Prostacyclin attenuates oxidative damage of myocytes by opening mitochondrial ATP-sensitive K⁺ channels via the EP₃ receptor

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We have found that cyclooxygenase (COX)-2, an inducible form of COX, mediates the cardioprotective effects of the late phase of ischemic preconditioning by increasing the synthesis of cardioprotective prostanooids such as prostacyclin (PGI₂) and PGE₂ (9, 42). A recent investigation demonstrated that COX-2-derived PGE₂ mediates not only ischemic, but also isoflurane-and diazoxide (Dx)-induced, preconditioning in vivo (3). PGI₂ and the PGE family have been shown to alleviate myocardial ischemia-reperfusion injury and limit oxidative damage. The cardioprotective effects of PGI₂ have been traditionally ascribed to activation of IP receptors. Recent advances in prostanoid research have revealed that PGI₂ can bind not only to IP, but also to EP, receptors, suggesting cross talk between PGI₂ and PGEs. The mechanism(s) whereby PGI₂ protects myocytes from oxidative damage and the specific receptors involved remain unknown. Thus fresh isolated adult rat myocytes were exposed to 200 μM H₂O₂ with or without carbaprostacyclin (cPGI₂), IP-selective agonists, and ONO-AE-248 (an EP₃-selective agonist). Cell viability was assessed by trypan blue exclusion after 30 min of H₂O₂ superfusion. cPGI₂ and ONO-AE-248 significantly improved cell survival during H₂O₂ superfusion; IP-selective agonists did not. The protective effect of cPGI₂ and ONO-AE-248 was completely abrogated by pretreatment with 5-hydroxydecanoate or glibenclamide. In the second series of experiments, the mitochondrial ATP-sensitive K⁺ (Kₐtp) channel opener diazoxide (Dx) reversibly oxidized flavoproteins in control myocytes. Exposure to prostanooids alone had no effect on flavoprotein fluorescence. A second application of Dx in the presence of cPGI₂ or ONO-AE-248 significantly increased flavoprotein fluorescence compared with Dx alone, but IP-selective agonists did not. This study demonstrates that PGI₂ analogs protect cardiac myocytes from oxidative stress mainly via activation of EP₃. The data also indicate that activation of EP₃ receptors primes the opening of mitochondrial Kₐtp channels and that this mechanism is essential for EP₃-dependent protection.

Flavoprotein fluorescence; IP receptor; oxidative stress; prostaglandin H₂O₂/PGI₂ — Human Subjects — Adult — Laboratory Animals — Antioxidants — ATP-Sensitive Channel/Ca²⁺ Channel family — Flavoprotein — G Proteins — Guinea Pig — Ischemia — Myocardium — Protein — Rats — Reperfusion — Site of Action — Topology

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strategies aimed at enhancing the beneficial effects of prostanoid without adverse effects.

**MATERIALS AND METHODS**

All procedures complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of Keio University School of Medicine.

**Materials.** Ds, 2,4-dinitrophenol (DNP), and 5-hydroxydecanoic acid sodium (5-HD) were purchased from Sigma-RBI (St. Louis, MO); glibenclamide (GLB) from Wako Pure Chemical Industries (Osaka, Japan); carboproxycyclin (cPGL) and polyclonal antibodies against EP1, EP2, EP3, and EP4 receptors from Cayman Chemical (Ann Arbor, MI); and polyclonal antibodies against the IP receptors from Santa Cruz Biotechnology (Santa Cruz, CA). ONO-AE-248, (Ann Arbor, MI); and polyclonal antibodies against the IP receptors from Keio University School of Medicine.

