Nitric oxide limits the eicosanoid-dependent bronchoconstriction and hypotension induced by endothelin-1 in the guinea-pig

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1 This study attempts to investigate if endogenous nitric oxide (NO) can modulate the eicosanoid-releasing properties of intravenously administered endothelin-1 (ET-1) in the pulmonary and circulatory systems in the guinea-pig.

2 The nitric oxide synthase blocker N^ω-nitro-L-arginine methyl ester (L-NAME; 300 μM; 30 min infusion) potentiated, in an L-arginine sensitive fashion, the release of thromboxane A₂ (TxA₂) stimulated by ET-1, the selective ET_B receptor agonist IRL 1620 (Suc-[Glu⁹,Ala^{11,15}]-ET-1(8-21)) or bradykinin (BK) (5, 50 and 50 nM, respectively, 3 min infusion) in guinea-pig isolated and perfused lungs.

3 In anaesthetized and ventilated guinea-pigs intravenous injection of ET-1 (0.1–1.0 nmol kg⁻¹), IRL 1620 (0.2–1.6 nmol kg⁻¹), BK (1.0–10.0 nmol kg⁻¹) or U 46619 (0.2–5.7 nmol kg⁻¹) each induced dose-dependent increases in pulmonary insufflation pressure (PIP). Pretreatment with L-NAME (5 mg kg⁻¹) did not change basal PIP, but increased, in L-arginine sensitive manner, the magnitude of the PIP increases (in both amplitude and duration) triggered by each of the peptides (at 0.25, 0.4 and 1.0 nmol kg⁻¹, respectively), without modifying bronchoconstriction caused by U 46619 (0.57 nmol kg⁻¹).

4 The increases in PIP induced by ET-1, IRL 1620 (0.25 and 0.4 nmol kg⁻¹, respectively) or U 46619 (0.57 nmol kg⁻¹) were accompanied by rapid and transient increases of mean arterial blood pressure (MAP). Pretreatment with L-NAME (5 mg kg⁻¹; i.v. raised basal MAP persistently and, under this condition, subsequent administration of ET-1 or IRL 1620, but not of U-46619, induced hypotensive responses which were prevented by pretreatment with the cyclo-oxygenase inhibitor indomethacin.

5 Thus, endogenous NO appears to modulate ET-1-induced bronchoconstriction and pressor effects in the guinea-pig by limiting the peptide's ability to induce, possibly *via* ET_B receptors, the release of TxA₂ in the lungs and of vasodilatory prostanoids in the systemic circulation. Furthermore, it would seem that these eicosanoid-dependent actions of ET-1 in the pulmonary system and on systemic arterial resistance in this species are physiologically dissociated.

Keywords: Pulmonary insufflation pressure; mean arterial blood pressure; ET_B receptors; eicosanoids; nitric oxide

Abbreviations: BK, Bradykinin; BQ-788, (N,cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-L-methoxy-carbonyltryptophanyl-D-norleucine); ET-1, Endothelin-1; INDO, Indomethacin; IRL-1620, (Suc-[Glu⁹,Ala^{11,15}]-endothelin-1(8-21)); L-NAME, N^ω-nitro-L-arginine methyl ester; MAP, Mean arterial pressure; NO, Nitric oxide; PBS, Phosphate-buffered saline; PG, Prostaglandin; PIP, Pulmonary insufflation pressure; TxA₂, Thromboxane A₂; TxB₂, Thromboxane B₂

Introduction

It is well established that eicosanoids contribute importantly to the pronounced bronchoconstrictor effects of ET-1 in the guinea-pig. Inhibition of eicosanoid production with the cyclo-oxygenase blocker indomethacin markedly attenuates the increase in pulmonary insufflation pressure (PIP) caused by intravenous (Payne & Whittle, 1988) and, to a lesser extent, aerosol administration of ET-1 (Lagente *et al.*, 1989; Pons *et al.*, 1991b). The fact that ET-1 effectively triggers release of TxA₂ and prostacyclin from guinea-pig perfused lungs (de Nucci *et al.*, 1988) further strengthens this view. Considering that TxA₂ receptor blockers effectively attenuate ET-1-induced contractions in guinea-pig isolated trachea, upper bronchi and parenchymal strips (Filep *et al.*, 1990), as well as increase in PIP *in vivo* (Noguchi *et al.*, 1993), TxA₂ seems to be the main eicosanoid mediator involved in the peptide's bronchoconstrictor effect.

