artery diameter by 50%.

Results. Although no agent alone caused vasospasm, the combination of pathophysiologic concentrations of serotonin and the stable thromboxane A₂ mimetic, U46619, injected through an IC catheter, synergistically caused coronary vasospasm on the second

or third challenge in five of seven monkeys. These drug-induced vasospasms were similar to vasospasms induced by mechanical injury followed by serotonin, and to those stimulated in human IC diagnostic tests, as judged by onset, appearance, kinetics and vasodilator reversal.

Conclusions. These studies in ovariectomized monkeys revealed that coronary vasospasm can be stimulated without preexisting vascular pathology, endothelial denudation or injury. Reproducible vasospasm of primate coronary arteries in response to these two endogenous pathophysiologic vasoconstrictors, which are thought to be precipitating stimuli in the etiology of vasospasm, suggests that structure-independent epicardial vasospasm can be an important element in serious cardiac ischemic events, particularly the focal, persistent vasospasms that occur without plaques or injury.

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Coronary vasospasm is a phenomenon identified by focal constrictions followed by downstream dilations in a characteristic hourglass-shaped pattern, with an underlying mechanism that is only partially explained (1). The focal constrictions in this signature pattern imply local vascular muscle hyperreactivity and persistent contraction as a definitive feature of vasospasm (1-6). Thromboxane A₂ and serotonin have been suggested as the initiating stimulus (7-9), a concept that is supported by animal and human studies (10). When injury or plaque rupture occurs, additional mediators include thrombin, adenosine diphosphate, free radicals of oxygen and platelet-activating factor, which would predispose toward initiation of vasospasm; furthermore, injury would simultaneously reduce potent endothelial relaxant factors (e.g., endothelial-dependent

relaxant factor, prostacyclin and tissue-type plasminogen activator) (10). Although platelet release products seem to be major factors in vasospasm (11,12), it is not clear that these substances can produce vasospasm in the absence of plaques or injury.

Although structural occlusion explains a major fraction of fatal ischemic events, the vasospasm phenomenon can be localized and episodic and is not always correlated with atherosclerotic plaques (1,2,13,14). Although plaque rupture is believed to be the initiating event in most cases of vasospasm and acute myocardial infarction, locally released vasoconstrictors are obligatory to an explanation of the segmental occlusion pattern and probably initiate vasospasm, particularly where plaques are either stable or absent.

Variant angina has been hypothetically explained by functional, putatively drug-induced vasospasms (1,2,6,15-17). Several agents, including serotonin, thromboxane A₂ (TxA₂), endothelin (ET1) and angiotensin II (AII) have been hypothesized (2,10,14,18) as vasoconstrictors responsible for vasospasm.

In human patients, coronary plaques are common and sometimes not correlated with vasospasm, which suggests that functional changes are a critical factor (13,14). However, the widespread occurrence of coronary atherosclerosis is itself a

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Abbreviations and Acronyms

Ang II	= angiotensin II
ACh	= acetylcholine
dP/dt	= rate of increase in left ventricular pressure
EC ₅₀	= effective concentration for 50% of a specified response
ET1	= endothelin-1
IC	= intracoronary
LAD	= left anterior descending coronary artery
ovx	= ovariectomized
TxA ₂	= thromboxane A ₂
VMC	= vascular muscle cell

complication for the analysis in humans (14,15,18,19). Hyperreactivity to vasoconstrictors has been shown (20) to precede atherosclerotic changes, and the decline in hyperreactivity to precede structural regression, in a primate model of diet-induced atherosclerosis. Thus, investigation of coronary hyperreactivity itself is greatly in need of additional study. Similarities of cardiovascular reflexes (21) and receptors (22) in nonhuman primates to those in humans and productive studies of atherosclerosis and coronary vasospasm in monkeys (20,23-25) led us to explore provoking coronary vasospasm in rhesus monkeys without coronary plaques or injury. This series of experiments explored whether a single agent or combination of vasoconstrictors together could stimulate the characteristic hourglass form of coronary vasospasm in the absence of injuries or plaques.

Our specific hypothesis was as follows. In the absence of protective factors (e.g., in the state of hypoestrogenism) and without arterial wall pathology or injury, localized regions of abnormal hyperreactivity in response to platelet vasoconstrictor products occur in coronary arteries; these foci of hyperreactivity can result in responses to platelet-released serotonin in combination with TxA₂ that include exaggerated, persistent contractions that fail to be reversed by normal inactivation processes of vascular muscle cell (VMC) and are sufficient to explain vasospasms. An important corollary is that neither structural occlusion nor injury is required for vasospasm, although plaque rupture and artery injury would be important components where they existed. A preliminary report of our results was presented previously (26).

Methods

Vasospasm definition. It is essential to differentiate 1) physiologic, autoregulatory constrictions that allow for a range of coronary blood flows versus 2) pathologic prolonged constrictions that result in ischemia and injury to the myocardium (2,6,27). Therefore, we define *coronary vasospasm* as epicardial coronary artery constriction to <25% of control diameter in focal areas with adjacent downstream dilation, with the hourglass pattern thus formed persisting for >5 min.

The characteristic hourglass appearance (see pp. 676 and 677) was the ultimate criterion used to define vasospasm.

Reactivity-based vasospasm is implied by enhanced responses to pharmacologic stimuli in the absence of predisposing structural factors and thus we hypothesize might be explained by local exaggerated constrictions to stimuli.

Animals. We studied 10 adult rhesus monkeys (*Macaca mulatta*) 7 to 19 years old (average 13.4) in the main protocols described below and 5 additional monkeys in preliminary experiments. For the 10 main study animals, we used 1 male (14 years old) weighing 10.3 kg and 9 ovariectomized (ovx) female monkeys weighing 5.3 to 8.2 kg (average 6.2), none of which had been exposed to high cholesterol diets and which were later verified (see Results) to lack evidence of cardiovascular disease. Ovx rhesus monkeys were entered into the study no less than 3 months after ovariectomy.