**Isolation of ventricular myocytes.** Single ventricular myocytes were isolated from Sprague-Dawley rats weighing 400 – 450 g by enzymatic digestion as previously described (18). Briefly, the isolated heart was mounted on a Langendorf apparatus and perfused with Ca$^{2+}$-free Tyrode buffer (in mmol/l: 134 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 1 MgCl$_2$, and 0.33 NaHPO$_4$, pH 7.4) for 3 min. Then the heart was perfused with Tyrode buffer containing 40 µM Ca$^{2+}$ and type 2 collagenase (0.5 mg/ml; Worthington Biochemical) for 12–15 min. The heart was rinsed with Tyrode buffer containing 200 µM Ca$^{2+}$ and no collagenase, and the left ventricle was cut into small pieces and digested in a shaking bath at 37°C for 20 min. The dispersed cells were filtered through a nylon mesh and stored at room temperature. The Ca$^{2+}$ concentration was gradually increased to 1.0 mM by addition of medium 199 without fetal bovine serum.

**RT-PCR of mRNA for prostanoid receptors.** Total cellular RNA was extracted from ventricular myocytes using the RNasey kit (Qiagen) according to the manufacturer’s protocol. To obtain an internal control of prostanoid receptor mRNA expression, total cellular RNA was also extracted from the kidney using the RNasey kit. RT-PCR was performed using 0.7 µg of total cellular RNA from each sample and the GeneAmp EZ rtHt RNA PCR kit (Applied Biosystems) according to the manufacturer’s protocol. PCR amplification of constitutively expressed GAPDH cDNA was used as a marker of the amount of input RNA. The primers and the amplification steps specific for the EP$_1$, EP$_2$, EP$_{3A/B}$, EP$_{3A/B}$-EP$_{3B}$, EP$_4$, and IP receptors and for GAPDH were determined according to previous reports (14, 36) and are summarized in Table 1. The PCR products (10 µl) were analyzed via electrophoresis in 2.5% agarose gels containing 0.5 µg/ml ethidium bromide.

**Western immunoblotting of prostanoid receptors.** Proteins were extracted from ventricular myocytes as described previously (41, 42). Briefly, ~1 × 10$^6$ isolated myocytes were homogenized in buffer A [25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 25 µg/ml leupeptin, 1 mM DTT, 25 mM NaF, and 1 mM Na$_3$VO$_4$] and centrifuged at 1,000 g for 10 min. The supernatant was carefully removed and recentrifuged at 16,000 g for 15 min to eliminate any contaminating pellet. The initial pellet was resuspended in a lysis buffer (buffer A + 1% Triton X-100) and incubated at 4°C for 2 h. Samples were centrifuged at 16,000 g for 15 min. The resulting supernatants were collected as membrane-rich fractions. Standard SDS-PAGE Western immunoblotting techniques assessed expression of the EP$_1$, EP$_2$, EP$_3$, EP$_4$, and IP receptors. Proteins (60 µg) from the membranous fraction were electrophoresed on a 12.5% denaturing gel and then electrophoretically transferred onto nitrocellulose membranes overnight at 4°C. To determine gel transfer efficiency, we made photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining. The membranes were incubated in 5% nonfat dry milk in a washing buffer [10 mM Tris-HCl (pH 7.2), 0.15 M NaCl, and 0.05% Tween 20] at room temperature for 2 h and then incubated with polyclonal antibodies (1:500 dilution) at 4°C overnight. After they were rinsed with washing buffer, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (1:2,500 dilution) at room temperature for 1.5 h and developed using the Vistra enhanced chemiluminescence Western blotting kit. The protein signals and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated by an image-scanning densitometer, and each protein signal was normalized to the corresponding Ponceau stain signal.

