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Red marine alga *Bryothamnion triquetrum* lectin induces endothelium-dependent relaxation of the rat aorta via release of nitric oxide

Ricardo F. Lima, David N. Criddle, Emmanuel P. Souza, Alexandre H. Sampaio, Kyria S. Nascimento, Benildo S. Cavada and Ana Maria S. Assreuy

Abstract

We have investigated the vascular relaxant effects of the lectin from a red marine alga *Bryothamnion triquetrum* (BTL), in particular, the endothelial-dependency and the participation of a specific glycoprotein-binding site. BTL (1–100 μg mL^{-1}) was applied to rat isolated aortic rings, with or without endothelium, tonically precontracted with phenylephrine (0.1 μM). Endothelium-dependent relaxation was assessed in the presence of indometacin (10 μM), L-nitro arginine methyl ester (L-NAME, 100 μM) and tetraethylammonium (TEA, 500 μM). For the involvement of the glycoprotein-binding site, BTL was assayed in presence of mucin (300 μg mL^{-1}) or *N*-acetyl D-glucosamine (GlcNAc; 300 μg mL^{-1}), a specific and non-specific lectin-binding sugar, respectively. BTL fully and concentration dependently relaxed preparations that possessed an intact endothelium (IC50 (concn producing 50% contraction) = 12.1 ± 1.6 μg mL^{-1}), whereas no significant relaxation was observed in endothelial-denuded tissue. L-NAME, but not indometacin or TEA, completely inhibited the lectin relaxation, suggesting the involvement of nitric oxide (NO). The lectin in association with mucin, but not with GlcNAc, inhibited BTL-induced relaxation, implicating the involvement of the lectin binding site. Our data suggest that the relaxant effect of the red marine alga *Bryothamnion triquetrum* lectin on isolated aorta occurs via interaction with a specific lectin-binding site on the endothelium, resulting in a release of NO.

Laboratório de Farmacologia dos Canais Iônicos – LAFACI, Departamento de Ciências Fisiológicas, CCS, Universidade Estadual do Ceará, Av. Paranjana 1700, 60740-000, Fortaleza-CE, Brazil

Ricardo F. Lima, David N. Criddle, Emmanuel P. Souza, Ana Maria S. Assreuy

MRC Secretory Control Research Group, The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, UK

David N. Criddle

Departamentos de Engenharia de Pesca e de Bioquímica e Biologia Molecular & BioMol-Lab, Universidade Federal do Ceará, C.P. 6043, 60455-970, Fortaleza-Ce, Brazil

Alexandre H. Sampaio, Kyria S. Nascimento, Benildo S. Cavada

Correspondence: D. N. Criddle, MRC Secretory Control Research Group, The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, UK.
E-mail: criddle@liv.ac.uk

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Introduction

Lectins are a structurally diverse class of glycoproteins distributed widely throughout nature, including marine algae (Beuth et al 1995), and have been utilised in the characterization of cell-surface glycoproteins in many cell types, such as endothelial cells (Weinhouse et al 1993). Lectins can interact with specific saccharide groups of glycoproteins to influence diverse biological processes, such as cell-to-cell signalling, host defence and cell differentiation, although often their precise roles remain poorly understood (Van Damme et al 1998; Beisel et al 1999; Assreuy et al 2003). Such glycoprotein binding can be blocked by specific sugars inhibiting function (Grulich-Henn et al 1988).

Previously it has been shown that extracts obtained from two red marine algae, *Bryothamnion triquetrum* and *B. seaforthii*, collected along the coast of Ceará (Brazil), exhibit strong haemagglutinating activity (Ainouz & Sampaio 1991; Ainouz et al 1992). The lectins derived from these algae have been isolated, purified, and characterized in some detail and interestingly their activity is not inhibited by simple sugars, but is sensitive to the glycoproteins fetuin, mucin and avidin (Ainouz et al 1995). *B. triquetrum* lectin (BTL) was the first algal lectin to have its primary structure fully elucidated and appears to be structurally arranged in several different forms: monomers, dimers and multimers, with each monomer > 10 kDa (Calvete et al 2000). This suggests that BTL does not conform to a previous view that algal lectins possess only a monomeric arrangement (Hori et al 1990). Since its amino-acid sequence bears no significant correlation with existing lectin sequences available in public databases, BTL may define a novel protein family (Calvete et al 2000).

