Selective Activation of the Prostanoid EP\textsubscript{3} Receptor Reduces Myocardial Infarct Size in Rodents

Kai Zacharowski, Antje Olbrich, Julie Piper, Gerd Hafner, Kigen Kondo, Christoph Thiemermann

Abstract—The cardioprotective effects of E-type prostaglandins (EPs) have been attributed to vasodilatation, inhibition of platelet and neutrophil function (EP\textsubscript{3} mediated), and an unknown "cytoprotective effect." We have hypothesized that selective activation of EP\textsubscript{3} receptors may cause cardioprotection. The prostanoid derivative ONO-AE-248 selectively binds to murine EP\textsubscript{3} receptors expressed in Chinese hamster ovary (CHO) cells (K\textsubscript{a}, 15 mmol/L) and prevents the rise in cAMP caused by forskolin in CHO cells (IC\textsubscript{50} \approx 1 mmol/L) in which the EP\textsubscript{3} receptor had been expressed. In anesthetized rats subjected to regional myocardial ischemia for 25 or 45 minutes and 2 hours of reperfusion, infusion of ONO-AE-248 (5 \mu g \cdot kg\textsuperscript{-1} \cdot min\textsuperscript{-1} IV) caused a significant reduction in infarct size, from 60±6% (n=8) to 36±6% (n=7) and from 78±2% (n=11) to 58±4% (n=9), respectively. The reduction in infarct size caused by ONO-AE-248 in rats subjected to 25 minutes of ischemia and reperfusion was abolished by a selective inhibitor of ATP-sensitive potassium (K\textsubscript{ATP}) channels, 5-hydroxydecanoate (n=6), and the protein kinase C inhibitors staurosporine (n=6) and chelerythrine (n=6). In anesthetized rabbits subjected to coronary artery occlusion for 45 or 60 minutes and 2 hours of reperfusion, infusion of ONO-AE-248 (5 \mu g \cdot kg\textsuperscript{-1} \cdot min\textsuperscript{-1} IV) caused a significant reduction in infarct size, from 61±2% (n=10) to 36±4% (n=8) and from 63±4% (n=7) to 42±4% (n=7), respectively. The reduction in infarct size caused by ONO-AE-248 in the rabbit was also abolished by 5-hydroxydecanoate. The cardioprotective effect of ONO-AE-248 in rats or rabbits was not associated with any hemodynamic effects. Selective activation of the prostanoid EP\textsubscript{3} receptor reduces myocardial infarct size in rodents by a mechanism(s) that may involve the activation of protein kinase C and the opening of K\textsubscript{ATP} channels. (Arterioscler Thromb Vasc Biol. 1999;19:2141-2147.)

Key Words: E-type prostaglandin–receptor agonist myocardial injury rodents protein kinase C ATP-sensitive potassium channels

The effects of E-type prostaglandins (EPs) are mediated by specific G protein–coupled receptors that activate intracellular signal transduction pathways. EP receptors have been classified into 4 subtypes, EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4}.\textsuperscript{1} In principle, EP\textsubscript{1} receptors may be coupled to G\textsubscript{a}, G\textsubscript{b}, and G\textsubscript{c} proteins.\textsuperscript{1} The G-protein coupling of EP\textsubscript{3} receptors depends on posttranscriptional splicing. The known splice variants (eg, EP\textsubscript{3b}) of this receptor family differ within their C-terminal amino acid domain, which appears to determine the class of G protein that responds to receptor stimulation.\textsuperscript{2-4} There is evidence that the EP\textsubscript{3a} and EP\textsubscript{3b} receptor are coupled to G\textsubscript{i}.\textsuperscript{5} Interestingly, EP\textsubscript{3} receptors are expressed on cardiac sarcolemmal membranes and appear to inhibit adenylyl cyclase activity via G\textsubscript{i}.\textsuperscript{6}