**Effect of prostanoid analogs on cell survival during oxidative stress by H$_2$O$_2$.** The effect of prostanoid analogs on cell survival under oxidative stress (200 µM H$_2$O$_2$) was evaluated using isolated adult rat ventricular myocytes. Isolated myocytes were placed in a flow-through chamber under an inverted microscope (Axiovert 100S TV, Zeiss, Oberkochen, Germany) and allowed to attach to the glass surface for 10 min. Then myocytes were superfused with HEPES buffer at a constant flow of 1.0 ml/min. In the control group (group I), after 5 min of washout perfusion with HEPES buffer containing 1.0 mM Ca$^{2+}$, the concentration of Ca$^{2+}$ was increased to 1.5 mM for 10 min. Then myocytes were exposed to 200 µM H$_2$O$_2$ in HEPES buffer containing 1.5 mM Ca$^{2+}$ (Fig. 1). In pilot studies, we found that most myocytes died within 10 min of superfusion with 500 µM H$_2$O$_2$. In

**Table 1. Primers, amplicon sizes, and primer annealing positions**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon Size, bp</th>
<th>Nucleotide Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP$_1$</td>
<td>Upstream</td>
<td>AGATGSCCAAGATACAGGGG</td>
<td>245</td>
<td>2396–2416</td>
</tr>
<tr>
<td>EP$_1$</td>
<td>Downstream</td>
<td>GGGTTTTTTAAAGTTGTGTGGC</td>
<td>2622–2641</td>
<td></td>
</tr>
<tr>
<td>EP$_2$</td>
<td>Upstream</td>
<td>TGCTTTGTTAATCTGCTTC</td>
<td>227</td>
<td>975–995</td>
</tr>
<tr>
<td>EP$_2$</td>
<td>Downstream</td>
<td>CCATTGGTTATCCTCCTGG</td>
<td>1181–1201</td>
<td></td>
</tr>
<tr>
<td>EP$<em>{3A/A}$ and EP$</em>{3A/B}$</td>
<td>Upstream</td>
<td>GGAATTCTGGATCGCTGGTTATCTTG</td>
<td>316‡</td>
<td>1006–1025*</td>
</tr>
<tr>
<td>EP$<em>{3A/A}$ and EP$</em>{3A/B}$</td>
<td>Downstream</td>
<td>GCTCTAGAGCAGCTGCTGTTTAAATGGC</td>
<td>227§</td>
<td>1285–1304†</td>
</tr>
<tr>
<td>EP$_{3B}$</td>
<td>Upstream</td>
<td>GGAATTCTGGATCGCTGGTTATCTTG</td>
<td>196</td>
<td>1006–1025</td>
</tr>
<tr>
<td>EP$_{3B}$</td>
<td>Downstream</td>
<td>CCAGGATTCTGCTGTTAAGCTCCTTGG</td>
<td>1164–1183</td>
<td></td>
</tr>
<tr>
<td>EP$_4$</td>
<td>Upstream</td>
<td>GATCAAGTGTCTCCTTCTCCG</td>
<td>148</td>
<td>1304–1324</td>
</tr>
<tr>
<td>IP</td>
<td>Upstream</td>
<td>TGCTTCAAGCATCCTACAGGC</td>
<td>222</td>
<td>182–201</td>
</tr>
<tr>
<td>IP</td>
<td>Downstream</td>
<td>GCTTTGGAGCATTGCGGCAAAA</td>
<td>384–403</td>
<td></td>
</tr>
</tbody>
</table>

