Pathogenesis of Coronary Artery Spasm in Miniature Swine With Regional Intimal Thickening After Balloon Denudation

Yusuke Yamamoto, Hitonobu Tomoike, Kensuke Egashira, Tadashi Kobayashi, Takayuki Kawasaki, and Motoomi Nakamura

Coronary artery spasm, an excessive and transient narrowing of the epicardial coronary artery, has been demonstrated angiographically and is now considered an underlying mechanism of the variant angina seen clinically. The pathogenesis of the focal excessive narrowing of the epicardial coronary artery has remained unclarified. Augmented constriction of the coronary artery, platelet aggregation, geometric factors related to coronary stenosis, and injury of small resistance vessels have been postulated to be mechanisms related to transient coronary stenosis. The augmented contraction of medium-sized arteries was noted in isolated ring or strip preparations when the sample had been exposed to electrolyte solution containing cholesterol, in cases where the sample had been taken from the hereditary hyperlipidemic rabbit, or where the vascular endothelium had been removed mechanically or chemically in vitro. Pharmacophysiological characteristics of the vessel wall were preferentially determined quantitatively in vitro. However, to elucidate the pathogenesis of coronary spasm in a clinical situation, the rationale of in vitro studies, such as whether coronary spasm in situ can be reproduced in vitro without serious modifications, has to be rigorously tested.

In previous studies, we provoked coronary artery spasm and coronary hypercontraction in Göttlingen miniature pigs and mongrel dogs, respectively, in which localized coronary narrowing was documented angiographically. Thus, our swine model should aid in clarifying differences in pharmacophysiological prop-

From Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka, 812 Japan.

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Address for reprints: Hitonoba Tomoike, MD, Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.

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properties of the coronary artery between in situ and in vitro conditions, in the same heart. This study examines whether regional augmented coronary narrowing in response to histamine can be reproduced in isolated hearts without blood constituents and neural components, and the pathogenesis of augmented responses to histamine was determined angiographically on isolated pig hearts perfused with electrolyte solution.

Materials and Methods

In Vivo Study

Preparation of Animal Model. Eighteen male Göttingen miniature pigs, 4–6 months old (5 ± 1 months; mean ± SE) and weighing 13–21 kg (18 ± 2 kg), were sedated with an intramuscular administration of ketamine hydrochloride (12.5 mg/kg i.m.) and anesthetized with an intravenous administration of 20 mg/kg sodium pentobarbital. The pigs were then intubated and ventilated with room air, and oxygen was supplemented via a positive pressure respirator (Shinano Inc., Tokyo, Japan). Under aseptic conditions, a preshaped green Kifa catheter (Kifa, Stockholm, Sweden) was inserted from the carotid artery into the orifice of the left coronary artery, under the guidance of the fluoroscopy of a C-arm X-ray system (Toshiba, Tokyo, Japan). After pretreatment with heparin (5,000 U i.v.), endothelial denudation about 2 cm long was performed on the left circumflex coronary artery (LCX) in 11 pigs and on the left anterior descending coronary artery (LAD) in 2 pigs using a balloon catheter (Edwards Laboratories, Santa Ana, Calif., 2F Fogarty Embolectomy Catheter) inserted into the Kifa catheter. Five other pigs serving as the controls were fed laboratory chow (Nippon Clea, Osaka, Japan) for 3 months and did not undergo endothelial denudation. Body weight increased from 18 ± 2 kg to 28 ± 4 kg after 3 months (p < 0.01).

Provocation of Coronary Artery Spasm. After three months, the denuded pigs were anesthetized with ketamine hydrochloride and sodium pentobarbital and were ventilated via a positive pressure respirator as described above. Under aseptic conditions, 5 ml of contrast medium (Urographin 76%, Nihon Schering, Osaka, Japan) was injected manually through the Kifa catheter, and coronary arteriograms were recorded on 35-mm cinefilm (CFS 746, Kodak, USA) at 48 frames/sec using a Toshiba 0.6-mm focal spot X-ray system. Histamine (10 μg/kg) and phenylephrine (1 μg/kg) were administered intracoronarily in random order. The injected volume of vasoactive agents dissolved in physiological salt solution was 1 ml, and the same amount of saline was used to flush the catheter. The injection time was 30 seconds. Thus, the estimated maximum concentration of histamine was no more than 5.2 × 10⁻⁵ M/l. Coronary arteriography was performed 1 and 2 minutes after i.c. administration of the drug, when hemodynamic variables recovered to the predrug state. To determine the diameter of the submaximally dilated coronary artery as a reference for evaluating vessel tone, coronary arteriography was performed 2 minutes after i.v. administration of nitroglycerin (20 μg/kg). Coronary artery spasm was defined as the focal excessive constriction of more than 50% stenosis of the diameter after nitroglycerin.

