Sustained Elevation of Serum Cortisol Level Causes Sensitization of Coronary Vasoconstricting Responses in Pigs In Vivo
A Possible Link Between Stress and Coronary Vasospasm

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Abstract—Vasospastic angina is induced by stress, for which cortisol secreted by activated hypothalamic/pituitary/adrenal axis may play an important role. However, direct evidence for this notion is still lacking. In this study, we examined whether sustained elevation of serum cortisol level sensitizes coronary vasoconstricting responses in pigs in vivo and, if so, whether Rho-kinase, which we found is a key molecule of coronary vasospasm, is involved. Oral administration of cortisol (20 mg/kg per day) increased its serum level to that seen in restraint stress in pigs. Thus, we examined coronary vasomotor responses in the following 4 groups: (1) control (without cortisol); (2) cortisol (20 mg/kg per day, PO) for 9 days; (3) cortisol plus RU38486 (a glucocorticoids receptor antagonist, 10 mg/kg per day, PO) for 9 days; and (4) cortisol for 9 days followed by 6-week withdrawal. Coronary angiography showed that intracoronary serotonin caused coronary hyperconstriction and reduction in coronary blood flow associated with ischemic ECG changes (coronary vasospasm) in only the cortisol group. All of these responses were abolished by hydroxyfasudil, a specific Rho-kinase inhibitor, in vivo. Organ chamber experiments demonstrated that serotonin concentration-dependently caused hypercontractions of coronary vascular smooth muscle associated with Rho-kinase activation (as evidenced by the enhanced phosphorylation of myosin binding subunit, a substrate of Rho-kinase) in only the cortisol group. All of these responses were again inhibited by hydroxyfasudil in vitro. These results indicate that sustained elevation of serum cortisol level sensitizes coronary vasoconstricting responses through Rho-kinase activation, suggesting the link between stress and coronary vasospasm. (*Circ Res*. 2006;99:767-775.)

Key Words: Rho-kinase ■ coronary vasospasm ■ cortisol ■ myocardial ischemia ■ stress

Physical and/or mental stress induces ischemic attacks in patients with coronary artery disease (CAD). Recent studies have demonstrated that psychological factors are involved in the pathogenesis of CAD[1–4] and that stress could induce coronary vasospasm and myocardial ischemia.[5–7] Indeed, stress test is clinically used to induce vasospastic angina.[8] However, it remains to be examined what component(s) and mechanism(s) of stress are responsible for coronary vasospasm. These points are important to develop an effective therapy to prevent stress-induced myocardial ischemia and sudden death.

Cortisol is among the major stress hormones and is secreted by the activated hypothalamic/pituitary/adrenal axis in physical and/or mental stress.[9–12] Cortisol also could cause endothelial and baroreflex dysfunction.[9,13–15] Short-term oral administration of a high-dose of cortisol impairs endothelial function even in healthy subjects.[14]

Rho-kinase/ROK/ROCK, one of the effectors of the small GTPase Rho, plays an important role in vascular smooth muscle cell (VSMC) contraction.[16–20] In a series of experimental and clinical studies, we have demonstrated that enhanced Rho-kinase activity plays a central role for coronary vasospasm in both animals and humans.[16–20] However, it remains to be examined whether Rho-kinase also is involved in the stress-induced coronary vasospasm.

In the present study, we thus examined whether sustained elevation of serum cortisol level sensitizes coronary vasoconstricting responses in pigs in vivo and, if so, whether Rho-kinase is involved in the molecular mechanism for the sensitization.

Materials and Methods
All procedures were approved by the Institutional Animal Care and Use Committee and were conducted according to the institutional guidelines of Kyushu University.

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Protocols

A total of 74 male domestic pigs (3 to 5 months, 25 to 30 kg) were used (Kyudo, Tosu, Japan).

Experiment 1: Determination of the Dose of Cortisol

Animals were implanted with a catheter in the jugular vein 5 days before the restraint stress test and were divided into the control, cortisol, and restraint stress groups (n=6 each). The cortisol group was given daily 20 mg/kg of cortisol in drinking water, whereas the other 2 groups were without any medication. The restraint stress group was tethered with a neck chain attached to the small pen ($40 \times 100 \times 80$ cm) from 8:00 AM to 8:00 PM, and were released from 8:00 PM to 8:00 AM the next morning.[21,22] Blood samples were collected at 3:00 AM, 9:00 AM, 3:00 PM, and 9:00 PM over 24 hours during the experiment. The hourly averages of area under the curve (AUC) of serum cortisol level were evaluated.[23]

Experiment 2: Coronary Vasomotor Responses to Chronic Cortisol Treatment

Pigs were used for coronary angiography (CAG) study (n=26), organ chamber experiments (n=26), and Western blot analysis (n=19). They were housed individually under a controlled room temperature. For CAG study, the animals were divided into the following 4 groups: (1) control group (n=8); (2) cortisol group treated with oral administration of cortisol (20 mg/kg per day) from day 1 to 7 and 9 and 10 (n=8); (3) RU group treated with cortisol (20 mg/kg per day) plus RU38486 (10 mg/kg per day), a glucocorticoid receptor antagonist,[24] on the same schedule as in the cortisol group (n=5); and (4) withdrawal group, which was treated as in the cortisol group, followed by withdrawal of cortisol from day 11 to 55. In the control, cortisol, and RU groups, CAG was performed on day 8, whereas it was performed on both day 8 and 52 in the withdrawal group (n=5). Organ chamber experiment was performed on day 11 in the control, cortisol, and RU groups (n=5 to 8) and on day 55 in the withdrawal group (n=5). All animals were fasted without any medication for 24 hours before the experiments.

Coronary Angiography

Animals were anesthetized with ketamine hydrochloride (15 mg/kg, IM) and pentobarbital (20 mg/kg, IV). They were then intubated and mechanically ventilated with room air. After systemic heparinization (5000 U/body), a preshaped 8F Judkins catheter was inserted through the carotid artery. CAG of the left coronary artery (LCA) was performed in a left oblique view with the cineangiography system (Toshiba Medical, Tokyo, Japan).[19,25,26] ECGs (leads I, II, and III) and arterial pressure were continuously monitored (Nihon Kohden, Tokyo, Japan).[19,25,26] Blood chemistry values (Na, K, Cl, blood urea nitrogen [BUN], creatinine [Cr], glutamate oxaloacetic transaminase [GOT], glutamate pyruvate transaminase [GPT], lactate dehydrogenase [LDH], creatine phosphokinase [CPK]) were also measured. End-diastolic frames were selected to measure coronary diameters. The measurement was made in a blind manner at the left anterior descending coronary artery (LAD), at both the large (just proximal to the first diagonal branch) and small (distal portion of the branch with a baseline diameter of ≈ 500 μm) coronary arteries.[19,25,26]

