Potent Constrictor Actions of Endothelin-1, Endothelin-2, and Endothelin-3 in Rat Isolated Portal Vein

Claudio Laurentino Guimaraes, Joao Batista Calixto, and Giles Alexander Rae

In rings of rat portal vein, endothelin-1, endothelin-2, and endothelin-3 caused graded slow contractions and potentiated spontaneous contractions. The apparent EC50 values and maximal responses to 30 nM endothelin were 1.4 nM and 0.96 g for endothelin-1, 5.2 nM and 0.65 g for endothelin-2, and 1.7 nM and 0.62 g for endothelin-3 (n=4-12). At concentrations producing half the contraction triggered by 80 mM KCl, the order of potencies was endothelin-1 > U46619 = angiotensin II > bradykinin > substance P > phenylephrine. Longitudinal portal-mesenteric vein preparations developed very modest contractions to endothelin-1 (0.13 g at 30 nM; n=5), but their responses to 80 mM KCl and phenylephrine were greater than those of rings. Responses of rings to endothelin-1 were profoundly reduced in Ca2+-free medium, but less inhibition was obtained after incubation with nicardipine (up to 1 μM) and/or nickel (up to 0.5 mM), phorbol (up to 0.3 μM), staurosporine (up to 10 nM), or cromakalim (3 μM). Indomethacin (5.6 μM) did not affect responses to endothelin-1. Cromakalim (0.1-3 μM) also relaxed rings constricted with 0.3 nM endothelin-1, and this effect was partially reversed by glibenclamide (3 μM). Thus, endothelins, especially endothelin-1, are potent constrictors of portal vein rings but not of portal-mesenteric vein strips. Their action appears to rely largely on Ca2+ influx from the external medium (only in part via L- and T-type Ca2+ channels) and activation of protein kinase C but not on eicosanoid generation. Also, both the development and maintenance of endothelin-1-induced venoconstriction can be inhibited by activation of hyperpolarizing ATP-dependent potassium channels. (Hypertension 1992;19[suppl II]:II-79-II-86)

Endothelial cells are well known to modulate vascular tone by releasing vasoactive substances such as prostacyclin and nitric oxide.1 It is now evident that these cells can also secrete endothelin-1 (ET-1), a remarkably potent vasoconstrictor peptide, which, together with endothelin-2 (ET-2) and endothelin-3 (ET-3), characterize a new class of endogenous bioactive substances.2,3 ET-1 exerts widespread actions in the cardiovascular system. When injected intravenously in the rat, it causes a short-lived lowering of systemic blood pressure promptly followed by a sustained pressor response mediated largely by prolonged arterial constriction of most vascular beds.2,4 The peptide also stimulates the heart,5 inhibits platelet aggregation,6 and can modulate sympathetic neurotransmission at both vascular4 and nonvascular junctions.8

Most of the studies on the effects of ET-1 on isolated blood vessels have used arterial preparations. However, it has been suggested that veins are more sensitive than arteries to the constrictor effects of ET-1.4 Indeed, potent constriction by ET-1 has been observed in rabbit jugular and mesenteric veins4-9 and in the retroperfused venous mesentery of the rat.10 Nevertheless, the peptide was found to be less active at constricting the guinea pig pulmonary vein11 and human pial veins12 and inactive on venules of the hamster cheek pouch13 and rabbit tenuissimus muscle.14 Altogether, these findings would suggest that ET-1 action in veins exhibits large tissue and species variability.

It is surprising, however, that two reports on the effects of ET-1 in the rat portal vein have yielded conflicting results. One study detected a remarkably intense and potent constrictor effect of the peptide in rings of the rat portal vein,15 whereas the other found that ET-1 caused a potentiation of the spontaneous contractions of longitudinal preparations of the rat portal vein but only a modest elevation in tone.16 In the current study, we have attempted to clarify this discrepancy and compare the profiles of activities of...
ET-1, ET-2, and ET-3 with that of other vasoconstrictors. Finally, we also present some functional evidence as to the possible mechanisms of ET-1 action in the smooth muscle of the portal vein.