Lectins may bind to vascular endothelial cells (Simionescu et al 1982; Mills & Haworth 1986) and a report has shown that wheat-germ agglutinin can release endothelium-derived relaxant factor in rat aorta (Kleha et al 1991). However, there remains little information available regarding the effects of lectins on blood vessels, and to the authors' knowledge no lectin derived from algae has yet been evaluated in the vasculature. In this study we have investigated the effects of BTL on isolated aortic smooth muscle and assessed the possible involvement of endothelium-derived factors in its action.

Materials and Methods

Drugs

The following drugs were used: acetylcholine (ACh), phenylephrine, L-nitro arginine methyl ester (L-NAME), indometacin, tetraethylammonium (TEA), mucin, *N*-acetyl D-glucosamine (GlcNAc) and papaverine. A stock solution of indometacin (10^{-2} M) was prepared in 70% ethanol, while all other agents were dissolved directly in distilled water. All the reagents were purchased from Sigma Chemical Company (St Louis, MO), Reagen (Rio de Janeiro, RJ, Brazil) or Vetec (Rio de Janeiro, RJ, Brazil).

Isolation and purification of *Bryothamnion triquetrum* lectin (BTL)

Specimens of the red marine algae *Bryothamion triquetrum* were collected on the Atlantic coast, NE Brazil (Pacheco Beach, Ceará). The purification process was followed according to the method of Ainouz et al (1995) and Calvete et al (2000). BTL is a 91-amino-acid-containing protein (MW 9 KDa), with four cysteine residues per lectin monomer, whose complete amino-acid sequence has previously been determined (Calvete et al 2000).

Tissue preparation

Institutional Ethical Committee approval (Universidade Estadual do Ceará) for experimental protocols used in this study was obtained. Male Wistar rats, 200–300 g, were killed by stunning and cervical dislocation. The thoracic aorta was quickly excised and placed in Tyrode solution (composition in mM: NaCl 136, KCl 5, $NaHCO_3$ 11.9, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, glucose 5.5). Tissues were then cleaned of connective tissue and cut into rings (3 mm). In certain experiments the endothelium was removed by mechanical rubbing of the intimal surface.

Measurement of contractile responses

Aortic rings were fixed in an organ bath chamber, filled with 10 mL Tyrode solution, bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. A resting tension of 2 g was applied and tissues were left to equilibrate for 60 min. The active tension was developed isometrically using a force transducer connected to a computerized data acquisition system (Chart 4.1; PowerLab AD Instruments, Inc., Australia). The presence of endothelium was determined by addition of ACh (1 μM) to phenylephrine (0.1 μM)-precontracted aortic rings. Tissues were considered to possess an intact endothelium when the relaxant response to ACh was greater than 75% of induced tone.

Evaluation of relaxant effects of BTL on aortic rings

BTL was added in cumulative concentrations (1–100 $\mu g\,mL^{-1}$) to tissues, with or without endothelium, pre-contracted with phenylephrine (0.1 μM). To investigate the possible involvement of endothelium-derived relaxant factors in the lectin-induced relaxation, an inhibitor of nitric oxide synthase (NOS), L-nitro arginine methyl ester (L-NAME; 100 μM), the cyclooxygenase inhibitor indometacin (10 μM), and the potassium-channel blocker tetraethylammonium (TEA, 500 μM) were added to pre-contracted tissues (possessing an intact endothelium) before performing cumulative concentration–response curves to BTL in the presence of these inhibitors. The reversibility of the lectin-induced relaxant effect was tested using a BTL submaximal effective concentration (30 $\mu g\,mL^{-1}$) on phenylephrine-precontracted tissues in the presence of indometacin (10 μM). Following a plateau relaxant response to BTL, L-NAME (0.1–100 μM) was cumulatively added to reverse this effect and papaverine (10 μM) subsequently used as a control to further relax the tissue.

To evaluate whether the effect of BTL was mediated via an interaction of its specific glycoprotein binding site, the lectin was pre-incubated with the specific binding glycoconjugate mucin (300 $\mu g\,mL^{-1}$) or with a non-specific binding sugar, *N*-acetylglucosamine (GlcNAc; 300 $\mu g\,mL^{-1}$), for 30 min at 37°C, before addition to the tissue. In separate experiments, BTL was heated at 90°C for 3 h before performing cumulative concentration–relaxation protocols to establish a possible dependence on the native protein conformation.