Protocols of CAG Study

First, CAG was performed under control conditions. Second, coronary vasomotor responses to serotonin (10 and 100 μg/kg, IC) were examined.[16,19,25,26] Serotonin induces coronary vasospasm that is similar to spontaneous spasm in humans compared with acetylcholine.[27] Third, endothelium-dependent vasodilating responses to bradykinin (0.1 μg/kg, IC) were examined.[25] Fourth, vasoconstricting responses to serotonin (100 μg/kg, IC) were examined after intracoronary infusion of hydroxyfasudil (30 and 100 μg/kg/min for 3 minutes), a specific Rho-kinase inhibitor.[26] Fifth, vasodilating responses to bradykinin (0.1 μg/kg, IC) were examined after intracoronary infusion of N^G-monomethyl-L-arginine (L-NMMA, 1

mg/kg for 10 minutes).[25] Finally, endothelium-independent vasodilating responses to nitroglycerin (10 μg/kg, IC) were examined.[25] CAG was performed 2 minutes after intracoronary administration of serotonin, bradykinin, or nitroglycerin, when the vasodilator or vasoconstrictor effect of each agent peaked.[19,25,26] Each protocol was performed with an interval of 20 to 30 minutes after the confirmation of disappearance of a drug effect by CAG and hemodynamic measurements. Each dose of drugs was diluted with 3 mL of physiological saline, except hydroxyfasudil, which was diluted with 3 mL of distillated water. The degree of coronary vasoconstricting response was expressed as percent change in luminal diameter and blood flow from the baseline values.[19,25,26]

Coronary Blood Flow Measurement

Coronary blood flow velocity was measured by a Doppler guide wire (FloWire, 12 MHz, Cardiometrics, Mountain View, Calif) at the same time with CAG. A 0.014-inch tip Doppler guide wire was advanced via the guiding catheter into the proximal LAD. The position of the Doppler guide wire was kept constant throughout the study. The time average of the spectral peak velocity (APV) was used for mean coronary blood flow velocity.[28] Coronary blood flow was calculated by multiplying a half APV by calculated coronary sectional area at the tip of the Doppler guide wire on the corresponding angiogram.[28]

Organ Chamber Experiments

Three days after the CAG study, animals were sedated with ketamine hydrochloride (15 mg/kg, IM), euphonized with a lethal dose of sodium pentobarbital (40 mg/kg, IV), and then the heart was excised. Epicardial and distal small (≈ 200 to 250 μm in inner diameter) right coronary arteries (RCA) were carefully dissected and cleaned of any perivascular connective tissue. We used RCA for organ chamber experiments to avoid any influence of CAG on LCA. We have previously confirmed that there are no differences in vascular responses (both in vivo and in vitro) between RCA and LCA,[29] which we also confirmed in the present study (Figure I in the online data supplement, available at http://circres.ahajournals.org). Epicardial RCA were cut into 8 rings (≈ 4 mm in length),[16,17,25,26] whereas distal small RCA into 8 rings (≈ 1 mm in length).[30,31] In 4 rings of both-sized arteries, the endothelium was removed by gentle rubbing of the luminal surface with a cotton swab.[17,25,30]

The rings were fixed vertically between hooks in an organ bath containing Krebs–Henseleit solution at 37°C with a mixture of 95% O_2/5% CO_2 and isometric tension was measured with force transducers (Nihon Kohden Inc). KCl solution (62 mmol/L) was applied every 15 minutes until the amplitude of contraction reached a constant value. The developed tension was expressed as a percentage of that attained in the last precontraction with 62 mmol/L KCl.[17,25,30] The presence and absence of the endothelium was confirmed by the presence and absence of relaxation to bradykinin (10^{-7} mol/L) during a contraction evoked by KCl, respectively.[25]

The direct VSMC vasocontracting effect of serotonin (10^{-9} to 10^{-5} mol/L) was evaluated in rings without endothelium.[17,26] The acute inhibitory effect of hydroxyfasudil on serotonin-induced VSMC contraction was examined after equilibration with hydroxyfasudil (10^{-6} and 3×10^{-6} mol/L, for 30 minutes) separately in different rings without endothelium.[17,26] Endothelium-dependent relaxations to bradykinin (10^{-11} to 10^{-6} mol/L) were examined in rings with endothelium during a contraction evoked by prostaglandin (PG) $F_{2\alpha}$ (2×10^{-6} mol/L). The extent of contraction in response to $PGF_{2\alpha}$ was adjusted to 50% to 70% of that induced by 62 mmol/L KCl.[25,30,31] The contribution of vasodilator PGs, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) to endothelium-dependent relaxations to bradykinin was evaluated by determining the inhibitory effect of indomethacin (10^{-5} mol/L), N^ω-nitro-L-arginine (L-NNA, 10^{-4} mol/L), and charybdotoxin (an inhibitor of large and intermediate-conductance KCa channels, 100 nmol/L) plus apamin (an inhibitor of small conductance KCa channels, 1 μmol/L), respectively.[30,31] Endothelium-independent relaxations to sodium nitroprusside (SNP) (10^{-10} to 10^{-5} mol/L) also were examined in rings without endothelium.
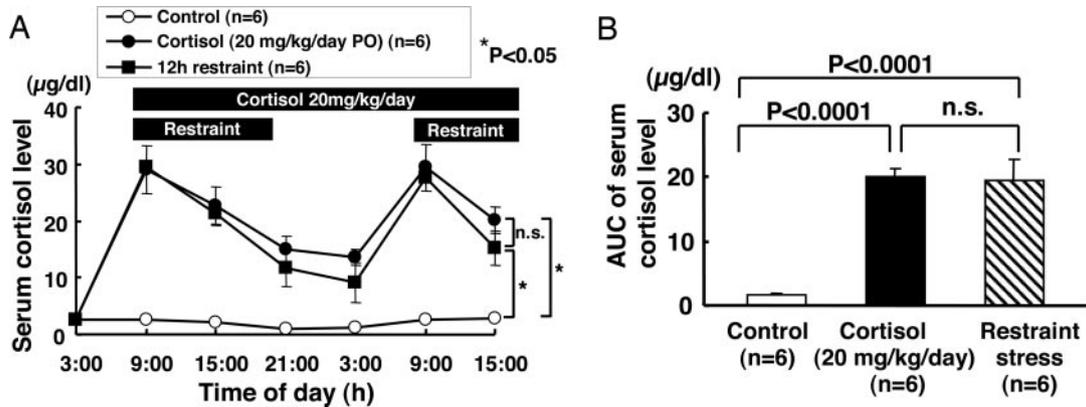
Figure 1. Daily profile (A) and areas under the curve (AUC) (B) of serum cortisol level during restraint stress and oral administration of the hormone. The oral administration of cortisol significantly increased serum cortisol level, equivalent to that in restraint stress. Results are expressed as mean±SEM. n.s. indicates not significant.