**Methods**

**Preparations**

Male Wistar rats (300–350 g) were stunned and killed by cervical dislocation. After insertion of a PE-50 polyethylene catheter into its lumen, the portal vein was excised and rolled around the catheter on damp filter paper to remove the endothelial cells. It was then placed in Krebs’ solution and freed of adhering tissues. The vessel was cut to yield a ring 3 mm long, which was placed over two steel stirrups and transferred to a double-jacketed organ bath containing 5 ml Krebs’ solution (millimolar composition: NaCl 118, KCl 4.7, KH₂PO₄ 0.9, CaCl₂ 2.5, NaHCO₃ 25, MgSO₄ 1.2, glucose 11; pH 7.4) at 37°C bubbled with 5% CO₂ in oxygen and connected to an isometric transducer coupled to a pen recorder. The preparation was distended to a basal tension of 0.5 g and allowed to equilibrate for at least 1 hour before pharmacological interventions, with frequent renewal of the bathing medium. Some experiments were performed with the portal vein together with the common mesenteric vein set up for recording of contractions along the longitudinal axis. Unlike the portal vein ring, this preparation develops marked spontaneous rhythmic contractions. For these later experiments, adhering tissues were removed in situ and the vessels cut out to yield a preparation approximately 1 cm long. After cotton threads were tied to each end, the preparation was placed vertically in the organ bath, under the same conditions as the ring preparations, for recording of isometric contractions along the longitudinal axis.

**Experimental Procedures**

After equilibration, portal vein rings were initially challenged with 80 mM KCl (by equimolar replacement of 74.4 mM NaCl by KCl in the medium) as a standard stimulus. After washout, replacement with normal medium, and complete relaxation (30 minutes), concentration–response curves were obtained to one of several agonists. Endothelins (ET-1, ET-2, and ET-3), Bay K 8644 (an activator of L-type calcium channels), and U46619 (a thromboxane A₂/prostaglandin H₂ mimetic) were added to the bath.

Responses to the endothelins and other agonists were studied at several concentrations. However, the concentrations of cromakalim (0.1–3 μM) were increased with increasing concentrations, for 2–3 minutes at 20-minute intervals. The longitudinal portal-mesenteric vein preparations were not challenged with 80 mM KCl before concentration–response curves were obtained, because preliminary experiments showed that this resulted in marked depression of the spontaneous contractions.

Another set of experiments was performed to assess the influence of external Ca²⁺ on ET-1-induced contractions of the portal vein ring. After exposure to 80 mM KCl, as before, the preparations were bathed in Ca²⁺-free Krebs’ solution containing 1 mM EGTA for 20 minutes, during which the solution was renewed every 5 minutes. This was followed by another 10 minutes in Ca²⁺-free solution without EGTA before the curve to ET-1 was initiated. Once the response to the highest concentration of ET-1 (30 nM) had stabilized, and still in the presence of the peptide, Ca²⁺ was reintroduced to the medium at 2.5 mM, and the response was recorded until a stable plateau was again reached.

Several sets of experiments were performed to determine the possible modes of ET-1 action in the portal vein. To investigate the roles played by L- and T-type Ca²⁺ channels, some preparations were exposed to the L-type channel blocker nicardipine (10 nM, 100 nM, or 1 μM), the T-type channel blocker NiCl₂ (0.25 or 0.5 mM), or both (1 μM+0.5 mM, respectively) 20 minutes before and during the construction of the ET-1 curve. Other preparations were tested against ET-1 in the presence of indomethacin (5.6 μM), incubated 30 minutes before additions of the peptide, to detect possible modulatory actions of cyclooxygenase-derived eicosanoids.

Another two sets of experiments were performed to assess the importance of protein kinase C activation in mediating responses to ET-1. Staurosporine (1 or 10 nM) or phorbol (30, 100, or 300 nM), an inhibitor and an activator, respectively, of protein kinase C, was added to the medium 30 minutes before construction of a curve to ET-1 in their presence. We also analyzed the effect of 10 nM staurosporine, incubated 30 minutes beforehand, on the contraction induced by 300 nM phorbol.

Finally, we investigated whether ET-1–induced contractions are susceptible to the inhibitory influence of ATP-dependent potassium (KATP) channels. In some of these experiments, the preparations were exposed to either cromakalim (3 μM) or glibenclamide (3 μM), an activator and a blocker, respectively, of KATP channels, for 20 minutes before and during construction of the ET-1 curve. In others, the preparations were first contracted with a low concentration of ET-1 (0.3 nM). Once the response to the peptide stabilized, and in its presence, increasing concentrations of cromakalim (0.1–3 μM) were added cumulatively to the medium to relax the preparation. At the end of these experiments, and still in the presence of both ET-1 and cromakalim, glibenclamide (3 μM) was added to the bath.

**Statistics**

Responses to the endothelins and other agonists are presented either as percentages of the response to 80 mM KCl or as the absolute tension developed per preparation. In either case, values shown represent mean±SEM. The EC₅₀ values are presented as the geometric means accompanied by their 95% confidence limits. Statistical analysis was performed by means of two-tailed paired and unpaired Student's
FIGURE 1. Typical isometric records of the effects of endothelin-1 on the rat isolated portal vein ring (panel A) and longitudinal portal-mesenteric vein preparation (panel B). Nanomolar concentrations of endothelin-1 are indicated in the figure. Similar records were obtained in another 11 (portal vein) and four (portal-mesenteric vein) experiments.

