Protease-Activated Receptor-2 Activation Causes EDHF-Like Coronary Vasodilation

Selective Preservation in Ischemia/Reperfusion Injury: Involvement of Lipoxygenase Products, VR1 Receptors, and C-Fibers

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Abstract—Activation of protease-activated receptor (PAR)-2 has been proposed to be protective in myocardial ischemia/reperfusion (I/R) injury, an effect possibly related to an action on the coronary vasculature. Therefore, we investigated the effects of PAR2 activation on coronary tone in isolated perfused rat hearts and elucidated the mechanisms of any observed effects. Although having a negligible effect on ventricular contractility, the PAR2 activating peptide SLIGRL produced an endothelium-dependent coronary vasodilatation (ED50=3.5 nmol). Following I/R injury, the response to SLIGRL was selectively preserved, whereas the dilator response to acetylcholine was converted to constriction. Trypsin also produced a vasodilator dose-response curve that was biphasic in nature (ED50 1=0.36 U, ED50 2=38.71 U). Desensitization of PAR2 receptors indicated that the high potency phase was mediated by PAR2. Removal of the endothelium but not treatment with L-NAME (300 μmol/L), indomethacin (5 μmol/L), or oxyhemoglobin (10 μmol/L) inhibited the response to SLIGRL and trypsin. Treatment with the K+ channel blockers TEA (10 mmol/L), charybdotoxin (20 nmol/L)/apamin (100 nmol/L), or elevated potassium (20 mmol/L) significantly suppressed responses. Similarly, inhibition of lipoxygenase with nordihydroguaiaretic acid (1 μmol/L), eicosatetraynoic acid (1 μmol/L), or baicalein (10 μmol/L), desensitization of C-fibers using capsazepine (3 μmol/L) inhibited the responses. This study shows, for the first time, that PAR2 activation causes endothelium-dependent coronary vasodilation that is preserved after I/R injury and is not mediated by NO or prostanoids, but involves the release of an endothelium-derived hyperpolarizing factor (EDHF), possibly a lipoxygenase-derived eicosanoid, and activation of VR1 receptors on sensory C-fibers. (Circ Res. 2002;90:465-472.)

Key Words: protease-activated receptors • nitric oxide • prostanoid • endothelium-derived hyperpolarizing factor • ischemia/reperfusion injury

Recent evidence suggests that protease-activated receptors (PARs), particularly PAR2 have a role to play in inflammatory cardiovascular disease.1–4 PAR2 are G protein–coupled receptors, the endogenous ligand for which is a tethered peptide (SLIGRL in rodents and SLIGKV in humans) that is exposed after enzymatic cleavage of the N-terminal 36 amino acids. Trypsin is thought to be the primary enzyme responsible for this proteolysis,5 although other possibilities include mast cell tryptase6,7 and the coagulation factor Xa.8,9 PAR2 is widely distributed throughout the cardiovascular system1 including endothelial10–13 and smooth muscle cells.13,14 This distribution, together with studies using synthetic activating peptides with a sequence corresponding to the endogenous tethered ligand, suggest that PAR2 may play a role in the regulation of blood pressure and vascular tone.1 PAR2 activation causes vasodilatation in both arteries and veins (eg, see references 2, 10, 15, and 16), which, in many cases, involves the release of nitric oxide (NO).3 However, when activated in vivo PAR2 causes a hypotensive response that is only partially blocked by NO synthase (NOS) inhibition,15 indicating the involvement of NO-independent mechanisms.

Although a role for PAR2 in normal physiology is uncertain, there is mounting evidence to suggest a role in cardiovascular pathology. Endothelial PAR2 expression is upregulated by inflammatory cytokines,3,17 lipopolysaccharide treatment,15 balloon-catheter injury,18 and myocardial ischemia/reperfusion (I/R) injury.19 This upregulation is associated with enhanced PAR2-mediated vasodilatation in vitro3,15 and hypotension in vivo.15 Furthermore, PAR2 mediates vasodilatation in isolated human coronary arteries only after incubation with inflammatory cytokines.3,12 PAR2 activation has
several proinflammatory effects including edema formation\textsuperscript{20,21} and leukocyte recruitment.\textsuperscript{22} However, PAR2 may also be protective in certain pathologies, and there is some debate concerning the functional consequences of PAR2 activation.\textsuperscript{3} For example, PAR2-mediated vasodilation is selectively preserved in the cerebral artery in spontaneously hypertensive rats (SHR) (relative to the impaired responses to acetylcholine and bradykinin), suggesting a beneficial role for PAR2 during chronic hypertension.\textsuperscript{23}

A recent study demonstrated that PAR2 activation is protective in myocardial I/R injury promoting a recovery of coronary flow and myocardial function.\textsuperscript{20} It is possible that the protective action of PAR2 in myocardial I/R injury involves an effect on the coronary circulation similar to that seen in SHR cerebral arteries; i.e., preservation of PAR2-mediated coronary vasodilation leading to enhanced coronary flow to the compromised myocardium. Therefore, we investigated the effects of PAR2 activation on coronary tone before and after I/R injury in isolated rat hearts and elucidated the underlying mechanisms involved in the vasoactive response.

