Vol. 185, No. 3, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 30, 1992 Pages 887-892

THE ENDOTHELIN RECEPTOR ANTAGONIST, BQ-123, INHIBITS ANGIOTENSIN II-INDUCED CONTRACTIONS IN RABBIT AORTA

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Received April 27, 1992

Summary: The purpose of this study was to examine the specificity of the cyclic pentapeptide ET_A receptor antagonist BQ-123. BQ-123 competitively antagonized endothelin-1-induced contractions in rabbit aorta, increases in inositol phosphates in cultured rat vascular smooth muscle A10 cells, and binding of [^{125}I]endothelin-1 to the cloned ET_A receptor cDNA expressed in Cos 7 cells. In contrast, BQ-123 was a weak antagonist of [^{125}I]endothelin-3 binding to rat cerebellar membranes and to membranes from Cos 7 cells transfected with the cloned ET_B receptor cDNA. BQ-123 shifted concentration-response curves in isolated rabbit aorta elicited by angiotensin II, but did not bind to angiotensin II receptors nor affect angiotensin II-induced increases in inositol phosphates. BQ-123 also did not affect contractions induced by KCl or norepinephrine. These data suggest that endothelin may play a role in angiotensin II-induced contractions of rabbit aorta.

ET, originally isolated from porcine endothelial cells (1), is a stimulant of smooth muscle (2,3), a positive inotrope and chronotrope in the myocardium (4), and a potent pressor agent in the renal vasculature (5). ET also stimulates the release of aldosterone from adrenoglomerulosa cells (6), and is mitogenic for cultured vascular smooth muscle cells (7), kidney mesangial cells (8) and Swiss 3T3 fibroblasts (9). Despite the numerous reports of these diverse effects and the intense interest in the ET family of peptides, the roles of these peptides in homeostasis and disease remain unknown.

Functional, pharmacological and molecular biological data substantiate the existence of two receptors for ET (10). The ET_A receptor displays several hundred fold selectivity in binding for ET-1 and ET-2 over ET-3 (11) while ET_B receptors bind ET-1, ET-2 and ET-3 with similar affinity (12). The discovery and development of ET receptor antagonists would aid in the understanding of the physiological and pathophysiological role for the ETs in vascular disease. Recently, the

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Abbreviations: ET, endothelin; SI-AII, Sarcosine[1],isoleucine[8]-angiotensin II; DMEM, Dulbecco'smodified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; DEAE, diethylaminoethyl; FCS, fetal calf serum; BSA, bovine serum albumin; IP, inositol phosphate; K_i, inhibition constant; VSM-A10, vascular smooth muscle A10; RASM, rat aortic smooth mucle; SEM, standard error of the mean; SD, standard deviation.

0006-291X/92 $4.00

cyclic pentapeptide BE18257A, cyclo(D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp), isolated from the fermentation products of *Streptomyces misakiensis*, was shown to be a selective ET_A receptor antagonist (13). BE18257A inhibited [^{125}]ET-1 binding in vascular smooth muscle (IC_{50} = 1.4 μM) and cardiac muscle (IC_{50} = 0.8 μM). In contrast, concentrations of BE18257A up to 100 μM did not inhibit [^{125}I]ET-1 binding in the kidney, adrenal gland or cerebellum. This peptide also inhibited vasoconstriction in rabbit iliac artery and pressor activity in the rat induced by ET-1. A related cyclic peptide BQ-123, cyclo(D-Asp-Pro-D-Val-Leu-D-Trp), has improved potency (IC_{50} = 7.3 nM) for inhibiting binding of [^{125}I]ET-1 in vascular smooth muscle (14). Preliminary studies on BQ-123 showed that it inhibited angiotensin II-induced contractions in isolated rabbit aorta (15). These findings raised the possibility that BQ-123 acted through a more complex mechanism of action. Thus, the purpose of this investigation was to examine the specificity of BQ-123.

Materials and Methods

Materials: [^{125}I]ET-1 (2,200 Ci/mmol), [^{125}I]ET-3 (2,200 Ci/mmol), and [^{125}I]SI-AII (2,200 Ci/mmol) were obtained from New England Nuclear. [^{3}H]myoinositol was from Amersham, ET-1, ET-3, angiotensin II, and SI-AII were purchased from Peninsula Labs, cell culture reagents from Gibco, Nuserum from Collaborative Research and transfection reagents were from Specialty Media. Rat VSM-A10 and Cos 7 cells were obtained from American Type Culture Collection. RASM cells were generously provided by Dr. Marschall Runge of Emory University. BQ-123 was synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute.