$t$ tests when appropriate; a value of $p<0.05$ was considered significant.

Drugs

The drugs used were ET-1 (porcine), ET-2 (human), and ET-3 (rat) (Peptide Institute Inc., Japan); Bay K 8644 (a kind gift from Bayer A.G., FRG); U46619 (Upjohn); cromakalim (BRL 34915, Beecham, UK); angiotensin II (synthesized by the Department of Biophysics of the Escola Paulista de Medicina, São Paulo, Brazil); staurosporine (Boehringer, FRG); nickel chloride (Riedel, FRG); and substance P (all from Sigma Chemical Co., St. Louis, Mo.). Most drugs were stored as 0.1-100 mM stock solutions at -20°C and diluted to the desired concentration in phosphate buffered saline solution just before use. Cromakalim was dissolved in 70% ethanol in water, the dihydropyridines, nicardipine, and Bay K 8644 were dissolved in absolute ethanol; and glibenclamide was dissolved in 5% glucose in 0.1N NaOH solution (to yield 1 mM stock solutions in all cases). Control preparations were always exposed to the vehicle used to dilute the test drug or drugs. Care was taken to avoid photodegradation of the dihydropyridines during the course of the experiments.

Results

Effects of Endothelins and Other Agonists on Portal Vein Rings

As shown in Figure 1, cumulative additions of ET-1 caused graded and sustained contractions of portal vein rings. These responses developed slowly over a period of 3–5 minutes and were also associated with a potentiation of the basal spontaneous rhythmic contractions. The contraction caused by the highest concentration tested (30 nM) reversed very slowly on washout of the peptide, usually taking more than 40 minutes to return to baseline. Also, marked tachyphylaxis was observed on construction of a second curve to ET-1, 1 hour after the first curve was ended ($n=3$; results not shown).

The other endothelins showed qualitatively similar profiles of action as ET-1. However, the mean results shown in Figure 2A and Table 1 indicate that ET-2 was less potent than ET-1 yet produced the same degree of contraction at the highest concentration tested (30 nM), whereas ET-3 was equipotent with ET-1 but caused significantly less contraction at 30 nM. In contrast, Bay K 8644 caused far less contraction of the portal vein rings than the endothelins (Figure 2A) but also potentiated rhythmic contractions.

With the exception of U46619, which like the endothelins caused sustained contractions of the portal vein ring, all other agonists tested induced rather phasic responses and had to be tested noncumulatively. Again, with the exception of U46619, all other agonists produced smaller maximal responses than ET-1 (Figure 2B and Table 1). Also, they were all less potent than ET-1, including U46619. At concentrations causing contractions equivalent to 50% of the response to 80 mM KCl, the rank order of potency was ET-1>U46619=angiotensin II>bradykinin>substance P>phenylephrine.

Effects of Endothelin-1 and Other Agonists in Longitudinal Portal-Mesenteric Vein Preparation

In contrast to portal vein rings, these preparations showed pronounced spontaneous rhythmic activity, which was increased in amplitude and frequency on exposure to ET-1 (Figure 1B). However, ET-1
TABLE 1. Responsiveness of Rat Isolated Portal Vein Rings and Longitudinal Portal-Mesenteric Vein Preparations to Various Venoconstrictors

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Portal vein ring</th>
<th>Portal-mesenteric vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50</td>
<td>Emax</td>
</tr>
<tr>
<td>KCl (80 mM)</td>
<td>...</td>
<td>468±17</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>1.4 nM (0.9-2.2)</td>
<td>961±126*†</td>
</tr>
<tr>
<td>Endothelin-2</td>
<td>5.2 nM (2.4-11.2)</td>
<td>645±144*†</td>
</tr>
<tr>
<td>Endothelin-3</td>
<td>1.7 nM (1.1-2.5)</td>
<td>625±86*†</td>
</tr>
<tr>
<td>Bay K 8644</td>
<td>7.3 nM (1.2-42.4)</td>
<td>90±26†</td>
</tr>
<tr>
<td>U46619</td>
<td>6.7 nM (4.8-9.3)</td>
<td>754±83†</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>4.3 nM (2.3-8.0)</td>
<td>725±68†</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>94.1 nM (26.2-334.3)</td>
<td>307±5†‡</td>
</tr>
<tr>
<td>Substance P</td>
<td>0.35 μM (0.28-0.44)</td>
<td>195±40†‡</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>2.8 μM (0.9-9.0)</td>
<td>422±80</td>
</tr>
</tbody>
</table>

EC50, geometric mean concentration producing half maximal response accompanied by 95% confidence limits; Emax, mean±SEM of maximal absolute tension (in milligrams) developed per preparation; n, number of experiments.