**Materials and Methods**

**Animal Preparation**

Following the Guide on the Operation of Animals (Scientific Procedures) Act 1986, male Wistar rats (250 to 300 g, Charles River, UK) were heparinized (sodium heparin, 250 iu, IP) and killed by cervical dislocation.

**Measurement of Coronary Hemodynamics and Cardiac Function**

Hearts were perfused in Langendorff mode as previously described\textsuperscript{24} (see also the expanded Materials and Methods online data supplement). Bolus doses (10 to 30 μL) of trypsin (0.03 to 30 U) or SLIGRL (0.3 to 100 nmol) were administered via the aorta and changes in coronary perfusion pressure (CPP) and left ventricular pressure (LVP) recorded.

PAR2 desensitization was achieved by an infusion of SLIGRL (20 μmol/L, 15 minutes) and confirmed by the reduced response to SLIGRL (30 nmol).

**Effects of I/R Injury**

After 20-minute equilibration, dilator responses to acetylcholine (3 nmol) and SLIGRL (100 nmol) were determined. Hearts maintained at 37°C were then subjected to global ischemia (25 minutes) by cessation of coronary flow. After restoration of flow, hemodynamic parameters were recorded for 60 minutes of reperfusion. The response to acetylcholine (3 nmol) and SLIGRL (100 nmol) on CPP were tested at 20-minute intervals.

**Mechanisms of PAR2 Responses**

The endothelium was removed by injection of Triton X-100 1% (60 μL).\textsuperscript{25} followed by a 20-minute recovery perfusion. Endothelium and vascular smooth muscle function were tested using bradykinin (0.03 nmol) and sodium nitroprusside (SNP, 10 nmol), respectively.

To investigate the involvement of NO and prostanoioids, dose-response curves were constructed in the absence and presence of either KCl (20 mmol/L),\textsuperscript{25} the nonselective K⁺ channel inhibitor, tetraethylammonium (TEA, 10 mmol/L), or the specific small- and intermediate-conductance K⁺ channel blockers charybdotoxin (CTX, 20 nmol/L) and apamin (100 nmol/L), respectively. To assess the involvement of lipoxynase products, hearts were treated with the nonselective lipoxynase inhibitors nordihydroguaiaretic acid (1 μmol/L),\textsuperscript{27} or eicosatetraynoic acid (1 μmol/L),\textsuperscript{28} The 12-lipoxygenase specific inhibitor, baicalein (10 μmol/L),\textsuperscript{29,30} was also tested.

To determine whether the effects of SLIGRL or trypsin involved activation of sensory C-fibers or vanilloid (VR1) receptors hearts were treated with capsaicin (1 μmol/L, 20 minutes,\textsuperscript{31} 30-minute recovery time) or capsazepine (3 μmol/L,\textsuperscript{32}) respectively.

**Reagents and Data Analysis**

For reagents and data analysis, refer to the expanded Materials and Methods section found in the online data supplement at http://www.circresaha.org.

**Results**

**Coronary Reactivity to SLIGRL and Trypsin**

SLIGRL (0.3 to 100 nmol) caused dose-dependent reductions in CPP (ED\textsubscript{50}=3.5 nmol [1.7 to 7.2]; E\textsubscript{max}=39.13 mm Hg [32.7 to 45.5], n=12; Figure 1A), without significantly affecting LV diastolic pressure, developed pressure, or heart rate (see Table). The nonactivating peptide LRGILS (0.1 to 100 nmol) had no effect on CPP (n=4). Trypsin (0.03 to 30 U) also caused dose-dependent reductions in CPP (Figure 1B). The dose-response curve to trypsin was analyzed using a nonlinear curve fitting program, and the model for an interaction with 2 independent sites provided a fit giving an \( R^2 \) value of 0.68 (better than the single site model, \( R^2=0.58 \)). The contribution of the interaction of trypsin with the high presence of either KCl (20 mmol/L),\textsuperscript{26} the nonselective K⁺ channel inhibitor, tetraethylammonium (TEA, 10 mmol/L), or the specific small- and intermediate-conductance K⁺ channel blockers charybdotoxin (CTX, 20 nmol/L) and apamin (100 nmol/L), respectively. To assess the involvement of lipoxynase products, hearts were treated with the nonselective lipoxynase inhibitors nordihydroguaiaretic acid (1 μmol/L),\textsuperscript{27} or eicosatetraynoic acid (1 μmol/L),\textsuperscript{28} The 12-lipoxygenase specific inhibitor, baicalein (10 μmol/L),\textsuperscript{29,30} was also tested.