EPs including prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) exert beneficial effects on biochemical, electrocardiographic, and functional indices of myocardial ischemia/reperfusion injury\textsuperscript{7-9} and reduce myocardial infarct size.\textsuperscript{10} The cardioprotective effects of these eicosanoids may be secondary to a reduction in afterload, an increase in coronary blood flow, inhibition of platelet function, and/or inhibition of the activation and extravasation of polymorphonuclear cells.\textsuperscript{11} All of these effects are secondary to the activation of EP\textsubscript{2} receptors, which activate G\textsubscript{i} and cause an activation of adenylyl cyclase.\textsuperscript{1} In addition, the protection of isolated cells or organs by prostaglandins has been attributed to an ill-defined "cytoprotective" or "cardioprotective" effect of these agents. The mechanism(s) or the prostanoid receptor(s) mediating this effect is unknown.\textsuperscript{12}

In 1995 through 1996, we discovered that the cardioprotective effects of EPs are due, at least in part, to activation of EP\textsubscript{1} or EP\textsubscript{3} receptors, which in turn leads to the opening of ATP-sensitive potassium (K\textsubscript{ATP}) channels.\textsuperscript{10,13} This hypothesis is supported by the following findings: (1) The cardioprotective effects of PGE\textsubscript{1} (nonselective agonist for all EP receptors) and sulprostone (selective agonist of EP\textsubscript{1} and EP\textsubscript{3} receptors) are abolished by inhibition of K\textsubscript{ATP} channels with glibenclamide or 5-hydroxydecanoate (5-HD).\textsuperscript{10,13} (2) Sulprostone causes cardioprotection without having any hemodynamic (EP\textsubscript{2}-mediated) effects.\textsuperscript{13} (3) Activation of EP\textsubscript{1} and...
EP$_3$ receptors may result in activation of protein kinase C (PKC) and the opening of K$_{ATP}$ channels.$^{1,14}$ Because EP$_3$ receptors are expressed on cardiomyocytes and are upregulated after ischemia of the heart,$^{6,15}$ we hypothesized that it is the activation of EP$_3$, rather than of EP$_1$, receptors that accounts for the cardioprotective and/or cytoprotective effects of EPs.

The overall aim of this study was to elucidate whether selective activation of prostanoid EP$_3$ receptors reduces myocardial infarct size in anesthetized rodents. We report the discovery of a prostanoid derivative, ONO-AE-248 (Figure 1), which selectively binds and activates EP$_3$ receptors in Chinese hamster ovary (CHO) cells in which this receptor has been expressed. Subsequently, we have investigated whether ONO-AE-248 reduces the infarct size caused by regional myocardial ischemia and reperfusion in anesthetized rats and rabbits. In addition, we have investigated whether the observed reduction in infarct size caused by ONO-AE-248 is attenuated by the K$_{ATP}$-channel blocker 5-HD (in rats and rabbits) or by the PKC inhibitors staurosporine and chelerythrine (rats).

**Methods**

**Receptor Binding Studies**

Cloned prostanoid receptors (EP$_1$, EP$_2$, EP$_{3a}$, EP$_a$, FP, TP, and IP) were expressed (separately) in CHO cells. The transfection of the respective cDNAs into CHO cells was performed as previously described.$^{16}$ In brief, CHO cells were cultured for 72 hours, and thereafter, membrane fractions were prepared.$^{16}$ The standard assay mixture used in the subsequent binding studies contained the following: (1) [H]PGE$_2$ or [H]PGF$_2\alpha$ (2.5 nmol/L) or [H]iloprost or [H]SQ29548 (5 nmol/L); (2) 10 to 30 mg of membranes obtained from CHO cells expressing the following prostanoid receptors: murine EP$_1$, EP$_2$, EP$_{3a}$, EP$_a$, and FP as well as human TP or IP; (3) 200 mL of either buffer A (used for the binding studies involving [H]PGE$_2$ or [H]PGF$_2\alpha$ and comprising 10 mmol/L potassium phosphate, 1 mmol/L EDTA, 10 mmol/L MgCl$_2$, and 100 mmol/L NaCl, pH 6.0) or buffer B (used for the binding studies involving [H]iloprost or [H]SQ29548 and comprising 10 mmol/L Tris·HCl and 100 mmol/L NaCl, pH 7.4). After incubation for 1 hour ([H]PGE$_2$ or [H]PGF$_2\alpha$) or 30 minutes ([H]iloprost or [H]SQ29548) at room temperature, the reaction was terminated by the addition of 2 mL of ice-cold buffer (A or B, as appropriate), after which the mixture was rapidly filtered through a Whatman GF/B glass filter to remove unbound label. The filter was then washed 3 times with the same buffer, and the radioactivity was measured by scintillation counting (ACS-II, Amersham). The specific binding was calculated by subtracting the nonspecific binding from the total binding. The dissociation constants (K$_d$) were calculated from Scatchard plots. The concentration of ONO-AE-248 that displaced 50% of the specific binding of the respective prostanoids (IC$_{50}$) was then calculated. The respective K$_d$ values (inhibition constant values) were calculated by using the following equation: $K_d=IC_{50}/(1+[C]/[R])$, in which [C] equals the concentration of the respective radioligand used.