*Anneling site in EP$_{3A/A}$. †Anneling site in EP$_{3A/B}$. ‡Anneling site in EP$_{3A/B}$. §Anneling site in EP$_{3A/B}$. ¶Anneling site in EP$_{3A/B}$.
contrast, most myocytes survived >60 min under 100 μM H2O2. Therefore, we selected 200 μM H2O2 in the present study. In the prostanoid-treated groups (groups II–VII), each prostanoid analog was added to the perfusate 5 min after initiation of the superfusion with HEPES buffer containing 1.5 mM Ca2+ and continued until the end of the experiment (Fig. 1). The prostanoid analogs used in the present study were cPGI2 (1.0 μM, group II), cicaprost (another PGI2 analog, 0.01–1.0 μM, group III), ONO-1301 (a relatively selective IP agonist, 0.1–10.0 μM, group IV), sulprostone (an EP1 and EP3 agonist, 0.1–10.0 μM, group V), ONO-AE-248 (an EP2-selective agonist, 0.01 μM, group VI), and ONO-8711 (an EP1-selective antagonist, 1.0 μM, administered without or with sulprostone, groups VII and VIII). In groups IX, X, XI, and XII, myocytes were treated with 5-HD (a selective mitochondrial KATP channel blocker) or GLB (a nonselective KATP channel blocker) before exposure to cPGI2 or ONO-AE-248. After 5 min of perfusion with HEPES buffer containing 1.5 mM Ca2+, isolated myocytes were superfused with HEPES buffer containing 100 μM 5-HD (groups IX and X) or 10 μM GLB (groups XI and XII) for 5 min, and then 1.0 μM cPGI2 or 0.01 μM ONO-AE-248 was added to the perfusate for 5 min and the myocytes were perfused with H2O2 (Fig. 1). Groups XIII and XIV were the drug-control groups of 5-HD and GLB (Fig. 1). All drugs except 5-HD and GLB were dissolved with DMSO, and the final concentration of DMSO was <0.1%. We confirmed that DMSO alone had no effect on cell viability in untreated myocytes. 5-HD and GLB were dissolved in HEPES buffer. Cell viability was assessed by trypan blue exclusion. At 30 min after H2O2 perfusion, myocytes were perfused with HEPES buffer containing 1% trypan blue for 5 min and then subjected to 5 min of washout perfusion. Without H2O2 perfusion, >80% of myocytes were trypan blue negative. The percentage of the unstained cells in a total of 500 cells was considered an index of cell survival. All data were obtained from at least four independent experiments.

Measurement of flavoprotein fluorescence. The mitochondrial redox state was monitored by recording the fluorescence of flavin adenine nucleotide-linked enzymes in mitochondria and served as an index of mitochondrial KATP channel activity (39, 46). Fresh isolated myocytes were placed in a flow-through chamber under an inverted microscope and superfused with glucose-free HEPES buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, with pH adjusted to 7.4 with NaOH, for 1 h at room temperature. Myocytes were excited at 488 nm by a mercury-arc lamp system with an epifluorescence attachment. Fluorescence was excited for 100 ms every 10 s. The emitted fluorescence at 520 nm was collected using a photomultiplier tube, and the data were analyzed as described previously (39, 46). The change of fluorescence was normalized using the baseline fluorescence and the maximum fluorescence obtained after exposure to 100 μM DNP at the end of the experiment. In the first series of experiments, the effect of Dx (200 μM) on flavoprotein fluorescence was evaluated (group XV; Fig. 2). In the second series of experiments, we assessed the effect of cPGI2, ONO-AE-248, cicaprost, or ONO-1301 alone (group XVI, XVII, XVIII, or XIX) and in combination with Dx (group XX, XXI, XXII, or XXIII) on flavoprotein fluorescence (Fig. 2).

Statistical analysis. Values are means ± SD. For intragroup comparisons, variables were analyzed by a two-way repeated-measures ANOVA (time and group) followed by Student’s t-tests for paired data with Bonferroni’s correction. For intergroup comparisons, data were analyzed by a one- or a two-way repeated-measures ANOVA (time and group), as appropriate, followed by Student’s t-tests for
unpaired data with Bonferroni’s correction. All statistical analyses were performed using the SAS software system.

RESULTS

Expression of prostanoid receptors in adult rat myocytes. Using RT-PCR, we found that adult rat myocytes express mRNA for EP1, EP4, and IP receptors (Fig. 3). It is known that, in the rat, there are three splicing variants of the EP3 receptor: EP3A/α, EP3A/β, and EP3B. In the present study, only mRNA for the EP3B variant was detected in adult rat myocytes (Fig. 4). Western immunoblotting revealed that the EP1 and EP3 receptor subtypes are abundant in adult rat myocytes (Fig. 5). The EP4 and IP receptors were also detected in adult rat myocytes, but we could not detect the EP2 receptor subtype at the mRNA or protein level.

