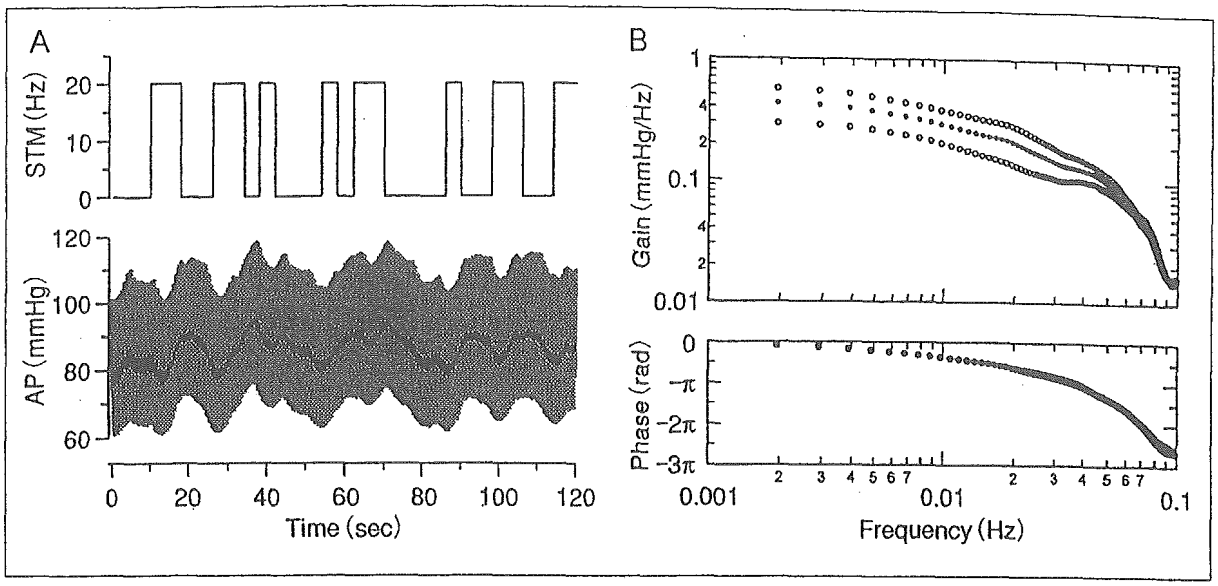

図3
A：不規則な脊髄交感神経刺激に対する血圧応答．刺激頻度(STM)を0か20ヘルツに不規則に変化させ，血圧応答(AP)を記録した．B：刺激頻度の変化に対する血圧応答の動的な特性を示す伝達関数．黒丸が平均値で白丸が平均±標準偏差(12例)を示す．

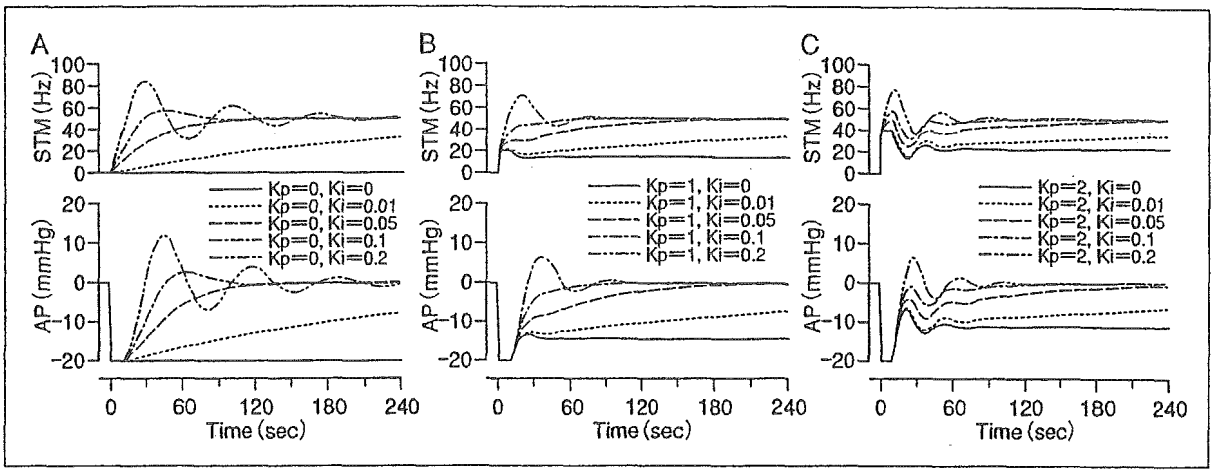

図4　人工的血管運動中枢の設計のためのシミュレーション
図2における比例補償係数(K_p)と積分補償係数(K_i)の組合わせを変えて，−20mmHgの外乱の影響がどのように表れるかを数値解析した．補償係数がともにゼロの場合には，外乱の影響はまったく抑制されない．

バイオニック動脈圧反射装置の有効性の検証

上記の結果を用いて，人工的血管運動中枢を設計し，試作したバイオニック動脈圧反射装置の有効性を検証した．検証にあたっては，起立性低血圧と同様，あるいは類似の血行動態変化による急激でかつ再現性のある低血圧モデルが理想的である．そこで，下肢人工関節置換術の際に止血目的で大腿部に圧迫帯を用いる症例に着目した．このような症例では，圧迫帯の解除時に急激な低血圧を生ずることが知られている[16)17)]．バイオニック動脈圧反射装置の作動中に圧迫解除を行った場合と，そうでない場合で，血圧がどのように変化するかを検討した．22例から得られた結果は，図5に示されている．大腿部の圧迫止血帯の急速解除に伴う血行動態は，解除後急激に血圧と中心静脈圧が低下した．これは，圧迫解除に伴う下肢への血液貯留により静脈還流が減少し心拍出量が減少したことと，大腿動脈の圧迫解除によって血管床の相対的増加がもたらされ，血管抵抗が減少したことを示

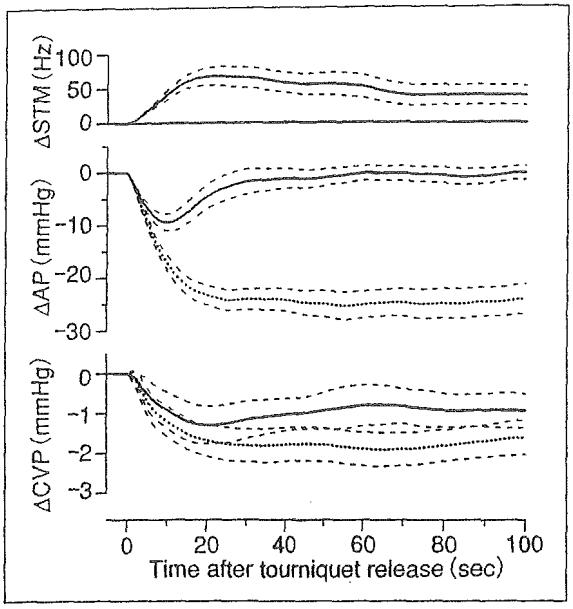

図5　圧迫止血帯の急激な解除に伴う急激な血圧低下
バイオニック動脈圧反射装置が作動していない場合(点線)には，圧迫止血帯の解除に伴い急激に血圧(AP)と中心静脈圧(CVP)が低下したが，バイオニック動脈圧反射が作動している場合(実線)には，両者ともに低下が抑制された．破線は平均±標準偏差(22例)を示す．

唆している．したがって，このような，10秒以内に血圧が20mmHg低下するモデルは，バイオニック動脈圧反射装置の有効性を評価するために妥当であると考えられた．

図から明らかなように，バイオニック動脈圧反射装置が作動している場合には，圧迫止血帯の解除に伴う急激な血圧低下は，数秒以内に食い止められ，止血帯解除前の血圧値にすみやかに回復した．以上のような結果から，バイオニック動脈圧反射装置は，本モデルのような血圧低下に対しては有用であると考えられた．

まとめ

起立性低血圧などのような血圧調節障害に対する治療法として，輸液・輸血法，薬物療法，機械的サポート法などが知られているが，本稿で紹介した手法はこれらとは異なり，自律神経系とインターフェイスして，医工学的に動脈圧反射機能を再建しようとするものである．その利点は，神経性であるがゆえに迅速な調節が可能であるだけでなく，オンデマンド的動作であるため，不要な臥位高血圧をまねく危険性が少ないことも期待される．

バイオニック動脈圧反射装置を臨床応用するにあたっては，長期使用の可能な交感神経刺激電極の開発や小型電気刺激装置が必要となるが，すでに難治性てんかんの治療用としてカフ型の迷走神経刺激電極や刺激装置が開発され，多くの症例に使用されている[18]．したがって，このような要素技術を応用すれば，バイオニック動脈圧反射装置が起立性低血圧の治療器として実用化できるかもしれない．

文　献

1) Robertson D. Diagnosis and management of baroreflex failure. Primary Cardiol 1995 ; 21 : 37.
2) The Consensus Committee of the American Autonomic Society and the American Academy of Neurology. Consensus statement on the definition of orthostatic hypotension, pure autonomic failure, and multiple system atrophy. Neurology 1996 ; 46 : 1470.
3) Ketch T, Biaggioni I, Robertson R, et al. Four faces of baroreflex failure : hypertensive crisis, volatile hypertension, orthostatic tachycardia, and malignant vagotonia. Circulation 2002 ; 105 : 2518.
4) Bannister R, da Costa DF, Hendry WG, et al. Atrial demand pacing to protect against vagal overactivity in sympathetic autonomic neuropathy. Brain 1986 ; 109 : 345.
5) Kristinsson A. Programmed atrial pacing for orthostatic hypotension. Acta Med Scand 1983 ; 214 : 79.
6) Sato T, Kawada T, Shishido T, et al. Novel therapeutic strategy against central baroreflex failure : a bionic baroreflex system. Circulation 1999 ; 100 : 299.
7) Sato T, Kawada T, Sugimachi M, et al. Bionic technology revitalizes native baroreflex function in rats with baroreflex failure. Circulation 2002 ; 106 : 730.
8) Yanagiya Y, Sato T, Kawada T, et al. Bionic epidural stimulation restores arterial pressure regulation during orthostasis. J Appl Physiol 2004 ; 97 : 984.
9) Guyton AC, Coleman TG, Granger HJ. Circulation : overall regulation. Ann Rev Physiol 1972 ; 34 : 13.
10) Sato T, Kawada T, Inagaki M, et al. New analytic

framework for understanding sympathetic baroreflex control of arterial pressure. Am J Physiol Heart Circ Physiol 1999 ; 276 : H2251.

11) Sunagawa K, Sato T, Kawada T. Integrative sympathetic baroreflex regulation of arterial pressure. Ann NY Acad Sci 2001 ; 940 : 314.

12) Kawada K, Sunagawa G, Takaki H, et al. Development of a servo-controller of heart rate using a treadmill. Jpn Circ J 1999 ; 63 : 945.

13) Hainsworth R, Karim F. Responses of abdominal vascular capacitance in the anaesthetized dog to changes in carotid sinus pressure. J Physiol (Lond) 1976 ; 262 : 659.

14) Carneiro JJ, Donald DE. Blood reservoir function of dog spleen, liver, and intestine. Am J Physiol Heart Circ Physiol 1977 ; 232 : H67.

15) Minson CT, Wladkowski SL, Pawelczyk JA, et al. Age, splanchnic vasoconstriction, and heat stress during tilting. Am J Physiol Regul Integr Comp Physiol 1999 ; 276 : R203.

16) Kahn RL, Marino V, Urquhart B, et al. Hemodynamic changes associated with tourniquet use under epidural anesthesia for total knee arthroplasty. Reg Anesth 1992 ; 17 : 228.

17) Sander-Jensen K, Mehlsen J, Secher NH, et al. Progressive central hypovolaemia in man–resulting in a vasovagal syncope? Haemodynamic and endocrine variables during venous tourniquets of the thighs. Clin Physiol 1987 ; 7 : 231.

18) Reid SA. Surgical technique for implantation of the neurocybernetic prosthesis. Epilepsia 1990 ; 31 Suppl 2 : S38.

* * *

Clinical and Experimental Pharmacology and Physiology (2005) **32**, 911–918

CONTRIBUTION OF BAROREFLEX SENSITIVITY AND VASCULAR REACTIVITY TO VARIABLE HAEMODYNAMIC RESPONSES TO COCAINE IN CONSCIOUS RATS

Michael Traub,* Tamiko Aochi,† Toru Kawada,‡ Toshiaki Shishido,‡ Kenji Sunagawa§ and Mark M Knuepfer

Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St Louis, Missouri, USA

SUMMARY

1. Baroreflex function is critical for short-term arterial pressure regulation and decreased baroreflex responsivity may predict a predisposition to hypertension and sudden cardiac death. In the present study, we assessed whether baroreflex sensitivity (BRS) and/or vascular reactivity covary with haemodynamic responsiveness to cocaine in vascular and mixed responders.

2. We assessed the heart rate index of BRS in resting animals. We examined dose–response relationships to pressor and depressor agents to determine cardiovascular reactivity. Subsequently, rats were given cocaine (5 mg/kg, i.v.) to classify them as vascular or mixed responders. Vascular responders ($n = 16$) were defined as those rats with a substantial (> 8%) decrease in cardiac output in response to cocaine owing to a larger increase in systemic vascular resistance. The remaining rats ($n = 8$) were mixed responders because they had smaller increases in vascular resistance and little change or an increase in cardiac output.

3. The BRS determined with angiotensin (Ang) II, but not with phenylephrine, was impaired in mixed responders compared with vascular responders. At equipressor doses, there were significantly greater reductions in cardiac output in vascular responders compared with mixed responders in response to phenylephrine or AngII. Methacholine produced greater decreases in heart rate in vascular responders, suggesting greater muscarinic responsivity.

4. We conclude that differences in vascular reactivity to AngII may contribute to differences in haemodynamic response profiles to cocaine in individual rats. More importantly, the differences in vascular responsivity and BRS do not appear to be primary determinants of haemodynamic response variability.

Key words: angiotensin II, cardiac output, cocaine, heart rate index of baroreflex sensitivity, methacholine, nitroprusside, phenylephrine, systemic vascular resistance, vascular responsiveness.