**Measurement of Forskolin-Stimulated cAMP Formation in CHO Cells**

Activation of EP$_{3a}$ or EP$_a$ receptors with specific agonists results in a reduction of the intracellular levels of cAMP (see introduction). To elucidate whether ONO-AE-248 attenuates the rise in cAMP caused by forskolin in CHO cells expressing murine EP$_3$ receptors, these cells were seeded (10$^5$ per well) in 24-well plates and cultured for 48 hours. Cells were washed with modified Eagle’s medium containing 1% (wt/vol) BSA and 10 mmol/L HEPES-NaOH buffer (pH 7.4) and subsequently preincubated (10 minutes at 37°C) in 450 mL of this buffer containing 3-isobutyl-1-methylxanthine (1 mmol/L). Modified Eagle’s medium (50 mL) containing 10 mmol/L forskolin and appropriate concentrations of ONO-AE-248 or PGE$_2$ were then added and incubated for 10 minutes at 37°C. The reaction was terminated by addition of 500 mL of 10% (wt/vol) trichloroacetic acid solution, and the generated cAMP was determined by radiomunnoassay. The amounts of cAMP generated by forskolin in the presence of PGE$_2$, or ONO-AE-248 were expressed as a percent of the amounts of cAMP generated by forskolin alone (control). The cDNAs of the prostanoid receptors were obtained from Dr S. Narumiya and Dr A. Ichikawa of Kyoto University, Kyoto, Japan.

**Rodent Models of Myocardial Infarction**

Ninety-five male Wistar rats (240 to 350 g; Tucks, Reyleigh, Essex, UK) were anesthetized (thiopentone sodium, 120 mg/kg IP). After tracheotomy and ventilation (70 strokes/min; tidal volume, 8 to 10 mL/kg; inspiratory oxygen concentration, 30%; positive end-expiratory pressure, 1 to 2 mm Hg resulting in PCO$_2$ values of 36 to 44 mm Hg and PO$_2$ values of >150 mm Hg), the animals were instrumented for the measurement of systemic hemodynamics. A thoracotomy was then performed and a suture placed around the left anterior descending coronary artery (LAD). The LAD was occluded (25 or 45 minutes) and reperfused for 2 hours. After injection of Evans blue dye (1 mL IV) to stain the area at risk (AR), the heart was removed and cut into 4 or 5 horizontal slices. The AR was determined by computer-assisted planimetry (Leica, Quantimed 500). Subsequently, the heart slices were weighed and incubated in p-nitro blue tetrazolium (NBT, 0.5 mg/mL; 20 minutes at 37°C) to stain the areas of viable and infarcted myocardium, which were quantified by planimetry. The AR and infarct size were automatically calculated and expressed as a percent of the left ventricle or of the AR, respectively.

In a separate study, 49 male New Zealand White rabbits (2.5 to 3.0 kg; Foxfield, Petersfield, Hampshire, UK) were premedicated (Hypnorm, 0.1 mL/kg IM containing 0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone) and anesthetized (pentobarbitone, 20 mg/kg IV). After tracheotomy and ventilation (36 to 40 strokes/min; tidal volume, 18 to 20 mL), a thoracotomy was performed and a suture placed around the left anterior descending coronary artery (LAD). The LAD was occluded (45 or 60 minutes) and opened to allow reperfusion (2 hours). The AR was determined by staining of the perfused myocardium (Evans blue dye), and infarct size was determined by staining of the AR with NBT as previously described.$^{10,17}$