Effect of prostanoid analogs on cell survival during H2O2 superfusion. During H2O2 superfusion, myocytes began to beat spontaneously and then became hypercontracted and died. The percentage of viable cells detected by trypan blue exclusion 30 min after H2O2 superfusion was 32% in control myocytes (Fig. 6). In contrast, >85% of untreated myocytes were viable after 30 min of HEPES buffer superfusion without H2O2.

The effects of prostanoid analogs on cell survival are also summarized in Fig. 6. Pretreatment with cPGI2 significantly improved cell survival during 30 min of H2O2 superfusion. The EP3-selective agonist ONO-AE-248 also improved cell survival to the same extent as cPGI2 treatment. In contrast, the IP-selective agonists that have greater affinity for IP than for EP3 receptors, cicaprost and ONO-1301, did not improve cell survival at any concentration used in the present study (only the data at 1.0 μM cicaprost and 1.0 μM ONO-1301 are presented in Fig. 6). The EP1 and EP3 agonist sulprostone failed to improve cell survival at any concentration used in the

![Fig. 2](image_url) Schematic diagram illustrating experimental protocols for the effect of prostanoid analogs on flavoprotein fluorescence (groups XV–XXIII). WO, washout; DNP, 2,4-dinitrophenol.

![Fig. 3](image_url) Representative RT-PCR showing expression of EP1, EP2, EP4, and IP receptor mRNA. mRNA from kidney tissue was used as an internal positive control.

![Fig. 4](image_url) Representative RT-PCR showing expression of EP3A/α, EP3A/β, and EP3B receptor, and GAPDH mRNA. mRNA from kidney tissue was used as an internal positive control.
present study (only the data at 0.1 μM are presented in Fig. 6), suggesting that the protective effects of EP3 stimulation were offset by detrimental effects of EP1 stimulation. This concept is further supported by the fact that the combination of 1.0 μM ONO-8711 (a selective EP1 antagonist) and 0.1 μM sulprostone improved cell survival, although ONO-8711 alone did not affect cell survival under oxidative stress (Fig. 6). Taken together, these results indicate that 1) the protective effects of cPGI2 are exerted via receptors other than the IP receptor (possibly EP3 receptors); 2) myocytes are protected against oxidative stress by activation of EP3, but not EP1, receptors; and 3) activation of EP1 receptors counteracts the salutary effects of EP3 receptor activation.

The salubrious effect of cPGI2 and ONO-AE-248 on cell viability was completely abolished by pretreatment with 100 μM 5-HD or 10 μM GLB, suggesting that mitochondrial K<sub>ATP</sub> channels are involved in this cytoprotection (Fig. 7). Neither 5-HD nor GLB had an effect on cell viability during H<sub>2</sub>O<sub>2</sub> superfusion (Fig. 7).

Effect of prostanoid analogs on flavoprotein fluorescence. Flavoprotein fluorescence was measured to evaluate mitochondrial redox state. D<sub>x</sub> reversibly oxidized flavoproteins via opening of mitochondrial K<sub>ATP</sub> channels. In fresh isolated adult rat myocytes, D<sub>x</sub> caused reversible flavoprotein oxidation to 25 ± 11% of the maximum value (**group XV**; Figs. 8 and 9). The effect of D<sub>x</sub> on flavoprotein fluorescence was completely abrogated by pretreatment with 100 μM 5-HD (data not shown). Subsequent exposure to cPGI2 (1.0 μM), ONO-AE-248 (0.01 μM), cicaprost (1.0 μM), or ONO-1301 (1.0 μM) alone had no effect on flavoprotein fluorescence (**group XVI**, **XVII**, **XVIII**, or **XIX**; Figs. 8 and 9). However, a second application of D<sub>x</sub> in the presence of cPGI2 or the EP3 agonist ONO-AE-248 increased flavoprotein fluorescence by 59% and 89%, respectively (**group XX** or **XXI**; Figs. 8B, 8C, and 9). In contrast, pretreatment with the selective IP agonist cicaprost or ONO-1301 before the second application of D<sub>x</sub> did not enhance flavoprotein fluorescence (**group XXII** or **XXIII**; Fig. 9). These data suggest that activation of EP3 receptors potentiates opening of mitochondrial K<sub>ATP</sub> channels and that the ability of cPGI2 to produce this effect is mediated by receptors other than the IP receptor (i.e., EP3 receptors).