INTRODUCTION

Baroreflex function is important for short-term cardiovascular regulation compensating for transient disturbances in arterial pressure. Increasing arterial pressure to stimulate baroreceptors evokes inhibition of sympathetic activity and enhances parasympathetic activity to minimize the magnitude of the pressor response.[1] Risk analysis studies suggest that decreased heart rate variability, one indicator of diminished baroreflex sensitivity (BRS), is an independent risk factor in humans for the development of arrhythmia[2] and sudden death in patients with congestive heart failure, diabetes mellitus and ischaemic heart disease.[3] Decreased BRS is also common in human subjects with essential hypertension.[4–6] Schwarz *et al.*[7] reported that dogs with coronary occlusion were either prone or resistant to ventricular fibrillation with exercise. Those dogs that were susceptible to fibrillation had reduced BRS, leading the authors to propose that BRS and autonomic imbalance may indicate a predilection to sudden cardiac death. Therefore, reduced BRS has been used to predict a predisposition to the development of cardiovascular disease and sudden cardiac death.

Cocaine evokes different patterns of cardiac output and systemic vascular resistance in rats.[8,9] Although cocaine elicits a pressor response in all rats, some respond with a substantial decrease in cardiac output and a large increase in systemic vascular resistance, whereas others have smaller decreases or an increase in cardiac output and smaller increases in systemic vascular resistance.[8,9] These groups have been designated vascular and mixed responders, respectively.[9] Similar varying haemodynamic response profiles have been observed in response to behavioural stress or amphetamine administration, suggesting that the differences in vascular responses to cocaine are not due to differences in the direct vascular actions of cocaine.[8,10] Because this response may be antagonized by prazosin, pentolinium and adrenal demedullation, the involvement of α-adrenoceptors and activation of the

Correspondence: Dr Mark M Knuepfer, Department of Pharmacological and Physiological Science, St Louis University School of Medicine, 1402 S Grand Blvd, St Louis, MO 63104, USA. Email: knuepfmm@slu.edu

*Present address: Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, Bronx, NY, USA.

†Present address: Arrowhead Regional Medical Center, Colton, CA, USA.

‡Present address: Department of Cardiovascular Dynamics at the National Cardiovascular Research Institute, Osaka, Japan.

§Present address: Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Received 15 December 2004; revision 3 June 2005; accepted 13 June 2005.

sympathoadrenal system has been implicated.[8,11] Vascular responders have a greater incidence and severity of cocaine-induced ultrastructural myocardial lesions and sudden cardiac death and are more prone to developing hypertension with repeated cocaine or stress.[8,9,12,13] Therefore, we proposed that haemodynamic response patterns to cocaine or behavioural stress may predict the predisposition to develop cardiovascular disease.[9] This may be an important model because clinical observations have suggested that haemodynamic response profiles to behavioural stress in humans vary similarly and those described as vascular responders to acute stress are more prone to develop heart disease and hypertension.[14–18]

Cocaine administration produces varying response patterns in conscious rats due, in part, to muscarinic receptor activation. Pretreatment with atropine methylbromide prevents cocaine-induced decreases in cardiac output and greater increases in systemic vascular resistance in vascular responders, making their haemodynamic responses similar to those in mixed responders.[19,20] Others have reported that cocaine has a direct effect on muscarinic receptors[21,22] or alters catecholamine release from sympathetic nerves.[23,24] Therefore, individual rats may have different haemodynamic response profiles owing to varying responsiveness of peripheral muscarinic receptors to activation by cholinergic agonists.

In the present study, we examined peripheral cardiovascular responsiveness to pressor and depressor agents and differences in the heart rate index of BRS to determine whether these contributed to varying haemodynamic response patterns in vascular and mixed responders. We proposed that vascular responders would have reduced BRS compared with mixed responders similar to humans predisposed to develop cardiovascular disease. We also examined whether vascular reactivity varies between vascular and mixed responders using graded doses of pressor and depressor agents. To produce pressor responses, we used the α-adrenoceptor agonist phenylephrine and the peptide angiotensin (Ang) II. To produce depressor responses, we used the endothelium-dependent muscarinic agonist methacholine and the endothelium-independent agonist nitroprusside. If the differences in haemodynamic responses were due to differences in peripheral vascular responsivity, vascular responders would have greater vasoconstrictor responses to pressor agents. If the differences were dependent on varying muscarinic receptor responsivity, we predicted that the muscarinic agonist, but not nitroprusside, would elicit more profound decreases in cardiac output and heart rate in vascular responders. We discovered that there were differences in BRS between vascular and mixed responders when tested with AngII and that vascular responsiveness to AngII, but not to phenylephrine, is enhanced in vascular responders.

METHODS

Surgical preparation

All experimental protocols were approved by the St Louis University Institutional Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington DC, 1996; http://www.nap.edu/readingroom/books/labrats/). Specific pathogen-free male Sprague-Dawley rats, weighing 285–415 g, were prepared surgically using aseptic techniques under pentobarbital sodium (50 mg/kg, i.p.) anaesthesia. A miniature pulsed Doppler flow probe was placed around the ascending aorta for cardiac output measurement via a midline thoracotomy, as described previously.[8] During recovery (10–14 days), rats were monitored for signs of normal locomotor activity and weight gain and were dosed with cefazolin (10 mg/kg, s.c.) for 1–3 days.

Subsequently, rats were re-anaesthetized with pentobarbital for insertion of sterile cannulas in the left femoral artery and vein for the measurement of arterial pressure and intravenous drug administration, respectively. The cannulas were directed subcutaneously to the posterior neck and sutured in place. Rats were also fitted with leads to measure electrocardiographic data. Multifiber wires (36 awg stainless steel) were implanted in each of the forelimbs, one in the left hind limb and one in the neck (for a ground). All leads were tunnelled subcutaneously to a socket on the skull to record the electrocardiogram. After a recovery period of 2–5 days, during which rats again received cefazolin and were monitored for weight loss, rats were acclimated to a Plexiglas test cage for 6 h. The following day, rats were again acclimated to the test cage for a minimum of 2 h before testing began. If rats displayed continued weight loss (>10% bodyweight) or abnormal ambulation for more than 2 days after either surgery, they were killed with pentobarbital (65 mg/kg, i.p.).

Baroreflex sensitivity determination

Baroreflex sensitivity was determined using phenylephrine, nitroprusside and AngII to raise or lower arterial pressure while measuring the change in heart rate. According to previously reported methods,[4,25,26] we estimated the cardiac index of BRS based on the dynamic response of heart rate to blood pressure alterations, comparing pulse intervals before the change in arterial pressure to a point just before peak arterial pressure change.[26] We avoided the maximum heart rate response because it occurred after the maximum change in arterial pressure and was likely influenced by neurohumoral responses to pressure changes. The slope of the line (heart rate *vs* arterial pressure in b.p.m./mmHg) is the heart rate index of BRS using 10–30 R-R intervals determined electronically. The R-R intervals were excluded if the change in rate between intervals was greater than 50 b.p.m., suggesting an arrhythmia. We attempted to investigate the early changing phase of the baroreflex function curve only by using lower doses of pressor and depressor agents. Therefore, we only studied the steepest part of the relationship. Owing to a lack of data with pressor and depressor responses of greater magnitude (plateau phase), we did not have the appropriate data to fit the logistic equation described by Head and McCarty.[27] The attempt to investigate the entire baroreflex function curve is arguably suspect because the time necessary to reach peak changes in arterial pressure varied between pressor and depressor agents. For example, pressor responses to phenylephrine and AngII occurred relatively quickly (5–7 s), whereas maximum depressor responses to nitroprusside occurred more slowly (10–20 s).

Vascular reactivity protocol

Rats were tested the first day by measuring mean arterial pressure, heart rate and cardiac output responses to a variety of pressor and depressor agents, namely phenylephrine, AngII, nitroprusside and methacholine. Bolus injections of three different doses of each agent were administered as follows: phenylephrine 0.5, 1 and 2 μg/kg; AngII 0.01, 0.05 and 0.1 μg/kg; nitroprusside 2, 3 and 6 μg/kg; methacholine 0.1, 0.5 and 1 μg/kg. Doses were given in alternating pairs, phenylephrine and nitroprusside at progressively larger doses and then AngII and methacholine at progressively larger doses. Each injection was followed by a 0.3 mL saline flush over 20–30 s. Control vehicle (0.9% saline) injections did not produce measurable haemodynamic or behavioural responses. Cardiovascular variables were allowed to return to normal for at least 2 min before administering subsequent doses of vasoactive compounds. To measure vascular reactivity to the drugs, the chart recordings of arterial pressure, heart rate and cardiac output were read before and at the peak change in arterial pressure produced by each drug so that the change in each of the parameters could be measured. From those data, changes in systemic vascular resistance were calculated using Ohm's law.

Cocaine administration

After characterising vascular responsiveness, rats were given cocaine (5 mg/kg, i.v., over 45 s) twice daily (with at least 3 h between dosings) for 2–3 days, as described previously.[8] Briefly, the maximal changes in cardiac output elicited in each trial were averaged in order to classify rats as vascular or mixed responders. Vascular responders were rats with a mean maximal decrease in cardiac output of 8% or more.[8] The remaining rats were classified as mixed responders.

Data acquisition

Ascending aortic blood flow was estimated using a 20 MHz pulsed Doppler flowmeter with a 100 kHz sampling frequency, anti-aliasing and auto-tracking (Department of Bioengineering, University of Iowa, Iowa City, IA, USA). Data were displayed continuously on a chart recorder. Percent changes in systemic vascular resistance and stroke volume were calculated using the mean arterial pressure or heart rate and the ascending aortic blood flow changes (kHz shift). The electrocardiogram (ECG) was recorded using a Grass amplifier (model 7P4) in a Grass Chart Recorder (model 7D; Grass Medical Instruments, Quincy, MA, USA) with a Vetter Data Recorder (model 420; AR Vetter, Rebersburg, PA, USA) to store the recordings before off-line analysis. Data were also displayed and recorded for computer analysis using a 16-channel data acquisition and analysis program (WINDAQ; DATAQ Instruments, Akron, OH, USA) for off-line analysis. The R-R intervals were recorded at 5000 samples/s per channel. Control ECG values were obtained in three separate recording series (each 5 min long) prior to experimentation.

Table 1 Initial baseline cardiovascular parameters

	Mixed responders	Vascular responders	*P*
MAP (mmHg)	114 ± 3 (8)	119 ± 3 (16)	0.3306, $t = 0.99$
HR (b.p.m.)	413 ± 11 (6)	408 ± 6 (16)	0.7057, $t = -0.38$
CO (kHz shift)	9.0 ± 0.8 (8)	10.4 ± 0.6 (16)	0.1565, $t = 1.47$

Data are the mean±SEM with *n* given in parentheses.
MAP, mean arterial pressure; HR, heart rate; CO, cardiac output.

Data analysis

We evaluated vascular reactivity using two-way analysis of variance (ANOVA). We compared cardiovascular responses in individual rats to three doses of each agonist using a paired approach (within group) and we compared the effects in mixed and vascular responders separately (between group). Post hoc analysis was performed using Bonferroni's (Dunn's) test. Occasional missing data points were interpolated by the software program. Unpaired *t*-tests with pooled variances were used to compare resting haemodynamic values and both the slope and *y*-intercepts of the baroreflex function relationship. Linear regression was performed on the heart rate–arterial pressure relationship in each rat in response to phenylephrine, nitroprusside and AngII. All statistical analyses were performed using GB Stat (Dynamic Microsystems, Silver Spring, MD, USA).

RESULTS

Differentiation of cardiovascular responses to cocaine

The resting values for haemodynamic parameters in freely moving, instrumented rats are given in Table 1. There were no significant differences at baseline between vascular and mixed responders. The cardiovascular responses to cocaine (5 mg/kg), as described previously,[8] consisted of a prominent but brief pressor response during the first minute (termed the peak response) and a modest sustained pressor response for several minutes thereafter. For each rat, the cocaine-induced change in cardiac output over the first 60 s for each trial was averaged in order to classify rats as vascular ($n = 16$) and mixed ($n = 8$) responders, as described previously.[8] Vascular responders had a greater reduction in cardiac output than mixed responders (−11.4 ± 1.6 *vs* −1.4 ± 0.5%, respectively; $P < 0.001$, $t = -4.59$) and a greater increase in systemic vascular resistance (46.5 ± 2.9 *vs* 33.1 ± 2.5%, respectively; $P < 0.005$,

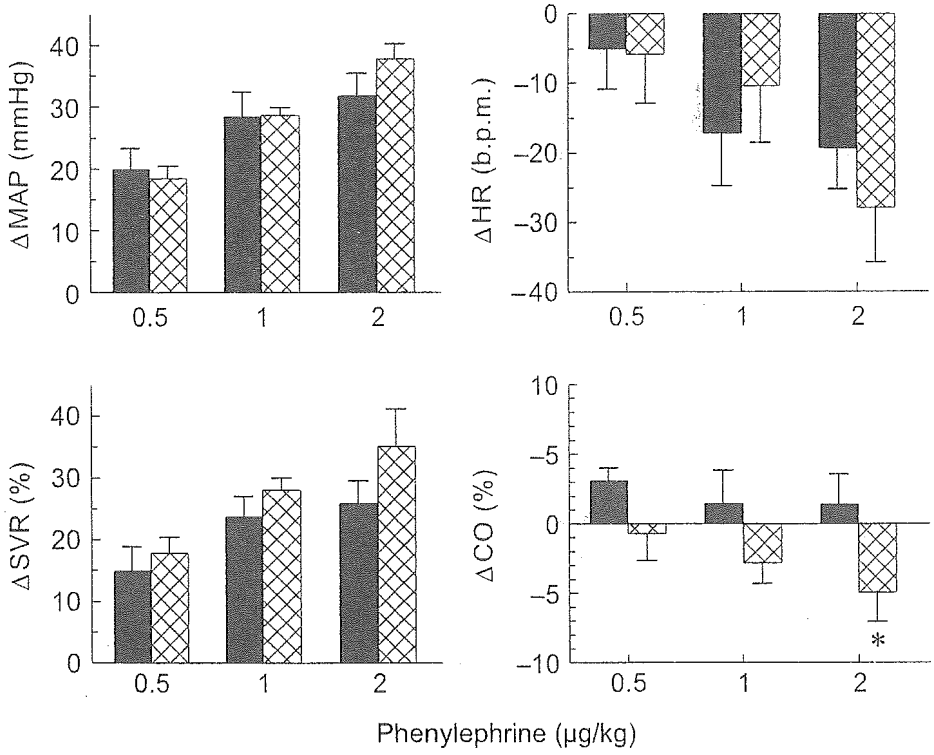

Fig. 1 Cardiovascular responses to graded doses of phenylephrine (0.5, 1 and 2 μg/kg) on mean arterial pressure (MAP), heart rate (HR), cardiac output (CO) and systemic vascular resistance (SVR). Data are the mean±SEM changes in parameters in mixed responders (■) and vascular responders (▨). All responses shown are dose related ($P < 0.0001$, MAP; $P = 0.0062$, HR; $P = 0.0408$, CO; $P = 0.0009$, SVR). Stroke volume was increased slightly but not dose related (data not shown). There was a significant difference in cardiac output responses between vascular and mixed responders ($P = 0.0391$) that was also significant ($P < 0.01$) at the highest dose by post hoc analysis (Bonferroni's test). An asterisk is used to signify significant differences ($P < 0.05$) in vascular responders compared with mixed responders.