Catheterization studies. Angiography at the Dotter Institute, after overnight fasting, began with preanesthesia with ketamine (10 mg/kg body weight intramuscular), intubation with an endotracheal tube and a light surgical plane of anesthesia with isoflurane (induced at 1.5% to 3%, maintained at 0.75% to 1.25%), vaporized with 33% nitrous oxide and 67% oxygen. Bilateral femoral intraarterial catheters allowed for coronary catheterization and continuous recording of systemic arterial blood pressure and heart rate, which were maintained close to the initial anesthetic value for each monkey. Intravenous heparin (1,000 U) was injected, and up to 120 ml of lactated Ringer's solution and 0 to 10 ml of dextran solution were used (as needed) to maintain diastolic blood pressure ≥ 60 mm Hg. A heating pad assisted in maintenance of a body temperature decrease $\leq 2^\circ\text{C}$ of preanesthesia body temperature (monitored by rectal thermometer). An electrocardiogram (ECG) recorded on a Gould eight-channel recorder, cutaneous arterial oxygen saturation, respiratory rate and end-tidal CO₂ were also monitored.

Entire experiments were recorded on videotape (with episodic fluoroscopic imaging and continuous voice annotation) to permit computerized quantitative coronary angiography. Protocol 1 (Table 1) evolved from a series of pilot studies in five additional monkeys in which we tested different combinations and doses of the four vasoconstrictors serotonin, TxA₂, ET1 and Ang II. Vasoconstrictor concentrations were incremented in log units because of the log-normal distribution of sensitivities in blood vessels (28). Because vasospasms were only observed in ovx monkeys and in response to serotonin and TxA₂ in combination, we focused on this combination.

Placement of the 3F catheter (except as noted later) was adjusted to provide sufficient filling with radioopaque contrast medium, limited reflux and isolation of a branch of the coronary arterial tree. Usually the left anterior descending coronary artery (LAD) was chosen, but the left circumflex (in two cases) or right coronary artery (in one case) was used instead, based on optimal placement and to avoid occlusion of blood flow. After adjustment of the camera angle to optimally image the coronary vascular tree, warm (35 to 38°C) 1- to 2-ml boluses of Hexabrix (Mallinckrodt) radioopaque contrast media were injected rapidly by hand to fill optimally. Fluoroscopic images were recorded on film using a Toshiba CAS-CP

Angiorex system with fluoroscopic C-arm, developed immediately and evaluated for suitability of subsequent analysis.

Every injection of drugs was made by intracoronary (IC) route, with a slow, constant flow of 1 ml over 30 ± 1 s. The time between drug injections was typically 7 to 10 min, and no less than 4 min, with pressures and heart rates allowed to return to $\leq 15\%$ change from baseline before the next injection. All concentrations given in the Methods section are syringe concentrations (uncorrected for dilution by coronary blood flow) to exactly describe the procedure. (The effective concentrations in the blood are presented in the Results section and in Table 3, as explained later.)

The drug-induced protocol, listed in Table 1, was the sequence of injections followed for the seven ovx monkeys. Step 1 established control coronary diameters; step 2 was slow IC injection of 1 ml of 100 nmol/liter of acetylcholine (ACh), a total of 0.018 μg , to validate endothelial function, as observed by vasodilation of large coronary arteries. Step 3 was IC injection of 1 ml of 1 $\mu\text{mol/liter}$ (a total of 0.18 μg) ACh. The higher ACh dose is about 500 times less than the 100 μg used for provoking vasospasm in susceptible patients (19,29).

The fourth step in protocol 1 (Table 1) was 1 ml of 100 $\mu\text{mol/liter}$ serotonin (a total of 17.6 μg), a vasodilator dose that provided an additional test of endothelial dilator function. The fifth step was to inject 1 mmol/liter serotonin to directly vasoconstrict VMC. The sixth and seventh steps were injection of 100 nmol/liter U46619 (0.035 μg) and 1 $\mu\text{mol/liter}$ U46619.

The eighth to tenth steps, which were 1-ml injections of 100 $\mu\text{mol/liter}$ serotonin and 1 $\mu\text{mol/liter}$ U46619 combined, were the critical steps. These concentrations of serotonin and TxA_2 injected in three repeated challenges provoked drug-induced vasospasm. Sensitivity was determined by response to the first, second or third stimulus, allowing at least a 7-min delay from one injection to the next. For three of these seven ovx monkeys, 1-nM ET1 (alone and plus serotonin) and 1- $\mu\text{mol/liter}$ AII (alone and plus serotonin) steps were administered between steps 10 and 11 (as additional challenges), but these extra steps never produced vasospasm. Step 11, used only in the two ovx monkeys in which vasospasm was not found by this point, was the triple combination (in 1 ml) of 100 $\mu\text{mol/liter}$ serotonin and 1 $\mu\text{mol/liter}$ U46619 with 1 nmol/liter ET1, which also did not produce vasospasm. Step 12 was a Ca^{2+} antagonist, either nitrendipine or mibefradil, as a vasodilator to relieve the vasospasm or cardiogenic shock that was the end point for step 11.

A separate mechanical injury protocol 2 (Table 2) was used in three additional monkeys (one male, two ovx). The injury step of protocol 2 was deliberate mechanical stretching of the LAD or left main coronary artery with a 5F catheter. (Except in these three monkeys with deliberate mechanical injury, the other seven [protocol 1] monkeys were studied with 3F catheters only to avoid mechanical injury, as verified by 1) lack of constriction at the catheter tip, 2) no occlusion of coronary flow by the catheter, and 3) no evidence of injury on dissection and histopathologic examination, as described later).