**Experimental Design (In Vivo Studies)**

The first study was designed to evaluate whether ONO-AE-248 reduces myocardial infarct size in the anesthetized rat. The following 4 experimental groups were studied: (1) LAD occlusion (45 minutes) and reperfusion (2 hours) plus infusion of vehicle (0.15% Tween-80/0.015% ethanol in saline), starting 10 minutes before LAD occlusion and maintained throughout the experiment (n = 11); (2) LAD occlusion (45 minutes) and reperfusion (2 hours) plus infusion of ONO-AE-248 (5 mg · kg$^{-1}$ · min$^{-1}$, n = 9), starting 10 minutes before LAD occlusion and maintained throughout the experiment;
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**TABLE 1. Results of Binding Assays for Various EPs**

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<td>(4%)</td>
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<tr>
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<td>(9%)</td>
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(3) sham operation (no LAD occlusion) and infusion of vehicle (n=3); and (4) sham operation and infusion of ONO-AE-248 (n=3).

The second study was designed to investigate whether a larger reduction in infarct size can be obtained with ONO-AE-248 when the ischemic period is reduced from 45 to 25 minutes. Having documented that ONO-AE-248 causes a substantial (~40%) reduction in myocardial infarct size under these experimental conditions, we subsequently investigated the role of the activation of potassium channels and/or PKC in this cardioprotective effect. To do this, the following additional 13 experimental groups were studied: (1) LAD occlusion (25 minutes) and reperfusion (2 hours) plus infusion of vehicle as above (n=8); (2) LAD occlusion and reperfusion plus infusion of ONO-AE-248 (5 μg·kg⁻¹·min⁻¹, n=7); (3) sham operation (no LAD occlusion) and infusion of vehicle (n=3); (4) sham operation and infusion of ONO-AE-248 (n=3); (5) LAD occlusion and reperfusion plus injection of 5-HD (5 mg/kg IV 10 minutes before LAD occlusion, n=6); (6) sham operation and injection of 5-HD (n=3); (7) LAD occlusion and reperfusion plus administration of 5-HD (as above) and ONO-AE-248 (as above, n=6); (8) LAD occlusion and reperfusion plus injection of staurosporine (1 μg/kg IV 10 minutes before LAD occlusion, n=6); (9) sham operation and injection of staurosporine (n=3); (10) LAD occlusion and reperfusion plus administration of staurosporine (as above) and ONO-AE-248 (as above, n=6); (11) LAD occlusion and reperfusion plus injection of chelerythrine (0.7 mg/kg IV 10 minutes before LAD occlusion, n=6); (12) sham operation and injection of chelerythrine (n=3); and (13) LAD occlusion and reperfusion plus administration of chelerythrine (as above) and ONO-AE-248 (as above, n=6).

In a third study, we investigated whether ONO-AE-248 reduces myocardial infarct size in the anesthetized rabbit. To do this, the following 3 experimental groups were studied: (1) LAL occlusion (45 minutes) and reperfusion (2 hours) plus infusion of vehicle (as above, n=10); (2) LAL occlusion and reperfusion plus infusion of ONO-AE-248 (5 μg·kg⁻¹·min⁻¹, n=8); and (3) sham operation (no LAL occlusion) and infusion of ONO-AE-248 (n=3).

The fourth study was designed to elucidate whether the reduction in infarct size afforded by ONO-AE-248 in the rabbit is attenuated by pretreatment of the animals with the Kₐ₅₆-channel blocker 5-HD. The following 4 experimental groups were studied: (1) LAL occlusion (60 minutes) and reperfusion (2 hours) plus infusion of vehicle (as above, n=7); (2) LAL occlusion and reperfusion plus infusion of ONO-AE-248 (as above, n=7); (3) LAL occlusion and reperfusion plus injection of 5-HD (5 mg/kg IV, n=7); and (4) LAL occlusion and reperfusion plus injection of 5-HD and ONO-AE-248 (n=7).