The modulatory properties of eicosanoids are not limited to the pulmonary effects of exogenous ET-1. The pressor effects of ET-1 in the anaesthetized rat are also strongly limited by the concomitant release of vasodilatory prostaglandins (de Nucci *et al.*, 1988), as well as NO (Warner *et al.*, 1989). Interestingly, the NO-releasing properties of ET-1 in many vascular circuits *in vitro* or *in vivo* (Webb, 1991; Warner *et al.*, 1993; D'Orléans-Juste *et al.*, 1994; Gellai *et al.*, 1997), as well as its ability to trigger TxA₂ release from guinea-pig perfused lungs (D'Orléans-Juste *et al.*, 1994) are both related to stimulation of endothelin ET_B receptors. Furthermore, these receptors also mediate ET-1-induced bronchoconstriction in the guinea-pig, as this effect is insensitive to blockade by the selective endothelin ET_A receptor antagonist BQ-123 (Noguchi *et al.*, 1993). The ET_B receptors coupled to NO release are most likely expressed on the vascular endothelium, but the cellular sources of ET_B-receptor mediated release of TxA₂ in the guinea-pig lung remain to be fully identified and could include tracheal epithelial cells, lung parenchyma, resident inflamma-

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tory cells and/or alveolar macrophages (Filep *et al.*, 1990, 1991; Ninomiya *et al.*, 1992; Fleisch *et al.*, 1996).

Cross-talk between cyclo-oxygenase and NO synthase activities in various *in vitro* systems have been suggested (Stadler *et al.*, 1993; Swierkosz *et al.*, 1995; Bishop-Bailey *et al.*, 1997), but similar studies in more complex *in vivo* systems are limited. It has been proposed that disruptions in the homeostatic balance between NO, eicosanoids and endothelins in the pulmonary circulation, involving deregulation of the synthesis and/or expression of these factors, may underlie disabling respiratory diseases such as cystic fibrosis and primary pulmonary hypertension (Giaid *et al.*, 1993a, 1995; Saleh *et al.*, 1997; Park *et al.*, 1997).

To our knowledge, there has been no report in the literature on the possible functional cross-talk between endothelins, NO and eicosanoids *in vivo* and, especially, between NO and ET-1-induced bronchoconstriction. To this effect, we have assessed the influence of an NO synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990), on ET-1-induced release of vasodilator and vasoconstrictor eicosanoids, as well as eicosanoid-mediated bronchoconstriction in the anaesthetized and ventilated guinea-pig. Our results lead us to conclude that indeed there is a significant interplay between NO, endothelins and arachidonic acid metabolites *in vivo*, whereby NO very effectively limits the ability of intravenous ET-1 to cause ET_B receptor-mediated eicosanoid release and hence bronchoconstriction and hypotension in this species.