To evaluate the acute effect of cortisol on coronary vasomotor responses, organ chamber experiments were also performed in rings with or without endothelium from normal coronary arteries in the absence and presence of cortisol (10 and 30 μg/dL for 60 minutes and 30 μg/dL for 120 minutes).

Histopathology

LCA were perfused via constant-pressure perfusion system (120 cm H_2O) with saline (1000 mL) and subsequently with 5% formaldehyde (1000 mL).[19,32] After fixation, LAD was cut transversely, dehydrated, embedded in paraffin, and cut into 5-μm-thick slices. These segments were stained with hematoxylin/eosin and van Gieson's elastic staining for histological analysis.

Western Blot Analysis for Rho-Kinase Activity

Rho-kinase activity can be evaluated by the extent of phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase, a substrate of Rho-kinase.[16,32–34] The regions containing MBS were visualized by ECL Western blotting luminal reagent (Santa Cruz Biotechnology, Santa Cruz, Calif).[16,17,32] Isolated RCA rings without endothelium were subjected to SDS-PAGE immunoblot analysis when serotonin-induced (10^{-6} mol/L) contraction reached a maximum.[16,17,32] Rho-kinase activity is expressed by the extent of MBS phosphorylation when normalized to total MBS.

Statistical Analysis

Results are expressed as mean±SEM. χ^2 test was used for comparison of ECG ST-segment changes. Results of CAG and blood flow were analyzed by 2-way ANOVA followed by Bonferroni test for multiple comparisons. Serum cortisol levels under control condition and during oral administration of cortisol and restraint stress, AUC of serum cortisol level, and the results of organ chamber experiments were analyzed by 2-way ANOVA, followed by Scheffe test for multiple comparisons. The results of Western blot analysis were analyzed by Dunnet test. A value of $P<0.05$ was considered to be statistically significant.

Results

Daily Profile of Serum Cortisol Level During Restraint Stress and Oral Administration

Daily profile (Figure 1A) and AUC (Figure 1B) of serum cortisol levels were comparable between restraint stress and oral administration of the hormone.

Coronary Vascular Responses to Serotonin In Vivo

Among the control, cortisol, and RU groups, hemodynamic variables (heart rate and blood pressure) (supplemental Table

I) and blood chemistry values (data not shown) were comparable. Because cortisol was stopped 24 hours before the CAG experiment, the serum cortisol level (μg/dL) was comparable among the 3 groups (2.4 ± 0.8 in the control, 1.9 ± 0.5 in the cortisol, and 2.1 ± 0.7 in the RU group, n=5 each).

There was no significant difference in baseline coronary diameter among the 3 groups (supplemental Table II). A low dose of serotonin (10 μg/kg, IC) caused mild coronary vasodilatation without any significant ECG changes in the control (Figure 2A through 2D) and RU (Figure 2I through 2L) groups but caused intense and diffuse vasoconstriction, especially at small coronary arteries, with ischemic ST changes in the cortisol group (ST elevation in 3/5 and ST depression in 1/5) (Figure 2E through 2H). By contrast, a high dose of serotonin (100 μg/kg, IC) caused coronary vasoconstriction in large (Figure 3G) and small (Figure 3H) arteries in all the 3 groups; however, the extent of the vasoconstriction was most prominent in the cortisol group, especially at small coronary arteries (Figure 3G and 3H). Intracoronary pretreatment with hydroxyfasudil dose-dependently suppressed the serotonin-induced vasoconstrictions in all the 3 groups (Figure 3).

Coronary blood flow was slightly increased in response to a low dose of serotonin but was decreased to a high dose of serotonin in the control and RU groups (Figure 3I). By contrast, in the cortisol group, coronary blood flow was decreased in response to both doses of serotonin, which was again dose-dependently inhibited and was converted to an increase in the flow by hydroxyfasudil, as seen in other 2 groups (Figure 3I).

Coronary Endothelial Vasodilator Function In Vivo

Bradykinin (0.1 μg/kg, IC) caused mild coronary vasodilatation in both-sized arteries and an increase in coronary blood flow in all the 3 groups (Figure 4). Pretreatment with intracoronary L-NMMA tended to inhibit the bradykinin-induced coronary vasodilatation in all groups, whereas nitroglycerin (10 μg/kg, IC) caused a comparable extent of coronary vasodilatation and increase in coronary blood flow in all groups (Figure 4).
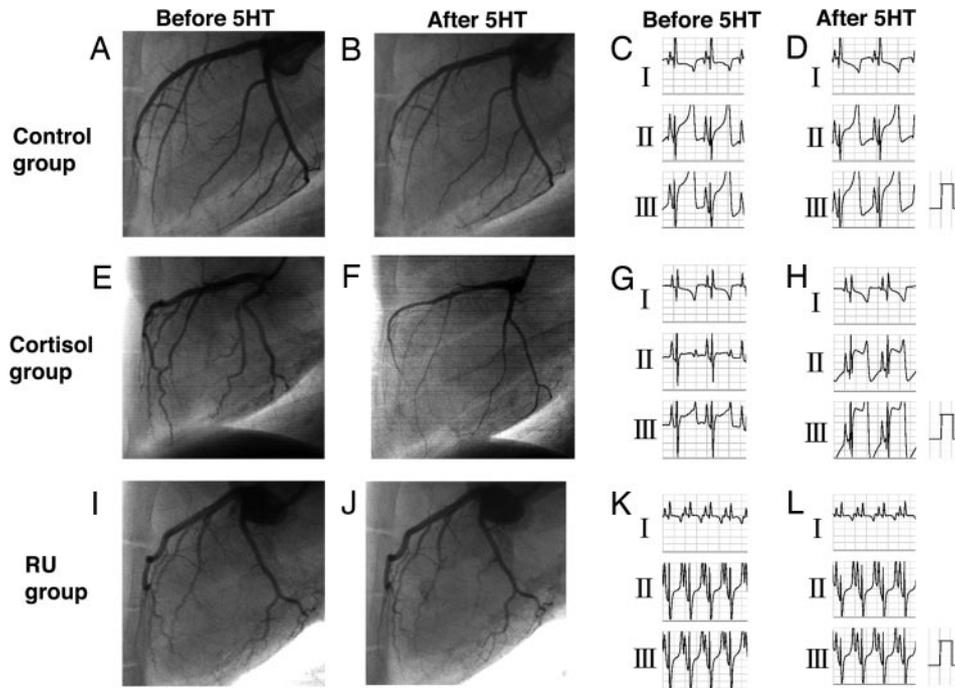
Figure 2. Coronary angiograms and ECG before and after a low dose of serotonin (5HT) (10 μg/kg, IC). Serotonin induced mild coronary vasodilatation with no ischemic ECG changes in the control (A through D) and RU (I through L) groups but induced diffuse and intense coronary vasoconstriction with ischemic ST changes in the cortisol group (E through H). Calibration on ECG, 1 mV.