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Cardiac Function and Coronary Hemodynamic Parameters

<table>
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<th>LVDP, mm Hg</th>
<th>LVDevP, mm Hg</th>
<th>HR, bpm</th>
<th>CPP, mm Hg</th>
<th>DEA/NO, Δmm Hg</th>
<th>Pinacidil, Δmm Hg</th>
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<td>242.2±4.8</td>
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Effects of Nω-nitro-L-arginine methyl ester (L-NNAME, 300 μmol/L), indomethacin (Indom, 5 μmol/L), capsaicin (1 μmol/L), capsazepine (3 μmol/L), nordihydroguaiaretic acid (NDG, 1 μmol/L), eicosatetraynoic acid (ETYA, 1 μmol/L), baicalein (10 μmol/L), tetrathylammonium (TEA, 10 mmol/L), KCl (K⁺, 20 mmol/L), charybdotoxin (CTX, 20 nmol/L)/apamin (100 nmol/L), ischemia/reperfusion (I/R) injury (25-minute ischemia/60-minute reperfusion), or SLIGRL (100 nmol) on left ventricular diastolic pressure (LVDP), left ventricular developed pressure (LVEDP), heart rate (HR), coronary perfusion pressure (CPP), and change (Δ) in CPP after pinacidil (10 nmol) or 2-(N,N-dimethylamino)-diazenolate-2-oxide (DEA/NO, 20 pmol) administration. N/A indicates not applicable. All values are expressed as mean±SEM for n=5 to 7 observations. *P<0.05 vs control.

Infusion of soybean trypsin inhibitor (5 μg/mL; Figure 2) completely abolished the responses to doses of trypsin below 3 U and inhibited the maximal response by 36% (n=4). HOE-140 (10 nmol/L) had no significant affect on the dose-response curve to trypsin (P>0.05, n=4; Figure 2).

Effects of I/R Injury

Prior to the induction of ischemia, acetylcholine and SLIGRL caused potent coronary vasodilation responses (Figure 3). After reperfusion, the response to acetylcholine was converted to vasoconstriction. In contrast, the vasodilator response to SLIGRL was largely maintained during reperfusion with full preservation observed at the end of the 60-minute reperfusion period (Figure 3). See Table for CPP and LV function values at the end of the reperfusion period.

Figure 2. Effects of PAR2 desensitization, trypsin inhibition, and B₂ receptor blockade on trypsin-induced coronary vasodilation. Vasodilator responses are expressed as change in coronary perfusion pressure (ΔCPP) in the rat isolated heart. Data obtained in the absence (○) or presence of PAR2 desensitization (●, SLIGRL, 20 μmol/L, 15 minutes), soybean trypsin inhibitor treatment (6 μg/mL, ■), or HOE 140 (10 nmol/L, ▲). Each point represents mean±SEM; n=5.

Figure 3. Effects of ischemia-reperfusion (I/R) injury on acetylcholine (ACh, 3 nmol) and SLIGRL (100 nmol)-induced coronary vasodilation. Vasodilator responses are expressed as change in coronary perfusion pressure (ΔCPP) prior to the induction of ischemia (control) and at 20, 40, and 60 minutes after reperfusion. Vasodilator responses to acetylcholine were converted to vasoconstriction after I/R injury, whereas responses to SLIGRL were largely preserved. Each bar represents mean±SEM; n=4.
Effects of nonspecific potassium channel blockade on (A) SLIGRL- and (B) trypsin-induced coronary vasodilatation. Vasodilator responses are expressed as change in coronary perfusion pressure (ΔCPP). Data obtained in the absence (●) or presence of TEA (○, 10 mmol/L) or elevated K⁺ (■, 20 mmol/L). All experiments were performed in the presence of L-NAME (300 μmol/L) and indomethacin (5 μmol/L). Each point represents mean±SEM; n=6.

Mechanisms of PAR2 Responses
Removal of the endothelium caused a significant reduction of the responses to SLIGRL (30 nmol) (from 21.7±2.9 to 0.6±0.6 mm Hg, n=4, P<0.05), trypsin (1 U) (from 29.7±1.9 to 3.2±0.9 mm Hg, n=4, P<0.05), and BK (0.03 nmol) (from 37.5±4.1 to 1.6±1.2 mm Hg, n=4, P<0.05) without affecting the response to SNP (10 nmol) (from 21.8±2.2 to 19.5±1.6 mm Hg, n=4, P>0.05).

L-NAME caused a small increase in CPP of 7.1±3.7 mm Hg (n=5; Table) and reduced the vasodilation response to acetylcholine (3 nmol) by 75±6.2% (P<0.05, n=4). L-NAME did not, however, modify the dose-response curves to SLIGRL or trypsin (P>0.05, n=5, 2-way ANOVA). The addition of indomethacin, without affecting CPP, had no effect on either SLIGRL or trypsin responses (n=5, P>0.05, 2-way ANOVA). Similarly, oxyhemoglobin had no effect on the curve to SLIGRL or the first phase of the trypsin dose-response curve (P>0.05, n=4). Oxyhemoglobin reduced the SNP response from 65.8±4.2 to 28.5±3.5 mm Hg (n=4, P<0.05).