Methods

Measurements of eicosanoids from isolated perfused guinea-pig lungs

Duncan Hartley guinea-pigs (250–350 g) (Charles River, St-Constant, Canada) of either sex were killed by cervical dislocation. Following thoracotomy, the lung artery was cannulated for perfusion of the lung circulation with a heparinized Krebs' solution (100 units ml⁻¹). The lungs were then removed and immediately suspended in a heated chamber (37°C) and perfused (5 ml min⁻¹) with an oxygenated (95% O₂, 5% CO₂) Krebs' solution. The lungs were left to stabilize for 45 min before two successive 3 min infusions (conducted 45 min apart) of ET-1 (5 nM), the selective ET_B receptor agonist IRL 1620 (Takai *et al.*, 1992) (50 nM), BK (50 nM; a potent eicosanoid releaser in this system, Bakhle *et al.*, 1985) or the stable TxA₂ analogue U 46619 ((15S)-hydroxy-11 α ,9 α -(epoxymethano)prosta-5Z,13E-dienoic acid; Coleman *et al.*, 1981) (10 nM). In a second series of experiments, one of the agonists was administered in each lung in a first infusion following which the NO synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME; 300 μ M, infused intraarterially (i.a.)) was applied prior to (for 30 min) and during the second infusion of the same agonist. The concentrations of these agonists and inhibitors were chosen according to a previously reported study (Lewis *et al.*, 1998). In a third set of experiments, L-arginine (6 mM) was infused (i.a.) 15 min before and then throughout treatment of the lungs with L-NAME. All agents were infused at a rate of 50 μ l min⁻¹. The effluent from the lungs was collected (1-min samples) before, during and after infusion of the various agents. The samples were stored at -20°C until determination of their levels of stable hydrolytic metabolites of prostacyclin (6-Keto-prostaglandin F_{1 α} (6-Keto-PGF_{1 α})) and thromboxane A₂ (thromboxane B₂(TxB₂)) by radioimmunoassay (Salmon, 1978).

In vivo experiments

Guinea-pigs of the same breed and characteristics as before were anaesthetized with ketamine/xylazine (90/15 mg kg⁻¹, intramuscular). Polyethylene catheters (PE-50) were inserted into the left external jugular vein and the right carotid artery for drug administration and recording of both mean arterial blood pressure (MAP) and heart rate, respectively. A cannula (PE-240) was then inserted into the trachea following thoracotomy to facilitate respiration. A blood pressure analyser (Micro-Med, Louisville, KY, U.S.A.) was used to monitor MAP and heart rate. The data were recorded at fixed 5-s time intervals throughout the entire experiment by an automated computer data acquisition system (Blood Pressure Analyzer Communications Program; MicroMed, Inc.) linked to a Compaq Prolinea 4/33 computer.

Thirty minutes after surgery, spontaneous breathing was suppressed with succinylcholine (5 mg kg⁻¹, s.c.) and each animal was connected through the tracheal cannula to a rodent respirator (Model 141, Valley Scientific Co. apparatus, Medway, MA, U.S.A.) and ventilated (6 ml kg⁻¹, 60 strokes min⁻¹). PIP was monitored with a pressure transducer (Statham, model P23AC) connected to a side arm of the tracheal cannula, according to the method of Konzett & Rössler (1940), and coupled to a Grass polygraph (model 7D) for the recording of bronchoconstrictor responses. The pharmacological responses to the various agonists were monitored for at least 40 min following their administration. Each animal received a single dose of one of the above-mentioned agonists (ET-1, IRL 1620, BK or U 46619). In some experiments, L-NAME (5 mg kg⁻¹, i.v.) was administered 3 min prior to agonist injection. Alternatively, this L-NAME treatment was preceded by an injection of either L-arginine (100 mg kg⁻¹, 5 min beforehand and with L-NAME, i.v.), the selective ET_B receptor antagonist, BQ-788 (N,cis-2,6-dimethyl-piperidinocarbonyl-L-L-methylleucyl-D-L-methoxy-carbonyl-tryptophanyl-D-norleucine; Ishikawa *et al.*, 1994) (0.25 mg kg⁻¹, 2 min beforehand, i.v.), or indomethacin (10 mg kg⁻¹, 15 min beforehand, i.v.).

In vivo experiments lasted no longer than 90 min and maintenance doses of anaesthetics were not required. Vehicles (phosphate-buffered saline (PBS) or Trizma base) were systematically tested and were found not to alter either basal MAP or PIP or the responses of the guinea-pig to the agonists used in the present study.