Phosphoinositide metabolism: VSM-A10 or RASM cells were cultured in 35 mm wells, and labelled to isotopic equilibrium with [^{3}H]myoinositol (2-4 μCi/ml) in inositol-free DMEM for 48 hours. Cells were washed free of [^{3}H]myoinositol and the medium changed to DMEM containing 10 mM LiCl to inhibit inositol-1 phosphatase. Following a 30 minute pre-incubation period with the test compound, cells were stimulated with agonist for 30 minutes. IPs were extracted in 2 mM boiling EDTA and separated using Dowex AG-1X8 anion exchange chromatography (16).

Preparation of VSM-A10 cell membranes: Confluent VSM-A10 cells in T75 flasks were trypsinized and collected in buffer A (DMEM plus 20 mM Hepes pH 7.4, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor) at a concentration of approximately 300 to 400 x 10^3 cells/ml. Cells were stored frozen at -70°C until use whereupon they were thawed in a 37°C water bath, homogenized with a Brinkman Polytron at setting 8 for 10 seconds, and centrifuged at 100,000 g for one hour at 4°C. The supernatant was discarded and the membrane pellet was resuspended in buffer A at a ratio of 10 ml per T75 flask. Membranes were homogenized, centrifuged and resuspended as described above and aliquots stored frozen at -70°C.

Preparation of rat cerebellar membranes: Brain tissue from Sprague-Dawley rats was homogenized for 30 seconds with a Brinkman Polytron (setting 8) in ice cold 50 mM Tris-HCl (pH 7.4) with 0.24 units/ml aprotinin and 1 mM EDTA. The crude particulate matter was removed by centrifugation at 750 g for 10 minutes at 4°C. The membranes were sedimented from the supernatant fraction by centrifugation at 48,000 g for 30 minutes. Membrane pellets were resuspended in the above buffer and stored in aliquots at -70° C until use.

Transient transfection and preparation of Cos 7 cell membranes: Transient transfection of Cos 7 proceeded as previously described (17). Briefly, cells were incubated with 2 - 6 μg of pCDM8/ET_A (17) or pCDM8/ET_B DNA (Dr. P. Rose, personal communication) and DEAE-dextran (250 μg/ml) mixture for 1.5 hours at 37°C in 4 ml DMEM. The DEAE-dextran/DNA mixture was removed and 3 ml of DMEM containing 10% FCS was added to each plate. Chloroquine was added to the medium and incubation proceeded for 2.5 hours at 37°C. Cells were washed with DMEM, growth medium (DMEM containing 10% FCS) added, and incubated at 37°C for 24 - 72 hours. Cos 7 cells in 100 mm dishes were collected in buffer A, homogenized with a Brinkmann Polytron, and centrifuged at 100,000 g for one hour at 4°C. The membrane pellet was resuspended in buffer A at a ratio of 10 ml per T75 flask. Membranes were homogenized, centrifuged and resuspended as described above and aliquots stored frozen at -70°C until assay.

Radioligand binding assays: ET receptor binding assays were conducted as described previously (18). Membranes (10 - 30 μg) from rat VSM-A10 cells, transfected Cos 7 cells, and

cerebellum were incubated with 50 pM [^{125}I]ET-1 or 0.1 nM [^{125}I]ET-3 in the absence or presence of 100 nM ET-1 or ET-3, respectively, in a final volume of 0.25 ml assay buffer (50 mM Tris-HCl pH 7.4, 0.1% BSA, 2 μM phosphoramidon) at 37°C for two hours.

[^{125}I]SI-AII binding was conducted as described (19). Membranes were incubated with 0.2 nM [^{125}I]SI-AII in the absence and presence of 1 μM angiotensin II in a final assay volume of 0.25 ml assay buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA) for 2 hours at 22°C.

Binding reactions were terminated by rapid filtration in a Tomtec™ cell harvester over a Filtermat B™ (Pharmacia LKB) pre-soaked for 1 hour in assay buffer ([^{125}I]ET-1 and [^{125}I]ET-3) or 0.1% polyethyleneimine ([^{125}I]SI-AII). The filtermat was rinsed with 150 mM NaCl, 5 mM Tris-HCl, pH 7.4 at 4°C, microwaved, and counted in a Betaplate™ liquid scintillation counter (Pharmacia LKB) in the presence of a Meltilex™ solid scintillant wax (Pharmacia LKB). Counting efficiency was 65% for [^{125}I] ligands. Data were analyzed by iterative curve fitting to a one or two binding site model, and K_i values calculated from IC_{50} values (20).

Force determinations: The thoracic aorta was removed from male New Zealand white rabbits (2 - 3 kg), cleaned of connective tissue, and cut into circumferential strips (3.5 - 5 mm wide). Each strip was mounted for isometric force recording between a micrometer for control of tissue length and a Grass FT 03 force transducer and Grass model 7D polygraph. The tissues were placed in individual chambers in a solution composed of (in mM): 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.9 $CaCl_2$, 25 $NaHCO_3$, 10.1 D-glucose, and 0.01 Na_2EDTA. The solution was warmed to 37°C and a pH of 7.4 was maintained by aeration with 95% O_2/5% CO_2. The strips were stretched to 4 g preload during a 1 hour equilibration period. Cumulative concentration response curves for angiotensin II and ET-1 were obtained in the absence and presence of the compounds of interest.