In the presence of L-NAME and indomethacin, vasodilator responses to SLIGRL and trypsin were attenuated in the presence of elevated K⁺ (20 mmol/L, n=6; Figure 4). TEA (10 mmol/L, n=6) suppressed SLIGRL responses and the high-potency responses to trypsin (P<0.05; Figure 4). TEA treatment did not affect LV function or the vasodilation to DEA/NO (20 pmol), but inhibited the response to pinacidil (10 nmol) (Table). Elevated K⁺ significantly affected LV function and reduced the vasodilation to both DEA/NO (20 pmol) and pinacidil (10 nmol) (Table). The combination of CTX (20 nmol/L) and apamin (100 nmol/L) blocked SLIGRL-induced vasodilatation (n=5; Figure 5) while not affecting basal cardiac function or responses to DEA/NO or pinacidil. The effects of elevated K⁺, TEA, CTX/apamin on LV function and CPP can be found in the Table.

Desensitization of C-fibers using capsaicin or blockade of VR1 receptors using capsazepine significantly reduced SLIGRL- and trypsin (first phase)-induced coronary vasodilatation (n=6; Figure 6). Capsaicin did not significantly affect LV function (Table) but caused an increase in CPP of 44.6±13.7 mm Hg (n=6), followed by a subsequent decrease of 32.8±9.5 mm Hg (n=6). Twenty minutes after cessation of capsaicin treatment, CPP had returned to its precapsaicin levels (Table). Capsazepine treatment had no significant effect on LV function or CPP (P>0.05, n=6; Table). The vasodilator responses to DEA/NO (20 pmol) or pinacidil (10 nmol) were not significantly affected by either capsaicin or capsazepine treatment (P>0.05, n=5; Table).

In the presence of L-NAME and indomethacin, inhibition of lipoxygenase using nordihydroguaiaretic acid, eicosatetraenoic acid, or baicalein, although not affecting basal LV function, CPP, or vasodilator responses to DEA/NO or pinacidil (Table), significantly reduced the coronary vasodilation responses (Figure 7, n=5).

Administration of 12-hydroperoxyeicosatetraenoic acid (HpETE) caused a small but dose-dependent reduction in coronary perfusion pressure (EC₅₀=1.1 nmol [0.05 to 25.1]; E₉⁰=−14.0±2.4 mm Hg, n=4). The dose-response curve to 12-HpETE was significantly attenuated in the presence of capsazepine (3 μmol/L) causing a 4.8-fold dextral shift in the dose-response curve and an 81.4% reduction in E₉⁰ (P<0.05, n=4).

Discussion
This study shows that PAR2 activating peptides and trypsin, without significantly altering LV function, cause endothelium-dependent vasodilation in the perfused coronary circulation. Our data support the concept that this response is...
mediated by a lipoxygenase-derived EDHF and independent of NO and prostanoids. In addition, the PAR2 responses are mediated in part via the activation of VR1 receptors located on sensory C-fibers (for review, see references 33 and 34) most likely by the activity of a lipoxygenase product. These findings show that PAR2 activation profoundly decreases coronary vascular resistance, which may be the property on which the recently identified protective effect of SLIGRL in myocardial I/R injury19 is dependent. Indeed, our data shows that the coronary vasodilation response to SLIGRL is selectively preserved after I/R injury despite the existence of confirmed endothelial dysfunction (conversion of acetylcholine-induced dilation to constriction). Thus these findings identify a novel pathway activated by PAR2 that may be important in protection against I/R injury and offers novel therapeutic targets in the treatment of ischemic heart disease.

SLIGRL is a highly selective PAR2 activator and this has been demonstrated in many systems including PAR2−/− mice.35 The effect of SLIGRL on coronary perfusion pressure was selective because the control peptide, LRGILS, had no effect. Similarly, trypsin produced potent dose-dependent coronary vasodilation. Interestingly, the dose-response curve to trypsin was biphasic in nature, due in part to PAR2 activation. PAR2 involvement was verified because desensitization of the receptors with SLIGRL suppressed the Emax of the high potency phase of the trypsin dose-response curve by approximately 73%. In contrast, the low potency phase of this curve was unaffected, indicating that this phase is mediated by a mechanism independent of PAR2. Confirmation that the activity of trypsin was due to proteolytic cleavage was provided by the studies using soybean trypsin inhibitor,10 which abolished the first phase responses to trypsin. This biphasic activity of trypsin is similar to that observed in other vascular preparations including porcine coronary arteries10 and rat pulmonary arteries,36 implying that trypsin is selective for PAR2 at low concentrations (<20 U/mL), but at high concentrations, other PAR2-independent mechanisms are involved. Proposed candidates for this component are trypsin-induced cleavage of PAR136 or kininogen cleavage57 leading to kinin synthesis. The latter possibility seems unlikely because the B2 antagonist, HOE-140, did not affect responses to trypsin. Thus, a major component of the vascular response to trypsin in the rat coronary circulation is mediated by activation of PAR2. However, definitive proof for a role for PAR2 awaits the availability of selective PAR2 antagonists.