$t = 3.11$) after cocaine administration. There were no significant differences in arterial pressure ($P = 0.86$, $t = 0.18$) or heart rate responses ($P = 0.49$, $t = 0.71$) to cocaine during the initial pressor response.

Vascular reactivity to pressor and depressor agents

Before characterising the responses to cocaine in individual rats, differences in vascular reactivity were measured in response to phenylephrine, AngII, nitroprusside and methacholine. Phenylephrine (0.5, 1 and 2 μg/kg, i.v.) elicited brisk pressor responses that were accompanied by a decrease in heart rate (presumably baroreflex mediated) and increases in systemic vascular resistance. In vascular responders, phenylephrine elicited a decrease in cardiac output, whereas it typically produced an increase in mixed responders (Fig. 1). There were no significant differences in the other parameters measured (Table 2). All haemodynamic responses were dose related (Table 2).

Table 2 *F* ratios and significance values for haemodynamic responses

	MAP	HR	CO	SVR
Phenylephrine				
MR versus VR	$F_{1,20} = 0.35$ $P = 0.563$	$F_{1,20} = 0.01$ $P = 0.922$	$F_{1,19} = 4.95$ $P = 0.039$	$F_{1,19} = 1.95$ $P = 0.179$
Dose	$F_{2,42} = 36.1$ $P < 0.0001$	$F_{2,42} = 5.83$ $P = 0.0062$	$F_{2,40} = 3.50$ $P = 0.0408$	$F_{2,40} = 8.57$ $P = 0.0009$
Nitroprusside				
MR versus VR	$F_{1,18} = 0.38$ $P = 0.546$	$F_{1,18} = 3.31$ $P = 0.086$	$F_{1,15} = 0.61$ $P = 0.447$	$F_{1,15} = 0.70$ $P = 0.417$
Dose	$F_{2,38} = 10.63$ $P = 0.0003$	$F_{2,38} = 1.78$ $P = 0.1828$	$F_{2,32} = 1.00$ $P = 0.38$	$F_{2,32} = 6.27$ $P = 0.0056$
Angiotensin II				
MR versus VR	$F_{1,16} = 0.02$ $P = 0.889$	$F_{1,18} = 3.32$ $P = 0.0863$	$F_{1,16} = 6.85$ $P = 0.0194$	$F_{1,16} = 5.66$ $P = 0.031$
Dose	$F_{2,34} = 103.7$ $P < 0.0001$	$F_{2,38} = 1.79$ $P = 0.1828$	$F_{2,34} = 14.81$ $P < 0.0001$	$F_{2,34} = 22.96$ $P < 0.0001$
Methacholine				
MR versus VR	$F_{1,17} = 0.96$ $P = 0.342$	$F_{1,17} = 7.34$ $P = 0.0155$	$F_{1,16} = 2.72$ $P = 0.1197$	$F_{1,16} = 0.85$ $P = 0.3702$
Dose	$F_{2,38} = 21.56$ $P < 0.0001$	$F_{2,36} = 2.13$ $P = 0.1353$	$F_{2,34} = 2.70$ $P = 0.0834$	$F_{2,34} = 17.23$ $P < 0.0001$

MR, mixed responder; VR, vascular responder; MAP, mean arterial pressure; HR, heart rate; CO, cardiac output; SVR, systemic vascular resistance.

Angiotensin II (0.01, 0.05 and 0.1 μg/kg, i.v.) also evoked an increase in arterial pressure in all rats that was dose related and due, primarily, to dose-dependent increases in systemic vascular resistance with concomitant decreases in heart rate. Vascular responders had greater dose-dependent increases in systemic vascular resistance and dose-dependent decreases in cardiac output compared with mixed responders (Fig. 2; Table 2).

Nitroprusside (2, 3 and 6 μg/kg, i.v.) elicited dose-related reductions in arterial pressure due to a decrease in systemic vascular resistance. There were small increases in heart rate and cardiac output that were not dose related. There were no differences between responses in vascular or mixed responders (Fig. 3, Table 2).

The muscarinic agonist methacholine was used to compare muscarinic receptor sensitivity in vascular and mixed responders. Methacholine (0.1, 0.5 and 1 μg/kg, i.v.) elicited dose-related depressor responses due to decreases in systemic vascular resistance. Vascular responders had greater increases in heart rate compared with mixed responders (Fig. 4; Table 2). No other differences in responsivity were observed.

Baroreflex sensitivity

A linear regression of the heart rate versus arterial pressure pulse determined at each R-R interval was used to estimate BRS (Fig. 5). Angiotensin II elicited a decreased slope and *y*-intercept of the BRS

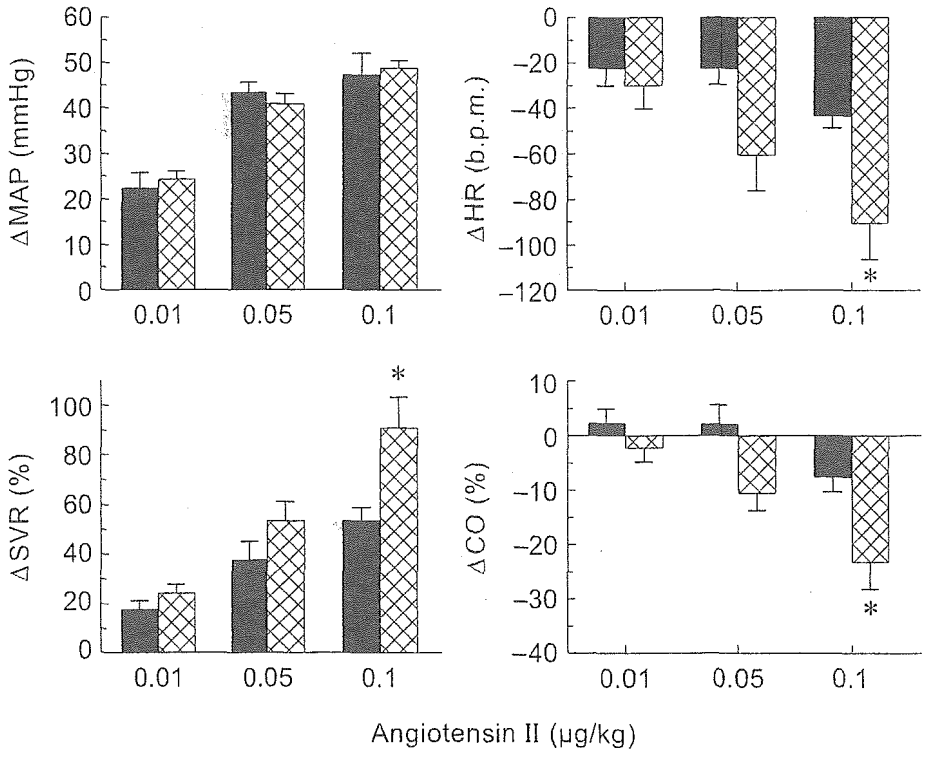

Fig. 2 Angiotensin II (0.01, 0.05 and 0.1 μg/kg, i.v.) elicited dose-related increases in mean arterial pressure (MAP; $P = 0.0003$) and systemic vascular resistance (SVR; $P = 0.0056$). Angiotensin also evoked dose-related reductions in heart rate (HR; $P = 0.0003$) and cardiac output (CO; $P < 0.0001$). The increase in systemic vascular resistance and the decrease in cardiac output were significantly greater in vascular responders (▨) compared with mixed responders (■), with $P = 0.031$ and $P = 0.0194$, respectively. The differences in HR ($P < 0.01$), SVR ($P < 0.05$) and CO ($P = 0.05$) were significant at the highest dose used, as indicated by asterisks.

relationship in mixed responders compared with vascular responders (Table 3). The BRS was not significantly altered in mixed responders in response to phenylephrine and nitroprusside compared with the BRS in vascular responders (Table 3).

DISCUSSION

In the present study, we did not observe differences in the heart rate index of BRS to an α-adrenoceptor agonist or to a direct vasodilator, but did see differences in BRS with AngII administration. There were also differences between vascular and mixed responders in vascular responsivity to AngII and in cardiac responsiveness to AngII, phenylephrine and methacholine administration. The differences in BRS and vascular responsiveness do not seem to be the primary cause of response variability in these animals, but suggest a possible contribution of angiotensin in response variability.

Angiotensin II administration alone produced greater reductions in cardiac output in vascular responders compared with mixed responders similar to the haemodynamic responses we recorded to

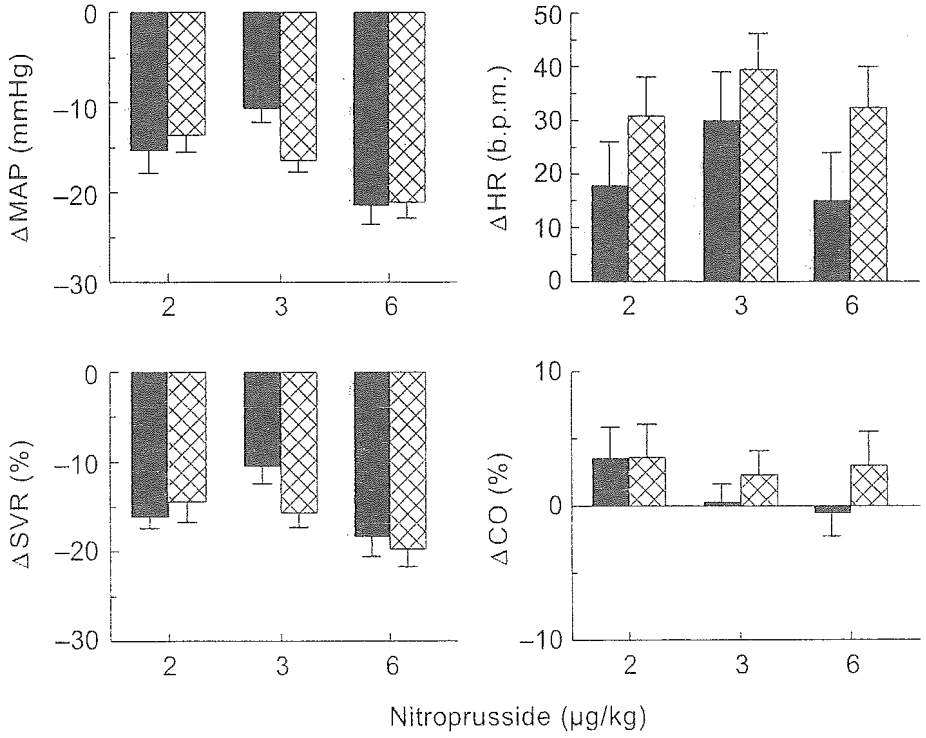

Fig. 3 The effects of nitroprusside (2, 3 and 6 μg/kg, i.v.) on haemodynamic responses in vascular responders (▨) and mixed responders (■). Nitroprusside elicited dose-related reductions in mean arterial pressure (MAP; $P = 0.0003$) due to dose-dependent decreases in systemic vascular resistance (SVR; $P = 0.0056$). There were no differences between vascular and mixed responders. HR, heart rate; CO, cardiac output.

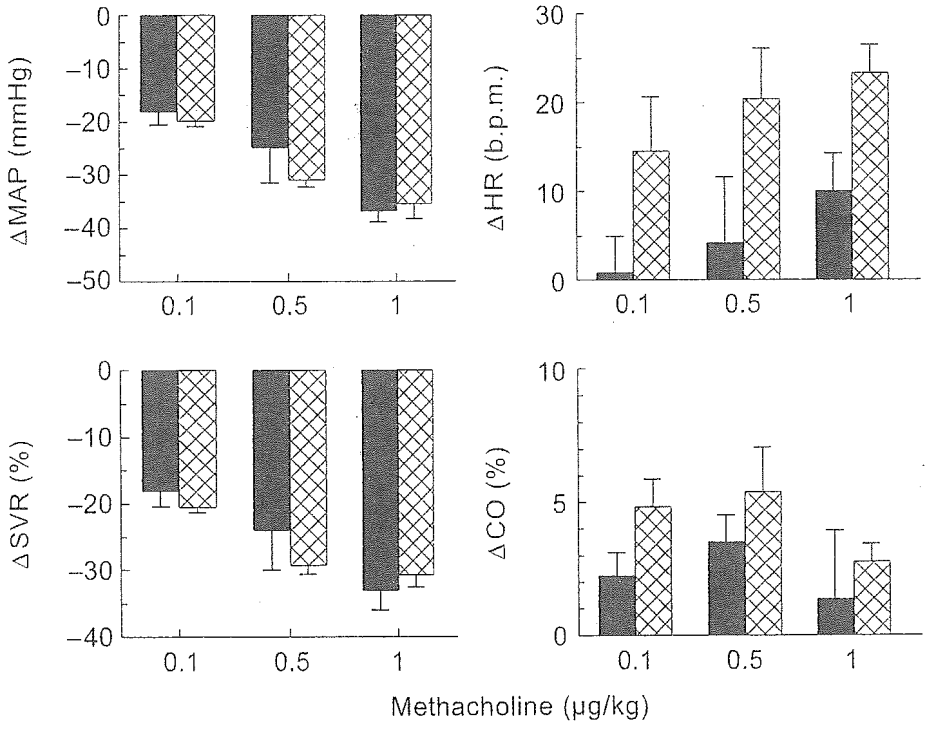

Fig. 4 Methacholine administration (0.1, 0.5 and 1 mg/kg, i.v.) evoked dose-related reductions in mean arterial pressure (MAP; $P < 0.0001$) due to dose-dependent falls in systemic vascular resistance (SVR; $P < 0.0001$). There was a significantly greater increase in heart rate (HR) in vascular responders (▨) compared with mixed responders ($P = 0.0155$; ■). CO, cardiac output.

cocaine (Fig. 1). In addition, we have reported that other psychoactive agents, such as amphetamine or ethanol, elicit a pressor response associated with greater decreases in cardiac output in vascular responders to cocaine.[8,9,28] This apparent anomaly is explained if greater increases in systemic vascular resistance occur in vascular responders in response to these diverse pharmacological stimuli. More importantly, we have noted that vascular responders to cocaine have more negative cardiac output responses and greater increases in systemic vascular resistance to conditional or unconditional behavioural stress.[10] Because it is unlikely that AngII elicits arousal or stress, these findings suggest a role for angiotensin in the mediation of these varying responses.