Free intracellular calcium ion determinations: Cultured VSM-A10 cells were loaded with the calcium indicator dye fura-2 by incubation with 2 μM fura-2 acetoxymethyl ester for 30 minutes at 37°C. Extracellular fura-2 acetoxymethyl ester was washed off the cells, then fluorescence experiments were carried out in a SPEX spectrofluoremeter at 37°C in quartz cuvettes as described elsewhere (19). The free intracellular calcium ion concentration was calculated from the fluorescence values as described (21).

Statistical analysis: Data are expressed as mean ± SD or SEM. Statistical comparisons were compared using Student's *t* test. The null hypothesis was rejected at $P < 0.10$.

Results and Discussion

ET-1 contracted isolated rabbit aortic strips in a concentration dependent manner (EC_{50} = 1 ± 0.2 nM). The addition of BQ-123 was associated with a rightward, parallel shift in the ET-1 concentration response curve with no significant depression of the maximum contraction. These data were consistent with competitive antagonism by BQ-123 at the ET receptor (Figure 1A). The calculated K_B for BQ-123 under these circumstances was 34 ± 3.9 nM which is similar to that obtained in isolated porcine left anterior descending coronary artery (14). In rat VSM-A10 cells, ET-1 stimulated a concentration dependent increase in IP. As with the rabbit aorta, the ET-1 concentration response curve was shifted to the right without suppression of the maximum effect in the presence of 100 nM BQ-123 (Figure 1B). The apparent K_B was 5 ± 0.4 nM. BQ-123 was also able to inhibit the transient increase in the free intracellular calcium ion concentration induced by 3 nM ET-1 stimulation of VSM-A10 cells (IC_{50} = 26 ± 7 nM).

BQ-123 inhibited [^{125}I]ET-1 binding to VSM-A10 cell membranes and membranes from Cos 7 cells transiently transfected with an ET_A receptor cDNA in a monophasic fashion and slope factors were not significantly different from unity (Table 1). By contrast, [^{125}I]ET-1 binding to rabbit saphenous vein membranes was inhibited in a biphasic manner indicative of two populations of receptors. BQ-123 was a weak inhibitor of [^{125}I]ET-3 binding to ET_B receptors in membranes from rat cerebellum and to membranes from Cos 7 cells transiently transfected with the human

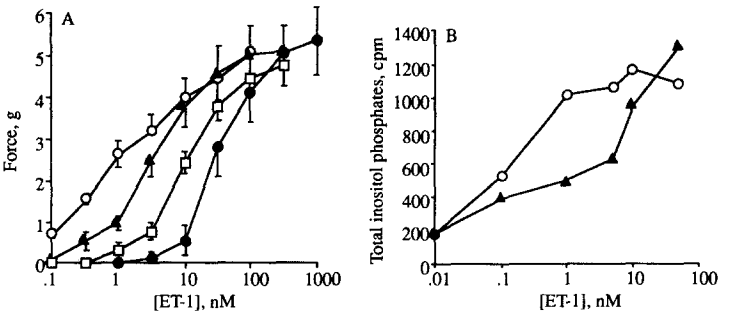

Figure 1. Effect of BQ-123 on ET-1 induced responses. A. Cumulative concentration response curves for force elicited by ET-1 in isolated rabbit aortic strips in the absence (○) and presence of 100 (▲), 300 (□), or 1000 (●) nM BQ-123. Values are means ± SEM, n = 4 for each curve. B. Concentration response curves for IP metabolism in VSM-A10 cells induced by ET-1 in the absence (○) and presence (▲) of 100 nM BQ-123. The curves are representative of two similar experiments.

placental ET_B receptor cDNA clone (Table 1). The binding affinities of BQ-123 in rat VSM-A10 cell and cerebellar membranes reported here are consistent with the IC_{50} values for BQ-123 inhibition of [^{125}I]ET1 binding in porcine aortic smooth muscle cells (7.3 nM) and cerebellar membranes (18 μM) (14).

To examine the selectivity of BQ-123 for force development elicited by ET-1, isolated strips of rabbit aorta were stimulated with cumulative concentrations of norepinephrine, KCl, or angiotensin II in the absence and presence of BQ-123. The EC_{50} values for norepinephrine and KCl were not affected by 300 nM BQ-123 (Table 2), however BQ-123 significantly ($P < 0.10$) shifted the EC_{50} value calculated for angiotensin II. Increasing concentrations of BQ-123 (0.3 - 10 μM) shifted the cumulative concentration response curves to angiotensin II to the right, but not in a completely concentration dependent manner (Figure 2). Moreover, the slope of the Schild plot (-0.51) was significantly different from unity suggesting that BQ-123 does not competitively inhibit ET-1 elicited contractions.