Coronary endothelial dysfunction is thought to be one of the critical events in the development of myocardial I/R injury.38–41 Reduced responses to endothelium-dependent dilators, including thrombin,38 acetylcholine,42 and serotonin,43 have been demonstrated following I/R injury. Unlike these ischemia-sensitive vasodilators, we demonstrate that PAR2 responses are resistant to I/R injury, and this may be the property on which the protection afforded by SLIGRL in I/R injury is dependent.19 The preservation of SLIGRL activity is not due to a nonspecific increase in coronary

Figure 6. Effects of C-fiber desensitization and vanilloid receptor (VR1) blockade on (A) SLIGRL- and (B) trypsin (first phase)-induced coronary vasodilatation. Vasodilator responses are expressed as change in coronary perfusion pressure (ΔCPP). Data obtained in the absence (○) or presence of capsaicin desensitization (●, 1 μmol/L, 20 minutes) or capsazepine treatment (▲, 5 μmol/L). All experiments were performed in the presence of L-NAME (300 μmol/L) and indomethacin (6 μmol/L). Each point represents mean ± SEM; n=6.

Figure 7. Effects of lipoxygenase inhibition with nordihydroguaiaretic acid, eicosatetraynoic acid, or baicalein on (A) SLIGRL- and (B) trypsin (first phase)-induced coronary vasodilatation. Vasodilator responses are expressed as change in coronary perfusion pressure (ΔCPP). Data obtained in the absence (○) or presence nordihydroguaiaretic acid (○, 1 μmol/L), eicosatetraynoic acid (●, 1 μmol/L), and baicalein (▲, 10 μmol/L). Each point represents mean ± SEM; n=5.
vascular smooth muscle sensitivity, as the response to the endothelium-independent coronary vasodilator SNP is unaffected by I/R injury in this model.42,43 One explanation for this selective preservation is that endothelial PAR2 expression is upregulated by I/R injury in rat isolated hearts,19 thus enhancing receptor reserve. Another possibility is that the mechanisms involved in PAR2 responses are resistant to I/R injury. To determine whether this latter possibility is the case, we dissected the mechanisms involved in PAR2 coronary vasodilation.41

Consistent with other studies showing endothelium-dependency,3 SLIGRL- and trypsin-induced vasodilator responses were inhibited by endothelium removal. This requirement for an intact endothelium was independent of either NO or prostanooids because inhibition of COX and NOS with well-established doses of indomethacin and L-NAME24 or scavenging of NO with oxyhemoglobin had no effect on the PAR2-mediated responses. This is in contrast to other studies where PAR2 vasodilatation is sensitive to NOS inhibition and therefore mediated by NO. A possible reason for this difference may relate to the size of the vessel studied. The majority of studies investigating the mechanisms of PAR2 vasodilatation have been conducted on primary order conduit vessels.10,16,23 Coronary perfusion pressure is, in the main, determined by the activity of the microvascular networks rather than the conduit arteries, and it is clear that the role of NO and prostanooids in endothelium-dependent responses diminishes as vessel diameter decreases.44 Indeed, other PAR2-mediated effects in the microvasculature are insensitive to NOS or COX inhibition. For example, SLIGRL-induced edema formation21 and hypotension,15 both responses occurring at the level of the resistance microvasculature, are not blocked by NOS or COX inhibition.

In most cases where NO and prostanooids have been excluded as mediators of endothelium-dependent relaxation, a third endothelium-derived substance, termed EDHF, is implicated.45 Accordingly, we have demonstrated that high extracellular K+, TEA (a nonselective K+-channel blocker), and the specific small- and intermediate-conductance Kcs channel blockers, apamin and CTX, treatments that inhibit K+ channel activity, membrane hyperpolarization and the subsequent vascular relaxation46,47 attributed to EDHF, significantly reduced the PAR2-mediated vasodilation. These data suggest that PAR2 vasodilator responses involve hyperpolarization of vascular smooth muscle cells and implicate EDHF as a mediator of this response. It is possible that the inhibitory effect of high K+ on the PAR2 response is due to a nonspecific effect on cardiac hemodynamics rather than its intended inhibition of vascular smooth muscle K+ channels. However, our data with other more specific K+ channel blockers TEA, CTX and apamin, which have no effect on other cardiac parameters but do block PAR2 responses, would suggest otherwise. The fact that the responses are EDHF-like and NO-independent provides a possible explanation for the preservation of the SLIGRL responses following I/R injury, ie, they are not affected by the impaired release of endothelial NO that occurs after I/R injury.48