Angiotensin II elicits a pressor response due to direct vasoconstriction[29,30] and increases noradrenaline release from sympathetic nerve terminals by actions in the ganglia and neuroeffector sites.[31–34] Dendorfer *et al.*[31] reported that intravenous administration of AngII as a bolus resulted in a rapid increase in arterial pressure, sympathetic nerve activity and plasma noradrenaline levels in pithed or ganglionic-blocked rats. Therefore, angiotensin elicits several acute responses to raise arterial pressure after bolus administration. Angiotensin II also elicits central sympathoexcitation due to actions on AT_1 receptors in the circumventricular organs, but this is likely to be a delayed effect and typically overcome by baroreflexes.[35,36] These data demonstrate that AngII has many actions that enhance sympathoexcitation centrally and peripherally. Our data suggest that the relative sensitivity to AngII may vary in vascular and mixed responders. Preliminary evidence from our laboratory suggests that angiotensin may be acting in the central nervous system (CNS).[37,38]

Angiotensin II suppresses baroreflex function by effects both in the CNS and in the carotid sinus.[39–41] Administration of angiotensin-converting enzyme inhibitors has been shown to increase BRS independently of pressure alterations, suggesting a CNS effect.[41,42] Others have suggested that systemic AngII inhibits baroreflex function by local vasoconstriction near nerve endings in the aortic arch of rats and rabbits.[43] Baroreflex function is depressed in response to AngII injected into area postrema or nucleus tractus solitarius.[39,40] Therefore, angiotensin plays a role in modulating BRS. Our data suggest that vascular responders may be less sensitive to this effect compared with mixed responders.

Others have reported that reduced BRS occurs in animals and humans that are prone to the development of hypertension or sudden cardiac death,[6,7] yet the heart rate index of BRS appears to be reduced in the population of rats that are less prone to develop cocaine- or stress-induced elevations in arterial pressure.[8,9,44] This may not, in fact, represent a lower BRS in mixed responders, but could reflect a greater sensitivity to AngII compared with vascular responders. In either case, this appears to be an exception to data described in humans and in dogs with coronary ischaemia.[6,7]

Table 3 Baroreflex responsivity

	Mixed Responders	Vascular Responders	*P*
Phenylephrine relationship			
Slope (b.p.m./mmHg)	−0.9826 ± 0.1828	−1.1245 ± 0.1324	0.2683, $t = 1.16$
Intercept (b.p.m.)	561 ± 26.8	571.0 ± 23.1	0.7758, $t = -0.29$
Nitroprusside relationship			
Slope (b.p.m./mmHg)	−1.2867 ± 0.2367	−1.6202 ± 0.1695	0.2532, $t = 1.19$
Intercept (b.p.m.)	595 ± 29.4	628 ± 17.7	0.3005, $t = -1.08$
Angiotensin II relationship			
Slope (b.p.m./mmHg)	−0.7969 ± 0.1411	−1.6288 ± 0.2714	0.0327,* $t = 2.44$
Intercept (b.p.m.)	520 ± 19.8	610 ± 28.6	0.0331,* $t = -2.44$

Data are the mean ± SEM.

Asterisks indicate significant differences ($P < 0.05$) as determined by unpaired Students *t*-test using pooled variances. Only baroreflex plots with $r > 0.40$ were included. Negative values reflect inverse relationships between changes in heart rate and pressure.

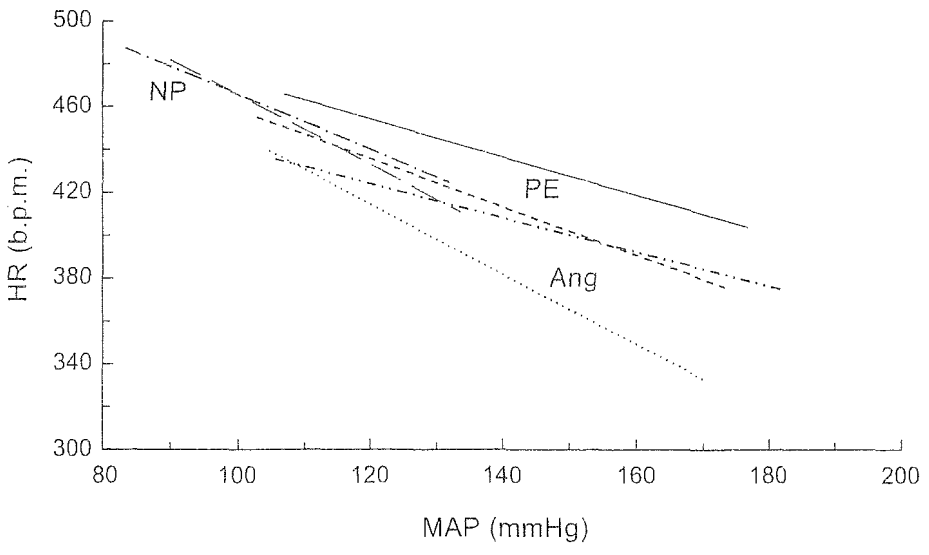

Fig. 5 Linear regression of baroreflex sensitivity in vascular and mixed responders (VR and MR, respectively) in response to bolus doses of nitroprusside (NP), phenylephrine (PE) and angiotensin (Ang) II. (——), MR-PE; (-----), VR-PE; (–·–·–), MR-NP; (– – –), VR-NP; (–·–·–), MR-AngII; (·····), VR-AngII. Statistical comparison of linear regressions is described in Table 3.

Stress activates the renin–angiotensin system in addition to the sympathoexcitatory response.[45] Because many of the haemodynamic and neuroendocrine responses to stress are similar to those of cocaine,[9,10] it is possible that the varying effects of angiotensin in these two groups represents a mechanism by which they have varying responses to cocaine and to acute behavioural stress. Alternatively, there may be an arousal or stress component to the administration of angiotensin intravenously. Cocaine has been shown to suppress the baroreflex control of heart rate.[46,47] We argued that this may be due to the actions of the psychostimulant[46] because behavioural stress or arousal suppress baroreflex function.[25,48] We and others have not reported behavioural arousal when angiotensin was administered. Therefore, it is unlikely that there is an arousal or stress component to the drug administration that is different from responses to intravenous administration of phenylephrine or saline. The suppression of baroreflex function by cocaine may also be due to the blockade of noradrenaline reuptake. Kawada *et al.* demonstrated that desipramine reduced the buffering ability of the baroreflex by inhibiting the natural frequency of the transfer function of sympathetic nerve activity to arterial pressure and heart rate responses.[49]

Phenylephrine elicited a greater decrease in cardiac output in vascular responders compared with mixed responders, corroborating an earlier observation.[50] Arterial pressure responses were not different in the present or earlier study. These data again correlate with clinical studies suggesting that differences in α-adrenoceptor activity/responsivity are not responsible for differences in the haemodynamic profile in response to stress in humans.[16]

Methacholine elicited a greater increase in heart rate in vascular responders compared with mixed responders. As a non-selective muscarinic agonist, methacholine elicits vasodilation and slows heart rate. The tachycardia that we recorded suggests that baroreflex-mediated sympathoexcitation overcame the direct effect of methacholine to increase potassium conductance and reduce nodal conduction in the heart. The results could be interpreted as either a greater sympathoexcitatory response in vascular responders or reduced cholinergic receptor sensitivity in cardiac nodal cells of vascular responders. The lack of difference observed with nitroprusside supports the latter conclusion. Moreover, we reported that vascular responders have greater sympathetic responsiveness to cocaine.[50,51] Baroreflex-mediated bradycardia in response to the phenylephrine-induced pressor response has been reported to be primarily dependent on parasympathetic innervation of the heart in conscious rats.[52] We can conclude that the endogenous parasympathetic nervous system does not appear to reflect differences in heart rate responsivity because responses to phenylephrine were not different. In contrast, the exogenous application of a muscarinic agonist does suggest varying sensitivity of cardiac cholinergic receptors.

Cocaine has been reported to act as a competitive inhibitor of M_2 muscarinic cholinergic receptors in the heart and brain.[21,22,53] This has been argued to be irrelevant, except after exposure to high doses, because significant cholinergic binding requires plasma concentrations 20-fold higher than those necessary to produce euphoria.[54] Our laboratory noted that atropine methylbromide reduced the decrease in cardiac output and the increase in systemic vascular resistance elicited by cocaine in vascular responders.[19,20] Muscarinic receptors on adrenergic nerve terminals have been reported to inhibit catecholamine release[23] and, therefore, may reduce cocaine-induced haemodynamic responses by inhibiting the sympathoexcitatory response at the nerve terminal. It has been argued that the pressor response to cocaine may also depend on central cholinergic receptors because intracisternal administration of hemicholinium-3 attenuates the pressor response to intravenous cocaine administration.[55] The present study suggests that there may be differences in the cardiac response to muscarinic agonists, but it is unclear whether the action is mediated centrally or peripherally.

In conclusion, the present studies demonstrate that there is a small but significant difference in the sensitivity of vascular responders to AngII and to methacholine administration. These findings may help explain why responses vary in vascular and mixed responders. If responsiveness to AngII contributes to differences in the haemodynamic response pattern, we should be able to prevent these with central or peripheral administration of angiotensin receptor antagonists. In fact, we have evidence that intracerebroventricular administration of losartan reduced the decrease in cardiac output observed selectively in vascular responders.[37] Indeed, acute cocaine administration has been shown to decrease both plasma renin concentration and activity through a CNS action.[56] Therefore, there is evidence suggesting a role for angiotensin receptors in the varying haemodynamic responsiveness to cocaine in vascular and mixed responders.

ACKNOWLEDGEMENTS

The authors thank Dr Qi Gan for excellent technical assistance in surgically instrumenting these animals. This work was supported by US Public Health Services grants DA05180 and DA13257.

REFERENCES

1. Dampney RAL, Coleman MJ, Fontes MAP *et al.* Central mechanisms underlying short- and long-term regulation of the cardiovascular system. *Clin. Exp. Pharmacol. Physiol.* 2002; **29**: 261–8.
2. Farrell TG, Bahir Y, Cripps T *et al.* Risk stratification for arrhythmic events in postinfarction patients based on heart rate variability, ambulatory electrocardiographic variables and the signal-averaged electrocardiogram. *J. Am. Coll. Cardiol.* 1991; **18**: 687–97.
3. Takase B, Kurita A, Noritake M *et al.* Heart rate variability in patients with diabetes mellitus, ischemic heart disease, and congestive heart failure. *J. Electrocardiol.* 1992; **25**: 79–88.
4. Bristow JD, Honour AJ, Pickering TG *et al.* Diminished baroreflex sensitivity in high blood pressure. *Circulation* 1969; **39**: 48–54.
5. Goldstein DS, Horwitz D, Keiser HR. Comparison of techniques for measuring baroreflex sensitivity in man. *Circulation* 1982; **66**: 432–9.
6. Korner PI, Head GA. Baroreflexes in hypertension. In: Kunos G, Ciriello J (eds). *Central Neural Mechanisms of Blood Pressure Regulation.* Birkhauser, Boston. 1992; 356–74.
7. Schwartz PJ, Vanoli E, Stramba-Badiale M, De Ferrari GM, Billman GE, Foreman RD. Autonomic mechanisms and sudden death: New insights from analysis of baroreceptor reflexes in conscious dogs with and without a myocardial infarction. *Circulation* 1988; **78**: 969–79.
8. Branch CA, Knuepfer MM. Causes of differential cardiovascular sensitivity to cocaine I: Studies in conscious rats. *J. Pharmacol. Exp. Ther.* 1994; **269**: 674–83.
9. Knuepfer MM, Mueller PJ. Review of evidence for a novel model of cocaine-induced cardiovascular toxicity. *Pharmacol. Biochem. Behav.* 1999; **63**: 489–500.
10. Knuepfer MM, Purcell RM, Gan Q, Le KM. Hemodynamic response patterns to acute behavioral stressors resemble those to cocaine. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001; **281**: R1778–86.