Table 1. Protocol 1: Drug-Induced Protocol for Coronary Vasospasm*

1. IC injection of contrast media to establish arterial diameters before drugs (control)
2. IC injection of 100 nmol/liter (0.018 μg) acetylcholine to show dilator capacity
3. IC injection of 1 $\mu\text{mol/liter}$ acetylcholine (0.18 μg) to show dilator capacity
4. IC injection of 100 $\mu\text{mol/liter}$ (17.6 μg) serotonin
5. IC injection of 1 mmol/liter (176 μg) serotonin
6. IC injection of 100 nmol/liter (0.035 μg) U46619 (stable thromboxane A_2 mimetic)
7. IC injection of 1 $\mu\text{mol/liter}$ (0.35 μg) U46619
8. Challenge 1 with combined 100 $\mu\text{mol/liter}$ (17.6 μg) serotonin and 1 $\mu\text{mol/liter}$ (0.35 μg) U46619
9. Challenge 2 with combined serotonin–U46619 stimulation
10. Challenge 3 with combined serotonin–U46619 stimulation
11. Triple combination with serotonin and U46619 and 1 nmol/liter endothelin 1 or serotonin and U46619 and $\mu\text{mol/liter}$ angiotensin II
12. Dilation 15 min later with 1 or 10 $\mu\text{mol/liter}$ nitrendipine or Ro 40-5967 (mibefradil)

*All concentrations are expressed as the undiluted solution in the injection syringe, but would have been diluted 15 times by coronary blood flow as the 1 ml was injected over 30 s. Coronary blood flow under these conditions was estimated at 30 ml/min (allowing for 50% flow obstruction by the catheter), as explained in Methods. This protocol was used in seven ovariectomized rhesus monkeys. IC = intracoronary.

Recovery and pathologic studies. Maximal experiment duration was ≤ 3 h. After the protocols, catheters and electrocardiographic electrodes were removed, and incisions were surgically closed. Isoflurane anesthesia was discontinued, the endotracheal tube was removed, and sedation was maintained using ketamine (15 mg/kg intramuscularly) until euthanasia in the necropsy room. Monkeys were euthanized by an overdose of intravenous Na^+ pentobarbital and exsanguination. Hearts were examined in situ for abnormalities in size and shape, removed and immediately dissected. The aorta and opened coronary arteries were further evaluated for narrowing, thickening or thrombosis, and myocardial slices were examined for color changes suggestive of lesions. Cross-sectional blocks of aorta and coronary arteries with adjacent myocardium were processed for glycol methacrylate embedding, and 2- μm sections were evaluated using hematoxylin–Lee, giemsa and Verhoeff stains.

Concentrations of drugs in coronary blood. Effective autacoid concentration (as diluted by blood flow) at the VMC membrane is critical but elusive. We based the analysis in Table 3 on the following assumptions. All injections were 1 ml, infused by hand steadily over a 30-s interval. The concentrations in the syringe would have been diluted instantaneously by coronary blood flow. Based on size, the 3F (1.0-mm outer diameter) catheter (area 0.785 mm^2) would occlude coronary blood flow in the average 1.35-mm diameter coronary artery (area 1.43 mm^2) by 54% (under the precontraction conditions). With coronary blood flow of 30 ml/min in this case, the concentration reaching a VMC would therefore be diluted about 15 times or more with distance from the injection point.

Table 2. Protocol 2: Mechanical Injury Protocol for Coronary Vasospasm*

1. IC injection of contrast media to establish arterial diameters before drugs (control)
2. IC injection of 100 nmol/liter (0.018 μ g) acetylcholine to show dilator capacity
3. IC injection of 1 μ mol/liter (0.18 μ g) acetylcholine to show dilator capacity
4. IC injection of 100 μ mol/liter (17.6 μ g) serotonin
5. IC injection of 1 mmol/liter (176 μ g) serotonin
6. IC injection of endothelin-1 (sequential doses to 10 nmol/liter)
7. IC injection of angiotensin II (sequential doses to 10 μ mol/liter)
8. Injury by stretching the coronary artery with a 5F catheter
9. IC injection of serotonin (sequential doses to 1 mmol/liter)
10. IC injection 15 min later with 1 or 10 μ mol/liter nitrendipine or Ro 40-5967 (mibefradil)
11. IC injection of U46619 (sequential doses to 10 μ mol/liter)
12. IC injection 15 min later with 1 or 10 μ mol/liter nitrendipine or Ro 40-5967

*This protocol was used in three rhesus monkeys. IC = intracoronary.

However, the blood flow through individual epicardial arteries (LAD or left circumflex) would have represented only part of total coronary flow, offsetting the downstream dilution. We compromised on the factor of 15 times for the Table 3 concentrations, although we acknowledge that this is only an approximation.

Coronary artery diameter measurement. Putative coronary vasospasm stimuli were judged primarily by actions on large coronary artery diameters, analyzed as diameter of the most proximal focus of vasospasm at end-diastole from videotaped images recorded at 30 frames/s. We used the image analysis program Image Pro to measure the diameters from single frames acquired with an Imagraph frame grabber in a Pentium computer. We used the 3F catheter tip ring (1.0 mm) for calibration and determined minimal and maximal diame-

ters just distal to the constriction. All responses to interventions were also X-ray imaged immediately (15 to 30 s after injection) and at exactly 3 min (and 15 min if there was vasospasm) after injection. Interobserver variability (coefficient of variation) was 7% on repeat measurements, as tested on selected samples.

Statistical analysis. Results are expressed as mean value \pm SEM for all variables measured. Statistical significance was determined by analysis of variance with Bonferroni protected paired *t* tests. Values of $p \leq 0.05$ were accepted as significant.