Effect of Withdrawal of Long-Term Treatment With Cortisol

In the withdrawal group, there was no significant difference in basal coronary diameters as compared with other 3 groups (supplemental Table I) or between day 8 (before cessation of the cortisol treatment) and day 52 (6 weeks after withdrawal) (supplemental Table III). On day 8, intracoronary serotonin again caused intense and diffuse coronary vasoconstriction (Figure 5A and 5B), whereas on day 52, the hyperconstrictions were no longer noted (Figure 5C and 5D). On day 8 and 52, hydroxyfasudil again inhibited the serotonin-induced coronary vasoconstriction and the decrease in coronary blood flow (Figure 5E through 5G).
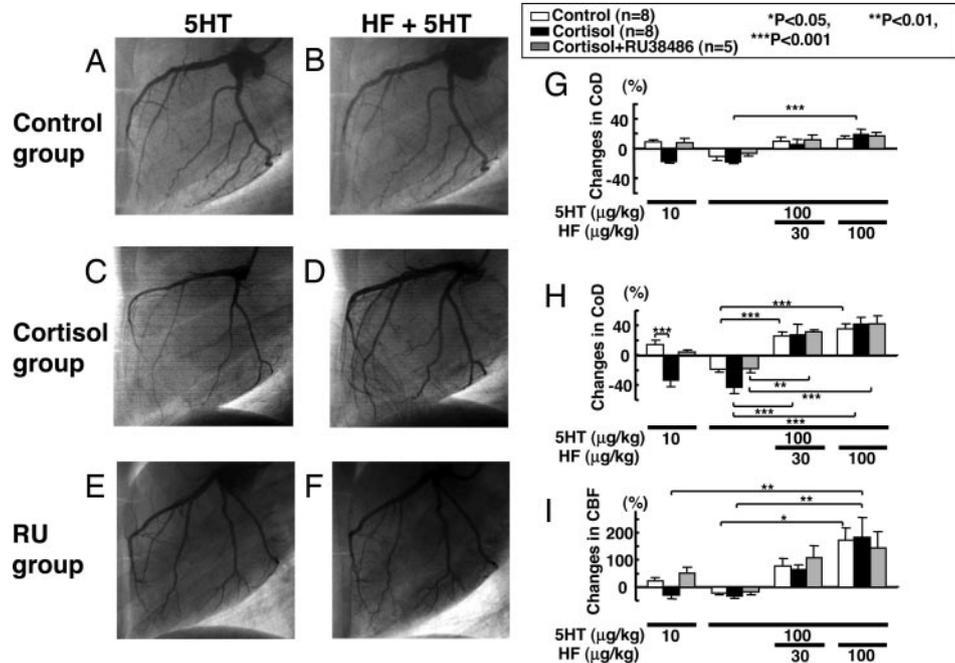
Figure 3. Inhibitory effects of hydroxyfasudil on serotonin-induced coronary vasospasm. Serotonin (5HT) (10 and 100 μg/kg, IC) caused hyperconstriction in both large (G) and small (H) coronary arteries in the cortisol (C) but not in the control (A) or RU (E) group. Hydroxyfasudil (HF) (100 μg/kg, IC) abolished the serotonin-induced vasoconstriction and converted the vasoconstriction to vasodilatation in all groups (B, D, F, G, and H). Similarly, HF converted the serotonin-induced reduction in coronary flow to an increase in the flow in the cortisol group (I). CoD indicates coronary diameter; CBF, coronary blood flow. Results are expressed as mean±SEM.
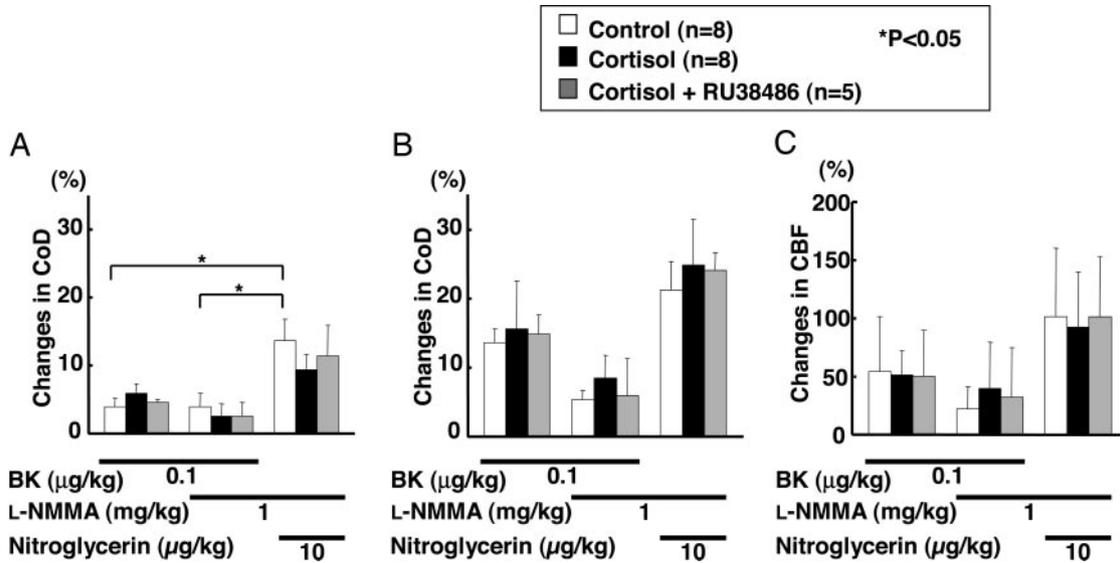
Figure 4. Assessment of coronary endothelial vasodilator functions in vivo. Bradykinin (BK) (0.1 μg/kg, IC) caused a comparable extent of coronary vasodilatation in both large (A) and small (B) coronary arteries and coronary flow (C) among the 3 groups. L-NMMA did not significantly inhibit the bradykinin-induced coronary vasodilatation in all groups. Intracoronary nitroglycerin (10 μg/kg) also caused a comparable extent of coronary vasodilatation among the 3 groups. CoD indicates coronary diameter; CBF, coronary blood flow. Results are expressed as mean±SEM.