In Vitro Study

One week after in vivo documentation of coronary artery spasm, 18 pigs were anesthetized with ketamine hydrochloride (12.5 mg/kg i.m.) and sodium pentobarbital (12.5 mg/kg i.v.), exsanguinated, and the hearts removed within 5 minutes. After being placed in cold oxygenated Krebs-Henseleit solution, a preshaped polyethylene cannula was inserted into the most proximal portion of the left main coronary artery and firmly secured by a ligature.

The hearts were then connected to the constant pressure perfusion system shown in Figure 1 and were perfused with Krebs-Henseleit solution at 37° C. Krebs-Henseleit solution contained (in mM) Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.6, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134.0, and glucose 11.5. The solution was aerated with 95% O₂ and 5% CO₂ and the pH was adjusted to 7.3–7.4.

Coronary arteriography in vitro. Coronary arteriography was performed by manually injecting 5 ml of contrast medium (Urographin 60%, Nihon Schering, Osaka, Japan) through a 19-gauge needle inserted into the perfusion line (Figure 1). The X-ray system, which was different from the one used in vivo, was equipped with an under-table X-ray tube with 0.6-mm focal spot (Toshiba, Tokyo, Japan). Arteriography was taken on a 35-mm cinefilm (CFS, Kodak, USA) at 9 frames/sec through an Arriflex cine camera (Arriflex, München, Federal Republic of Germany) at 90 mA, 100 kV, and 2 msec/frame. The distance between the heart and the image intensifier was kept constant during the experiment.

Experimental protocol. After connecting the isolated heart to the constant pressure perfusion system, 20 ml KCl (40 mM) solution was infused into the left coronary artery, and the heart was arrested. The heart was then continuously perfused with Krebs-Henseleit solution at 37° C. After 90 minutes of equilibration, the following studies were performed at the arrested state.

Coronary angiograms were taken before and after the infusion of vasoactive agents such as histamine, phenylephrine, and nitroglycerin. These agents were administered continuously into the left coronary artery using a constant micro volume pump (Tokyo-Rikakai, Tokyo, Japan) for 2 minutes (Figure 1). The volume injected was limited to < 1% of the total coronary flow. The final drug concentration in the perfusion medium was calculated in molar units.

Guanethidine (3 × 10⁻⁶ M), atropine (10⁻⁶ M), and tetrodotoxin (3 × 10⁻⁷ M) were applied to suppress the effects of neurotransmitters released from the nerve endings. Before, and 20 minutes after, pretreatment with these nerve-ending blockers, histamine (10⁻⁷ M–10⁻³ M) was infused (n = 7). To block histamine-H₁ receptor activity, mepyramine (10⁻⁶ M) was administered to 6 pigs 20 minutes before the infusion of histo-
The perfusion system is characterized by the following: First, a constant pressure perfusion system was applied using a Starling resistor. The perfusion pressure was maintained at 90 mm Hg and was monitored through a thin polyethylene tube 0.5 mm o.d. (Igarashi, Fukuoka, Japan) with a Statham P23Db pressure transducer. To monitor accurate perfusion pressure, the tip of the tube was placed 1–2 mm outside the ostium of a cannulation device. Second, the perfusate was not recirculated. Third, a peristaltic pump (Watson-Marlow, England) through a depulsator generated minimized pulsatile flow. K-H solution = Krebs-Henseleit solution; EMF = electromagnetic flow probe; II = image intensifier.

mine (10^{-5} M). Ca^{2+}-free solution containing EGTA (2 mM) was perfused 5 minutes before histamine (10^{-5} M) or nitroglycerin (10^{-5} M). Nitroglycerin (10^{-5} M) infusion in Ca^{2+}-free solution was used to determine the level of the maximal coronary dilation. The degree of coronary dilation by nitroglycerin (10^{-5} M) was similar in the presence (2.6 ± 0.1 and 2.5 ± 0.1% at the intact and denuded areas) or absence (2.6 ± 0.1 and 2.4 ± 0.1% at the intact and denuded areas) of 2.6 mM Ca^{2+} in the perfusate. Thus, the degree of coronary constriction to vasoactive stimuli was expressed as the percent increase or decrease of the diameter observed after nitroglycerin administration (10^{-5} M).