**DISCUSSION**

The major findings of this study are as follows: 1) in adult rat cardiac myocytes, the EP<sub>1</sub>, EP<sub>3</sub>, EP<sub>4</sub>, and IP receptors are expressed at the mRNA level, but the EP<sub>1</sub> and EP<sub>3</sub> receptors are the most abundant at the protein level. 2) Stimulation of the EP<sub>3</sub> receptor protects cardiac myocytes against...
oxidative damage, whereas stimulation of the IP receptor does not. 3) The beneficial effects of EP3 stimulation are abrogated by concomitant EP1 stimulation, suggesting opposing effects of EP1 and EP3 receptors. 4) In contrast to IP receptor-selective agonists, cPGI2 improves survival during oxidative stress, suggesting that this beneficial effect is mediated by activation of EP3, rather than IP, receptors. 5) Opening of mitochondrial KATP channels is involved in EP3-dependent protection during oxidative stress. 6) The ability of cPGI2 to open mitochondrial KATP channels appears to be mediated by EP3, rather than IP, receptors.

**Expression of prostanoid receptors in adult rat myocytes.** Hohlfeld et al. (25) demonstrated that myocardial receptors for PGE1 belong to the EP3 subtype, inhibit adenylyl cyclase, and are upregulated during myocardial ischemia in the pig. However, it remains unclear whether other subtypes of the EP receptor exist in the myocardium. Sugimoto et al. (45) showed by Northern blotting that mRNA for the EP3 and EP4 receptors was expressed in the adult mouse heart. Recently, Mendez and LaPointe (33) reported that EP1, EP3, and EP4 subtypes could be detected by Western immunoblotting of membrane-enriched preparations of neonatal ventricular rat myocytes. They also suggested that EP1 and EP3 receptors were involved in myocyte hypertrophy, because AH-6809, an EP1- and EP3-selective antagonist, inhibited only partially the effect of sulprostone, an EP1- and EP3-selective agonist, on protein synthesis (33). The range of EP receptors expressed in the heart is likely to be affected by developmental changes (adult vs. neonatal myocytes), species differences, and the relatively low sensitivity of Northern blotting for low-abundance mRNA.

Our results demonstrate, for the first time, that the EP3B receptor is the EP3 variant expressed in adult rat cardiac myocytes, whereas EP3A and EP3AB are not detectable by RT-PCR. The specific variant expressed is important, because different EP3 variants couple to different G proteins. The EP3 receptor can couple to Go, Go, or Go (11, 20, 34, 45). The class of G protein is determined by the COOH-terminal amino acid domain of the EP3 receptor. Although EP3A and EP3AB reportedly couple to Go (11, 20), it is unknown which G protein is coupled to rat EP3B. Further investigations are necessary to clarify the signaling pathways downstream of EP3B in rat ventricular myocytes.

**Effect of prostanoid analogs on cell survival under oxidative stress.** Increasing evidence demonstrates that endogenous production of prostaglandins plays a role in myocardial ischemia-reperfusion injury and/or development of ischemic preconditioning. Camitta et al. (12) demonstrated that targeted disruption of the COX-1 or COX-2 gene exacerbates myocardial ischemia-reperfusion injury but does not abrogate development of the acute phase of ischemic preconditioning in mice. Gres et al. (19) reported that endogenous production of prostaglandins is involved in the acute phase of ischemic preconditioning in pigs. The discrepancy between these studies may be due, at least in part, to species difference and/or the difference in the strength of the ischemic preconditioning stimulus. Therefore, it is likely that endogenous prostaglandins play a role in the defense system against oxidative stress. However, we cannot distinguish the effect of each prostanoid receptor from others by administration of the COX inhibitor. Unfortunately, specific antagonists for the EP3 or IP receptors are not available. Our data show that administration of ONO-9711 did not affect cell survival under oxidative stress, suggesting that endogenous PGE2 has no effect on cell survival under oxidative stress via stimulation of the EP1 receptor. Myocytes isolated from specific prostanoid receptor-knockout mice would be very useful to elucidate the role of each prostanoid receptor in the myocardium.