Drugs

ET-1 and IRL 1620 were purchased from American Peptide Company Inc. (Sunnyvale, U.S.A.). BQ-788 was purchased from Peptides International (Louisville, U.S.A.). BK was synthesized in our laboratory. U 46619 (dissolved in methyl acetate and then freshly diluted in phosphate-buffered saline, pH 7.4) was purchased from Cayman Chemical Company (Ann Arbor, U.S.A.). L-NAME, L-arginine, heparin, PBS, 6-Keto-PGF_{1 α} , TxB₂, 6-Keto-PGF_{1 α} and TxB₂ antisera were obtained from Sigma (St. Louis, U.S.A.). Tritiated 6-Keto-PGF_{1 α} and TxB₂ were purchased from Amersham (Oakville, Canada). The TxB₂ antiserum has a 100% cross-reactivity with authentic TxB₂, less than 2% cross-reactivity with prostaglandin D₂ (PGD₂) and prostaglandin F_{1 α} (PGF_{1 α}) and less than 0.1% cross-reactivity with 6-Keto-PGF_{1 α} , prostaglandin E₁ (PGE₁) and prostaglandin E₂ (PGE₂). The 6-Keto-PGF_{1 α} antiserum has 100% cross-reactivity with authentic 6-Keto-PGF_{1 α} , 23% with PGE₁, 4% with PGE₂,

7% with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and less than 1% with TxB_2 . Both 6-Keto- $PGF_{1\alpha}$ and TxB_2 radioimmunoassays have a detection limit of 0.4 ng ml^{-1} . The eicosanoid antisera employed in the present study did not cross-react with any of the agonists or inhibitors used. All agents were dissolved in PBS, except for indomethacin which was dissolved in Trizma-base (0.2 M).

Data analysis

Results are shown as means $\pm$ s.e.mean for n experiments. Statistical comparisons between the release of eicosanoids from the perfused lungs triggered by the first (control) and second infusions (in presence of L-NAME, with or without L-arginine) of each agonist were made by paired Student's t -test. Since the pressor and bronchoconstrictive properties of ET-1 were very poorly reversible, guinea-pigs were not used as their own controls for the *in vivo* experiments with inhibitors or antagonists. Statistical comparisons between responses of guinea-pigs pretreated or not with L-NAME, L-arginine, indomethacin or BQ-788 *in vivo* were made using unpaired Student's t -test. Changes in MAP, increases in PIP or increased release of eicosanoids from the perfused lung when compared to basal at time 0, were analysed by ANOVA followed by Dunnett's test for multiple comparisons. Differences in which $P < 0.05$ were considered significant.

Ethics

The care of animals and all the research protocols conformed to the guiding principles for animal experimentation, as enunciated by the Canadian Council on Animal Care and approved by the Ethical Committee on Animal Research of the University of Sherbrooke.

Results

L-NAME selectively potentiates TxA_2 release induced by ET-1, IRL 1620 or BK from the guinea-pig perfused lung

A first control 3-min infusion of ET-1 (5 nM) or BK (50 nM) triggered a significant release of prostacyclin and TxA_2 from the guinea-pig perfused lung (Figures 1a,b and 2). In contrast, the selective ET_B receptor agonist IRL 1620 (50 nM) markedly increased only the release of TxA_2 (Figure 1c,d), confirming previous findings (D'Orléans-Juste *et al.*, 1994). The TxA_2 -mimetic U 46619 (50 nM) failed to alter eicosanoid release (TxA_2 release: from 0.72 ± 0.07 to peak of $0.74 \pm 0.09 \text{ ng ml}^{-1}$; prostacyclin release: from 0.65 ± 0.08 to peak of $0.73 \pm 0.05 \text{ ng ml}^{-1}$, $n = 3$).

The TxA_2 -releasing properties of ET-1 and IRL 1620 were quite reproducible, as a second control infusion with either

Figure 1 Profile of prostacyclin (measured as 6-keto- $PGF_{1\alpha}$) and thromboxane A_2 (measured as TxB_2) release from the isolated perfused guinea-pig lung induced by ET-1 (5 nM) (a, b) and IRL 1620 (50 nM) (c, d) in the absence or presence of L-NAME ($300 \text{ } \mu\text{M}$). The 6-keto- $PGF_{1\alpha}$ and TxB_2 release was monitored for 24 min after the interruption of a 3 min infusion of the agonist through the lung (t.l.) arterial vasculature. (b) Basal levels of eicosanoid were measured before the infusion of the agonist. Arrows represent the time points (min) at which a first infusion of ET-1 or IRL-1620 induce a significant release of eicosanoids ($P < 0.05$) when compared to basal. Each value represents the mean $\pm$ s.e.mean of 4–8 experiments. *, $P < 0.05$ when compared with the levels of eicosanoid from the lungs without added L-NAME at each time point.