The stability of the resting coronary diameter repeatedly confirmed in 14 pigs and the reproducibility of the response to histamine (10^{-5} M) during the experiment in 6 pigs. All these procedures were completed within 270 minutes from the start of perfusion.

Data Analysis

Cinefilms were projected (Tagarno 35-CX, Tagarno, Horsens, Denmark) and in the case of in vivo angiograms, the end-diastolic frame was selected for measurement by ECG-R waves recorded on the cinefilm. Photocopies (13 × 18 cm) were made to measure the diameter. With a caliper, the diameter was measured to 1/20 mm and the absolute diameter (mm) was calculated by referencing a Kifa catheter and a cannula for calibrations in vivo and for the isolated heart, respectively. Reproducibility of the measurements of coronary artery diameter was evaluated by comparing the data measured by individual observers with the correlation between repeated measurements (r = 0.99, p < 0.001) and between different observers (r = 0.99, p < 0.001) in both in vivo and in vitro coronary angiograms. Sites of the coronary diameter measurements were the area where a focal excessive constriction was maximal and an area of the contralateral artery with a similar diameter as seen in the control resting state. The degree of constrictive response was expressed as a percent change of the luminal diameter from the level after nitroglycerin 10^{-5} M administration in both in vivo and in vitro angiograms.

After the in vitro experiments, a barium and gelatin mixture was injected into the coronary artery at a perfusion pressure of 90 mm Hg, and the heart was fixed in 20% formaldehyde. Four transverse sections of the heart were radiographed stereoscopically, as previously described.19 Anatomical risk area of the left coronary artery was determined on the X-ray film and was measured by a computerized planimeter (n = 5).

Morphological Study

After fixation with 20% formaldehyde, branches of the left coronary artery were sectioned at 5-mm intervals. These were stained with hematoxylin-eosin and
van Gieson's elastic stain. Each block was numbered and correlated with angiograms in vivo as well as in vitro. To determine the area of maximal constriction, radiopaque markers were placed just above the spastic site of the epicardial coronary artery for topological identification. The maximum thickness of the intima and the mean thickness of the media was measured under a microscope with a micrometer scale. The ratio of intimal to medial thickness was calculated as an index of atherosclerotic changes of the coronary artery.

**Drugs**

The following drugs were used: histamine hydrochloride and 1-phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), tetrodotoxin (TTX) (Sankyo), nitroglycerin (NG) (Nihon Kayaku, Japan), ethyleneglycolbis (β-aminoethylether)-N, N'-tetraacetic acid (EGTA) (Dozin Lab, Japan), mepyramine maleate (May & Baker Ltd, England), and guanethidine sulfate (Ciba-Geigy, Japan).

**Statistics**

The data were expressed as mean ± SE. Multiple comparisons were made by an analysis of variance, and paired data were analyzed by Student's t test. A p value less than 5% was considered statistically significant.

**Results**

**Characteristics of Coronary Artery Spasm in Vivo**

Coronary artery spasm was documented in vivo at 14 sites in 13 pigs with endothelial denudation. Spasm did not occur in 5 control pigs with an intact endothelium. As shown in the representative angiograms (Figure 2, upper panel), histamine, i.e., provoked ECG-ST changes and angiographical coronary spasm at the area denuded 3 months earlier. The degree of luminal reduction after intracoronary administration of histamine (10 µg/kg) was 63 ± 3% in the denuded area and 26 ± 2% in the non-denuded area (p < 0.01 between these areas). Phenylephrine constricted the denuded and contralateral coronary arteries by 22 ± 6 and 21 ± 4%, respectively, but did not produce an enhanced constriction of the denuded arteries. Coronary arteriography after nitroglycerin showed no significant organic narrowing (Figure 2, upper right panel).