Results

Protocols that failed to induce coronary vasospasm. *Single-drug challenges.* Coronary catheterization laboratory studies of vasoconstriction in male or ovx rhesus monkeys were begun by individually testing serotonin, Tx_A₂ mimetic (U46619), ET1 or AII by injection in increasing (half-log units) concentrations to find the reduction of artery diameter by 50%. We used 10 times as the multiplier (where practical) to reach the maximal dose to be tried (Table 3). No vasoconstrictor alone caused coronary vasospasm, even though there were transient reductions to 20% of control diameter. The first injection in protocol 1 was a low dose of 6.7 nmol/liter ACh to test endothelial integrity, as judged by vasodilation, which was sufficient in sensitive arteries (three females, one male), whereas 67 nmol/liter ACh produced immediate vasodilation within 15 s in all monkeys.

Intracoronary injections of serotonin from 67 nmol/liter to 667 μ mol/liter were vasoactive in all animals. The 6.7 μ mol/liter concentration (step 4 in the protocol) caused dilation of the larger arteries (in 4 of 10), whereas 67 μ mol/liter (step 5) produced variable vasoconstriction of large arteries (mild constriction in 5, but no change in 5), and small arteries in all 10, indicated by increased time to clear radiocontrast media.

Table 3. Sensitivity to Vasospasm Stimuli

	ED ₅₀ (-log mol/liter)	EC ₅₀ (-log mol/liter)	Max Dose (-log mol/liter)	Spasms/N	Protocol
Single stimulus					
Serotonin*	2.5	3.8	2	0/8	1†
U46619	5.5	6.8	5	0/8	1†
Endothelin 1	8.5	9.8	8	0/3	2
Angiotensin II	5.5	6.8	5	0/3	2
Combined stimuli					
Endothelin 1+serotonin‡	9	10.3	8	0/3	2
Angiotensin II+serotonin‡	6	7.3	5	0/3	2
Mechanical injury+serotonin‡	NA	NA	NA	3/3	2
U46619+serotonin‡	6	7.3	5	5/7	1

*1 mmol/liter. †One of the protocol 2 animals also was tested with serotonin alone and U46619 alone, after the angiotensin II step, accounting for the total of eight in the first two rows. Vasospasms all consisted of reduction to $\leq 25\%$ of control diameter with downstream dilation (hourglass shape) that persisted for ≥ 15 min. ‡100 μ mol/liter. EC₅₀ = estimated actual coronary artery blood concentration in -log mol/liter based on a 15-fold dilution, (for details, see Methods); ED₅₀ = dose in μ mol/liter (as the concentration in the syringe, without allowing for any dilution due to coronary blood flow) of the named vasoconstrictor that caused a 50% reduction of diameter from control; Max Dose = maximal dose tried (syringe concentration) in -log mol/liter for each vasoconstrictor; N = number of animals in a protocol; NA = not applicable; Spasms = number of animals with vasospasm.

These serotonin-induced contractions appeared uniform along the length of the arteries, with no evidence of the characteristic vasospasm hourglass profile. Estimated EC_{50} concentrations (allowing for 15 times dilution of syringe concentrations) for 50% diameter reduction (EC_{50}) was 200 $\mu\text{mol/liter}$ (Table 3).

Intracoronary injection of the stable TxA_2 mimetic U46619 in concentrations from 6.7 to 2,000 nmol/liter caused constriction of arteries in all 10 monkeys, predominantly due to constriction of small arteries and markedly increased total coronary resistance as indicated by fluoroscopy (resistance to injection and slow clearance of the radioopaque contrast media). Thromboxane A_2 at 6.7 nmol/liter (step 6) substantially reduced intramural small-vessel diameter and impaired cardiac contractility (decreased maximum rate of increase in pulse [dP/dt]). As opposed to injections of serotonin, which characteristically increased heart rate and blood pressure, TxA_2 injections increased blood pressure with decreased or unchanged heart rate. At 67 nmol/liter, TxA_2 caused vasoconstriction of predominantly small-resistance arteries, evidenced by pronounced ischemic narrowing, decreases in blood pressure and increased time to clear radiocontrast media in all monkeys (step 7). The estimated EC_{50} for 50% diameter reduction was 200 nmol/liter (Table 3). Although blood pressure and cardiac contractility decreased with repeat injections of high TxA_2 doses, there was no coronary vasospasm with TxA_2 alone.

Endothelin 1 alone or with serotonin. Endothelin 1 in concentrations up to 2 nmol/liter did not cause vasospasm. Cardiac systolic function was depressed (decreased dP/dt), and there were 50% reductions in diameter of only two arteries to ET1 plus serotonin, but there was no coronary vasospasm. Addition of 6.7 $\mu\text{mol/liter}$ serotonin increased sensitivity by three times; the estimated ET1 EC_{50} of the responsive ovx monkeys was 200 pmol/liter without and 67 pmol/liter with serotonin (Table 3). The triple combination with 67 nmol/liter U46619, used in the two ovx monkeys that failed to show vasospasm by this point, produced cardiogenic shock but not vasospasm; diastolic blood pressure fell to ≤ 20 mm Hg, and the heart was severely hypokinetic as observed by fluoroscopy.

Angiotensin II alone or with serotonin. Angiotensin II in concentrations up to 200 nmol/liter did not cause vasospasm but did increase systemic blood pressure. Coronary diameters were not obviously affected, except in one ovx monkey, from which a maximal sensitivity of 200 nmol/liter without and 67 nmol/liter with serotonin was estimated (Table 3). Although the combination with serotonin increased heart rate, there was no coronary vasospasm (Table 3).