**Fig. 9.** Comparison of flavoprotein oxidation among groups XV–XXII. Flavoprotein oxidation is expressed as a percentage of maximal value obtained by DNP perfusion.
for this purpose. Further investigations are needed to clarify the role of endogenous prostaglandins under oxidative stress.

PGI₂ analogs, such as iloprost, have been reported to protect rat myocytes from oxidative stress (1, 15), but the mechanism(s) for this effect has not been clarified. cPGI₂ potently activates the IP receptor but activates the EP₃ receptor as well (11, 35). In contrast, cicaprost and ONO-1301 are selective activators of the IP receptor (11, 35). Therefore, a comparison of the effects of cPGI₂ and cicaprost should be useful to distinguish the role of IP receptors from that of EP₃ receptors. Surprisingly, our results suggest that stimulation of the EP₃ receptor prolongs cell survival under oxidative stress, whereas stimulation of the IP receptor does not.

We chose the concentrations of cicaprost and ONO-1301 so that they could be used as relatively selective IP receptor agonists (11, 35). When the concentration of cicaprost was increased to 10 μM, cell viability during H₂O₂ superfusion improved (data not shown), suggesting that high concentrations of cicaprost protected myocytes via simultaneous stimulation of the EP₃ receptor. However, the effect of cicaprost (10 μM) was still less than that of cPGI₂, and there was no further improvement in cell survival when the concentration of cicaprost was increased to 50 μM (data not shown). High concentrations of cicaprost can also bind to the EP₁ receptor (11, 35). The protective effect of the EP₃ receptor would be cancelled out by the simultaneous stimulation of the EP₁ receptor. In addition, cPGI₂, but not cicaprost, binds to the EP₅ receptor and peroxisome proliferator-activated receptor (PPAR) (11, 35). Stimulation of the EP₅ receptor has recently been reported to protect from myocardial ischemia-reperfusion injury (51). Stimulation of PPAR-δ may protect myocytes from oxidative stress just as the stimulation of PPAR-α or -γ does (49). Therefore, activation of the EP₅ receptor or PPAR-δ might contribute, in part, to the cytoprotection afforded by cPGI₂.

Our results were obtained in the setting of oxidative stress in isolated cells, and selective stimulation of the IP receptor still has potential as a therapeutic target in vivo and ex vivo. Recent evidence indicates that myocardial ischemia-reperfusion injury is aggravated in IP⁻/⁻ mice in vivo and ex vivo, suggesting an important role of the IP receptor in myocardial ischemia-reperfusion injury (50). Most of the available evidence suggests that the IP receptor signals via stimulation of cAMP generation (11, 35, 38, 45). Thus possible mechanisms whereby stimulation of the IP receptor provided cardioprotection include the activation of Ca²⁺-activated K⁺ channels and the activation of sarcolemmal KₐTP channels in a cAMP- and PKA-dependent manner (8, 10, 40, 52).

Effect of prostanoid analogs on mitochondrial KₐTP channels. Our results demonstrate that stimulation of the EP₃ receptor protects myocytes from oxidative damage. Inhibition of adenylyl cyclase (23, 25, 31, 53, 54) may contribute, at least in part, to the cytoprotective effect against oxidative stress elicited by selective stimulation of the EP₃ receptor. However, the present study strongly suggests that opening of mitochondrial KₐTP channels is involved in this cytoprotective effect, because the EP₃ selective agonist ONO-AE-248 enhanced Dx-induced flavoprotein fluorescence, and the protective effects of this drug were abrogated by 5-HD or GLB. Our finding that cicaprost or ONO-1301, a relatively selective IP agonist, did not enhance flavoprotein oxidation suggests that mitochondrial KₐTP channels are affected to a lesser extent by stimulation of the IP receptor than by stimulation of EP₃ receptors.