**Measurement of the Plasma Levels of Troponin T (TnT) in the Rat**

At the end of the experiment (study 2, groups 1 [n=8], 2 [n=7], and 3 [n=3]), a 1-mL blood sample was obtained from the carotid cannula and centrifuged to obtain plasma. The concentration of TnT was determined by the short turn-around-time assay (STAT provided by Boehringer Mannheim) using an Elecsixs system 2010.

**Drugs**

Chelerythrine and staurosporine were dissolved in dimethyl sulfoxide (final concentration in vivo <0.2% for dimethyl sulfoxide), ONO-AE-248 (prostanoid acid derivative, molecular weight of 380.5) was dissolved in ethanol, Tween-80, and saline (final concentration in vivo, <0.1% for ethanol and 0.1% for Tween-80), 5-HD was dissolved in saline. Unless otherwise stated, all compounds were obtained from Sigma Chemical Co. Thiopentone sodium (Intralva) was obtained from May & Baker Ltd. Chelerythrine and staurosporine were from Calbiochem.

**Statistical Analysis**

All values in the text, figures, and tables are expressed as the mean±SEM of n observations. Statistical analysis was performed by 1-way ANOVA followed, if appropriate, by Bonferroni’s test for multiple comparisons. A value of P<0.05 was considered statistically significant.

**Results**

**Specific Binding of ONO-AE-248 to Membranes of CHO Cells Expressing EP, FP, TP, or IP Receptors**

In CHO cells expressing the murine EP₃ receptor, ONO-AE-248 caused a concentration-related displacement of [³H]PGE₂ with a Kᵢ of 15 nmol/L (Table 1). In contrast, the Kᵢ values for the displacement of [³H]PGE₂ from murine EP₁, EP₂, or EP₅ receptors ranged from 3.7 (EP₂) to >10 μmol/L (Table 1). In contrast to sulprostone, which potently binds to murine EP₁ receptors (Kᵢ of 42 nmol/L), ONO-AE-248 caused only a small displacement of [³H]PGE₂ from the murine EP₅ receptor (15% at 10 μmol/L). At concentrations of 10 μmol/L, ONO-AE-248 caused only (1) a 1% displacement of [³H]PGE₂ from the murine FP receptor, (2) a 9% displacement of [³H]SQ29548 from the human TP receptor, and (3) a 4% displacement of [³H]prolactin from the human IP receptor (Table 1). Thus, ONO-AE-248 binds selectively at concentrations <1 μmol/L to EP₃, PGE₂, and IP₃ receptors.

ONO-AE-248 Attenuates the Forskolin-Induced Rise in Intracellular cAMP in CHO Cells Transfected With the Murine EP₃ Receptor

To elucidate whether binding of ONO-AE-248 to the EP₃ receptor activates signal transduction events (ie, reduction in intracellular cAMP), whole CHO cells in which the EP₃ receptor had been expressed were challenged with forskolin, which increases the intracellular levels of cAMP, in the absence (control) or presence of PGE₂, a nonselective agonist of all EP receptors; sulprostone, an EP₂ and EP₃-receptor agonist; or ONO-AE-248. PGE₂ (IC₅₀ ~0.1 nmol/L), sulprostone (IC₅₀ ~0.1 nmol/L), or ONO-AE-248 (IC₅₀ ~1 nmol/L) caused a concentration-dependent inhibition of the rise in intracellular cAMP caused by forskolin. At a concentration of 10 nmol/L, the rise in cAMP caused by forskolin was abolished by sulprostone, reduced to 12±2% of control by PGE₂, and reduced to 42±2% of control by ONO-AE-248 (n=3). Thus, activation of EP₃ receptors with ONO-AE-248, sulprostone, or PGE₂ attenuates the rise in cAMP caused by.
ONO-AE-248 Reduces Myocardial Infarct Size in Rats

**Study 1 in Rats: 45 Minutes of LAD Occlusion**

Values for MAP, HR, and PRI measured during the course of the experiment (study 1) are given in Table 2. Baseline hemodynamic data were similar (P>0.05) in all groups studied. In rats subjected to sham operation or LAD occlusion and reperfusion, ONO-AE-248 did not cause any significant hemodynamic effects. Moreover, none of the other interventions studied (5-HD, staurosporine, or chelerythrine) caused any significant hemodynamic effects when given to either sham-operated rats or rats subjected to LAD occlusion and reperfusion, in either the absence or presence of ONO-AE-248.