*Response to 30 nM (highest concentration tested).
†p<0.05 (Student's t test) relative to contraction induced by 80 mM KCl.
‡Response to 1 μM (highest concentration tested).

caused only a very discrete increase in tension, much smaller than that detected in portal vein rings (Table 1). A similar pattern of action was seen with U46619, although this compound caused greater sustained contractions than did ET-1 (Table 1). In contrast, phenylephrine at low concentrations (0.1-1 μM) enhanced rhythmic contractions and caused phasic responses. At higher concentrations (3-30 μM), phenylephrine inhibited spontaneous activity but induced tonic contractions (Table 1). The effects of ET-2, ET-3, angiotensin II, bradykinin, and substance P were not tested in the longitudinal portal-mesenteric vein preparations.

Effects of Calcium Removal, Nicardipine, and Nickel on Endothelin-1 Action

The constrictor effect of ET-1 was severely depressed in portal vein rings bathed in Ca²⁺-free medium (Figure 3A). Nevertheless, the response to 30 nM ET-1 was fully restored to normal when the cation was reintroduced to the bathing medium (after application of the peptide) at 2.5 mM.

Preincubation of portal vein rings with nicardipine (10 nM, 100 nM, or 1 μM) significantly attenuated, but by no means abolished, ET-1–induced responses (Figure 3B). The depressor effect of nicardipine was clearly observed with as little as 10 nM and peaked at 100 nM. Figure 3C shows the inhibitory effect of NiCl₂ (0.25 or 0.5 mM) on ET-1–induced contractions, which was clearly concentration dependent and somewhat greater than that induced by nicardipine. Simultaneous preincubation with both nicardipine (1 μM) and NiCl₂ (0.5 mM) did not cause greater inhibition of ET-1–induced contractions than that observed with NiCl₂ alone (0.5 mM; Figure 3C). Finally, neither nicardipine (1 μM) nor NiCl₂ (0.5 mM) relaxed rings preconstricted with ET-1 (30 nM; n=3; results not shown).

Effects of Phorbol, Staurosporine, and Indomethacin on Endothelin-1 Action

Rings exposed to phorbol (30, 100, or 300 nM) expressed a graded slowly developing tonic contraction (reaching 47.5±2.5% of the response to 80 mM...
KCI after 300 nM phorbol; n = 3) that waned back to baseline after 10–20 minutes. After incubation with phorbol for 30 minutes and in its continued presence, the responsiveness of the preparations to low concentrations of ET-1 (0.01–0.1 nM) were potentiated (particularly by 100 nM phorbol), whereas responses to concentrations of ET-1 in excess of 0.3 nM were clearly depressed in a concentration-dependent fashion (Figure 4A). On the other hand, preincubation with staurosporine (1 or 10 nM) for 30 minutes, which unlike phorbol did not affect basal tone, caused graded inhibitions of responsiveness to ET-1 over the full range of the concentration–response curve (Figure 4B). Also, as shown in Figure 4C, staurosporine (10 nM), incubated for 30 minutes, caused similar depressions of the contractions induced by equieffective concentrations of both phorbol (300 nM) and ET-1 (0.3 nM).

Unlike phorbol and staurosporine, preincubation with indomethacin (5.6 μM) for 30 minutes failed to alter responses of portal vein rings to ET-1 (0.01–30 nM; n = 5; results not shown).

Effects of Cromakalim and Glibenclamide on Endothelin-1 Action

Preincubation of portal vein rings with the K<sub>ATP</sub> channel activator cromakalim (3 μM) 20 minutes before ET-1 was added resulted in a rightward shift (approximately 10-fold) of the curve to the peptide. However, because of the limited availability of ET-1, it was not possible to test if this inhibition was also accompanied by a significant depression of the max-
pared with ET-1 alone, plus 3 μM cromakalim. Partial reversal by glibenclamide (GBC, 3 μM) of the relaxation induced by 3 μM cromakalim. Each bar represents the mean of seven observations and vertical lines the SEM. *p<0.05 compared with ET-1 alone, plus 3 μM cromakalim.