Statistics

All results are presented as mean ± s.e.m. of n experiments. Comparisons were made using Student's *t*-test (paired or unpaired as appropriate) or one-way analysis of variance followed by post-hoc Tukey multiple comparison test and values taken to be significantly different from controls when $P < 0.05$.

Results

BTL induces endothelium-dependent relaxation of rat isolated aortic rings

Phenylephrine (0.1 μM) induced stable, tonic contractions of rat aortic rings of 0.27 ± 0.03 g in preparations possessing an intact endothelium (n = 7; Figure 1A) and of 0.72 ± 0.11 g in those in which the endothelium had been

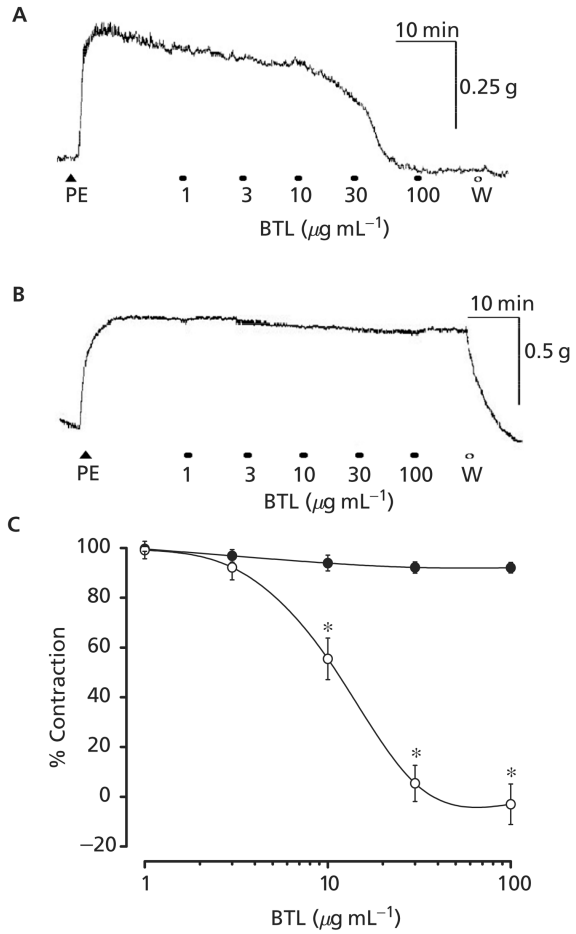

Figure 1 *Bryothamnion triquetrum* lectin (BTL) induces endothelium-dependent relaxation of rat isolated aorta. Typical traces showing the effects of BTL (1–100 $\mu g\,mL^{-1}$) on aortic rings precontracted with 0.1 μM phenylephrine (PE) with intact endothelium (A) or without endothelium (B). W indicates washing of the preparation with Tyrode's solution. C. Mean data from these experiments comparing the responses in tissues with (○, n = 7) and without (●, n = 6) endothelium. Values are expressed as mean ± s.e.m. % changes from initial PE-induced tonic contraction; $*P < 0.05$, endothelially-intact vs -denuded tissues.