Organ Chamber Experiments

Serotonin caused concentration-dependent contractions of coronary rings without endothelium from both-sized arteries (Figure 6). The extent of the contractions was significantly greater in the cortisol group compared with other 3 groups in both-sized coronary arteries (Figure 6A and 6D). Hydroxy-fasudil significantly suppressed the serotonin-induced hyper-contractions of both-sized arteries (Figure 6B and 6E). No
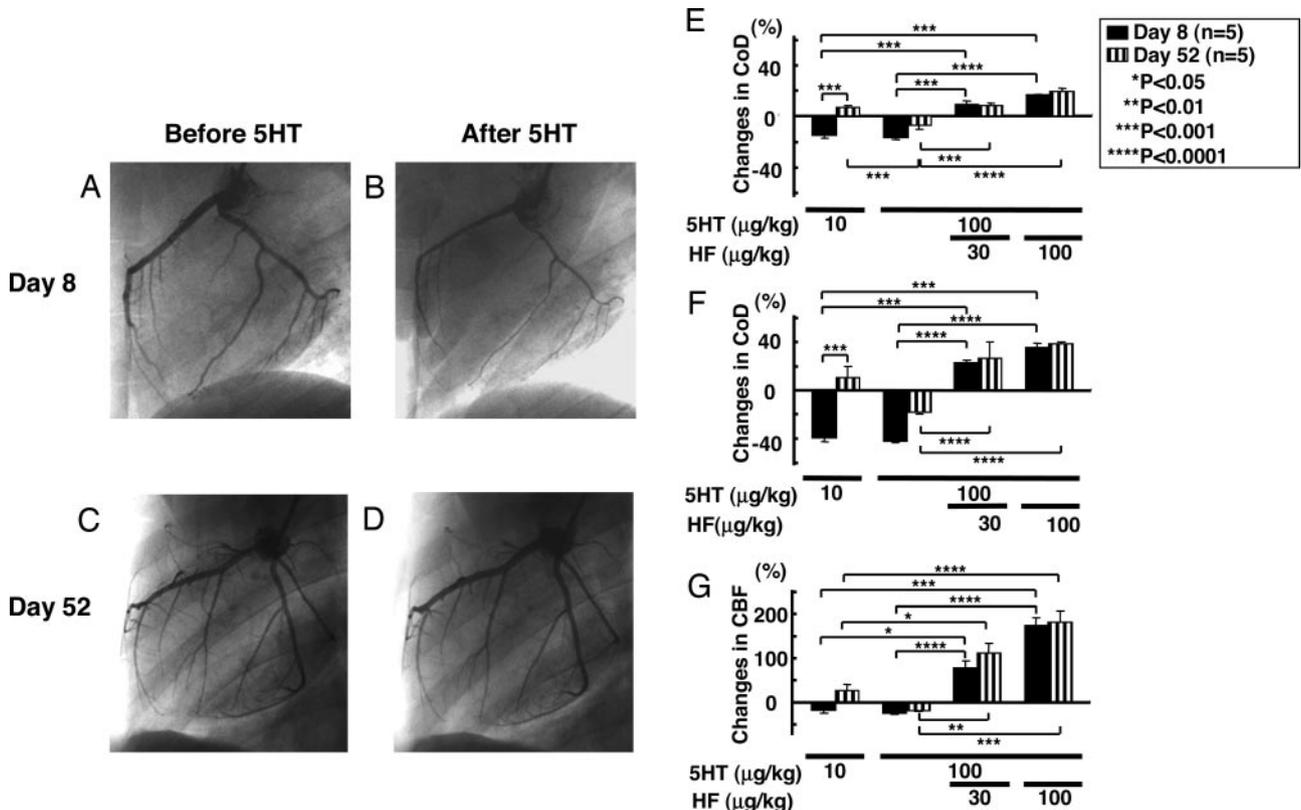
Figure 5. Effects of cessation of chronic cortisol treatment. After confirmation of the serotonin (5HT)-induced coronary vasospasm on day 8 (A and B), the cortisol treatment was stopped. On day 52, the serotonin-induced vasospasm was no longer noted (C and D). Comparisons between day 8 and 52 are shown for coronary vasoconstriction in large (E) and small (F) coronary arteries and coronary flow (G). CoD indicates coronary diameter; CBF, coronary blood flow; HF, hydroxyfasudil. Results are expressed as mean±SEM.
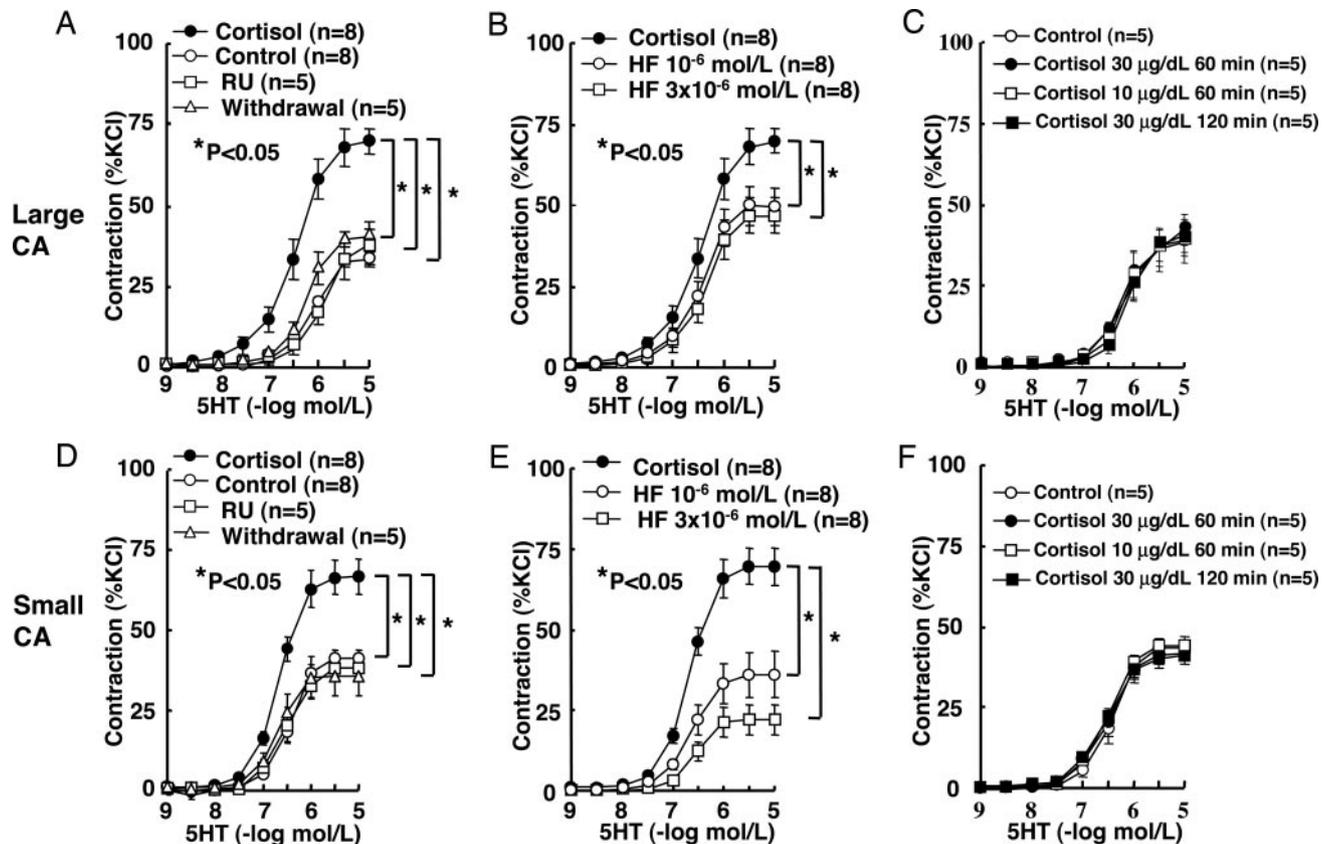
Figure 6. Acute and chronic effects of cortisol on serotonin-induced coronary VSMC contractions in vitro. The chronic cortisol treatment enhanced serotonin-induced VSMC contractions in both large (A) and small (D) coronary arteries (CA) compared with other 3 groups, which were significantly inhibited by hydroxyfasudil (HF) in both large (B) and small (E) coronary arteries. By contrast, no acute effects of the hormone were noted in large (C) or small (F) coronary arteries. Results are expressed as mean±SEM.