Recent evidence demonstrates that primary sensory afferent neurons express PAR2; the activation of which stimulates the release of neuropeptides.49 We tested the hypothesis that SLIGRL-induced coronary vasodilation response is mediated by this neurogenic mechanism. Indeed, SLIGRL- and trypsin-induced coronary vasodilation were attenuated in hearts that had been subjected to a capsaicin treatment protocol that desensitizes sensory C-fibers.31 The response is possibly mediated by the direct activation of PAR2 expressed on C-fibers and the subsequent release of sensory neuropeptides (eg, calcitonin gene-related peptide [CGRP] and substance P). However, a direct action on neuronal PAR2 is unlikely for several reasons. First, the known pharmacology of the sensory neuropeptides is inconsistent with the above hypothesis. Substance P–induced coronary vasodilator responses are NO-mediated.34,50 If SLIGRL were acting at PAR2 on C-fibers to release substance P, one would expect this to involve NO, which is at odds with our data showing NO-independence. Furthermore, in contrast to the marked response to PAR2 activation, substance P is only weakly active in the rat perfused coronary circulation41 (unpublished observation, 2001). On the other hand, CGRP is a potent coronary vasodilator,51 but if this peptide were released in this manner, it would be expected to act in an endothelium-independent manner,34,50 again contrary to our data showing that the response is dependent on an intact endothelium. Another possible explanation is provided by the recently identified interaction between endothelium-derived substances and the VR1 expressed on C-fibers.33,34 Indeed, SLIGRL- and trypsin-induced vasodilation were profoundly attenuated by the VR1 selective antagonist capsazepine,32 which to our knowledge is not a PAR2 antagonist. These data support a sequence of events whereby SLIGRL (and trypsin) acts at PAR2 expressed on the coronary endothelium, causing the release of an endothelium-derived substance that in turn activates VR1 on C-fibers. It is unlikely that trypsin directly activates C-fibers because it is a large protein and not able to act extravascularly.

Recently, it has been demonstrated that lipoxygenase-derived eicosanoids activate VR1 receptors.27,52 Among these eicosanoids, the 12-lipoxygenase–derived product, 12-hydroxyeicosatetraenoic acid (HpETE) is the most potent. This eicosanoid is released by the endothelium and exhibits EDHF-like properties.26 In light of this, we investigated whether PAR2 coronary vasodilation may be secondary to release of an endothelial-derived lipoxygenase product. Indeed the structurally unrelated lipoxygenase inhibitors nordihydroguaiaretic acid27,28 and eicosatetraynoic acid28 suppressed PAR2-mediated coronary vasodilation, without affecting the response to DEA/NO or pinacidil. Furthermore, the flavonoid baicalein,29 a specific 12-lipoxygenase inhibitor, suppressed SLIGRL-induced vasodilation. In addition, 12-HpETE produced a moderate dilator response that, such as SLIGRL, is sensitive to capsazepine. Anandamide is also a candidate mediator given that it is a vasodilator released from the endothelium and can activate VR1 receptors on C-fibers.33 However, in the present study anandamide was not released into the coronary effluent following SLIGRL administration (unpublished observation, 2001), suggesting that this
substance is unlikely to be involved in the PAR2 response. Taken together, these data suggest that a lipoxigenase-derived eicosanoid is released from the endothelium following PAR2 activation that activates either the vascular smooth muscle directly and/or the VR1 receptor on C-fibers to produce the resultant dilator response. Further studies are required to confirm which of the lipoxigenase-derived eicosanoids is involved in PAR2-mediated coronary vasodilation; however, 12-HpETE, being the most potent activator of the VR1 receptor, a likely candidate.

Interestingly, both the 12-lipoxygenase pathway and C-fibers have been implicated in endogenously activated mechanisms of protection against I/R injury and possibly provide a rational for the protective effect of SLIGRL in I/R injury. Whether PAR2 is activated by its endogenous proteases during I/R in vivo is an interesting possibility and warrants further investigation. Our data show that SLIGRL is a potent coronary vasodilator, and this response is selectively preserved after I/R injury, thus suggesting that PAR2-mediated coronary vasodilation is an important factor in the protection afforded by SLIGRL. This selective preservation of SLIGRL activity is possibly related to the mechanism identified (ie, NO-independent) and thus not affected by the impaired release of endothelial NO that occurs after I/R injury. PAR2-mediated vasodilation is also selectively preserved (relative to the impaired response to acetylcholine and bradykinin) in the cerebral artery in spontaneously hypertensive rats. However, the PAR2-mediated response in this vessel is NO-mediated, raising the possibility that PAR2 responses are selectively preserved in pathologies associated with endothelial dysfunction, irrespective of the mechanism involved.

In summary, this study describes a PAR2-mediated, endothelium-dependent coronary vasodilation in the rat isolated heart that is selectively preserved following I/R injury. The responses to both SLIGRL and trypsin are independent of NO or prostanooids. However, the PAR2 response is sensitive to treatment with CTX and apamin and TEA and high extracellular potassium, indicating involvement of EDHF. C-fibers also play a role in the response because capsaicin and capsazepine inhibited the responses. It is likely that the endothelium-derived mediator is a lipoxigenase-derived eicosanoid because treatment with 3 structurally unrelated lipoxigenase inhibitors (nordihydroguaiaretic acid, eicosatetraynoic acid, and baicalein) suppressed the response. The present study suggests that PAR2 activation contributes to NO-independent regulation of coronary tone and identifies a novel vasodilator mechanism of action. The protective role of PAR2 in I/R injury, together with the observed preservation of PAR2-mediated dilation during reperfusion, suggests a beneficial role for this receptor in I/R injury. Future studies will determine whether PAR2 is involved in the regulation of the human coronary circulation and whether the receptor is involved in human I/R injury.