**Histamine-Induced Augmented Constriction of Coronary Artery in Vitro**

The left coronary perfusion flow at the basal state was gradually decreased from 1.73 ± 0.12 ml/min/g at the beginning of the study to 1.26 ± 0.11 ml/min/g at the end. Focal augmented constriction was re-documented in vitro at 13 of the 14 sites previously provoked in vivo, as shown in the lower panel of Figure 2. The degree of focal constriction, as a percent reduction

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**Figure 2.** Representative coronary angiograms in vivo (upper panel) and in vitro (lower panel). Arrow indicates the site of coronary spasm. ECG lead II was recorded in vivo. The estimated concentration of histamine 10 µg/kg in bolus intracoronary administration was maximally 3.2 × 10⁻⁵ M/L. CAG = coronary arteriography.
of the coronary artery from the mean diameter of the proximal and distal coronary arteries to the spastic area, was 63 ± 4 and 57 ± 3% in vivo (10 μg/kg bolus, i.e., which is close to 5.2 × 10⁻³ M) and in vitro (10⁻⁵ M i.e.), respectively (no significant difference).

In 2 cases, the coronary branch distal to the spastic area was cannulated for the injection site or for monitoring peripheral coronary pressure. A proximal injection of histamine (10⁻⁵ M) into the left coronary artery induced focal spasm (Figure 3, Panel B) and produced transient pressure gradient by 13-18 mm Hg (15-20%) between the proximal and distal cannulas to the spastic area. In contrast, peripheral injection of the same dose of histamine caused neither focal coronary spasm nor pressure gradient (Figure 3, Panel C). This evidence suggests that the coronary artery responsible for the spasm was in the area of stenosis viewed angiographically.

In 5 cases, coronary angiograms were recorded immediately, at 30 seconds, and at 90 seconds after 10⁻⁵ M histamine infusion; diameter reductions were 68 ± 2, 67 ± 3, and 56 ± 3% at the spastic area and 48 ± 2, 29 ± 4, and 27 ± 9% at the nondenuded area, respectively. Thus, the enhanced contraction was more persistent at the spastic area than the nondenuded area.

**Pharmacophysiological Characteristics of Histamine-Induced Augmented Coronary Constriction**

The concentration-response relation to histamine (10⁻⁷ M-10⁻⁵ M) is summarized in Figure 4 for both denuded and nondenuded portions of the coronary artery. Histamine (10⁻⁷ M-10⁻⁵ M) reduced the coronary diameter in relation to the dose of the denuded area more than the nondenuded area (p<0.01 in 10⁻⁶ M-10⁻⁵ M).

Pharmacological examination revealed that the intimal thickening was limited to the denuded site. The maximum thickness of the intima in the denuded site (80 ± 8 μm) was significantly thicker than that in the nondenuded site (18 ± 3 μm) (p<0.01), whereas the medial thickness in the denuded portion (154 ± 15 μm) was similar to that of the nondenuded area (150 ± 12 μm). Endothelial cells were microscopically detected on both the normal and thickened intima.

**Morphometry of Coronary Arteries**

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FIGURE 4. Dose-response relation of percent luminal reduction to histamine. Resting coronary diameter at the denuded area remained constant for 3 hours from 2.22 ± 0.09 to 2.21 ± 0.07 mm and at the nondenuded area from 2.34 ± 0.03 to 2.33 ± 0.03 mm. Constrictive responses to histamine were repeatedly examined at the nondenuded and denuded areas, and the degree of constriction was reproducible throughout the experiment, from 64 ± 2% to 64 ± 2% and from 34 ± 3 to 34 ± 3% in denuded and nondenuded areas, respectively.