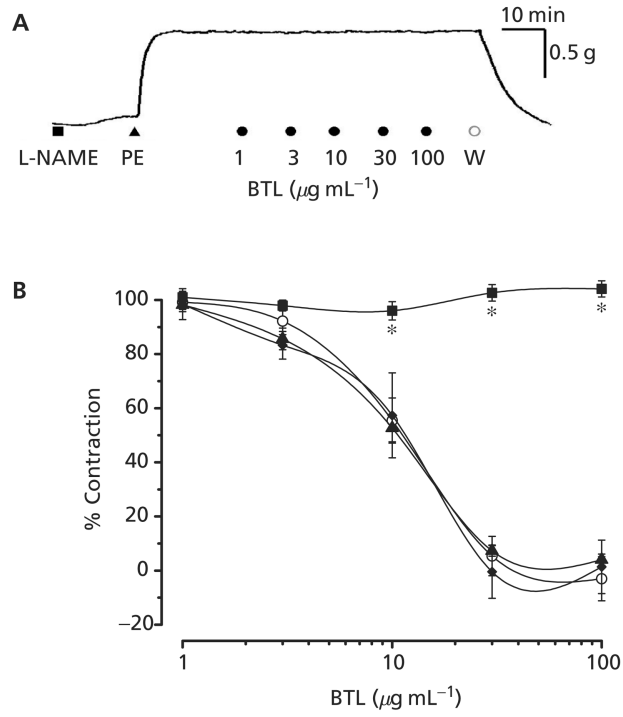

Figure 2 BTL-induced relaxation of rat isolated aorta is blocked by L-NAME, but not by TEA or indometacin. A. Typical trace showing the lack of relaxant effect of BTL in 0.1 μM phenylephrine (PE)-precontracted aortic rings with intact endothelium in the presence of L-NAME (100 μM). W indicates washing of the preparation with Tyrode's solution. B. Comparative effects of BTL (1–100 $\mu g\,mL^{-1}$) on PE-induced contractions of aortic rings, with an intact endothelium, in the absence (○, n = 7) or presence of 10 μM indometacin (●, n = 7), 500 μM TEA (▲, n = 5) or 100 μM L-NAME (■, n = 6). Values are expressed as mean ± s.e.m. % changes from initial PE-induced tonic contraction; $*P < 0.05$, vs control.

mechanically removed (n = 6; Figure 1B). Cumulative addition of BTL to precontracted tissues induced a significant, concentration-dependent relaxation of preparations with preserved endothelium, starting at 10 $\mu g\,mL^{-1}$, with complete reversal evident at 100 $\mu g\,mL^{-1}$ (IC50 (concn producing 50% contraction) = 12.08 ± 1.63 $\mu g\,mL^{-1}$; Figures 1A and 1C). No relaxant effect, however, was observed in tissues in which the endothelium had been removed (Figures 1B and 1C). Additionally, in separate experiments no significant effect of BTL (1–100 $\mu g\,mL^{-1}$) was seen on tissue basal tonus (n = 5; data not shown).

BTL-induced relaxation is blocked by L-NAME, but not by indometacin or TEA

The relaxant effect of BTL was totally blocked by the NOS inhibitor L-NAME (100 μM; n = 6; Figures 2A and 2B) in aortic rings with an intact endothelium. In contrast, there was no significant inhibition of BTL-induced relaxation observed in the presence of the non-selective potassium-channel blocker TEA (500 μM; IC50 = 11.33 ± 2.05 $\mu g\,mL^{-1}$; n = 5) or the cyclooxygenase inhibitor indometacin (10 μM; IC50 = 10.17 ± 1.27 $\mu g\,mL^{-1}$; n = 7; Figure 2B).

Additionally, the reversibility of lectin-induced relaxation was evaluated by cumulative application of L-NAME (0.1–100 μM) following the response to a submaximal (30 $\mu g\,mL^{-1}$) concentration of BTL in phenylephrine-precontracted preparations. Thus, following a stable plateau response to BTL, addition of L-NAME reversed this effect in a concentration-dependent manner, starting at 0.1 μM and attaining a complete reversal at 1 μM (n = 5; Figures 3A and 3C). Interestingly, L-NAME increased aortic tone in excess of the initial tonic contraction induced by phenylephrine at higher concentrations, reaching 389 ± 19% of the initial induced tone at a concentration of 100 μM. A further addition of papaverine (10 μM) was able to relax the tissue in the continued presence of BTL (30 $\mu g\,mL^{-1}$) and L-NAME (100 μM), showing that