Protocols that induced coronary vasospasm. Mechanical injury. As a basis for comparison, we used deliberate injury to the coronary arteries inflicted by stretching with a catheter with deliberately roughened tip, in one male and two ovx monkeys (protocol 2, Table 2). The stretch maneuver would cause injury to both endothelium and media and induce local vasoconstriction. Intracoronary injection of 67 $\mu\text{mol/liter}$ serotonin subsequent to injury caused coronary vasospasm at multiple sites, as demonstrated in the male monkey (Fig. 1). Before injury,

arteries were open and well filled (Fig. 1A). The 5F catheter was then advanced to produce injury by a single stretch at the point marked by the radioopaque tip, which produced transient focal narrowing to 20% of control, with recovery to $>50\%$ in 4 min. We injected 67 $\mu\text{mol/liter}$ serotonin (IC) 12 min later as 1 ml over 30 s (as explained in Methods), causing vasospasm evident at three focal points made prominent by three distal dilations (Fig. 1B) forming the classical hourglass pattern that mimics human coronary vasospasm. Vasospasm was relieved with injection of 10 $\mu\text{mol/liter}$ nitrendipine (no dilution allowed for in this low blood flow condition), as shown by return to nearly control dimensions (except at the injury region near the catheter tip) in Figure 1C. Subsequent injection of 67 nmol/liter U46619 caused another vasospasm with the same foci as that for serotonin (Fig. 1D). Even in the deliberate catheter injury experiment shown in Figure 1, the vasospasm induced was not simply a local response to direct mechanical injury because vasospasm occurred not only at the point of artery wall injury, but also at foci several millimeters distal to the injury. Such characteristic focal contractions appeared at least 10 to 25 mm beyond the furthest point ever reached by guide wires or catheters. Focal constriction and downstream dilation occurred that would be consistent with punctate release or sites of hyperreactivity, or both, because there was no evidence of emboli.

The injury in Figure 1 was imposed with a 5F catheter that stretched the left main coronary artery. Vasospasm was stimulated by subsequent IC serotonin or 1 nmol/liter U46619 but not by ≤ 10 nmol/liter ET1 or ≤ 10 $\mu\text{mol/liter}$ AII. With 4F catheters, there was also injury due to stretching and flow occlusion in these 25- to 45-g hearts. Mechanical injury was also used in two ovx monkeys (protocol 2), with multiple vasospasms similar to the results in the male monkey shown in Figure 1. However, in the remaining seven ovx monkeys, only 3F catheters along with protocol 1 were used to avoid injury to study purely drug-induced coronary vasospasm.

Combined serotonin and TxA_2 . With the combination of 6.7 $\mu\text{mol/liter}$ serotonin and 67 nmol/liter U46619, we found coronary vasospasm in five of seven ovx monkeys, even though vasospasm could not be found in response to 6.7 or 200 $\mu\text{mol/liter}$ serotonin alone or 6.7 to 2,000 nmol/liter U46619 alone in the same seven animals (Table 3). None of the seven monkeys responded to the first serotonin and U46619 combination injection; two responded to the second injection; and 3 responded to the third injection with vasospasm. The two that did not show vasospasm nevertheless showed severe vasoconstriction and are included in the hemodynamic data in Table 4. Sample angiograms showing vasospasm induced by the combination of serotonin and U46619 are displayed in Figure 2. The control condition (Fig. 2A) shows a normally patent left main coronary artery filled with radioopaque contrast media. Constriction 15 s after the second injection of serotonin and U46619 is shown in Figure 2B and after 3 min in Figure 2C. All vasospasms observed in protocol 1 occurred >5 mm beyond the most distal point ever reached by either catheter or guide wire.

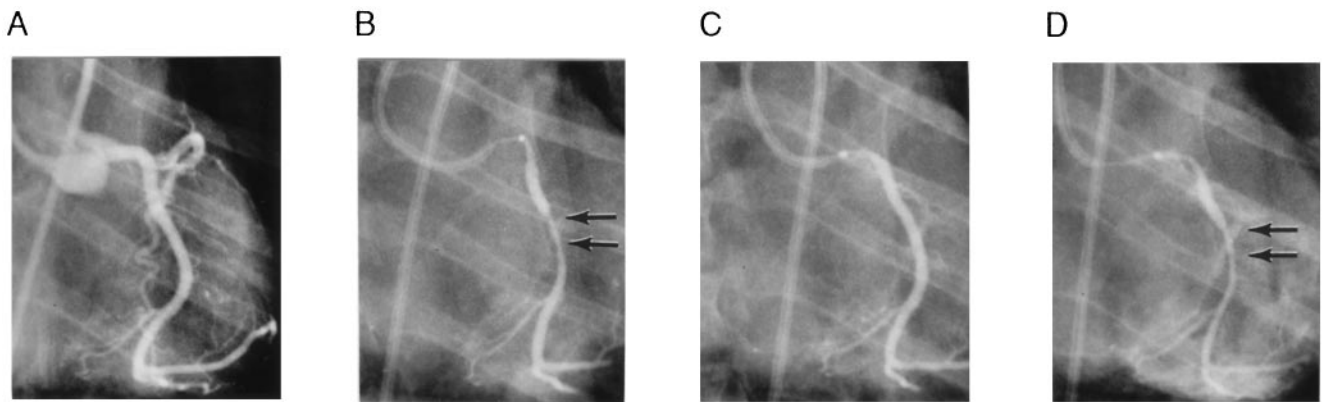


Figure 1. Injury-induced coronary vasospasms with focal constrictions and downstream dilations (hourglass pattern) are shown in the left circumflex coronary artery of a 14-year old male rhesus monkey. The control angiogram (A) shows well dilated coronary arteries before injury. After injury by stretching with the 5F catheter (the injured region extends to the radioopaque tip of the catheter) and infusion of 1 ml of 1 mmol/liter serotonin over a 30-s interval (67 μ mol/liter after dilution), there was vasospastic constriction, with branches and smaller coronary arteries disappearing from the image (B). Arrows mark two sites of vasospasm >5 mm beyond the farthest extent of stretching. After infusion of 1 ml of 10 μ mol/liter nitrendipine (not assuming any dilution because of the ischemia) over a 30-s interval, there was relief of vasospasm and an increase in the diameter of the artery (C). Injection of 1 μ mol/liter U46619 (estimated to be 67 nmol/liter after dilution) caused redevelopment of constriction and vasospasm at the same focal areas (arrows) (D).