acute effects of cortisol on serotonin-induced contractions of normal coronary rings were noted (Figure 6C and 6F).

Endothelium-dependent relaxations to bradykinin were comparable among the 4 groups in both-sized arteries under the 4 different conditions (supplemental Table IV), and no acute effects of cortisol on endothelium-dependent relaxations of normal coronary rings were noted (supplemental Table V). Endothelium-independent relaxations to SNP also were comparable among the 4 groups in both-sized coronary arteries (supplemental Table VI).

Histopathology

There were no obvious histological changes (eg, intimal thickening or inflammatory cell infiltration) in any of the 4 groups (n=5 each, data not shown).

Western Blot Analysis

The extent of MBS phosphorylation, a marker of Rho-kinase activity, was enhanced in response to serotonin only in the cortisol group, and was dose-dependently inhibited by hydroxyfasudil (Figure 7). By contrast, in other 3 groups, the serotonin-induced Rho-kinase activation was absent and no inhibitory effects of hydroxyfasudil were noted (Figure 7). There was a significant positive correlation between the extent of MBS phosphorylation (Rho-kinase activity) and that of the serotonin-induced contractions among the 4 groups studied (Figure 8A) and the inhibitory effects of hydroxyfa-

sudil on the serotonin-induced activation of Rho-kinase was confirmed (Figure 8B).

Discussion

The novel findings of the present study are that sustained elevation of serum cortisol level causes sensitization of coronary VSMC constricting responses to serotonin both in vivo and in vitro and that Rho-kinase–mediated pathway is substantially involved in the molecular mechanisms for the sensitization. To the best of our knowledge, this is the first study that demonstrates the link between elevated serum cortisol level and hyperconstriction of coronary VSMCs through Rho-kinase activation in vivo.

Sustained Elevation of Serum Cortisol Level and Rho-Kinase Activity

We have demonstrated that Rho-kinase is upregulated in spastic coronary segment, which leads to inhibition of myosin light chain (MLC) phosphatase with resultant enhancement of MLC phosphorylation and VSMC contraction.[16,18,26,35] In the present study, we were able to demonstrate that sustained elevation of serum cortisol level enhances coronary vasospastic activity through Rho-kinase activation. Serotonin exerts 5-HT$_{2A}$ serotonergic receptor– and Rho-kinase–mediated direct vasoconstrictor effects and 5-HT$_{1B}$ serotonergic receptor–mediated endothelium-dependent relaxations in porcine coronary arteries.[36] In the present study, a specific Rho-kinase
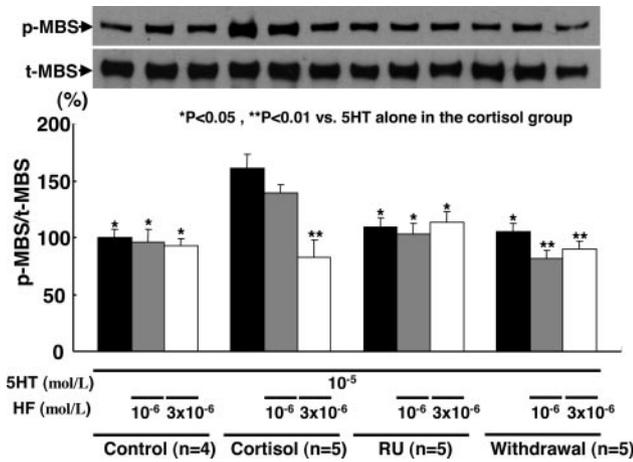
Figure 7. Cortisol-induced Rho-kinase activation in coronary VSMCs. Rho-kinase activity, as evaluated by phosphorylated MBS/total MBS ratio, was significantly enhanced in the cortisol group during serotonin-induced contraction compared with other 3 groups, which was concentration-dependently inhibited by hydroxyfasudil (HF). Results are expressed as mean±SEM.

inhibitor, hydroxyfasudil, unmasked endothelium-dependent vasodilatation and increase in coronary blood flow in response to serotonin through inhibition of the Rho-kinase–mediated direct vasoconstrictor effects of the monoamine.

In this study, the cortisol treatment was stopped 24 hours before the experiment to avoid any acute effects of the hormone. Moreover, cortisol had no acute effect on coronary vasoconstricting responses in vitro. Thus, the sensitization of coronary vasoconstricting responses is apparently attributable to chronic effects of cortisol on Rho-kinase activity in coronary VSMCs.[16,18,26,35] The finding that withdrawal of cortisol resulted in the disappearance of coronary hyperconstricting responses both in vivo and in vitro associated with normalization of coronary Rho-kinase activity further supports this notion.