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Protease-Activated Receptor-2 Activation Causes EDHF-Like Coronary Vasodilation: Selective Preservation in Ischemia/Reperfusion Injury: Involvement of Lipoxygenase Products, VR1 Receptors, and C-Fibers

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**Materials and Methods**

**Animal Preparation**

Experiments were performed in accordance with the ‘Guide on the Operation of Animals (Scientific Procedures) Act 1986’. Male Wistar rats (250 - 300g, Charles River, UK) were heparinized (sodium heparin, 250iu, i.p.) and killed by cervical dislocation. Hearts were rapidly excised and placed in ice-cold perfusion solution (constituents below) bubbled with 95% O₂ / 5% CO₂ prior to coronary perfusion pressure (CPP) measurement.

**Measurement of Coronary Hemodynamics**

Hearts were perfused in Langendorff mode as previously described\(^1\). Briefly, the aorta was cannulated and perfused retrogradely under constant flow (10 ml/min) using a calibrated roller pump (Gilson, Minipuls-2) with a buffer solution of the following composition (mM): D(+)glucose, 11.1; CaCl₂, 1.4; NaCl, 118.5; NaHCO₃, 25.0; MgSO₄, 1.2; NaH₂PO₄, 1.2 and KCl, 4.0; gassed with 95% O₂/5% CO₂, (pH 7.4) warmed to 37°C. The CPP in the aortic line was monitored by a Statham Spectramed pressure transducer connected to a PowerLab. Air temperature was maintained by means of a heated (37°C) water jacket. On establishing a stable CPP (20-30 min following cannulation), bolus doses (10-30 µl) of either trypsin (0.03-30 U), or the PAR2 activating peptide SLIGRL (0.3-100 nmol) were administered via the aorta and changes in CPP recorded. Hearts with a CPP less than 60 mmHg were excluded. Endothelial integrity was established with a bolus injection of the endothelium-dependent vasodilator bradykinin (0.03nmol) given before the coronary reactivity experiments. Other substances were infused at 0.1 ml/min.

**Measurement of Left Ventricular Pressure**

Left ventricular pressure and dP/dT were continually recorded using a fluid filled latex balloon inserted in to the left ventricle (LV) via the left atrium and mitral valve. The LV diastolic pressure was set at a level to generate the maximum developed isovolumetric pressure
(developed pressure = systolic – diastolic pressure). LV diastolic pressure was generally set between 5-10 mmHg.

**SLIGRL- and Trypsin-induced vasodilation**

To determine whether the actions of trypsin involved PAR2 activation a receptor desensitization protocol was utilised. Desensitization was achieved by an infusion of SLIGRL (20µM, 15 min). A bolus dose of SLIGRL (30 nmol) was given before and after infusion to confirm PAR2 desensitisation. Trypsin dose-response curves (0.03 – 30 U) were then constructed in hearts in which PAR2 was desensitised.

Trypsin dose-response curves were also constructed in the presence and absence of Soybean trypsin inhibitor (5µg/ml) to confirm that activity of trypsin was proteolysis-dependent.

To determine whether the actions of trypsin were due to kinin release dose-response curves to trypsin were constructed in the presence or absence of the kinin B receptor antagonist HOE-140 (10nM). Bradykinin (0.03nmol) was given before and after equilibration with HOE-140 to confirm receptor blockade.

**Effects of ischemia-reperfusion injury on PAR2 responses.**

After a 20-min equilibration period, the dilator responses to acetylcholine (3nmol) and SLIGRL (100 nmol) were recorded. Hearts were then subjected to 25 minutes of global ischemia by cessation of coronary flow and maintained at 37°C. Following restoration of flow hemodynamic parameters were recorded for 60 min of reperfusion. The response to acetylcholine (3nmol) and SLIGRL (100 nmol) on CPP were tested at 20 min intervals during reperfusion

**Mechanisms of PAR2 responses**

*Determination of endothelium-dependency.* The endothelium was removed by injection of a bolus dose of Triton X-100 1%, 60 µl) into the perfusion line followed by a 20 min recovery
perfusion. Endothelium and vascular smooth muscle function were tested before and after Triton-X treatment using bradykinin (0.03 nmol) and the endothelium-independent vasodilator SNP (10 nmol) respectively. Trypsin- and SLIGRL-induced responses were determined before and after Triton-X treatment.