11. Knuepfer MM, Gan Q, Mueller PJ. Mechanisms of hemodynamic responses to cocaine in conscious rats. *J. Cardiovasc. Pharmacol.* 1998; **31**: 391–9.
12. Knuepfer MM, Branch CA, Gan Q, Fischer VF. Cocaine-induced myocardial ultrastructural alterations and cardiac output responses in rats. *Exp. Mol. Pathol.* 1993; **59**: 155–68.
13. Williams JB, Keenan SM, Gan Q, Knuepfer MM. Hemodynamic response profile predicts susceptibility to cocaine-induced toxicity. *Eur. J. Pharmacol.* 2003; **464**: 189–96.
14. Brod J. Haemodynamic basis of acute pressor reactions and hypertension. *Br. Heart J.* 1963; **25**: 227–45.
15. Eliot RS. Stress and the heart: Mechanisms, measurement and management. *Postgrad. Med.* 1992; **92**: 237–48.
16. Girdler SS, Hinderliter AL, Light KC. Peripheral adrenergic receptor contributions to cardiovascular reactivity: Influence of race and gender. *J. Psychosom. Res.* 1993; **37**: 177–93.
17. Krantz DS, Manuck SB. Acute psychophysiologic reactivity and risk of cardiovascular disease: A review and methodologic critique. *Psychol. Bull.* 1984; **96**: 435–64.
18. Sherwood A, Hinderliter AL, Light KC. Physiological determinants of hyperreactivity to stress in borderline hypertension. *Hypertension* 1995; **25**: 384–90.
19. Knuepfer MM, Gan Q. Role of cholinergic receptors and cholinesterase activity in hemodynamic responses to cocaine in conscious rats. *Am. J. Physiol.* 1999; **276**: R103–12.
20. Knuepfer MM. Muscarinic cholinergic and β-adrenergic contribution to hindquarters vasodilation and cardiac responses to cocaine. *J. Pharmacol. Exp. Ther.* 2003; **306**: 515–22.
21. Miao L, Qiu Z, Morgan JP. Cholinergic stimulation modulates negative inotropic effect of cocaine on ferret ventricular myocardium. *Am. J. Physiol.* 1996; **270**: H678–84.
22. Sharkey J, Ritz MC, Schenden JA *et al.* Cocaine inhibits muscarinic cholinergic receptors in heart and brain. *J. Pharmacol. Exp. Ther.* 1988; **246**: 1048–52.
23. Lavallée M, de Champlain J, Nadeau RA, Yamaguchi N. Muscarinic inhibition of endogenous myocardial catecholamine liberation in the dog. *Can. J. Physiol. Pharmacol.* 1978; **56**: 642–9.
24. Shannon RP, Stambler BS, Komamura K *et al.* Cholinergic modulation of the coronary vasoconstriction induced by cocaine in conscious dogs. *Circulation* 1993; **87**: 939–49.
25. Brooks D, Fox P, Lopez R, Sleight P. The effect of mental arithmetic on blood pressure variability and baroreflex sensitivity in man. *J. Physiol.* 1978; **280**: P75–6 (Abstract).
26. Smyth HS, Sleight P, Pickering GW. Reflex regulation of arterial pressure during sleep in man: A quantitative method of assessing baroreflex sensitivity. *Circ. Res.* 1969; **24**: 109–21.
27. Head GA, McCarty R. Vagal and sympathetic components of the heart rate range and gain of the baroreceptor–heart rate reflex in conscious rats. *J. Auton. Nerv. Syst.* 1987; **21**: 203–13.
28. Mueller PJ, Gan Q, Knuepfer MM. Ethanol alters hemodynamic responses to cocaine in conscious rats. *Drug Alcohol Depend.* 1997; **48**: 17–24.
29. Peach MJ. Renin–angiotensin system: Biochemistry and mechanisms of action. *Physiol. Rev.* 1977; **57**: 313–70.
30. Timmermans PB, Wong PC, Chiu AT *et al.* Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.* 1993; **45**: 205–51.
31. Dendorfer A, Thornagel A, Raasch W, Grisk O, Tempel K, Dominiak P. Angiotensin II induces catecholamine release by direct ganglionic excitation. *Hypertension* 2002; **40**: 348–54.
32. Szabo B, Hedler L, Schurr C, Starke K. Peripheral presynaptic facilitatory effect of angiotensin II on noradrenaline release in anesthetized rabbits. *J. Cardiovasc. Pharmacol.* 1990; **15**: 968–75.
33. Aiken JW, Reit E. Stimulation of the cat stellate ganglion by angiotensin. *J. Pharmacol. Exp. Ther.* 1968; **159**: 107–14.
34. Farr WC, Grupp G. Ganglionic stimulation: Mechanism of the positive inotropic and chronotropic effects of angiotensin. *J. Pharmacol. Exp. Ther.* 1971; **177**: 48–55.
35. Sanderford MG, Bishop VS. Angiotensin II acutely attenuates range of arterial baroreflex control of renal sympathetic nerve activity. *Am. J. Physiol. Heart Circ. Physiol.* 2000; **279**: H1804–12.
36. Philips MI. Functions of angiotensin in the central nervous system. *Annu. Rev. Physiol.* 1987; **49**: 413–35.
37. Knuepfer MM, Rowe K, Schwarz JA, Lomax L. Central peptides responsible for the sympathomimetic effects of cocaine. *Regul. Pept.* 2005; **127**: 1–10.
38. Reilly NS, Lomax LL, Knuepfer MM. AT_1 receptors in the median preoptic area (mnPOA) are responsible for regulating hemodynamic responses to cocaine and to behavioral stress. *FASEB J.* 2004; **18**: A675–6 (Abstract).
39. Bishop VS, Sanderford MG. Angiotensin II modulation of the arterial baroreflex: Role of the area postrema. *Clin. Exp. Pharmacol. Physiol.* 2000; **27**: 428–31.
40. Boscan P, Allen AM, Paton JFR. Baroreflex inhibition of cardiac sympathetic outflow is attenuated by angiotensin II in the nucleus of the solitary tract. *Neuroscience* 2001; **103**: 153–60.
41. Heesch CM, Crandall ME, Turbek JA. Converting enzyme inhibitors cause pressure-independent resetting of baroreflex control of sympathetic outflow. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 1996; **270**: R728–37.
42. Kumagai K, Suzuki H, Ryuzaki M *et al.* Effect of antihypertensive agents on arterial baroreceptor reflexes in conscious rats. *Hypertension* 1992; **20**: 701–9.
43. Munch PA, Longhurst JC. Contrasting effects of vasopressin and angiotensin II on rabbit aortic baroreceptors. *Am. J. Physiol.* 1991; **260**: H811–20.
44. Muller JR, Le KM, Haines WR *et al.* Hemodynamic response pattern predicts susceptibility to stress-induced elevation in arterial pressure in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001; **281**: R31–7.
45. Aguilera G, Young WS, Kiss A, Bathia A. Direct regulation of hypothalamic corticotropin-releasing hormone neurons by angiotensin II. *Neuroendocrinology* 1995; **61**: 437–44.
46. Knuepfer MM, McCann RK, Kamalu L. Effects of cocaine on baroreflex control of heart rate in conscious rats. *J. Auton. Nerv. Syst.* 1993; **43**: 257–66.
47. Trouvé R, Nahas G, Latour C. Inhibition by cocaine of the baroreflex in the rat. *Proc. Soc. Exp. Biol. Med.* 1992; **201**: 215–18.
48. Schlor KH, Stumpf H, Stock G. Baroreceptor reflex during arousal induced by electrical stimulation of the amygdala or by natural stimuli. *J. Auton. Nerv. Syst.* 1984; **10**: 157–65.
49. Kawada T, Miyamoto T, Uemura K *et al.* Effects of neuronal norepinephrine uptake blockade on baroreflex neural and peripheral arc transfer characteristics. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2004; **286**: R1110–20.
50. Branch CA, Knuepfer MM. Causes of differential cardiovascular sensitivity to cocaine II: Sympathetic, metabolic and cardiac effects. *J. Pharmacol. Exp. Ther.* 1994; **271**: 1103–13.
51. Purcell RM, Gan Q, Knuepfer MM. Variable renal sympathetic responses to cocaine in rats. *FASEB J.* 2001; **15**: A801 (Abstract).
52. Stornetta RL, Guyenet PG, McCarty RC. Autonomic nervous system control of heart rate during baroreceptor activation in conscious and anesthetized rats. *J. Auton. Nerv. Syst.* 1987; **20**: 121–7.
53. Flynn DD, Vaishnav AA, Mash DC. Interactions of cocaine with primary and secondary recognition sites on muscarinic receptors. *Mol. Pharmacol.* 1992; **41**: 736–42.
54. Schneider DJ. Cardiac ramifications of cocaine abuse. *Coron. Artery Dis.* 1991; **2**: 267–73.
55. Buccafusco JJ, Davis JA, Shuster LC, Buccafusco CJ, Gattu M. The importance of brainstem cholinergic neurons in the pressor response to cocaine. *J. Pharmacol. Exp. Ther.* 2005; **312**: 179–91.
56. Van de Kar LD, Levy AD, Rittenhouse PA *et al.* Cocaine-induced suppression of renin secretion is mediated in the brain: Investigation of cardiovascular and local anesthetic mechanisms. *Brain Res. Bull.* 1992; **28**: 837–42.

Circ J 2005; **69:** 1466–1471

Vasospastic Angina and Microvascular Angina are Differentially Influenced by *PON1* A632G Polymorphism in the Japanese

Junko Mashiba, MD; George Koike, MD; Hitoshi Kamiunten, MD;
Manami Ikeda; Kenji Sunagawa, MD

Background Ethnicity and smoking are well-known risk factors for the pathogenesis of coronary vasospasm. Oxidative stress induced by smoking plays a crucial role in coronary vasospasm, but is not enough to account for the pathogenesis of coronary vasospasm, indicating that genetic factors are strongly involved.
Methods and Results The study group comprised 162 vasospastic angina patients (VSAs), 61 microvascular angina patients (MVAs) and 61 non-responders (NRs) diagnosed by acetylcholine provocation test. Four polymorphisms of the oxidative stress related genes, cytochrome b-245, α polypeptide gene (*CYBA*) C242T and A640G, paraoxonase 1 gene (*PON1*) A632G, phospholipase A2 group VII gene (*PLA2G7*) G994T were genotyped. Allele frequency of *PON1* 632-G was significantly higher in both the VSA with dominant fashion and the MVA with recessive fashion compared with NR. This association was strongly influenced by gender in the MVA only. There were no significant associations between the other polymorphisms and coronary vasospasm. In addition, the allele frequency of *PON1* 632-G in the Japanese was higher than in Caucasians.
Conclusions There was a significant association between *PON1* A632G polymorphism and MVA as well as VSA, but the impact of this on VSA and MVA is different in the Japanese. (*Circ J* 2005; **69:** 1466–1471)

Key Words: Coronary vasospasm; Oxidative stress; Polymorphism; *PON1* (paraoxonase 1 gene); Smoking

Coronary vasospasm is a major cause of the ischemic heart disease[1] and previous studies have demonstrated a significantly higher prevalence in the Japanese than in Caucasians, suggesting an important role of genetic factors in the pathogenesis[2,3]

Smoking is also a crucial risk factor for coronary vasospasm[4,5] In humans it causes oxidative modification of biological molecules to form superoxide anions[6] which injure the endothelium and inactivate nitric oxide (NO), a major biological vasodilator, by forming peroxynitrite, resulting in impaired endothelium-dependent vasorelaxation[6,7] Antioxidants, such as vitamins C and E, restrain coronary vasospasm[8–10] However, smoking is not sufficient to account for the pathogenesis of coronary vasospasm because not all smokers suffer from coronary vasospasm,[11] indicating that genetic factors are strongly involved.

Recent progress in molecular genetics has made it possible to search for the genes responsible for coronary vasospasm. Oxidative-stress-related genes are attractive candidates in terms of the pathogenesis of coronary vasospasm and several genetic studies have been carried out. One study reported that cytochrome b-245, α polypeptide gene (*CYBA*) C242T polymorphism is associated with coronary vasospasm,[12] and another reported that paraoxonase 1 gene (*PON1*) A632G polymorphism is also![13] However, each study failed to confirm the others results. Phospholipase A2 group VII gene (*PLA2G7*) G994T polymorphism is also an attractive candidate, but no significant association with coronary vasospasm has been reported![12,13]

Coronary vasospasm has 2 clinical entities: spasm occurring in angiographically visible epicardial coronary arteries (vasospastic angina: VSA) and that in angiographically invisible small coronary arteries (microvascular angina: MVA)![14,15] The mechanisms of these are similar, but not identical,[16] and the clinical features also differ![17] The difference in the pathogenesis of these 2 entities could be related to differences in genetic and non-genetic factors, but there have not been any studies addressing these issues by comparing VSA and MVA.

In this study, to elucidate genetic factors relating to oxidative stress in the pathogenesis of coronary vasospasm, genetic analysis was carried out for 4 polymorphisms of oxidative-stress-related genes (*CYBA* C242T and A640G, *PON1* A632G, *PLA2G7* G994T) among 3 groups: VSA patients, MVA patients and non-responders (NR) based on their response to intracoronary acetylcholine (ACh) administration.

Methods

Study Population

The study population comprised 284 patients with VSA, MVA or NR who were admitted to Kyushu University Hospital between September 1994 and February 2005. All patients studied were Japanese, and had angina symptoms. All underwent coronary angiography followed by ACh provocation test for diagnosis as previously described![15] Briefly, a graded dose of ACh (maximum 100 mg) was in-

(Received April 22, 2005; revised manuscript received September 16, 2005; accepted September 28, 2005)
Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
Mailing address: George Koike, MD, PhD, Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: koike@cardiol.med.kyushu-u.ac.jp

Table 1 Clinical Parameters of the Study Patients

	VSA (n=162)	MVA (n=61)	NR (n=61)
Male/Female	94/68	11/50††	31/30
Age (years)	60.22±10.21	62.90±10.33	59.70±9.74
BMI (kg/m²)	23.90±3.69	22.77±3.43	22.96±3.18
Current smoking status	87/162 (54%)*	9/61 (15%)†	19/61 (31%)
Diabetes	20/162 (12%)	12/61 (20%)	6/61 (10%)
Hypertension	73/162 (45%)	26/61 (43%)	22/61 (36%)
Hypercholesterolemia	45/162 (28%)	24/61 (39%)	19/61 (31%)

Values (age, BMI) are means±SD. Two-tailed unpaired t-tests (for continuous values) and χ^2 analyses with Fisher's exact probability (for categorical variables) were used to compare the values for the VSA and NR, as well as the MVA and NR.
**p<0.05 (VSA vs NR), †p<0.05 (MVA vs NR), ††p<0.01 (MVA vs NR).*
VSA, vasospastic angina; MVA, microvascular angina; NR, nonresponder; BMI, body mass index.