The onset of constriction to the serotonin and U46619 combination injection began immediately on arrival and was evident on the first film (15 s after the injection). The contraction progressed over the next 2 min, reaching a peak between 2 and 4 min from the end of injection. The number of vasospasm regions in a major artery averaged three (range one to six). When there was more than one vasospasm in an artery, the second and subsequent vasospasms occurred 5 to 25 mm distal to the first focal vasospasm. The hourglass pattern in Figure 2C is similar to that in the mechanical injury plus serotonin vasospasm (Fig. 1, B and D).

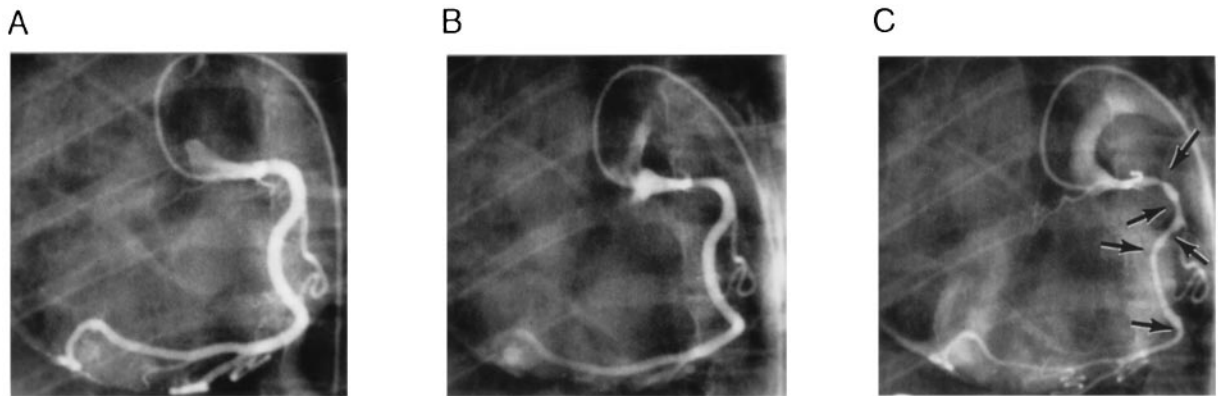
Life-threatening reductions in blood pressure and cardiac contractility resulted from such severe coronary ischemia (Table 4). Diastolic blood pressure fell to <20 mm Hg in several monkeys, correlated with more than fivefold decreases in dP/dt values (cardiac contractility) and faint ventricular contractions observed fluoroscopically. Systolic blood pressure correspondingly fell to <40 mm Hg, and in several cases heart rate fell to <60 beats/min. Minimal diameter was severely reduced, in some loci to <0.1 mm (<8%), and downstream dilation was more than five times the diameter at the vasospasm focus. All variables except maximal diameter were significantly different from control values, as indicated by daggers in Table 4.

Relief of coronary vasospasm. To allow monkeys to recover and prevent death due to cardiogenic shock, we followed

combined serotonin and U46619 stimulation with an IC Ca^{2+} antagonist, either nitrendipine or mibefradil, at the end of the 15-min vasospasm, except for that in Figure 2. Intracoronary injection of 1 ml of 10 μ mol/liter nitrendipine or 1 μ mol/liter mibefradil (without allowing for dilution due to severely reduced blood flow) caused improved cardiac performance, beginning ~1 to 3 min after injection, with an increase in systolic and diastolic blood pressure and improved cardiac contractility (increased dP/dt). In monkeys with severe vasoconstriction and low heart rates (<90 beats/min), Ca^{2+} antagonists effected increases to 90 to 200 beats/min due to reversal of atrioventricular conduction blocks (evident in the recovery of heart rate shown in Table 4). Hemodynamic variables returned to levels not significantly different from control with the help of Ca^{2+} antagonists, although there was a trend toward increased heart rate.

Nitrendipine (1 to 10 μ mol/liter not allowing for dilution) injected IC was effective in eliminating the vasospasm, even in animals that had diastolic blood pressures as low as 20 mm Hg. Mibefradil (Ro 40-5967) IC injections at 0.1 to 10 μ mol/liter (without allowing for any dilution by blood flow) were also immediately effective in relieving vasospasm and returning blood pressures from diastolic levels as low as 10 to 20 mm Hg back to normal ranges (minimums of 100/60 mm Hg for systolic/diastolic levels). Figure 3 shows an experiment in which vasospasm was induced by serotonin and U46619 combination injection and relieved by Ca^{2+} antagonist. Figure 3A is the control circulation, which contrasts with early vasospasm in Figure 3B (at 15 s) and the fully developed vasospasm in Figure 3C. Relief of the vasospasm is shown in Figure 3D and by hemodynamic variables in Table 4. In all monkeys, Ca^{2+} antagonists relieved vasospasm, allowing recovery of blood pressures, heart rate and contractility toward normal.

Pathologic examinations. Gross structural abnormalities were not seen in any of the hearts. There was also no visible evidence of acute injury or preexisting lesions in coronary arteries or the myocardium at or near the spasm foci. Multiple small fibrous plaques were present in the aortas of two animals (the 18-year old ovx monkey in Fig. 3 and another 13-year old ovx monkey), which both showed vasospasm. However, the lesions were minor, confined to the aortic arch and would not



have influenced the vasospasms reported here. The same monkey showed a small microscopic focus of intimal thickening in an apical portion of the LAD, and there was segmentally denuded endothelium in one small area in this and one other 13-year old ovx monkey that did not show vasospasm. There were small thrombi composed of fibrin and platelets in only this one monkey with vasospasm. The 18-year old ovx monkey in Figure 2, which was the only monkey in this series that died as the result of vasospasm, showed acute cytolytic changes consisting of wavy fibers and sarcoplasmic vacuolation accompanied by interstitial edema in the left ventricle, probably as a result of the fatal vasospasm.