In the 13 experimental groups studied, the AR ranged from 45±2% to 56±2% and was not different between groups (P>0.05). When compared with vehicle-treated control animals, infusion of ONO-AE-248 caused a significant reduction in infarct size, from 60±3% (control, n=11) to 58±4% (P<0.05, n=9) of the AR.

**Study 2 in Rats: 25 Minutes of LAD Occlusion**

MAP, HR, and PRI were measured during the course of the experiment (study 2, data not shown). Baseline hemodynamic data were similar (P>0.05) in all groups studied. In rats subjected to LAD occlusion for 25 minutes plus 2 hours of reperfusion, ONO-AE-248 did not cause any significant hemodynamic effects. Moreover, none of the other interventions studied (5-HD, staurosporine, or chelerythrine) caused any significant hemodynamic effects when given to either sham-operated rats or rats subjected to LAD occlusion and reperfusion, in either the absence or presence of ONO-AE-248.

In the 13 experimental groups studied, the AR ranged from 45±2% to 56±2% and was not different between groups (P>0.05). When compared with vehicle-treated control animals, infusion of ONO-AE-248 caused a significant reduction in infarct size, from 60±3% (control, n=8) to 58±4% (P<0.05, n=7) of the AR.

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staurosporine (Figure 2b) and chelerythrine (Figure 2c). Neither staurosporine nor chelerythrine, however, affected the infarct size caused by regional myocardial ischemia and reperfusion in the rat (Figure 2b and 2c).

To exclude the possibility that ONO-AE-248 interferes with the NBT staining procedure, 3 rats were subjected to regional myocardial ischemia (25 minutes) and reperfusion (2 hours). The heart was removed, and the AR was incubated with NBT (as above) in the presence of 10 μmol/L ONO-AE-248. The infarct size in these experiments was 62±5% (n=3) and hence, not different from control. Thus, ONO-AE-248 does not interfere with the staining procedure.

ONO-AE-248 Reduces Myocardial Ischemia–Mediated TnT Increases in the Rat

In rats subjected to sham operation (surgical procedure only), the plasma levels of TnT were below the detection limit of the assay (<0.01 μg/L, n=3). In contrast, LAD occlusion (25 minutes) and reperfusion (2 hours) resulted in a significant increase in the plasma levels of TnT, to 65±14 μg/L (n=8). Treatment of rats subjected to regional myocardial ischemia and reperfusion with ONO-AE-248 attenuated this rise in the plasma levels of TnT to 21±6 μg/L (P<0.05, n=7).

ONO-AE-248 Reduces Myocardial Infarct Size in Rabbits

Study 3 in Rabbits: 45 Minutes of LAL Occlusion

Values for MAP, HR, and PRI measured during the course of the experiment (study 3) are given in Table 2. Baseline hemodynamic data were similar (P>0.05) in all groups studied. In rabbits subjected to LAL occlusion for 45 minutes plus 2 hours of reperfusion, ONO-AE-248 did not cause any significant hemodynamic effects. The AR was similar in rabbits subjected to LAL occlusion for 45 minutes and treated with either vehicle (control, 40±2%, n=10) or ONO-AE-248 (48±3%, n=8). When compared with vehicle-treated control animals, infusion of ONO-AE-248 caused a significant reduction in infarct size, from 61±2% (control, n=10) to 36±4% (P<0.05, n=8) of the AR.

Study 4 in Rabbits: 60 Minutes of LAL Occlusion

MAP, HR, and PRI were measured during the course of the experiment (study 4, data not shown). Baseline hemodynamic data were similar (P>0.05) in all groups studied. In rabbits subjected to LAL occlusion for 60 minutes plus 2 hours of reperfusion, ONO-AE-248 did not cause any significant hemodynamic effects. The AR ranged from 38±4% to 47±2% and was not different between groups (P>0.05). When compared with control animals, infusion of ONO-AE-248 caused a significant reduction in infarct size, from 63±4% (control, n=7) to 42±4% (P<0.05, n=7) of the AR (Figure 3). Pretreatment with 5-HD abolished the reduction in infarct size afforded by...
ONO-AE-248 (Figure 3). However, 5-HD alone did not affect the infarct size when compared with vehicle control (Figure 3).