In the present study, endothelial vasodilator function per se was preserved, whereas VSMC contraction was enhanced in response to the chronic elevation of serum cortisol level in both-sized coronary arteries. However, the decrease in coronary flow secondary to enhanced vasoconstriction in coronary resistance vessels could, at the same time, amplify the

Figure 8. Correlation between the extent of MBS phosphorylation and that of serotonin-induced contractions in the control, cortisol, RU, and withdrawal groups (A) and in the absence (cortisol) and presence of hydroxyfasudil (HF) (B). There were significant positive correlations between the 2 values.

net vasoconstriction by reducing shear stress induced vascular relaxation. The mechanism for the enhanced coronary vasoconstricting responses caused by sustained elevation of serum cortisol level is apparently attributable to VSMC hyperreactivity that exceeds endothelial vasodilator capacity, a consistent finding with our previous studies.[25,35,36] In the present study, coronary endothelial vasodilator function in response to bradykinin was relatively resistant to the blockade of NO synthesis in both-sized arteries both in vivo and in vitro. This finding also is consistent with our previous findings that endothelium-dependent relaxation to bradykinin is largely mediated by EDHF.[20,37] However, it remains to be examined whether EDHF-mediated responses also are impaired in response to a long-term increase in serum cortisol levels.
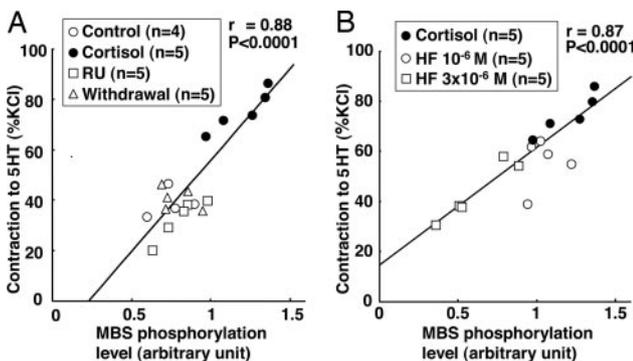
Cortisol and CAD
There is a line of evidence for the link between cortisol and CAD. The 5-year incidence of cardiovascular events was significantly higher in men with abnormal cortisol secretion compared with those with a normal pattern.[38] In addition, increased serum cortisol level in patients with depression enhances prothrombotic state[2] and increases the density of 5-HT$_2$ serotonergic receptors in platelets, a useful index of platelet activation.[39] Cortisol also could accelerate atherosclerotic process[40]; however, no atherosclerotic changes were noted in the present study, probably because of the relatively short treatment period.

Role of Cortisol in the Pathogenesis of Stress-Induced CAD
Environmental and/or psychological factors contribute to the pathogenesis of CAD.[1–4] It is known that serum levels of cortisol are frequently and chronically elevated in humans with stress and are also normalized after stress is resolved.[41–44] Indeed, natural and/or social disasters have been associated with a transient increase in ischemic cardiac events after the disasters.[45–48]

Coronary vasospasm could be induced in either a focal form or a diffuse form.[36] The latter form may be more frequently associated with myocardial ischemia because of more increased coronary vascular resistance.[36] In the present study, the sustained increase in serum cortisol level sensitized coronary VSMC constricting responses and caused a diffuse form of coronary vasospasm with myocardial ischemia, suggesting an increased risk of myocardial ischemia and sudden death in stress.

Cortisol is secreted by the activated hypothalamic/pituitary/adrenal axis and plays a key role in stress.[11,12] Elevated plasma and urinary levels of corticosteroids and a disturbed diurnal cortisol rhythm have been documented in a variety of diseases with mental stress, including depression, which is among the important risk factors of cardiovascular disease.[10–12] Stress may be closely related to vasospastic angina[6,8]; however, no direct evidence has yet been provided for the link between stress and coronary vasospasm. The present study suggests that Rho-kinase–mediated sensitization of coronary VSMC constricting responses caused by elevated

serum cortisol level is involved in the increased risk of CAD in stressful environments.

Limitations of the Study

Several limitations should be mentioned for the present study. First, we did not directly examine the effects of stress on coronary artery responsiveness. In a preliminary study, we actually attempted to induce a long-lasting stress in pigs; however, continuous restraint in a small cage did not cause sustained elevation of cortisol, indicating that the animals adapted to the restraint stress. In addition, intermittent restraint stress caused elevation of cortisol level; however, this elevation also declined in several days. This point should be examined in a future study. Second, because we only examined the coronary vasomotor responses in pigs, the vascular responsiveness in different organs, different species, and different stage of vascular disease remain to be examined. Third, the detailed molecular mechanisms for the cortisol-induced Rho-kinase activation remain to be examined. Fourth, the effects of cortisol on coronary vascular responses to agonists other than serotonin remain to be examined. However, the use of serotonin was justified as a vasoconstrictor of the coronary arteries in humans.[49] Moreover, because we have previously demonstrated that serotonin and many other vasoconstrictors use Rho-kinase pathway for their vasoconstrictor effects,[19,38] it is possible that coronary vasoconstricting responses to many other agonists also are enhanced when serum cortisol level is chronically elevated.

Clinical Implications

The present study provides the direct evidence for the role of sustained elevation of serum cortisol level in the pathogenesis of coronary vasospasm through activation of Rho-kinase. Importantly, the cessation of the cortisol administration normalized both the coronary vasoconstricting responses and Rho-kinase activity. Thus, the present results suggest that effective management of stress is crucial for the prevention of coronary vasospasm and that a specific Rho-kinase inhibitor may be useful to inhibit the stress-induced ischemic cardiovascular events in humans.

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Disclosures

None.

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Sustained Elevation of Serum Cortisol Level Causes Sensitization of Coronary Vasoconstricting Responses in Pigs In Vivo: A Possible Link Between Stress and Coronary Vasospasm

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Online Figure Legend

Online Figure 1.

No differences in coronary VSMC contractions in vitro between RCA and LCA.   There were no differences in serotonin-concentration dependent contractions of coronary rings without endothelium in both large (**A**) and small (**C**) normal coronary arteries (CA) between right coronary artery (RCA) and left coronary artery (LCA).   No differences between serotonin-induced VSMC contractions in both large (**B**) and small (**D**) CA in the cortisol group between RCA and LCA were also noted.   After equilibration with hydroxyfasudil (10^{-6} and 3×10^{-6} mol/L, for 30 min) separately in different rings without endothelium, there were also no differences in both large (**B**) and small (**D**) CA between RCA and LCA.   Results are expressed as mean±SEM.

Online Tables

Online Table 1. Hemodynamic variables

	Control (n=8)	Cortisol (n=8)	RU (n=5)	Withdrawal		P value
				Day 8 (n=5)	Day 52 (n=5)	
HR (bpm)	129 ± 10	135 ± 13	133 ± 20	122 ± 7	124 ± 10	n.s.
SBP (mmHg)	117 ± 14	122 ± 4	125 ± 12	120 ± 11	121 ± 10	n.s.
DBP (mmHg)	84 ± 2	92 ± 6	83 ± 5	83 ± 4	86 ± 6	n.s.