Discussion

These results show that even in the absence of injury or plaques, stimulation by the combination of serotonin and a mimetic of TxA₂ evokes coronary vasospasm in ovx rhesus monkeys. The radiologic appearance and pharmacologic responses closely mimic characteristics of human coronary vaso-

Figure 2. Drug-induced vasospasm is shown by angiograms from an 18-year old ovx monkey right coronary artery (without injury). Multiple vasospastic hourglass patterns formed in response to the second challenge with the combination serotonin-TxA₂ provocation. The control condition is shown in A. After the second injection of the combination of 6.7 μ mol/liter serotonin and 67 nmol/liter U46619, after an identical first challenge by 10 min, loci of constrictions began to form, evident as decreases in diameter interspersed among regions of almost normal diameter, as shown here at 30 s (B). These regions already identified where spasms would occur at 5 min, at which time diameters were <25% of control at the points indicated by five arrows (C). This was the only monkey in this study group that did not survive vasospasm. In this case, recovery by direct myocardial stimulation was unsuccessfully attempted with 1 ml of 100 μ mol/liter epinephrine (not allowing for dilution) injected IC over 30 s (instead of vasodilation by a Ca²⁺ antagonist).

Table 4. Hemodynamic Variable Changes at Control, Serotonin and U46619 Combination and Recovery Steps in Drug-Induced Vasospasm Protocol in Seven Ovariectomized Rhesus Monkeys*

	Control (mean \pm SEM)	S+U (mean \pm SEM)	Recovery (mean \pm SEM)†
Systolic BP (mm Hg)	122 \pm 7	83 \pm 14‡	114 \pm 10
Diastolic BP (mm Hg)	84 \pm 6	43 \pm 15‡	76 \pm 11
dP/dt max	1,653 \pm 437	305 \pm 136‡	1,285 \pm 548
Heart rate (beats/min)	107 \pm 5	74 \pm 18‡	126 \pm 22
Min diameter (mm)	1.33 \pm 0.45	0.22 \pm 0.11‡	1.08 \pm 0.76
Max diameter (mm)	1.35 \pm 0.44	1.12 \pm 0.68	1.21 \pm 0.77

*All seven monkeys without mechanical injury are included in these data, whether showing vasospasm (5 monkeys) or not (2 monkeys). The three monkeys that received mechanical injury are excluded from the table. †The monkey that did not recover (Fig. 2) is not included in these data. ‡All serotonin and U46619 combination data except maximal diameter are significantly different from control and recovery data by Bonferroni protected *t* tests (*p* < 0.05). BP = blood pressure; diameter = point of greatest vasoconstriction (Min) and downstream dilation (Max) (in mm) before, during the peak of the serotonin and U46619 combination vasospasm and after recovery; dP/dt max = maximal rate of increase of left ventricular blood pressure measured from the intraarterial blood pressure catheter in mm Hg/s.

spasm found in diagnostic provocative testing with single injections of ergonovine, histamine or high dose ACh (12,14,16,19,29-32). The lack of epicardial vasospasm in response to single agonists is remarkable and probably accounts for the difficulty in provoking vasospasm in animals. The requirement for a combination of synergistic stimuli would suggest that the vasospasm phenomenon is complex. These results support the hyperreactivity hypothesis for vasospasm that had been deduced earlier from observations of human angiograms (5,17) and demonstrate a useful animal model of human vasospasm.

Comparison with human vasospasm. Based on initiating stimulus, characteristic hourglass pattern of focal constriction followed by downstream dilation, extended time course of profound vasoconstriction and pharmacology of vasospasm reversal, this rhesus monkey coronary vasospasm model closely mimics human coronary vasospasm. The serotonin and U46619 combination stimulus caused a similar pattern of vasospasm to that found in human patients (1,2,6,13) or caused by mechanical injury (with the catheter tip), as shown by comparison of Figure 1 with Figures 2 and 3. Vasospasm (as defined here) required stimulation by serotonin, even after mechanical injury, in all monkeys studied. The requirement for a vasoconstrictor in both drug-induced and mechanical protocols emphasizes the importance of blood vessel reactivity. Hyperreactivity, whether due to endothelial denudation, appears to be an