Discussion

In studies in pigs, angiographic evidence of coronary spasm was obtained from two different in situ and in vitro preparations in the same heart. The major new findings are 1) there is reproduction of augmented constriction of the epicardial large coronary artery by histamine in the isolated perfused heart, similar in degree and area to that observed angiographically in vivo, which eliminates the pathogenetic role of blood constituents and neural control in the provocation of vascular hypercontraction; 2) there is no significant influence of neurotransmitter blockade on the dose-response relation of the coronary artery to histamine; 3) there is an absence of enhanced constriction of the denuded portion of the coronary artery by histamine (10⁻⁵ M) in calcium-free perfusate; and 4) histamine-induced augmented constriction was blocked after pretreatment with mepyramine (10⁻⁶ M), an H₁ blocker. Potassium chloride (40 mM) and phenylephrine (10⁻⁵ M) did not augment constriction at the denuded site, which suggests that coronary spasm is a unique situation in which the vessel wall contracts in the presence of autacoids such as histamine. Histological studies revealed that the augmented constriction was limited to the area with intimal thickening and where the endothelium was seen microscopically as the nondenuded site.

Feasibility of in Vitro Studies on Coronary Spasm

Coronary artery spasm was repeatedly provoked along the area of balloon-denudation of the epicardial coronary artery. Therefore, such a localized phenomenon may best be documented by coronary arteriography, which also facilitates simultaneous determination of the degree of constriction of the epicardial large intact vessel.

We applied a coronary arteriographic technique not only in situ but also to the isolated heart perfused with oxygenated electrolyte solution, under a constant perfusion pressure (90 mm Hg). Thus, the role of blood constituents and neural control is readily discernible, according to differences in coronary spasm that occur both in situ and in the same heart isolated and perfused with electrolytes. Since the coronary artery diameter depends on the level of arterial pressure, a constant perfusion pressure system has advantages for simple analysis of changes in diameter. In the isolated pig hearts, Krebs-Henseleit solution was perfused at 90 mm Hg, a level similar to the mean arterial pressure noted in situ, and therefore presumed physiological. In this potassium-chloride-arrested heart, coronary blood flow was also stable during the experimental period. Thus, we were able to eliminate the effect of flow-dependent coronary dilation.

FIGURE 5. Effects of nerve ending blockade of guanethidine, 3 × 10⁻⁶ M; atropine, 10⁻⁶ M/L; and tetrodotoxin, 3 × 10⁻⁷ M/L on histamine-induced percent luminal reduction at the intact and denuded areas. KH = Krebs-Henseleit solution.

Intact portion

- KH
- Guanethidine 3 × 10⁻⁵ M/L
- Atropine 10⁻⁶ M/L
- Tetrodotoxin 3 × 10⁻⁷ M/L

Denuded portion

- KH
- Histamine -log M
- Control 7 (n=6) 6 (n=6) 5 (n=6)
Several questions can be raised with regard to this perfusion apparatus. First, under the present experimental conditions, the conduit vessels might be influenced by vasodilating substances produced from the adjacent myocardium. Second, the oxygen-carrying capacity of oxygenated Krebs-Henseleit solution is far below the level of the blood-perfused system.

Since the conduit coronary artery was continuously perfused with a nonrecirculating system, under a pressure of 90 mm Hg, the vasodilator substance produced in the myocardium rendered ischemic due to electrolyte perfusion would rapidly be washed away. In the present study, physiological salt solution was used as the perfusate and a focal-augmented constriction in the present animal model is not related to \( \alpha \)-adrenergic activity or to an abnormality in nerve endings.

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Clinical evidence suggests a close topological relation between the sites of coronary artery spasm and organic coronary stenosis, i.e., the site of coronary atherosclerosis. Our present observations support this proposal. Enhanced constriction at the site with intimal thickening may be explained by the increase in coronary narrowing due to a geometric effect and/or changes in the vascular responses to specific vasoactive agents such as histamine, as demonstrated in the present study. Ginsburg et al noted the augmented contraction to histamine in coronary strips obtained at autopsy on patients with atherosclerosis. Experimental studies on vascular tone in vitro demonstrated augmented contraction to vasoconstrictive stimuli in vascular strips from subjects with arteriosclerosis or atherosclerosis. Thus, the atherosclerosis itself may potentiate the vascular response. In a foregoing paper, it was shown that the degree of constriction was always greater than that predicted from the equation, such as in the formula proposed by Freedman et al. In the present study, KCl (40 mM) equally constricted intact vessels and those with intimal thickening.