Results are expressed as mean±SEM.   Control, control group; Cortisol, cortisol group; RU,

RU group.   HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.   n.s.,

not significant.

Online Table 2. Baseline Coronary Artery Diameters

	Control (n=8)	Cortisol (n=8)	RU (n=5)	P value
Large CA (mm)	2.52 ± 0.68	2.64 ± 0.37	2.56 ± 0.47	n.s.
Small CA (mm)	0.47 ± 0.11	0.48 ± 0.10	0.48 ± 0.12	n.s.

Results are expressed as mean±SEM.　Control, control group; Cortisol, cortisol group; RU,

RU group.　CA, coronary arteries.　n.s., not significant.

Online Table 3. Baseline Coronary Artery Diameters in the Withdrawal Protocol

	Day 8 (n=5)	Day 52 (n=5)	P value
Large CA (mm)	2.53 ± 0.37	2.53 ± 0.16	n.s.
Small CA (mm)	0.46 ± 0.23	0.47 ± 0.24	n.s.

Results are expressed as mean±SEM.   CA, coronary arteries.

Day 8, before the cessation of the cortisol treatment.   Day 52, 6 weeks after withdrawal.

n.s., not significant.

Online Table 4. Endothelium-Dependent Relaxations to Bradykinin in Vitro

Inhibitor(s)	Control (n=8)		Cortisol (n=8)		RU (n=5)		Withdrawal (n=5)	
	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max
Large CA								
No inhibitor	8.3 ± 0.1	96 ± 2	8.3 ± 0.1	99 ± 1	8.2 ± 0.1	99 ± 1	8.1 ± 0.1	99 ± 1
I	8.1 ± 0.1	98 ± 1	8.0 ± 0.1	97 ± 1	8.2 ± 0.1	98 ± 2	8.2 ± 0.1	97 ± 1
I+L	7.7 ± 0.1	81 ± 3	7.8 ± 0.1	77 ± 8	7.8 ± 0.1	84 ± 4	7.8 ± 0.1	72 ± 7
I+L+Apamin+CTx	ND	2 ± 1	ND	2 ± 1	ND	3 ± 1	ND	2 ± 0.4
Small CA								
No inhibitor	8.6 ± 0.1	99 ± 0.4	8.7 ± 0.1	96 ± 1	8.6 ± 0.1	98 ± 1	8.7 ± 0.2	99 ± 1
I	8.6 ± 0.1	99 ± 1	8.5 ± 0.1	96 ± 1	8.7 ± 0.1	98 ± 3	8.6 ± 0.1	99 ± 2
I+L	8.3 ± 0.1	96 ± 3	8.4 ± 0.1	96 ± 5	8.5 ± 0.2	96 ± 3	8.6 ± 0.1	98 ± 5
I+L+Apamin+CTx	8.0 ± 0.1	48 ± 5	8.3 ± 0.1	48 ± 7	8.2 ± 0.3	47 ± 10	8.4 ± 0.2	47 ± 11

Results are expressed as mean±SEM.   Control, control group; Cortisol, cortisol group; RU, RU group; Withdrawal, withdrawal group. EC_{50}, negative logarithm of half-maximal effective concentration of bradykinin (mol/L); Max, maximal relaxation to bradykinin (%); ND, not determined because of the markedly inhibited relaxation; I, indomethacin; L; N^{G}-monomethyl-L-arginine; CTx, charybdotoxin.

Online Table 5. Endothelium-Dependent Relaxations to Bradykinin after Acute Exposure to Cortisol in Vitro

Inhibitor(s)	Control (n=8)		Cortisol 30 µg/dL for 60 min (n=6)		Cortisol 10 µg/dL for 60 min (n=6)		Cortisol 30 µg/dL for 120 min (n=6)	
	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max
Large CA								
No inhibitor	8.1 ± 0.1	100 ± 0	8.2 ± 0.1	100 ± 0	8.2 ± 0.1	98 ± 1	8.1 ± 0.1	99 ± 0
I	8.1 ± 0.2	95 ± 3	8.1 ± 0.0	97 ± 1	8.1 ± 0.1	98 ± 1	8.2 ± 0.1	97 ± 1
I+L	7.7 ± 0.1	77 ± 4	7.9 ± 0.2	75 ± 11	7.8 ± 0.1	83 ± 3	7.8 ± 0.1	75 ± 5
I+L+Apamin+CTx	ND	3 ± 3	ND	1 ± 2	ND	3 ± 1	ND	2 ± 0
Small CA								
No inhibitor	8.6 ± 0.1	100 ± 0	8.6 ± 0.1	98 ± 1	8.6 ± 0.0	8.6 ± 0.1	100 ± 0	8.6 ± 0.1
I	8.5 ± 0.1	98 ± 1	8.5 ± 0.1	97 ± 1	8.6 ± 0.1	8.5 ± 0.1	98 ± 1	8.5 ± 0.1
I+L	8.4 ± 0.1	89 ± 4	8.5 ± 0.1	86 ± 5	8.5 ± 0.2	8.4 ± 0.1	89 ± 4	8.5 ± 0.1
I+L+Apamin+CTx	8.0 ± 0.2	50 ± 7	8.2 ± 0.1	69 ± 6	8.1 ± 0.3	8.0 ± 0.2	50 ± 7	8.2 ± 0.1

Results are expressed as mean±SEM.   Control, control group; Cortisol, cortisol group; RU, RU group; Withdrawal, withdrawal group. EC_{50}, negative logarithm of half-maximal effective concentration of bradykinin (mol/L); Max, maximal relaxation to bradykinin (%); ND, not determined because of the markedly inhibited relaxation; I, indomethacin; L; N^G-monomethyl-L-arginine; CTx, charybdotoxin.

Online Table 6. Endothelium-Independent Relaxations to Sodium Nitroprusside in Vitro

	Control (n=8)		Cortisol (n=8)		RU (n=5)		Withdrawal (n=5)	
	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max
Large CA	7.4 ± 0.1	98 ± 1	7.3 ± 0.1	96 ± 1	7.5 ± 0.1	96 ± 1	7.5 ± 0.1	98 ± 1
Small CA	7.9 ± 0.1	97 ± 1	7.8 ± 0.1	96 ± 2	7.7 ± 0.2	97 ± 1	7.8 ± 0.2	99 ± 1

Results are expressed as mean±SEM.   Control, control group; Cortisol, cortisol group; RU, RU group; Withdrawal, withdrawal group.   CA, coronary arteries; EC_{50}, negative logarithm of half-maximal effective concentration of sodium nitroprusside (mol/L); Max, maximal relaxation to sodium nitroprusside (%).

A Control Large CA

B Cortisol Large CA

Large CA

D Control Small CA

E Cortisol Small CA

Small CA