Inhibition of [^{125}I]SI-AII binding to angiotensin II receptors by BQ-123 was conducted in membranes from the rat adrenal cortex. BQ-123 up to concentrations of 100 μM failed to inhibit specific [^{125}I]SI-AII binding to rat adrenocortical membranes or to rabbit aortic membranes. Additionally, BQ-123 (100 μM) did not affect angiotensin II induced IP generation in RASM cells. Thus, the effect of BQ-123 on angiotensin II induced force development in isolated rabbit aortic strips cannot be explained by a direct effect of BQ-123 on the angiotensin II receptor.

Table 1. K_i values and slope factors for BQ-123 inhibition of [^{125}I]ET-1 or [^{125}I]ET-3 binding

Tissue or Cell	K_i (nM)	Slope factor
VSM-A10	18 ± 4.2	0.9 ± 0.1
pCDM8/huET$_A$ Cos 7	2.4 ± 0.1	0.9 ± 0.1
Rabbit saphenous vein	7.6 ± 1.3 (75 ± 6%)	1.0*
	1,900 ± 1,200 (25 ± 6%)	1.0*
Rat cerebellum	7,000 ± 42	1.4 ± 0.1
pCDM8/huET$_B$ Cos 7	32,000 ± 1,200	0.8 ± 0.02

Values represent means ± SD, n = 2. *Slope constrained to 1.0 for two-site analysis.

Table 2. EC_{50} values for agonist stimulation of isolated rabbit aorta

Agonist	Agonist alone	Agonist + 300 nM BQ-123
ET-1	1.0 ± 0.23 nM	10 ± 1.2 nM*
Norepinephrine	290 ± 61 nM	420 ± 100 nM
KCl	25 ± 3.8 mM	25 ± 1.0 mM
Angiotensin II	0.73 ± 0.07 nM	1.2 ± 0.26 nM*

Values represent means ± SEM, n = 4. *Denotes statistical significance at P<0.10.

It is unlikely that BQ-123 nonspecifically depressed force elicited by angiotensin II, because it had no effect on contractions induced by norepinephrine or KCl. This finding appears to rule out effects of BQ-123 on intracellular targets (such as the contractile proteins) or on ion channels. It is possible that angiotensin II induced the release of ET-1 which participated in the contractile response in rabbit aorta. Angiotensin II has been reported to stimulate the release of ET-1 in cultured endothelial cells (22) and in endothelial cells *in situ* (23). ET-1 is generally believed to be synthesized in precursor form as preproendothelin immmediately prior to release (1). Preproendothelin is hydrolyzed to big ET-1 by various intracellular proteases, however the subsequent conversion of big ET-1 to ET-1 is due to the action of phosphoramidon-sensitive endothelin converting enzyme (1,24). Therefore, the effect of phosphoramidon on contractions induced by angiotensin II was examined. At 1 μM, phosphoramidon alone did not affect the angiotensin II concentration response curve in rabbit aorta, nor did it potentiate the effect of BQ-123. Thus, it is unlikely that the processing of big ET-1 is involved in the angiotensin II induced force development under these conditions. The possibility that ET-1 is stored in the rabbit aorta and is released upon stimulation with angiotensin II must be considered.

In summary, BQ-123 is a potent and specific blocker of ET_A receptors in a number of different tissues and cells. The ability of BQ-123 to block the effects of ET-1 in rabbit aorta suggests that the contractile effects of ET-1 are due to stimulation of ET_A receptors. BQ-123 depressed angiotensin II-induced contractions in rabbit aorta, but the effect was unlikely to be due

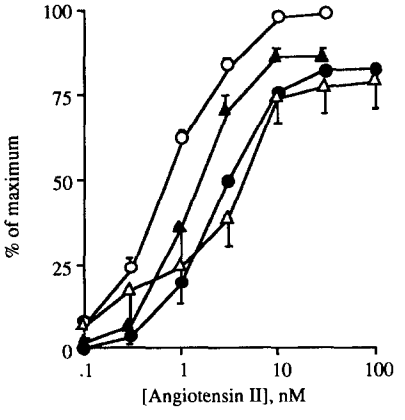

Figure 2. Effect of BQ-123 on angiotensin II induced contractions in isolated rabbit aorta. Cumulative concentration response curves were obtained in the absence (○) and presence of 0.3 (▲), 3 (●), and 10 (Δ) μM BQ-123. Values are means ± SEM, n = 4 for each curve.

to its binding to angiotensin II receptors or to other nonspecific vasorelaxant actions. It is possible that angiotensin II elicits the release of stored ET-1 from rabbit aorta and that this ET-1 participates in the contractile responses induced by angiotensin II.

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