**Mechanism of Coronary Artery Spasm**

Recently, double blind studies on \( \alpha \)-blockers given to patients with variant angina did not support the presence of augmented \( \alpha \)-adrenergic activities. Coronary artery spasm was also redocumented in patients with complete autonomic denervation of the heart (autotransplantation). In the present study, the exogenously added \( \alpha \)-adrenergic agonist, phenylephrine, did not augment the constrictive response at the denuded area in vivo. Furthermore, the histamine-induced augmented constriction at the denuded area was not modified by treatment with nerve transmitter blockers. Thus, the coronary spasm seen in the present animal model is not related to \( \alpha \)-adrenergic activity or to an abnormality in nerve endings.

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ing. Therefore, enhanced constriction at the site of intimal thickening is selectively hypersensitive to histamine. Accordingly, vascular changes related to atherosclerosis seem to play a crucial role in the pathogenesis of coronary artery spasm. In the present study, enhanced constriction of the denuded vessel may also represent a defective relaxation because coronary constriction at the denuded area was persistent at least for 90 seconds. However, the mechanisms and the functional significance of the abnormality in relaxation remained to be clarified.

Pathogenesis of Coronary Spasm

In a previous in vivo study, histamine was infused intracoronarily to the denuded area at the proximal and distal sites to determine the primary focus of the histamine-induced vasoconstriction followed by myocardial ischemia. It became clear that regional dysfunction and a decrease in the peripheral coronary pressure occurred only after the proximal injection of histamine in vivo. Similar phenomena were observed in 2 cases in vitro in the present experiment. Therefore, the primary focus of the histamine-induced coronary artery spasm was the denuded area of the epicardial vessel and not the resistance vessel.

It has been suggested that endothelial cells release vasodilating substances, called endothelium-derived relaxing factors (EDRF), in response to various vasoactive stimuli. PGJ₂, a potent vasodilating substance, is also produced in endothelial cells as well as the inner side of the media, and its production is reduced in the presence of atherosclerosis. Therefore, disruption of the vascular endothelium may augment responses to vasoactive stimuli such as histamine and serotonin. Observations using microscopy revealed that the denuded portion was completely covered by a regenerated endothelium at 3 months after denudation. Therefore, the functional state of the regenerated endothelium remains to be assessed.

Ginsburg et al reported provocation by histamine of coronary artery spasm in patients with variant angina. In human coronary arterial strips, histamine produced a greater contractile tension than did carbachol, serotonin, phenylephrine, and ergonovine. Moreover, the contractile response to histamine, but not carbachol and calcium, was augmented in atherosclerotic vessels. Supersensitivity to histamine and serotonin was repeatedly noted in our studies on pigs. The number of H₁ receptors was found to be increased at the atherosclerotic segments of the coronary artery and aorta, and this is another possible explanation for the supersensitivity to H₁ receptor stimulation. Therefore, histamine is probably one of the most likely vasoactive agents linked to coronary artery spasm, in which the large epicardial artery primarily contracts and results in myocardial ischemia. This possibility has recently been documented in a considerable percent of patients with vasospastic angina with or without coronary artery disease.

Barger et al reported that the vasa vasorum of human coronary arteries was rich in the atherosclerotic portion, determined by cinematography following silicone polymer injection. With microvasculature, this may result in a higher tissue concentration of vasoactive substances to these areas and contribute to the production of enhanced focal constriction by circulating vasoactive substances. However, in the present study, the microvasculature and vasa vasorum were not detectable by a conventional histological technique at the thickened intima composed of fibromuscular proliferation.

Our study also revealed that histamine causes arterial constriction in the presence of Ca²⁺-free solution, but no focal-augmented constriction in the electrolyte-perfused isolated heart. The degree of contraction at the denuded area was similar to that at the intact area in Ca²⁺-free solution. Therefore, we propose that the enhanced influx of Ca²⁺ via H₁ receptors at the denuded vessel plays a major role in coronary artery spasm.

Perspective

The present study correlating the findings under in vivo and in vitro conditions, using the same heart in which coronary artery spasm occurred, provides meaningful information with regard to quantitative pharmacological responses of spastic vessels. The lack of any significant difference in the characteristics of coronary artery spasm between in vivo and in vitro conditions will pave the way for further studies in vitro.

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Y Yamamoto, H Tomoike, K Egashira, T Kobayashi, T Kawasaki and M Nakamura

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