**Involvement of Endothelial Mediators.** In order to investigate the involvement of NO and prostanoids in mediation of the responses to SLIGRL and trypsin, dose-response curves were constructed in the absence and presence of either N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, 300 µM, 20 min pre-treatment) to inhibit NOS and/or the cyclooxygenase inhibitor indomethacin (5 µM, 20 min pre-treatment) to inhibit prostaglandin synthesis. The NO-scavenger oxyhaemoglobin (10 µM) was also used to investigate possible NOS-independent NO pathways. All subsequent experiments were conducted in the presence of L-NAME and indomethacin. To assess the involvement of endothelium-derived hyperpolarising factor (EDHF)-like pathways curves were constructed in the absence and presence of either KCl (20 mM),\textsuperscript{3} the non-selective K\textsuperscript{+} channel inhibitor, tetraethylammonium (TEA, 10mM) or the specific small- and intermediate-conductance K\textsubscript{Ca} channel blockers charybdotoxin (CTX, 20 nM) and apamin (100 nM) respectively. To assess the possible involvement of lipoxygenase products hearts were treated with the non-selective lipoxygenase inhibitors nordihydroguaiaretic acid (1 µM)\textsuperscript{4} or eicosatetraynoic acid (1 µM)\textsuperscript{5}. The 12-lipoxygenase specific inhibitor, baicalein (10 µM)\textsuperscript{6,7} was also tested.

**Involvement of C fibres and vanilloid receptors.** To determine whether the effects of SLIGRL or trypsin involved activation of sensory C fibres or vanilloid (VR\textsubscript{1}) receptors hearts were treated with capsaicin (1 µM, 20 min,\textsuperscript{8} 30 min recovery time) or capsazepine (3 µM,\textsuperscript{9}) respectively.

The specificity of each of the various agents was determined by testing the vasodilator effects of the potassium channel opener pinacidil (10nmol) or the NO-donor 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO, 20 pmol) in the absence and presence of inhibitor.
Data Analysis

**Description of dose-response curves** The responses to trypsin and SLIGRL are expressed as a percentage of their individual maxima. ED$_{50}$ values were calculated from the 50% response level and expressed as geometric mean with 95% confidence limits in parentheses using a non-linear curve fitting analysis (Graph Pad Prism 2.01). The biphasic dose-response curves to trypsin were described as follows: For each fraction of the response contributing to the total response, the maximum was calculated and expressed as $E_{\text{max-1}}$ and $E_{\text{max-2}}$. The doses of trypsin that produced half of $E_{\text{max-1}}$ and $E_{\text{max-2}}$ were denoted $ED_{50-1}$ and $ED_{50-2}$ respectively. Monophasic concentration-effect curves were analysed by fitting a four parameter logistic equation to the data to obtain location and slope parameters. The equation is:

$$y = a + b/\{1 + 10^{-d(c+\log[A])}\},$$

Biphasic curves were analysed by fitting a modified four parameter logistic equation:

$$y = a + b\{(f / 1 + 10^{c1+\log[A]}) + (1 - f / 1 + 10^{c2+\log[A]})\}$$

where $A$ is the agonist concentration, $a$ is the basal value, $b$ is the vertical range, $c$ is the pED$_{50}$ ($c1$ is pED$_{50-1}$ and $c2$ is pED$_{50-2}$), $d$ is the mid-point slope and $f$ is the fraction of the receptors that are activated with a potency described by $c1$. The remainder of the receptors are activated with a potency described by $c2$. The data points were fitted to the equations using a non-linear curve fitting analysis program (Graph Pad Prism, Graph Pad Software Inc., San Diego, USA).

All other data are given as arithmetic mean±s.e. mean or geometric mean with 95% confidence limits. The number of observations is indicated by $n$. The significance of differences between values was determined by use of Student’s paired or unpaired $t$ test. Dose-response curves were compared using two-way ANOVA (analysis of variance, Graph Pad Prism, Graph Pad Software Inc., San Diego, USA). The criterion for statistical significance was $P<0.05$.

Reagents

Oxyhaemoglobin was prepared as follows: 1mM of commercial human haemoglobin was reduced using an excess of sodium dithionate (Na$_2$S$_2$O$_4$). This reducing agent converted methaemoglobin into oxyhaemoglobin and was subsequently removed by column separation.
using sephadex (G-25). SLIGRL was a generous gift from Dr Nathalie Vergnolle (University of Calgary, Canada). Indomethacin was dissolved in 1% sodium carbonate (Na₂CO₃) solution. Capsaicin and capsazapine were dissolved in 100% ethanol to 10 mM, diluted to 1 mM in 50% ethanol and further diluted in saline. Nordihydroguaiaretic acid was dissolved in 95% ethanol (100 mM) and diluted in saline. Pinacidil was diluted in DMSO (10mM) and further diluted in saline. Charybdotoxin and apamin were obtained from Alomone labs (Jerusalem, Israel). L-NAME, indomethacin, TEA, human haemoglobin, bradykinin, sodium nitroprusside, capsaicin, capsazepine, nordihydroguaiaretic acid, pinacidil and soybean trypsin inhibitor were purchased from Sigma Chemical Company (Dorset, UK). 2-(N-N-diethylamino)-diazenolate-2-oxide (DEA/NO) was from Cayman Chemicals (Alexis, UK). 12(S)-HpETE (Cayman Chemical) was obtained in ethanol (50 µg/500 µl), protected from light, and administered undiluted.

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