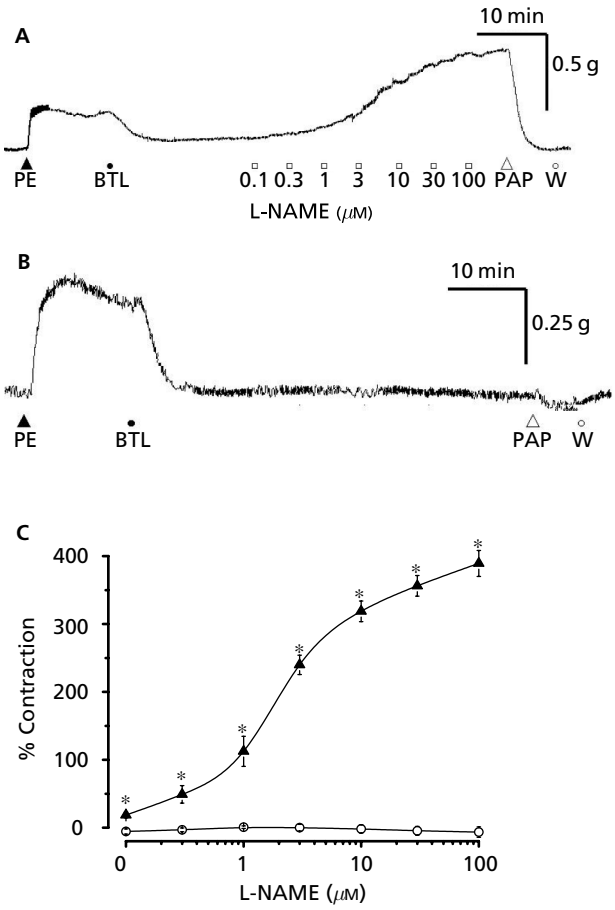

Figure 3 BTL-induced relaxation is reversed by L-NAME. A. Typical trace showing the reversal of 30 μg mL^{-1} BTL-induced relaxation by L-NAME (0.1–100 μM) in phenylephrine (PE)-precontracted rat aortic rings with intact endothelium. Papaverine (PAP, 10 μM) was added at the plateau of the L-NAME response to show that relaxation was still viable in the preparation. W indicates washing of the preparation with Tyrode's solution. B. Typical trace showing the stability of 30 μg mL^{-1} BTL-induced relaxation in PE-precontracted aortic rings with intact endothelium. C. Mean data comparing the effects of 30 μg mL^{-1} BTL on vascular tonus in the absence (○; time control, n = 6) and presence of L-NAME (▲; 0.1–100 μM, n = 5). Values are expressed as mean ± s.e.m. % changes from initial PE-induced tonic contraction; *P < 0.05, vs absence of L-NAME.