agonist produced similar magnitudes of release as the first (peak releases: ET-1: first infusion 4.78 ± 0.43 ng ml⁻¹, second infusion 3.77 ± 0.34 ng ml⁻¹, $n=6$; IRL 1620: first infusion 6.84 ± 0.48 ng ml⁻¹, second infusion 7.24 ± 1.25 ng ml⁻¹, $n=4$). In contrast, TxA₂ release triggered by BK showed significant tachyphylaxis (peak releases: first infusion 2.44 ± 0.36 ng ml⁻¹, second infusion 1.2 ± 0.52 ng ml⁻¹, $n=7$, $P<0.05$). Conversely, release of prostacyclin was reproducible when triggered by BK (peak releases: first infusion 8.54 ± 1.99 ng ml⁻¹, second infusion 5.48 ± 1.26 ng ml⁻¹, $n=7$; $P>0.05$), but as previously reported (D'Orléans-Juste *et al.*, 1994) not by ET-1 (peak releases: first infusion 3.40 ± 0.61 ng ml⁻¹, second infusion 1.22 ± 0.55 ng ml⁻¹, $n=16$, $P<0.05$). Finally, two successive infusions of IRL-1620 did not induce a significant release of PGI₂ (peak releases: first infusion 0.8 ± 0.2 ; second infusion: 0.9 ± 0.1 ng ml⁻¹, $n=3$).

Figure 2 Profile of prostacyclin (measured as 6-keto-PGF_{1α}) (a) and thromboxane A₂ (measured as TxB₂) (b) release from the isolated perfused guinea-pig lung induced by BK (50 nM) in the absence or presence of L-NAME at 300 μM. The 6-keto-PGF_{1α} and TxB₂ release was monitored for 24 min after the interruption of a 3 min infusion of the agonist through the lung (t.l.) arterial vasculature. (b) Basal levels of eicosanoid were measured before the infusion of the agonist. Arrows represent the time points (min) at which a first infusion of bradykinin induces a significant release of eicosanoids when compared to basal ($P<0.05$). Each value represents the mean $\pm$ s.e.mean of eight experiments. *, $P<0.05$ when compared with the levels of eicosanoid from the lungs without added L-NAME at each time point.

When L-NAME (300 μM) was infused into the perfused lung for 30 min prior to a second infusion of ET-1, IRL 1620 or BK, the release of TxA₂ induced by each agonist was sharply potentiated (Figures 1b,d and 2b). It is noteworthy that the potentiating influence of L-NAME on TxA₂ release stimulated by ET-1 and IRL 1620 was more pronounced and longer-lasting than that triggered by BK. On the other hand, L-NAME pretreatment did not significantly alter the magnitude of prostacyclin released by IRL-1620 and BK whereas the inhibitor delayed by 2 min the ET-1-induced release of the same prostanoid, when compared to the amounts measured following the first infusion (Figures 1a,c and 2a). The ability of L-NAME to enhance TxA₂ release stimulated by ET-1, IRL 1620 or BK was almost fully prevented when the lungs were infused with L-arginine (6 mM) for 15 min before starting infusion with the NO-synthase blocker (Figure 3). Worthy of

Figure 3 Profile of thromboxane A₂ release (measured as TxB₂) from the isolated perfused guinea-pig lung induced by ET-1 (5 nM) (a), IRL 1620 (50 nM) (b) or BK (50 nM) (c) in the absence or presence of 300 μM of L-NAME alone or with 6 mM of L-arginine. The TxB₂ release was illustrated at six time points (for ET-1 and IRL 1620: 0, 2, 5, 10, 15, 25 min; for BK: 0, 2, 3, 4, 10, 15 min) following the beginning of a 3 min infusion of the peptide through the lung arterial vasculature. (b) Basal levels of TxB₂ were measured before the infusion of the peptide. Each value represents the mean $\pm$ s.e.mean of at least five experiments. *, $P<0.05$ when compared with the levels of TxB₂ from the control value at each time point.

notice is the fact that L-NAME *per se* did not affect the basal release of 6-keto-PGF_{1 α} nor TxB₂ (Figures 1 and 2).