Table 2 Primers and Restriction Endonucleases Used for Genotyping of *CYBA* C242T and A640G, *PON1* A632G and *PLA2G7* G994T Polymorphisms

Polymorphism	Sense primer	Anti-sense primer	Restriction endonuclease	Reference
CYBA C242T	*5'-TGCTTGTGGGTAAACCAAGGCCGGTG-3'*	*5'-AACACTGAGGTAAGTGGGGGTGGCTCCTGT-3'*	*Rsa I*	*20*
CYBA A640G	*5'-AGGTCCCCAGGTCAACCCCATCC-3'*	*5'-CGCTGCGTTTATTGCAGGTGGGTGC-3'*	*Dra III*	*20*
PON1 A632G	*5'-TATTGTTGCTGTGGGACCTGAG-3'*	*5'-CACGCTAAACCCAAATACATCTC-3'*	*Alw I*	*21*
PLA2G7 G994T	*5'-CTATAAATTTATATCATGCT-3'*	*5'-TTTACTATTCTCTTGCTTTAC-3'*	*Mae II*	*22*

fused into the left coronary artery, after which systemic arterial pressure, heart rate, and 12-lead electrocardiogram (ECG) trace and coronary cineangiogram were recorded. If ACh infusion into the left coronary artery did not induce angina, ECG changes, or coronary spasm, a further graded dose of ACh (maximum 50 mg) was infused into the right coronary artery. Whenever angina, ischaemic ECG changes, or coronary spasm were observed, the infusion was stopped and arterial pressure, 12-lead ECG trace, and coronary cineangiogram were recorded and 1 mg isosorbide dinitrate (ISDN) was infused into the coronary artery. VSA was defined as >75% diameter reduction compared with the post- ISDN infusion diameter; MVA was defined as induction of angina and ischemic ECG changes without coronary artery spasm; NR was defined as no induction of angina, ischemic ECG or coronary artery spasm. Patients with significant organic stenosis (>50% luminal diameter) after ISDN administration were excluded, as were patients with acute myocardial infarction, hypertrophic cardiomyopathy, severe valvular disease, severe angina, congestive heart failure or chronic renal failure requiring hemodialysis.

Other clinical parameters were collected: age, gender, body mass index, current cigarette smoking, hypertension, diabetes mellitus, and hypercholesterolemia (Table 1). Definition of current cigarette smoking was a patient who was currently smoking or had quit smoking within the past 2 years because the risk of coronary events in ex-smokers declines to the level of non-smokers within 2 years after quitting.[18,19] Hypertension was defined as blood pressure higher than 140/90 mmHg or taking antihypertensive drugs. Diabetes mellitus was defined as blood glucose levels greater than 126 mg/dl at fasting, greater than 200 mg/dl at 2 h in an oral glucose tolerance test, taking hypoglycemic drugs or using insulin. Hypercholesterolemia was defined as total cholesterol level equal to or more than 220 mg/dl, low-density-lipoprotein (LDL) level equal to or more than 140 mg/dl or taking lipid-lowering drugs.

The study protocol was approved by the Institutional Human Research Committee of Kyushu University. All patients gave written informed consent before enrolment.

DNA Extraction

A 10-ml sample of peripheral blood was collected from each subject, and immediately stored at 4°C until extraction of genomic DNA from peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany).

Genotyping of Polymorphisms

Genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) procedures according to previous reports with minor modification.[20–22] PCR primers and restriction endonucleases used in this study are summarized in Table 2. After PCR-RFLP procedures, reaction mixtures were then electrophoresed on 5.0% agarose gel (2.0% Seakem agarose and 3.0% Nusieve agarose) and visualized by ethidium bromide staining.

Statistical Analysis

Continuous variables were compared by two-tailed unpaired t-tests. Categorical variables were compared by χ^2 analyses with Fisher's exact probability test. Odds ratios (ORs) were calculated as an index of the association of *CYBA* C242T (C/C type, C/T type, T/T type) and A640G (A/A type, A/G type, G/G type) polymorphisms, *PON1* A632G (A/A type, A/G type, G/G type) polymorphism and *PLA2G7* G994T (G/G type, G/T type, T/T type) polymorphism with the phenotypes of VSA and MVA. For each OR, we calculated two-tailed probability values and 95% confidence intervals (CIs). The effect of a mutant allele was assumed to be additive, dominant, or recessive. Values for additive effect were predicted by Hardy-Weinberg equilibrium.

Multiple logistic regression analysis was carried out using SPSS 9.0J for Windows (SPSS, Tokyo, Japan). Independent variables were coded as the following dummy variables: gender, 0 for female and 1 for male; age, 0 for <60 years and 1 for ≥60 years; body mass index, 0 for <26 kg/m² and 1 for ≥26 kg/m², current cigarette smoking, 0 for non-smokers and 1 for smoker; hypertension, 0 for

Table 3 Frequencies of the *CYBA* C242T and A640G, *PON1* A632G and *PLA2G7* G994T Polymorphisms

	VSA (n=162)	MVA (n=61)	NR (n=61)
CYBA C242T			
C/C	131/162 (81%)	49/61 (80%)	48/61 (79%)
C/T	31/162 (19%)	11/61 (18%)	11/61 (18%)
T/T	0/162 (0%)	1/61 (2%)	2/61 (3%)
C allele	0.9	0.89	0.88
T allele	0.1	0.11	0.12
CYBA A640G			
A/A	12/162 (7%)	6/61 (10%)	5/61 (8%)
A/G	122/162 (75%)	39/61 (64%)	44/61 (72%)
G/G	28/162 (18%)	16/61 (26%)	12/61 (20%)
A allele	0.45	0.42	0.44
G allele	0.55	0.58	0.56
PON1 A632G			
A/A	19/162 (12%)	7/61 (11%)	14/61 (23%)
A/G	64/162 (40%)	18/61 (30%)	23/61 (38%)
G/G	79/162 (49%)	36/61 (59%)	24/61 (39%)
A allele	0.31	0.26	0.42
G allele	0.69	0.74	0.58
PLA2G7 G994T			
G/G	114/162 (70%)	43/61 (70%)	38/61 (62%)
G/T	46/162 (29%)	17/61 (28%)	22/61 (36%)
T/T	2/162 (1%)	1/61 (2%)	1/61 (2%)
G allele	0.85	0.84	0.8
T allele	0.15	0.16	0.2

See Table 1 for abbreviations.

Table 4 Odds Ratio (OR) and 95% Confidence Interval (CI) was Calculated of the Each Genotypes With the Phenotype of the VSA and MVA, With the Effects of *CYBA* 242-T, *CYBA* 640-G, *PON1* 632-G and *PLA2G7* 994-T Allele as Being Additive, Dominant or Recessive

	VSA vs NR		MVA vs NR	
	OR (95%CI)	p-value	OR (95%CI)	p-value
CYBA C242T				
Additive	1.32 (0.69–2.55)	0.40	1.18 (0.53–2.59)	0.69
Dominant	0.87 (0.42–1.81)	0.72	2.03 (0.18–23.04)	0.82
Recessive	–	–	1.11 (0.46–2.67)	0.50
CYBA A640G				
Additive	1.03 (0.68–1.58)	0.88	0.90 (0.54–1.50)	0.70
Dominant	0.90 (0.30–2.66)	0.84	1.22 (0.35–4.24)	0.75
Recessive	1.17 (0.55–2.48)	0.68	0.69 (0.29–1.61)	0.39
PON1 A632G				
Additive	1.56 (1.02–2.40)	0.040	2.02 (1.18–3.47)	0.010
Dominant	2.24 (1.04–4.82)	0.035	2.3 (0.86–6.17)	0.093
Recessive	1.47 (0.81–2.67)	0.21	2.22 (1.08–4.58)	0.030
PLA2G7 G994T				
Additive	0.75 (0.43–1.27)	0.28	0.75 (0.39–1.46)	0.40
Dominant	0.70 (0.37–1.29)	0.25	0.69 (0.32–1.47)	0.34
Recessive	0.75 (0.07–8.42)	0.61	1.00 (0.06–16.36)	1.0

See Table 1 for abbreviations.

normotension and 1 for hypertension; diabetes mellitus, 0 for absence and 1 for presence; hypercholesterolemia, 0 for absence, 1 for presence. Statistical significance was defined as $p<0.05$.

Results

Clinical Parameters of the Study Population

Clinical parameters of each patient group are summarized in Table 1. Each parameter was compared between the VSA and NR groups as well as between the MVA and NR groups. In the comparison of the VSA and NR groups, the frequency of smoking was higher in the VSA, which was compatible with previous studies[4,17] In the comparison of the MVA and NR groups, the frequency of females was significantly higher ($p<0.01$), and the frequency of current smoking status was significantly lower ($p<0.05$) in the MVA than in the NR, which was also compatible with results from a previous study![17]

Genetic Analysis of the Relationship of Oxidative-Stress-Related Gene Polymorphisms With Coronary Vasospasm

Polymorphisms of 4 oxidative-stress-related genes, *CYBA* C242T and A640G, *PON1* A632Gand *PLA2G7* G994T, were genotyped for the 3 groups of patients (Table 3). There was a Hardy-Weinberg equilibrium in the allele frequencies of *CYBA* C242T, *PON1* A632G and *PLA2G7* G994T polymorphisms, verified by chi-square analyses with Fisher's exact probability test. In contrast, the allele frequency of the *CYBA* A640G polymorphism

Table 5 Multiple Logistic Analysis for Comparison Between VSA and NR

	β Coefficient	SE	Wald	p-value
PON1 632 A/G and G/G genotype	*0.176*	*0.084*	*2.096*	*0.037*
Gender	*0.004*	*0.068*	*0.062*	*0.950*
Age	*0.063*	*0.062*	*1.010*	*0.314*
BMI	*0.037*	*0.070*	*0.534*	*0.594*
Smoking	*0.129*	*0.069*	*1.874*	*0.062*
Diabetes	*0.003*	*0.095*	*0.036*	*0.971*
Hypertension	*0.040*	*0.064*	*0.625*	*0.533*
Hypercholesterolemia	*–0.015*	*0.066*	*–0.230*	*0.819*
Constant	*0.463*	*0.099*	*4.668*	*0.0001*

SE, standard error; Wald multiple logistic regression analysis.
See Table 1 for other abbreviations.

Table 6 Multiple Logistic Analysis for Comparison Between MVA and NR

	β Coefficient	SE	Wald	p-value
PON1 GG genotype	*0.174*	*0.088*	*1.982*	*0.049*
Gender	*–0.344*	*0.106*	*–3.247*	*0.002*
Age	*0.043*	*0.096*	*–0.445*	*0.657*
BMI	*0.007*	*0.113*	*0.063*	*0.950*
Smoking	*–0.076*	*0.119*	*–0.643*	*0.529*
Diabetes	*0.184*	*0.184*	*1.466*	*0.146*
Hypertension	*0.097*	*0.092*	*1.054*	*0.294*
Hypercholesterolemia	*0.037*	*0.036*	*0.404*	*0.687*
Constant	*0.668*	*0.108*	*6.183*	*0.0001*

SE, standard error; Wald multiple logistic regression analysis.
See Table 1 for other abbreviations.

was not in agreement with those predicted by the Hardy-Weinberg equilibrium for each group ($p<0.05$).

Proportion test was carried out next between the VSA and NR groups and between the MVA and NR groups. As summarized in Table 4, there was a significant association of *PON1* A632G polymorphism in both comparisons. The genetic impact of *PON1* 632-G allele was dominant and additive (dominant: OR 2.24, 95% CI 1.04-4.82, $p=0.035$; additive: OR 1.56, 95% CI 1.02–2.40, $p=0.040$) in the comparison of the VSA and NR. In contrast, the genetic impact of *PON1* 632-G allele was recessive and additive in the comparison of the MVA and NR (recessive: OR 2.22, 95% CI 1.08–4.58, $p=0.030$; additive: OR 2.02, 95% CI 1.18–3.47, $p=0.010$). This is the first identification of a gene responsible for the pathogenesis of MVA.

In contrast, there were no significant associations with the *CYBA* C242T/A640G or *PLA2G7* G994T polymorphisms in either comparison (Table 4).

Detailed Analysis of Relationship of PON1 A632G Polymorphism With Coronary Vasospasm

Multivariate analysis that included *PON1* A632G genotype, age, gender, current smoking status, hypertension, diabetes, and hypercholesterolemia was carried out and is summarized in Tables 5 and 6. In the comparison of the VSA and NR, the G allele of the *PON1* A632G polymorphism had a significant association with VSA ($p=0.037$), which was independent of current smoking status ($p=0.44$, chi-square analyses with Fisher's exact probability test with Smoking and PON1 G allele), indicating that the effect of the *PON1* A632G polymorphism was not influenced by smoking in the pathogenesis of VSA.

In the comparison of the MVA and the NR, the G allele of the *PON1* A632G polymorphism also had a significant association with MVA ($p=0.049$). Interestingly, female gender had a more significant association with MVA ($p=0.002$), so multivariate analysis after correction by gender was carried out for the MVA and NR, and the G allele of the *PON1* A632G polymorphism no longer had a significant association with MVA, indicating that its effect in the pathogenesis of MVA was significantly influenced by gender.

Ethnic Difference in PON1 A632G Polymorphism

The allele frequency of the *PON1* A632G polymorphism in the general Japanese population has not previously been reported, so we genotyped 102 healthy volunteers. The *PON1* 632A/A, A/G, and G/G genotypes were observed in 10, 54, and 38 subjects, respectively, and based on this result, the estimated allele frequencies of *PON1* 632-A and 632-G are 0.363 and 0.637, respectively. In contrast, the allele frequencies in Caucasian are 0.726–0.826 and 0.174–0.274, respectively (NCBI dbSNP ss5111592 and ss5586846). This ethnic difference in the allele frequency of *PON1* A632G polymorphisms may account for the difference in the prevalence of coronary vasospasm.

Discussion

Smoking is known to be a crucial risk factors for coronary vasospasm[4,5] because it generates oxidative stress, such as oxygen free radicals, which inactivates NO and damages endothelial cells[6,7] causing impaired endothelium-dependent vasodilatation that leads not only to VSA[5,23–25] but also MVA.[16] Based on this, it is plausible that the oxidative-stress-related genes are candidates for the pathogenesis of VSA and MVA. In the present study, 3 genes, *CYBA*, *PON1* and *PLA2G7*, were studied based on their function and previous genetic studies of cardiovascular diseases.

CYBA encodes p22-phox[26] a critical component of the NADH/NADPH oxidase, which generates superoxide in both endothelial and smooth muscle cells within vascular tissue[27,28] *CYBA* C242T polymorphism results in amino acid substitution (H72Y)[29] and CYBA with the 242-T allele reduces NADH/NADPH oxidase activity in human blood vessels, suggesting that this genetic variation plays a significant role in modulating superoxide production[30] It has been reported that *CYBA* C242T polymorphism is associated with a reduced risk of coronary artery disease (CAD) in the Japanese[20]

PON1 protects LDL from oxidation by hydrolysis of biologically active lipoperoxides[31] which injures the blood vessel endothelium[32] *PON1* A632G polymorphism results in amino acid substitution (Q192R) and *PON1* with the 632-G allele reduces its antioxidant activity[33] It has been previously reported that there is a significant association between the *PON1* 632-G allele and VSA[13]

PLA2G7 encodes platelet-activating factor acetylhydrolase, which retards the oxidation of LDL by preventing the generation of phospholipid hydroperoxides[34,35] *PLA2G7* G994T polymorphism results in an amino acid substitution (V279F), and *PLA2G7* with the 994-T allele loses its catalytic activity[22] It has been reported that PLA2G activity is associated with angiographic CAD[36]

We studied 4 polymorphisms, *CYBA* C242T and A640G, *PON1* A632G and *PLA2G7* G994T polymorphisms, and there was only a significant association between the *PON1* A632G polymorphism and coronary vasospasm (Table 4). As it has been previously demonstrated that *PON1* 632-G allele causes lower antioxidant activity[13,33] *PON1* could be a causal gene for coronary vasospasm.