Discussion

The current study possibly represents the first direct comparison between the constrictor effects of ET-1 and those of ET-2 and ET-3 in rings of the rat portal vein. ET-2 displayed a similar efficacy as ET-1 but was considerably less potent. In contrast, the partial reversals by glibenclamide (GBC, 3 μM) of the relaxation induced by 3 μM cromakalim. Each bar represents the mean of seven observations and vertical lines the SEM. *p<0.05 compared with ET-1 alone, plus 3 μM cromakalim.

Figure 5. Bar graph shows effects of cromakalim on rat portal vein rings preconstricted with endothelin-1 (ET-1). Open bar indicates response to ET-1 (0.3 nM) before addition of cromakalim; hatched bars show graded relaxations induced by cumulative additions of cromakalim; closed bar illustrates partial reversal by glibenclamide (GBC, 3 μM) of the relaxation induced by 3 μM cromakalim. Each bar represents the mean of seven observations and vertical lines the SEM. *p<0.05 compared with ET-1 alone, plus 3 μM cromakalim.

The finding that ET-1–induced contractions were severely blunted in portal vein rings bathed in Ca2+-free medium but were promptly restored to normal when the cation was reintroduced to the bath demonstrates that influx of Ca2+ from the extracellular environment plays a major role in the development of responses to ET-1. Other researchers have reported similar findings in arterial smooth muscle.19–21 We also attempted to determine the channels mediating ET-1–induced Ca2+ influx. Prior incubation with up to 1 μM of the L-type Ca2+ channel blocker nicardipine caused only a modest (though significant) inhibition of ET-1–induced contractions. This finding, together with the ability of ET-1 to cause much greater contractions than the L-type channel activator Bay K 8644, suggests that these channels play only a minor role in ET-1–induced contraction. Although similar results have been obtained in strips of rat aorta19 and rabbit jugular vein,9 activation of L-type Ca2+ channels may contribute more expressively to the development of ET-1 responses at least in some vessels, such as the porcine coronary artery.2 We also observed that the relatively selective T-type Ca2+ channel blocker NiCl2 was capable of inhibiting ET-1–induced contractions. Its inhibitory effect was
greater than that of nicardipine but was by no means complete. The partial inhibition afforded by NiCl₂ in the portal vein ring also conforms with previous evidence, obtained in porcine coronary artery and rat aorta, for a significant role of T-type Ca²⁺ channels in triggering responses to ET-1. Nevertheless, the fact that neither nicardipine nor NiCl₂ relaxed portal vein rings precontracted by 30 nM ET-1 strongly suggests that, once the response to a high concentration of the peptide has developed, Ca²⁺ influx through L- and T-type Ca²⁺ channels is no longer needed to maintain contraction.

Interestingly, portal vein rings bathed in medium containing both nicardipine and NiCl₂ simultaneously developed greater contractions to ET-1 than those incubated solely with NiCl₂. Although we cannot explain this rather paradoxical finding at present, it clearly indicates that influx of Ca²⁺ through L- and T-type Ca²⁺ channels did not account for the full expression of ET-1-induced venoconstriction. Perhaps part of the Ca²⁺ influx triggered by the peptide occurred through nonselective cation channels, as has been demonstrated in aortic smooth muscle, or via a receptor-gated Ca²⁺ channel, as has been suggested for rat glomerular mesangial cells.

Another interesting finding of the present study was that responses to ET-1 were also depressed after activation and subsequent uncoupling of protein kinase C with phorbol, or inhibition of protein kinase C with staurosporine. This suggests that ET-1–induced contractions of the rat portal vein ring are coupled, at least in part, to protein kinase C–mediated mechanisms. Indeed, ET-1 has been shown to stimulate phospholipase C and the phosphoinositide cascade in rabbit aortic smooth muscle. Such an action of ET-1, via generation of inositol phosphates, diacylglycerol, or both, has been shown to mobilize Ca²⁺ from intracellular stores and to sensitize the contractile machinery to Ca²⁺. It should be noted that, unlike phorbol, staurosporine displays only a limited selectivity of action toward protein kinase C; 3) do not involve eicosanoid generation; and 4) can be reduced by activation of KATP channels.

In conclusion, the present study demonstrates that endothelins are exceptionally potent constrictors of the rat portal vein ring but are less effective in constricting longitudinal portal-mesenteric vein preparations. We also present functional evidence that suggests that responses of the rat portal vein ring to ET-1 depend largely on influx of Ca²⁺ from the external medium, only a fraction of which occurs through L- and T-type Ca²⁺ channels; 2) are mediated, at least in part, by activation of protein kinase C; 3) do not involve eicosanoid generation; and 4) can be reduced by activation of KATP channels.

References


KEY WORDS • endothelin • vascular smooth muscle • portal vein • nicardipine • glibenclamide • nickel • phorbols • calcium
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