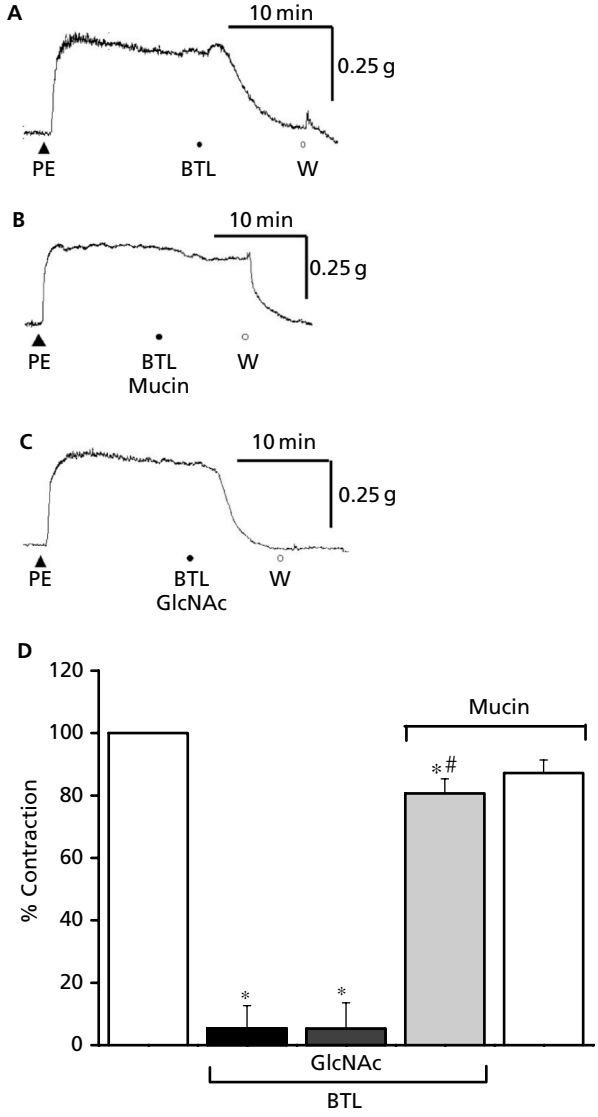

Figure 4 BTL-induced relaxation involves a specific lectin-binding site. Typical traces showing the relaxant effects of 30 μg mL^{-1} BTL on phenylephrine (PE)-precontracted rat aortic rings with intact endothelium in the absence of prior incubation (A), following pre-incubation with mucin (300 μg mL^{-1}) (B) and following pre-incubation with *N*-acetyl D-glucosamine (GlcNAc; 300 μg mL^{-1}) (C). W indicates washing of the preparation with Tyrode's solution. D. Mean data from these experiments showing control relaxant effect of BTL (black bar, n = 6), effect after pre-incubation with GlcNAc (dark grey bar, n = 5), effect after pre-incubation with mucin (light grey bar, n = 6) and effect of mucin alone in the absence of BTL (open bar, n = 5). *P < 0.05, vs PE control (100% contraction); #P < 0.05, vs BTL alone.

the lectin was not impairing endothelium-independent relaxation of the tissue. In time-matched controls, in the absence of L-NAME, 30 μg mL^{-1} BTL-induced relaxation was maintained and no spontaneous reversal of the phenylephrine-induced contraction was evident (n = 5; Figures 3B and 3C).

The lectin vasorelaxant effect involves its specific glycoprotein binding site

To assess the importance of the carbohydrate binding site for lectin-mediated effects, BTL-induced aortic relaxation was measured as previously described except that the lectin was pre-incubated with either mucin or GlcNac (at a ratio of 1/10), 30 min before addition to the organ bath. A sub-maximal concentration of BTL (30 μg mL^{-1}) alone caused a significant relaxation of the phenylephrine-induced contractions (94.61 ± 7.28%; n = 6) as previously observed (Figures 4A and 4D). A similar magnitude of

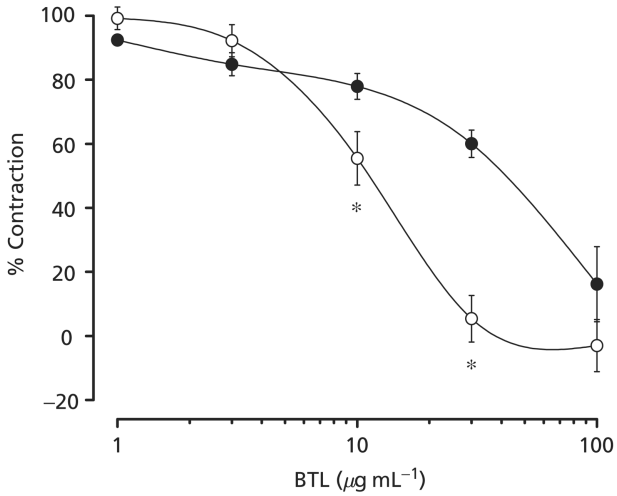

Figure 5 Thermal denaturation does not abolish the relaxant effect of BTL but reduces its potency. Comparative relaxant effects of untreated BTL (○, n = 7) and denatured lectin (●, n = 6) in phenylephrine (PE)-precontracted aortic rings with intact endothelium. Values are expressed as % changes from initial PE-induced tonic contraction; $*P < 0.05$, vs denatured BTL.

response was observed with BTL co-incubated with GlcNac (n = 5; Figures 4C and 4D). In contrast, pre-incubation with mucin significantly reduced the relaxation elicited by BTL to only 12.8 ± 4.21% of the phenylephrine-induced tone (n = 6; Figures 4B and 4D), while mucin per se showed no significant effect upon the tonic contractions (n = 5; Figure 4D).

Thermal denaturation does not abolish the BTL-induced relaxation but reduces its potency

Following heating at 90°C for 3 h, BTL was added in increasing cumulative concentrations (1–100 $\mu g\,mL^{-1}$) to phenylephrine-precontracted aortas with an intact endothelium and the response compared with those of non-treated lectin. Denaturation of BTL resulted in a decrease in potency, with an approximate 3-fold shift of the concentration–relaxation curve to the right ($IC50 = 34.28 \pm 3.4\,\mu g\,mL^{-1}$; n = 6; Figure 5). However, the maximal effect was not altered by such treatment, with no significant difference detected between the relaxation elicited at the highest concentration tested ($100\,\mu g\,mL^{-1}$) of denatured and untreated BTL (Figure 5).