There are common characteristics of VSA and MVA: (1) Ca-channel blockers are effective[37,38] and (2) endothelial dysfunction is thought to be primary cause[17,39] Therefore, it is likely that a single gene, such as *PON1*, is involved in the pathogenesis of both entities, as demonstrated in this study. On the other hand, there are also unique characteristics of each type of coronary vasospasm. VSA sometimes results in myocardial infarction, whereas the prognosis of MVA tends to be good[17] Angiotensin-converting enzyme inhibitors and statins are effective only in some cases of MVA[40–42] Therefore, it is also plausible that the mode of inheritance of the *PON1* A632G polymorphism has an influence on the clinical expression of VSA and MVA.

Previous studies demonstrate that several factors, such as hypertension, hypercholesterolemia and smoking, contribute to the microvascular endothelial dysfunction that results in MVA[43] Recently, estrogen deficiency has been recognized as a cause of endothelial dysfunction in MVA[44] and estrogen replacement therapy has been shown to restore the impaired vasodilator response in women with MVA[45,46] Interestingly, in this study the association between the G allele of *PON1* A632G polymorphism and MVA was significantly influenced by female gender, but was not of the same level of significance with VSA, indicating that genetic impact of *PON1* A632G polymorphism on MVA is strongly influenced by gender. It is highly likely that the pathogenesis of MVA with the *PON1* A632G polymorphism could involve an X chromosome effect or some female-specific gene expression. There were no significant associations between the other polymorphisms and coronary vasospasm. Recently, Murase et al reported that there is a significant association between *CYBA* C242T polymorphism and VSA only in males;[12] however, there are crucial differences between their study and ours. They used ergonovine (Erg) provocation to induce coronary vasospasm and used 90% stenosis as significant after Erg provocation, whereas we used ACh provocation and 75% stenosis was significant. More importantly, they used subjects without chest pain as controls and did not perform cardiac catheter examination.

Study Limitations

The study population is relatively small for this type of study, which could potentially lead to a false-positive result. However, the diagnostic procedure was very precise and accurate, and ethnic divergence relatively small because the study included only Japanese. Therefore, it is more likely to detect a true genetic effect. Regarding the significant association between *PON1* A632G polymorphism and VSA, this study confirms previous results[13] suggesting that it is highly likely that this is a true positive. In contrast, the association between *PON1* A632G polymorphism and MVA has to be confirmed by other studies.

In conclusion, the allele frequency of *PON1* 632G was significantly higher in both VSA and MVA patients compared with NR. This is the first study to identify a gene responsible for the pathogenesis of MVA. The genetic impact of *PON1* A632G polymorphism on MVA was significantly affected by female gender. These results indicate that *PON1* A632G polymorphism has an impact on the pathogenesis of coronary vasospasm, but mechanisms of its effect on VSA and MVA are different.

Acknowledgments

This study was supported by health science research grants from the Ministry of Health, Labor and Welfare, Tokyo, Japan, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (16590696), by research grants from the Uehara Memorial Foundation, Tokyo, Japan, from Kanae Foundation for Life & Socio-Medical Science, and from Japan Heart Foundation/Zeria Pharmaceutical grant for Research on Molecular Cardiology.

We thank members of the cardiac catheterization laboratory at Kyushu University Hospital for diagnosing the subjects, Dr Makoto Usui for assisting with the blood sampling, and Dr Miyuki Tsuchihashi-Makaya for assisting with the statistical analysis.

References

1. Cannon CP, Braunwald E. Unstable angina and non-ST elevation myocardial infarction. *In*: Zipes DP, Libby P, Bonow RO, Braunwald E, editors. Heart disease, 7th edn. Philadelphia: Elsevier Saunders; 2005; 1243–1279.
2. Beltrame JF, Sasayama S, Maseri A. Racial heterogeneity in coronary artery vasomotor reactivity: Differences between Japanese and Caucasian patients. *J Am Coll Cardiol* 1999; **33:** 1442–1452.
3. Pristipino C, Beltrame JF, Finocchiaro ML, Hattori R, Fujita M, Mongiardo R, et al. Major racial differences in coronary constrictor response between japanese and caucasians with recent myocardial infarction. *Circulation* 2000; **101:** 1102–1108.
4. Sugiishi M, Takatsu F. Cigarette smoking is a major risk factor for coronary spasm. *Circulation* 1993; **87:** 76–79.
5. Kugiyama K, Yasue H, Ohgushi M, Motoyama T, Kawano H, Inobe Y, et al. Deficiency in nitric oxide bioactivity in epicardial coronary arteries of cigarette smokers. *J Am Coll Cardiol* 1996; **28:** 1161–1167.
6. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, et al. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers: Smoking as a cause of oxidative damage. *N Engl J Med* 1995; **332:** 1198–1203.
7. Wei EP, Kontos HA, Christman CW, DeWitt DS, Povlishock JT. Superoxide generation and reversal of acetylcholine-induced cerebral arteriolar dilation after acute hypertension. *Circ Res* 1985; **57:** 781–787.
8. Miwa K, Miyagi Y, Igawa A, Nakagawa K, Inoue H. Vitamin E defi-

ciency in variant angina. *Circulation* 1996; **94:** 14–18.
9. Kugiyama K, Motoyama T, Hirashima O, Ohgushi M, Soejima H, Misumi K, et al. Vitamin C attenuates abnormal vasomotor reactivity in spasm coronary arteries in patients with coronary spastic angina. *J Am Coll Cardiol* 1998; **32:** 103–109.
10. Motoyama T, Kawano H, Kugiyama K, Hirashima O, Ohgushi M, Tsunoda R, et al. Vitamin E administration improves impairment of endothelium-dependent vasodilation in patients with coronary spastic angina. *J Am Coll Cardiol* 1998; **32:** 1672–1679.
11. Yasue H, Kugiyama K. Coronary artery spasm: Japanese view. *Coron Artery Dis* 1990; **1:** 668–673.
12. Murase Y, Yamada Y, Hirashiki A, Ichihara S, Kanda H, Watarai M, et al. Genetic risk and gene-environment interaction in coronary artery spasm in Japanese men and women. *Eur Heart J* 2004; **25:** 970–977.
13. Ito T, Yasue H, Yoshimura M, Nakamura S, Nakayama M, Shimasaki Y, et al. Paraoxonase gene Gln192Arg (Q192R) polymorphism is associated with coronary artery spasm. *Hum Genet* 2002; **110:** 89–94.
14. Chilian WM, Kuo L, DeFily DV, Jones CJ, Davis MJ. Endothelial regulation of coronary microvascular tone under physiological and pathophysiological conditions. *Eur Heart J* 1993; **14**(Suppl I): 55–59.
15. Mohri M, Koyanagi M, Egashira K, Tagawa H, Ichiki T, Shimokawa H, et al. Angina pectoris caused by coronary microvascular spasm. *Lancet* 1998; **351:** 1165–1169.
16. Egashira K, Inou T, Hirooka Y, Yamada A, Urabe Y, Takeshita A. Evidence of impaired endothelium-dependent coronary vasodilatation in patients with angina pectoris and normal coronary angiograms. *N Engl J Med* 1993; **328:** 1659–1664.
17. Masumoto A, Mohri M, Takeshita A. Three-year follow-up of the Japanese patients with microvascular angina attributable to coronary microvascular spasm. *Int J Cardiol 2001*; **81:** 151–156.
18. Rosenberg L, Palmer JR, Shapiro S. Decline in the risk of myocardial infarction among women who stop smoking. *N Engl J Med* 1990; **322:** 213–217.
19. Dobson AJ, Alexander HM, Heller RF, Lloyd DM. How soon after quitting smoking does risk of heart attack decline? *J Clin Epidemiol* 1991; **44:** 1247–1253.
20. Inoue N, Kawashima S, Kanazawa K, Yamada S, Akita H, Yokoyama M. Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. *Circulation* 1998; **97:** 135–137.
21. Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE. The molecular basis of the human serum paraoxonase activity polymorphism. *Nat Genet* 1993; **3:** 73–76.
22. Stafforini DM, Satoh K, Atkinson DL, Tjoelker LW, Eberhardt C, Yoshida H, et al. Platelet-activating factor acetylhydrolase deficiency: A missense mutation near the active site of an anti-inflammatory phospholipase. *J Clin Invest* 1996; **97:** 2784–2791.
23. Okumura K, Yasue H, Matsuyama K, Ogawa H, Kugiyama K, Ishizaka H, et al. Diffuse disorder of coronary artery vasomotility in patients with coronary spastic angina: Hyperreactivity to the constrictor effects of acetylcholine and the dilator effects of nitroglycerin. *J Am Coll Cardiol* 1996; **27:** 45–52.
24. Soejima H, Miyamoto S, Kojima S, Hokamaki J, Tanaka T, Kawano H, et al. Coronary spastic angina in patients with connective tissue disease. *Circ J* 2004; **68:** 367–370.
25. Takagi S, Goto Y, Hirose E, Terashima M, Sakuragi S, Suzuki S, et al. Successful treatment of refractory vasospastic angina with corticosteroids: Coronary arterial hyperactivity caused by local inflammation? *Circ J* 2004; **68:** 17–22.
26. Dinauer MC, Pierce EA, Bruns GA, Curnutte JT, Orkin SH. Human neutrophil cytochrome b light chain (p22-phox): Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. *J Clin Invest* 1990; **86:** 1729–1737.
27. Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK. p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem 1996*; **271:** 23317–23321.
28. Munzel T, Hink U, Heitzer T, Meinertz T. Role for NADPH/NADH oxidase in the modulation of vascular tone. *Ann NY Acad Sci* 1999; **874:** 386–400.
29. Parkos CA, Dinauer MC, Walker LE, Allen RA, Jesaitis AJ, Orkin SH. Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b. *Proc Natl Acad Sci USA* 1988; **85:** 3319–3323.
30. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Functional effect of the C242T polymorphism in the NAD(P)H oxidase p22phox gene on vascular superoxide production in atherosclerosis. *Circulation* 2000; **102:** 1744–1747.
31. Mackness MI, Arrol S, Durrington PN. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett* 1991; **286:** 152–154.
32. Anderson TJ, Meredith IT, Yeung AC, Frei B, Selwyn AP, Ganz P. The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. *N Engl J Med* 1995; **332:** 488–493.
33. Mackness B, Mackness MI, Arrol S, Turkie W, Durrington PN. Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by high density lipoprotein against low density lipoprotein oxidative modification. *FEBS Lett* 1998; **423:** 57–60.
34. Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, et al. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response: Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest* 1995; **96:** 2758–2767.
35. Watson AD, Navab M, Hama SY, Sevanian A, Prescott SM, Stafforini DM, et al. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J Clin Invest* 1995; **95:** 774–782.
36. Winkler K, Winkelmann BR, Scharnagl H, Hoffmann MM, Grawitz AB, Nauck M, et al. Platelet-activating factor acetylhydrolase activity indicates angiographic coronary artery disease independently of systemic inflammation and other risk factors: The Ludwigshafen Risk and Cardiovascular Health Study. *Circulation* 2005; **111:** 980–987.
37. Rosenthal SJ, Ginsburg R, Lamb IH, Baim DS, Schroeder JS. Efficacy of diltiazem for control of symptoms of coronary arterial spasm. *Am J Cardiol* 1980; **46:** 1027–1032.
38. Sutsch G, Oechslin E, Mayer I, Hess OM. Effect of diltiazem on coronary flow reserve in patients with microvascular angina. *Int J Cardiol* 1995; **52:** 135–143.
39. Zeiher AM, Drexler H, Wollschlager H, Just H. Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. *Circulation* 1991; **84:** 1984–1992.
40. Kaski JC, Rosano G, Gavrielides S, Chen L. Effects of angiotensin-converting enzyme inhibition on exercise-induced angina and ST segment depression in patients with microvascular angina. *J Am Coll Cardiol* 1994; **23:** 652–657.
41. Iriarte M, Caso R, Murga N, Boveda J, Saenz R, Lopez de Argumedo M, et al. Enalapril-induced regression of hypertensive left ventricular hypertrophy, regional ischemia, and microvascular angina. *Am J Cardiol* 1995; **75:** 850–852.
42. Kayikcioglu M, Payzin S, Yavuzgil O, Kultursay H, Can LH, Soydan I. Benefits of statin treatment in cardiac syndrome-X1. *Eur Heart J* 2003; **24:** 1999–2005.
43. Kaski JC, Aldama G, Cosin-Sales J. Cardiac syndrome X: Diagnosis, pathogenesis and management. *Am J Cardiovasc Drugs* 2004; **4:** 179–194.
44. Hayward CS, Kalnins WV, Kelly RP. Acute effects of 17beta-estradiol on ventricular and vascular hemodynamics in postmenopausal women. *Am J Physiol Heart Circ Physiol* 2000; **279:** H2277–H2284.
45. Rosano GM, Peters NS, Lefroy D, Lindsay DC, Sarrel PM, Collins P, et al. 17-beta-Estradiol therapy lessens angina in postmenopausal women with syndrome X. *J Am Coll Cardiol* 1996; **28:** 1500–1505.
46. Roque M, Heras M, Roig E, Masotti M, Rigol M, Betriu A, et al. Short-term effects of transdermal estrogen replacement therapy on coronary vascular reactivity in postmenopausal women with angina pectoris and normal results on coronary angiograms. *J Am Coll Cardiol* 1998; **31:** 139–143.