**Discussion**

This study demonstrates that the prostanoid derivative ONO-AE-248 selectively binds to \( \text{EP}_3 \) receptors expressed in CHO cells. In these cells, ONO-AE-248 also attenuates the rise in cAMP caused by forskolin, suggesting that ONO-AE-248 activates \( \text{G}_i \) in these cells. Thus, ONO-AE-248 is a selective agonist of the \( \text{EP}_3 \) receptor. We also report that ONO-AE-248 reduces the myocardial infarct size in rats and rabbits subjected to coronary artery occlusion and reperfusion. The reduction in infarct size caused by ONO-AE-248 was determined by staining of the viable myocardium within the AR with the formazan dye NBT. The reduction in infarct size caused by ONO-AE-248 as determined by this staining procedure is indeed due to a reduction in myocardial tissue injury, as ONO-AE-248 also attenuated the increase in the plasma levels of TnT caused by regional myocardial ischemia in the rat. There is good evidence that a rise in the plasma levels of cardiac TnT are specific for myocardial tissue injury. Unlike plasma levels of creatine phosphokinase or lactate dehydrogenase, which are elevated in open-chest models of myocardial infarction due to the surgical procedure (K.Z., and C.T., unpublished observations, 1998), the thoracotomy employed here did not result in any detectable rise in the plasma levels of TnT. Because ONO-AE-248 also did not interfere with the NBT staining procedure used, our data strongly support the conclusion that ONO-AE-248 does indeed cause a significant reduction in myocardial infarct size.

What, then, is the mechanism(s) by which ONO-AE-248 causes a significant reduction in the degree of necrosis caused by myocardial ischemia and reperfusion? Clearly, a reduction in blood pressure and hence, myocardial oxygen consumption due to the activation of EP \( \text{sub}_3 \) receptors expressed in CHO cells is in excess of 10 \( \text{mmHg} \). Because ONO-AE-248 did not affect the hemodynamic effects in either rats or rabbits. This also supports the notion that at the doses used in vivo, ONO-AE-248 is a selective \( \text{EP}_3 \)-receptor agonist and does not activate \( \text{EP}_2 \) receptors, which mediate the hypotension caused by EPs. This finding is important, because it demonstrates that the cardioprotective effects of ONO-AE-248 are, unlike those of prostacyclin, iloprost, or (at higher doses) PGE \(_1\), not limited by hemodynamic side effects.

We suggest that the observed reduction in infarct size by ONO-AE-248 is due to the activation of \( \text{EP}_3 \) receptors in vivo. It is unlikely that the dose of ONO-AE-248 used in this study is sufficient to activate other EP receptors for the following reasons. We demonstrated that the \( K_i \) value of ONO-AE-248 at the \( \text{EP}_3 \) receptor is 0.015 \( \text{mmol/L} \), whereas those for the \( \text{EP}_1 \) and the \( \text{EP}_2 \) receptors are in the range of 3 to 4 \( \text{mmol/L} \), and those for the \( \text{EP}_4 \), FP, TP, and IP receptors are in excess of 10 \( \text{mmol/L} \). Because ONO-AE-248 did not cause a significant fall in blood pressure, it is extremely unlikely that the dose of ONO-AE-248 chosen here was sufficient to activate \( \text{EP}_2 \) receptors. Thus, we propose that it is most likely that the observed effects of ONO-AE-248 are due to the activation of \( \text{EP}_3 \) receptors. Hohlfeld and colleagues\(^ {19} \) have reported in abstract form that the \( \text{EP}_3 \)-receptor agonist M&B28767 also causes a substantial (50%) reduction in infarct size in pigs subjected to regional myocardial ischemia. Taken together, these findings support the view that activation of \( \text{EP}_3 \) receptors does indeed cause a reduction in infarct size.