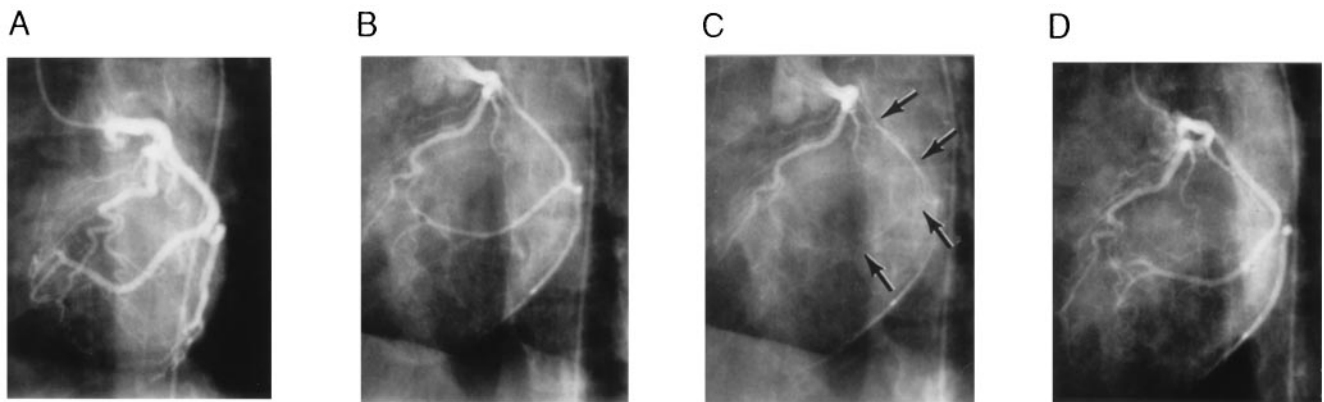


Figure 3. Drug-induced coronary vasospasm (in uninjured coronary arteries of an 18-year old ovx monkey) and relief by the Ca^{2+} antagonist mibefradil (Ro 40-5967) are shown by this series of angiograms. Control left circumflex and LAD coronary arteries are shown in **A**. Regions of strong constriction began 15 s after stimulation with the second challenge (7 min after an identical first challenge) with serotonin and U46619 combination, as shown in **B**. Focal constrictions followed by dilations (vasospasms) are demonstrated at 3 min in **C**. Several sites showed reductions of 90% compared with control diameter, as indicated by the four **arrows**. Relief of vasospasm by 10 $\mu\text{mol/liter}$ mibefradil (no dilution assumed because of markedly reduced blood flow) is shown in **D**. IC injection of mibefradil reversed vasospasm and returned blood pressure, which had dropped $<40/20$ mm Hg, to $>100/60$ mm Hg due to improved cardiac function (observed fluoroscopically as improved contraction strength and more rapid radiocontrast medium clearance).

important component in the etiology (5,17) and is a logical deduction from the hourglass shaped constriction, although this point has been vigorously debated (2).

Platelet release substances provoke vasospasm. The results add to growing support for the hyperreactivity hypothesis that spontaneously occurring functional vasospasm is closely associated with sensitivity to stimulation by the combination of serotonin and TxA_2 . Similar serotonin-thromboxane correlations have been made in patients and were suggested to provide an important pathophysiologic mechanism (4,7,12,14,33). Our findings are consistent with those from human angioplasty, in which serotonergic receptor stimulation was essential, as shown by block with serotonin antagonists (e.g., methysergide or LY 53587) (9,19,20,34).

The concentrations of serotonin used here are similar to those for serotonergic agonists used to provoke vasospasm in patients but are much higher than circulating levels measured in the coronary sinus (32,35). However, platelet volume is extremely small, and the release of products is local; thus, release site/circulating ratios $>10^6$ times would be anticipated. The 5 ng/ml of serotonin measured in flow-diluted coronary sinus blood (12,32) could thus represent up to 5 mmol/liter released in the vicinity of the activated platelet. If so, concentrations injected in the present studies would represent similar conditions to platelet local release. The injections here exposed coronary arteries to an estimated concentration of 67

$\mu\text{mol/liter}$ serotonin (see Methods) for 30 s, which may even be only a fraction of stimulus duration when released by platelets. Our IC injection would have been immediately washed out by blood flow instead of being anchored to a site, as a thrombus would have been. Immediate washout offers a plausible explanation for the requirement for simultaneous stimuli to trigger drug-induced vasospasm. Serotonin amplified responses to U46619 and other vasoconstrictors, causing a $\frac{1}{2}$ log unit lower EC_{50} in each instance (Table 3).

Vasospasm due to increased reactivity. To our knowledge, we demonstrated for the first time in an animal that vasospasm can be drug induced, even without stenosis or atherogenic diets, which are known to increase coronary reactivity (23,25,36-38). The hypothesis is thus supported (i.e., thromboxane A_2 supersensitivity of VMC causes vasospasm in the setting of concomitant serotonin). The combination of local platelet release of TxA_2 and serotonin with supersensitivity (28) of small groups of VMC can explain focal contraction areas, perhaps implicating increased TxA_2 receptor numbers, as reported in myocardial infarction and unstable angina pectoris (39). With both protocols, monkey coronary vasospasm had the same familiar hourglass pattern as in human patients (Figs. 1 to 3).

Virtues and limitations of primate model. Vasoconstrictor stimulation allows the purely functional aspect of vasospasm to be separated from structural mechanisms. This simplification of the vasospasm process would be unlikely, or at least extremely difficult, in humans, where lack of atherosclerotic plaques is rare (2,33). Rhesus monkeys in which hormonal status, diet and medical history are defined and controlled allow a rare and definitive test of specific mechanisms for induction of functional (rather than structural) coronary vasospasm. Unlike other models, the ovx rhesus monkeys required no crush or other injury or restriction of coronary arteries. There is no chance of smoking, stress, lipids or other complications of human vasospasm, and heart disease is rare in rhesus macaques (25,36). A hypoestrogenic state was a necessary factor for hyperreactivity sufficient to allow vasospasm to exist because vasospasm was not found with a similar protocol in monkeys that had physiologic levels of estrogen and progesterone (40). However, we recognize that there may be un-

known differences in coronary artery characteristics between rhesus monkeys and humans that could limit the extrapolation of this hyperreactivity mechanism. Injection of combined serotonin and U46619 produced small-artery constriction greater than might occur in variant vasospasm. However, rhesus monkey coronary arteries appear more relevant to human coronary arteries than those of other animals (e.g., pig, dog, rabbit or pigeon) in appearance and dynamics of vasospasm (18) and appear to be particularly useful for comparative study of mechanisms hypothesized to underlie functional vasospasm (2,7,10,14,20).

Questions raised. These data support the reports that platelet release products are important in the etiology of coronary vasospasm (7,8,41) and thus support the thrombus vasoconstrictor release/hyperreactivity hypothesis. There can be little doubt that platelet aggregation is intimately involved in vasospasm based on success of the monoclonal antibody 7E3 against glycoprotein IIb/IIIa receptor, effectively blocking platelet aggregation and relieving vasospasm in patients (42). Platelet Ca^{2+} vasoconstrictor abnormalities are also thought to contribute to the increased risk of vasospasm in hypertension (43). Because thrombus formation and dissolution of plaques is a dynamic process (9), spasm occurrence may be determined by sensitivity of VMC to vasoconstrictor release. There remain several fundamental questions. Is the site of vasospasm defined by a localized increase in vasoconstrictor sensitivity? Which changes in platelets are necessary for triggering the vasospasm? Although these and other puzzles remain to be solved, we conclude that vasospastic (structure independent) vasospasm can be stimulated in hyperreactive coronary arteries by combined platelet release products.

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