Coronary Microvascular Dysfunction in Patients With Microvascular Angina

Analysis by TIMI Frame Count

Hongtao Sun, MD, Yoshihiro Fukumoto, MD, Akira Ito, MD, Hiroaki Shimokawa, MD, and Kenji Sunagawa, MD

Abstract: We have previously reported that angina pectoris persists in patients with coronary microvascular spasm (MVS) even on calcium channel blockers. Because measurement of myocardial lactate production in the coronary sinus is necessary to diagnose MVS, a more feasible diagnostic method needs to be developed. In this study, we examined the diagnostic significance of Thrombolysis in Myocardial Infarction (TIMI) frame count, a marker of coronary blood flow, in 131 consecutive patients who underwent provocation test for coronary spasm with acetylcholine (ACh). Epicardial coronary spasm (ES) was diagnosed as more than 75% of ACh-induced vasoconstriction noted by coronary angiography. MVS was diagnosed as ACh-induced myocardial ischemia (chest pain, ischemic ECG changes, and myocardial lactate production) without ES. TIMI frame count was significantly increased in patients with MVS alone (n = 35) and those with ES + MVS (n = 16) compared with those with ES alone (n = 53) or those with no myocardial ischemia (Normal, n = 27) either before and after intracoronary ACh and even after intracoronary isosorbide dinitrate (ISDN) in both the left anterior descending (LAD) and the left circumflex coronary artery (LCX). TIMI frame count in LAD correlated well to that in LCX in patients with MVS, suggesting diffuse impaired coronary microcirculation in the myocardium. These results suggest that increased TIMI frame count in response to ACh reflects microvascular dysfunction in MVS and that ISDN may not be enough to relieve MVS. Thus, TIMI frame count may be useful to diagnose MVS without requiring coronary sinus catheterization or myocardial lactate production measurement.

Key Words: microvascular spasm, TIMI frame count, ISDN, coronary spasm

(*J Cardiovasc Pharmacol*™ 2005;46:622–626)

Chest pain with a normal coronary arteriogram remains a dilemma for diagnosis of coronary spasm including epicardial spasm (ES) and/or microvascular spasm (MVS) or noncardiac chest symptoms.[1–3] Calcium channel blockers (CCBs) are effective to diminish chest symptoms for patients with ES; however, MVS persists even on CCBs, resulting in an impaired quality of life.[1] Coronary MVS can cause myocardial ischemia even in the absence of epicardial coronary stenosis or obstruction in humans.[1,4] Furthermore, we have recently demonstrated that coronary MVS contributes to the occurrence of angina in a quarter of patients with ES.[5] At present, MVS can be diagnosed only by provocation test with intracoronary acetylcholine (ACh) or ergonovine, with measurement of lactate production in the coronary sinus, but these invasive methods limit the usefulness of the provocation test. Thus, a more feasible diagnostic method for MVS needs to be developed.

Thrombolysis in Myocardial Infarction (TIMI) frame count is a simple, reproducible, objective, and quantitative index of coronary blood flow.[6] However, no studies have ever examined whether or not TIMI frame count is useful to diagnose MVS. Thus, in the present study, we examined the usefulness of TIMI frame count to diagnose MVS.

METHODS

This study was reviewed and approved by the Ethical Committee of our hospital. An informed consent for provocation test for coronary spasm with acetylcholine (ACh) was given by all patients.

Study Population

We examined 131 consecutive patients (62 men and 69 women) with chest pain and normal coronary arteriograms (CAG) who underwent an ACh provocation test with myocardial lactate production measurement between January 1995 and July 2000. All cardiovascular medications, except sublingual nitroglycerin, were discontinued at least 24 hours before the test, and no patient had received long-acting CCBs before the procedure.

Definition of ES and MVS

Epicardial coronary spasm (ES) was defined when graded doses of intracoronary ACh (10, 30, and 100 μg) induced more than 75% vasoconstriction by coronary angiography.[7] The degree of vasoconstriction was normalized by the diameter obtained after intracoronary administration of isosorbide dinitrate (ISDN) administration, which was measured with calipers in a blind manner.[7] For measurement of myocardial lactate production, paired 2-mL samples of blood were

Received for publication May 22, 2005; accepted August 1, 2005.
From the Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan.
Reprints: Yoshihiro Fukumoto, Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan (e-mail: yofukumoto-circ@umin.ac.jp).

collected simultaneously from the ascending aorta and the coronary sinus. MVS was diagnosed when 10 or 30 μg of intracoronary ACh induced myocardial ischemia (2 or more signs out of chest pain, ischemic ECG changes, and myocardial lactate production), as previously reported.[5] The myocardial lactate extraction ratio was calculated as the ratio of the coronary arterial–venous difference in lactate concentration to the arterial concentration. Myocardial lactate production (negative extraction ratio) was considered to be evidence of myocardial ischemia.

Data Collection

Baseline demographic information (including age and sex), coronary risk factors (hypercholesterolemia, smoking, hypertension, diabetes mellitus, and family history of ischemic heart disease) were recorded for each patient. Hypercholesterolemia was defined as total cholesterol ≥220 mg/dL. Hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg or the use of antihypertensive drug(s). Diabetes was defined as fasting blood sugar ≥140 mg/dL, blood sugar during a 75 g oral glucose tolerance test ≥200 mg/dL, or the use of antidiabetic drug(s).

TIMI Frame Count

TIMI frame count was assessed by a single observer (H.S.), who was blinded to clinical diagnosis of MVS or ES. All coronary angiograms were filmed in a speed of 30 frames per second.[6] TIMI frame count was measured to first reach a standardized distal landmark in the left anterior descending (LAD) and the left circumflex coronary artery (LCX), using a frame counter on a cineviewer, in a blind manner.[6] We diagnosed MVS by TIMI frame count as 60 counts or more in LAD and 45 or more in LCX.

Statistical Analysis

Continuous variables were expressed as mean ± SD. Comparisons among groups were made by use of 1-way ANOVA followed by post-hoc test with Stat View (SAS Institute, Cary, NC). P values less than 0.05 were considered to be statistically significant.

RESULTS

Characteristics of Patients

Depending on the results of the ACh provocation test, the subjects were divided into 4 groups, including no myocardial ischemia (Normal), epicardial spasm alone (ES), microvascular spasm (MVS) alone, and both (ES + MVS) (Table 1). There was a predominance of women in MVS and ES + MVS (Table 1). Current smoking was more prevalent in ES group, but the prevalence of other coronary risk factors was comparable among the 4 groups. Ischemia signs were observed during the ACh provocation test in ES, MVS, and ES + MVS groups but not in the Normal group (Table 1).

Correlation With TIMI Frame Count and Myocardial Ischemia of Microvascular Origin

TIMI frame count was significantly increased in the MVS and ES + MVS groups compared with the Normal or ES group both under control conditions (Fig. 1) and after ACh (Fig. 2) in both left coronary arteries. Importantly, ISDN did not improve TIMI frame count in the MVS or ES + MVS group (Fig. 3).

When all patients were divided into 4 groups by the number of ischemia signs (chest pain, ischemic ECG changes, and myocardial lactate production), 29 patients had no ischemia signs, 10 had 1 sign, 55 had 2 signs, and 37 had all 3 ischemia signs (Fig. 4). TIMI frame count in control CAG was significantly increased in accordance with the number of ischemia signs (Fig. 4). Furthermore, the TIMI frame count in LAD significantly correlated with that in LCX in patients with MVS, suggesting the diffuse impaired coronary microcirculation in the myocardium (Fig. 5).

When MVS was diagnosed by increased TIMI frame count with 60 or more in LAD and 45 or more in LCX, control

TABLE 1. Characteristics of Patients

	Normal	ES	ES + MVS	MVS	P Value
n	27	53	16	35	
Age	57.4 ± 14.2	60.7 ± 8.5	59.0 ± 10.9	60.8 ± 9.8	N.S
Sex (male/female)	12/15	35/18	5/11†	10/25††	<0.01
Coronary risk factors					
Hypertension	44%	42%	38%	49%	N.S
Diabetes	7%	19%	6%	11%	N.S
Smoking	30%	51%	19%†	26%†	<0.05
Hypercholesterolemia	19%	40%	44%	31%	N.S
Family history	33%	17%	6%	14%	N.S
Ischemia sign					
Symptom	4%	89%*	94%**	80%**	<0.01
ECG changes	0%	77%**	88%**	74%**	<0.01
Lactate production	4%	36%**	63%**	83%**	<0.01
Lactate extraction ratio	0.13 ± 0.17	0.02 ± 0.33	−0.26 ± 0.44**††	−0.13 ± 0.21*†	<0.01

Continuous variables are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs. Normal, †P < 0.05, ††P < 0.01 vs. ES, statistically analyzed by one-way ANOVA followed by post-hoc test.

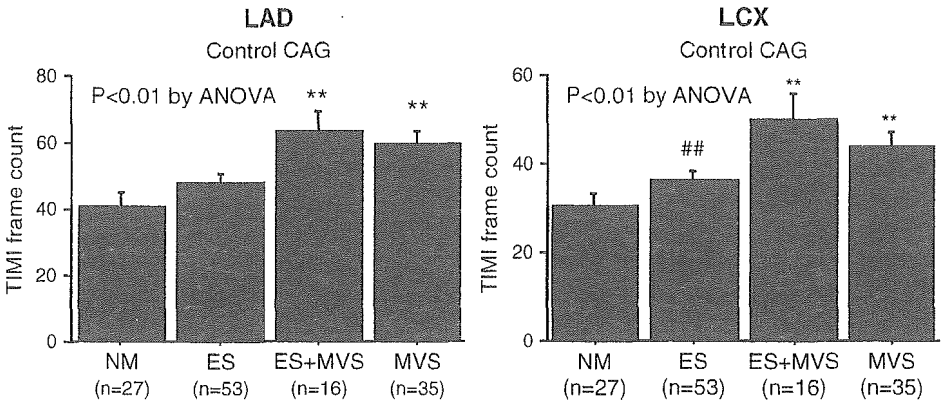

FIGURE 1. TIMI frame count in control coronary arteriograms (CAG) among the 4 groups. TIMI frame count was significantly increased in patients with both epicardial and microvascular spasm (ES + MVS) and MVS alone (MVS) compared with those with no myocardial ischemia (Normal) and epicardial spasm alone (ES) under control conditions in both LAD and LCX (**$P < 0.01$ versus Normal or ES; ##$P < 0.01$ versus Normal).

CAG had a sensitivity of 45% for LAD and 47% for LCX and a specificity of 77% for LAD and 75% for LCX (Fig. 6).

DISCUSSION

The novel findings of the present study were that (1) TIMI frame count, a marker of coronary blood flow, was significantly increased in MVS irrespective of the presence or absence of ES, (2) TIMI frame count was significantly increased in accordance with the number of ischemia signs, and (3) TIMI frame count had an acceptable specificity to diagnose MVS. Thus, the present study suggests that TIMI frame count may reflect the severity of microvascular dysfunction and therefore may be useful to diagnose the disorder. Our group has previously reported that basal coronary tone is elevated in patients with variant angina.[7] Therefore, the combination of basal coronary tone and TIMI frame count may be helpful in diagnosing a patient with ES and/or MVS without performing coronary sinus catheterization or myocardial lactate production measurement. TIMI frame count also has been demonstrated to be useful in detecting the coronary flow changes in patients with stent implantation[8] or the impaired coronary microcirculation in patients who have intracoronary thrombus, impaired flow, and increased burden of coronary atherosclerosis.[9]

The present study also demonstrates that ISDN can reduce symptoms caused by ACh but fails to improve TIMI frame count in patients with MVS. This result suggests that ISDN alone can not effectively ameliorate myocardial ischemia caused by MVS sufficiently because nitrates are great large vessel dilators but not very good at dilating resistance arteries, which is compatible with the previous studies.[10,11] To improve quality of life in patients with MVS, we usually prescribe CCBs, angiotensin-converting enzyme inhibitors, or nicorandil, alone or in combination, with the limited effectiveness.[2]

The precise mechanism of MVS still remains unclear. It has been demonstrated that hypersensitivity to vasoactive substances in MVS is partially mediated by thromboxane A_2[12] and that myocardial ischemia enhances contractile response of coronary arterioles to serotonin, probably through vasoconstrictor prostaglandin release (eg, thromboxane A_2) by upregulated cyclooxygenase (COX)-2.[13] We have recently demonstrated that the Rho/Rho-kinase pathway also plays an important role in the pathogenesis of MVS.[14–16] Therefore, it remains to be examined whether Rho-kinase inhibitor is useful for treating MVS.

Limitations of the Study

Several limitations should be mentioned for the present study. First, we did not directly examine coronary flow velocity using a flow wire technique. However, the validity of TIMI frame count to represent coronary flow velocity has previously been confirmed.[6,8,9,17,18] Second, because we used only ISDN as a vasodilator, we were unable to correlate TIMI

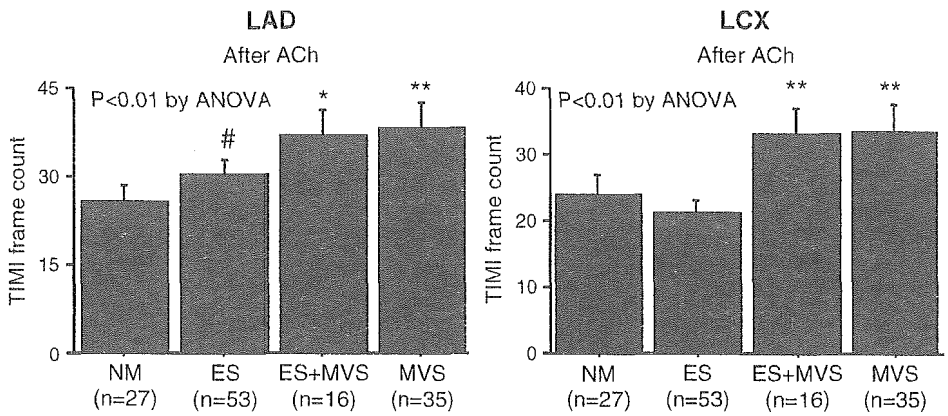

FIGURE 2. TIMI frame count in coronary arteriograms after intracoronary ACh among the 4 groups. TIMI frame count was significantly increased in patients with both epicardial and microvascular spasm (ES + MVS) and MVS alone (MVS), compared with those with no myocardial ischemia (Normal) and epicardial spasm alone (ES) under control conditions and after intracoronary ACh in both LAD and LCX (**$P < 0.01$ versus Normal or ES, *$P < 0.05$ versus Normal or ES, #$P < 0.05$ versus Normal).