What, then, is the mechanism(s) by which activation of \( \text{EP}_3 \) receptors reduces infarct size? The reduction in infarct size caused by either PGE \(_1\) or the \( \text{EP}_2 / \text{EP}_4 \)-receptor agonist sulprostone is at least in part due to the activation and opening of \( \text{K}_{\text{ATP}} \) channels.\(^ {10,13} \) Here we report that the cardioprotective effect of the selective \( \text{EP}_3 \)-receptor agonist ONO-AE-248 is, in rats and rabbits, abolished by pretreatment of the animals with 5-HD, a selective blocker of \( \text{K}_{\text{ATP}} \) channels. These findings suggest that activation of \( \text{EP}_3 \) receptors leads to opening of \( \text{K}_{\text{ATP}} \) channels, which in turn results in cardioprotection. The signal transduction events involved in the cardioprotective effects of ONO-AE-248 are reminiscent of the ones that mediate the potent anti-ischemic effects of "ischemic preconditioning."\(^ {20} \) Preconditioning of the heart and other organs or tissues with ischemia results in the release of adenosine and other mediators (eg, bradykinin, endothelin-1, angiotensin II, and catecholamines), which activate G protein–coupled receptors resulting in (1) translocation of PKC from the cytosol to the cell membrane, (2) activation of PKC, (3) opening of \( \text{K}_{\text{ATP}} \) channels, and (4) ultimately, cardioprotection.\(^ {20,21} \) The reduction in infarct size afforded by ischemic preconditioning in the rabbit model of myocardial ischemia and reperfusion used here is also abolished by 5-HD.\(^ {13} \) Thus, we have hypothesised that activation of \( \text{EP}_3 \) receptors by ONO-AE-248, like ischemic preconditioning, may lead to activation of PKC, opening of \( \text{K}_{\text{ATP}} \) channels, and ultimately cardioprotection. To support this hypothesis, we have demonstrated that the reduction in infarct size caused by ONO-AE-248 in the rat is abolished by 2 chemically distinct inhibitors of PKC, namely, staurosporine, a nonselective PKC inhibitor, and chelerythrine, a selective PKC inhibitor. The doses of staurosporine and chelerythrine employed here have been reported to specifically inhibit the activation of PKC in vivo and ex vivo\(^ {23-24} \) and also abolish the cardioprotective effects of ischemic preconditioning in rodents.\(^ {22,25} \)

The cytoprotective properties of prostaglandins were first described as the ability of these agents to protect the gastric mucosa against noxious stimuli (eg, ethanol, acid, and hot water).\(^ {26} \) Although the mechanism of these cytoprotective effects of prostaglandins has been elusive, these cytoprotective effects have been suggested to contribute to or account for the protection of certain organs or tissues, including the heart, by prostaglandins against a variety of different noxious stimuli.\(^ {12,26} \) Thus, cytoprotection is the protection of a tissue by prostaglandins in the absence of alterations in blood flow, inhibition of platelet function, or inhibition of polymorphonuclear cell activation mediated by EP \(_3\) receptors. Activation of \( \text{EP}_3 \) receptors leads to protection of the myocardium against ischemia, without causing alterations in systemic hemodynamics (vide infra) and presumably, inhibition of the function of blood-borne cells. Thus, activation of these receptors may contribute to or even account for the protection of cardiomyocytes caused by EPs.

In conclusion, this study demonstrates for the first time that (1) ONO-AE-248 selectively binds to \( \text{EP}_{\text{sub}3} \) receptors, (2) ONO-AE-248 causes a reduction in intracellular cAMP in
CHO cells, in which the EP₃ receptor had been expressed, and challenged with forskolin, (3) ONO-AE-248 reduces infarct size in rats and rabbits subjected to regional myocardial ischemia and reperfusion, (4) the cardioprotective effects of ONO-AE-248 are abolished by the K⁺-ATP-channel blocker 5-HD (rats and rabbits), and (5) the cardioprotective effects of ONO-AE-248 are abolished by the PKC inhibitors staurosporine and chelerythrine (rats). We speculate that the cytoprotective or cardioprotective effects of EPs are at least in part due to activation by these agents of EP receptors. Our discovery of a selective agonist of the EP₃ receptor provides an important pharmacological tool to elucidate the role of the prostanoïd EP₃ receptors in physiology and pathophysiology.

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References
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