Discussion

Our results show for the first time that a lectin derived from the red marine alga *Bryothamnion triquetrum* induces reversible, concentration-related relaxation of the rat isolated aorta. Importantly, BTL-induced relaxation of phenylephrine-precontracted tissues was strictly dependent on the presence of an intact endothelium and was thus completely inhibited by either mechanical removal of the endothelial layer or by the NOS inhibitor L-NAME (Rees et al 1990). Nitric oxide (NO), the principal mediator of endothelium-dependent relaxation in rat aorta, is produced from arginine by the endothelial isoform of NOS (eNOS) in response to agonists such as ACh, bradykinin and substance P (Fleming & Busse 1999). These agonists cause intracellular calcium elevation and consequent activation of calmodulin that stimulates eNOS. However, eNOS can also be activated by a calcium-independent mechanism(s) (Fleming et al 1998; Venema 2002). A general characteristic of NO-mediated events in blood vessels is their inhibition by L-arginine analogues, such as L-NAME (Moncada et al 1991; Tare et al 2000). Since L-NAME was able to both inhibit and reverse BTL-induced relaxation in this study it is likely that NO is being released by interaction of the lectin with the endothelial cells, leading to activation of smooth muscle guanylate cyclase and consequent vasorelaxation (Moncada et al 1991; Karaki et al 1997).

In contrast, the effects of BTL were not inhibited by the non-selective potassium-channel blocker TEA, excluding possible involvement of endothelium-dependent hyperpolarizng factor (Edwards & Weston 1998; Edwards et al 1998; Hill et al 2000). Similarly prostacyclin, the major product of the cyclooxygenase pathway in vascular endothelial cells, does not appear to mediate the observed effects of BTL in rat aorta. This substance is rapidly released and synthesized in response to intracellular calcium elevation (Moncada et al 1976; Nilius & Droogmans 2001) and induces smooth muscle relaxation via elevation of cAMP (cyclic adenosine monophosphate) and subsequent protein kinase A activation (Karaki et al 1997). Many agonists, such as ACh, bradykinin and adenosine triphosphate, which stimulate the release and synthesis of NO, can also elicit prostacyclin release (Nilius & Droogmans 2001). However, the relaxant effect of BTL on rat isolated aorta was not blocked by the cyclooxygenase inhibitor indometacin (Connolly et al 1998), suggesting that prostaglandins do not participate in this action.

Lectins are glycoproteins that possess at least one carbohydrate-binding site at which they are able to reversibly bind to specific sugars (Peumans & Van Damme 1995). Thus, a variety of lectin-induced pro- and anti-inflammatory effects are reversed by their specific sugar inhibitors (Assreuy et al 1999, 2003; Alencar et al 1999, 2003). Similarly, the GLcNAc ligand has been shown to inhibit both an increase in vascular permeability (Northover & Northover 1994) and the endothelium-dependent relaxation induced by wheat-germ agglutinin (WGA) in rabbit aorta (Kleha et al 1991). In the search for BTL-specific carbohydrate ligands, it was discovered that this particular lectin's agglutinating activity cannot be inhibited by simple monosaccharides, but rather is potently reduced by glycoproteins such as avidin, fetuin and mucin (Ainouz et al 1995). In this study, co-incubation with mucin profoundly inhibited the vasorelaxation induced by BTL, whereas similar pretreatment with GLcNAc (a non-specific inhibitor) did not, suggesting that the specific lectin site is essential. Thus, our results show that BTL exhibits a profile of action distinct from lectins previously reported to exert endothelium-dependent relaxant effects

(Kleha et al 1991, 1993), and is likely to possess a novel pharmacology. Further detailed study of the interaction of BTL with the vascular endothelium is thus indicated to more fully understand the mechanism whereby this lectin can modulate liberation of NO from blood vessels, with a perspective to uncover novel approaches for vascular control. Interestingly, denaturation of the lectin significantly decreased the potency of BTL to relax the blood vessel, although a maximal response was still obtainable at higher lectin concentrations. This may suggest that although lectin binding was hindered by denaturation, a specific part of the BTL molecule might be involved in activation of endothelial NOS.

In conclusion, our data show that the relaxant effect of the red marine alga *Bryothamnion triquetrum* lectin on isolated aortic rings occurs via interaction with a specific lectin-binding site on the endothelium, resulting in a release of NO.

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