The mechanism whereby opening of mitochondrial KₐTP channels protects myocytes from oxidative stress remains unclear. Two reports have demonstrated that Dx protects myocytes from oxidative stress-induced apoptosis (2, 26). Akao et al. (2) demonstrated that opening of mitochondrial KₐTP channels by Dx inhibited the loss of Δψ and the release of cytochrome c induced by H₂O₂. They also showed that the cardioprotective effect of Dx against oxidative stress was inhibited by 5-HD. Opening of mitochondrial KₐTP channels by Dx increases the production of reactive oxygen species (17), which could preclude myocytes against oxidative stress.

Hide et al. (21, 22) demonstrated that PGE₁, PGE₀, and sulprostone reduce infarct size by activation of mitochondrial KₐTP channels. Ma et al. (32) demonstrated that administration of PGE₁ could induce early and late phases of the cardioprotection via the GLB-sensitive mechanism. Zacharowski et al. (54) reported that selective activation of the EP₃ receptor by ONO-AE-248 reduced infarct size via mechanisms that may involve the activation of PKC and the opening of mitochondrial KₐTP channels in rats and rabbits. In contrast, Vesper and Schror (48) reported that the cardioprotective effect of iloprost could not be abrogated by the administration of GLB. We think that the report by Vesper and Schror does not contradict our findings, because iloprost can also bind to the EP₁ receptor (11, 35). Their conclusions regarding the involvement of mitochondrial KₐTP channels in IP₃-mediated cardioprotection were based on indirect evidence, i.e., the fact that 5-HD abolished cardioprotection. The present study provides two independent lines of evidence that stimulation of the EP₃ receptor affects the opening status of KₐTP channels.

How stimulation of the EP₃ receptor modulates mitochondrial KₐTP channels remains unknown. The classical signaling pathway downstream of the EP₃ receptor is coupling to a G protein (11, 20). Sato et al. (39) demonstrated that stimulation of the A₁ receptor, which couples to Gi, primes the opening of the mitochondrial KₐTP channel in a similar experimental model. Besides its regulatory effect on adenylyl cyclase, the EP₃ receptor could signal via the small G protein (4, 29, 43). PKC (6, 54), phosphatidyl inositol signaling (34), and cAMP-response element-mediated gene transcription (7). The signaling pathways linking the EP₃ receptor to the mitochondrial KₐTP channels represent an important area for future investigations.

Conclusions. We demonstrate that activation of the EP₃ receptor protects myocytes from oxidative damage via a mechanism that involves opening of mitochondrial KₐTP channels and that the cytoprotective effects of PGI₂ analogs are due, at least in part, to activation of EP₃ receptors by these agents. To the best of our knowledge, these concepts have not been previously suggested or demonstrated.

Although in isolated ventricular myocytes the effect of IP receptor stimulation on cell survival under oxidative stress was marginal, in the clinical setting PGI₂ analogs may also be potentially useful. For patients with hypotension and fixed coronary stenosis, administration of an EP₃-selective agonist might be useful, because it primes the opening of mitochondrial KₐTP channels without adverse effects on hemodynamics. For patients with unstable plaques, administration of a PGI₂ analog, which can bind to the IP and EP₃ receptors, should be
considered, because it could correct pathophysiological features of unstable angina, such as platelet activation and neutrophil infiltration around injured vessels. A detailed understanding of the actions of prostanoids on various receptors and of the specific receptor-dependent effects on myocardial ischemia-reperfusion injury may have implications for the development of new therapeutic strategies predicated on the use of targeted prostanoid receptor agonists or gene transfer of prostanoid receptor genes.

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