L-NAME in vivo modifies PIP and MAP responses to ET-1, IRL 1620 or BK, but not U 46619

Basal PIP and MAP in naïve anaesthetized and ventilated control guinea-pigs was averaged at 8 ± 0.3 mmHg ($n = 40$) and 57 ± 0.7 mmHg ($n = 31$), respectively. Intravenous injection of L-NAME (5 mg kg^{-1}) did not alter PIP (L-NAME-treated PIP 9 ± 0.5 mmHg, $n = 45$), but increased MAP rapidly and persistently, starting within 30 s of injection and reaching a sustained plateau within 2–3 min, with a peak Δ MAP of 31 ± 0.8 mmHg ($n = 45$), which remained unchanged throughout the full remainder of the experiment (Table 1). In untreated guinea-pigs, ET-1 ($0.25 \text{ nmol kg}^{-1}$, i.v.) triggered transient increases in PIP and MAP, whereas in L-NAME treated animals it induced far greater increases in PIP allied to a sustained hypotension (Figure 4 and Table 1). Although IRL 1620 (0.4 nmol kg^{-1} , i.v.) was as effective as ET-1 ($0.25 \text{ nmol kg}^{-1}$, i.v.) in increasing PIP in untreated control animals (Figure 5), the peak of its transient hypertensive effect was significantly smaller than that caused by the latter peptide (Table 1), confirming previous studies (Pons *et al.*, 1991a; Gratton *et al.*, 1995). Nevertheless, when injected into L-NAME-treated guinea-pigs, IRL 1620 caused, like ET-1, greater PIP responses and prolonged decreases in MAP.

Like ET-1 and IRL 1620, BK (1 nmol kg^{-1} , i.v.) increased PIP in control untreated guinea-pigs (Figure 5). However, unlike those peptides, it induced a clear decrease in MAP which was greatest at 2 min (Δ MAP of -18.6 ± 3.8 mmHg, $n = 4$) and reversed to baseline at 10 min. L-NAME treatment potentiated both BK-induced increases in PIP as well as the magnitude and duration of its hypotensive effect (Δ MAP of -46.5 ± 4.5 mmHg, $n = 4$). In sharp contrast, the TxA₂-mimetic U 46619 ($0.57 \text{ nmol kg}^{-1}$, i.v.) caused increases in both PIP and MAP in control animals which were unaffected by treatment with L-NAME (Figure 6 and Table 1). As shown in Figure 6, the failure of L-NAME to affect PIP responses to U 46619 was confirmed over a broad range of doses of this particular agonist, and is in clear contrast to the significant potentiation of bronchoconstriction triggered by submaximally effective doses of ET-1, IRL 1620 or BK. It is also noteworthy that doses higher than 1 and 1.6 nmol kg^{-1} of ET-1 or IRL 1620, respectively, were found to be lethal under these experimental conditions.

Influence of indomethacin, L-arginine and BQ-788 on the responses to of ET-1, IRL 1620 or BK in L-NAME-treated guinea-pigs

Pretreatment with indomethacin (10 mg kg^{-1}), L-arginine (100 mg kg^{-1}) or BQ-788 (0.25 mg kg^{-1}) did not affect basal

Figure 4 Original recordings showing the effects of an intravenous administration of ET-1 ($0.25 \text{ nmol kg}^{-1}$) alone or in the presence of L-NAME (5 mg kg^{-1} , injected 3 min before ET-1) on mean arterial pressure (MAP) and increase in pulmonary insufflation pressure (Δ PIP) in anaesthetized and ventilated guinea-pigs. The first arrow indicates administration of vehicle or L-NAME at time point -3.

Table 1 Influence of L-NAME (5 mg kg^{-1}), indomethacin (10 mg kg^{-1}) or BQ-788 (0.25 mg kg^{-1}) on the mean arterial blood pressure (MAP) following an administration (at time point 0) of ET-1 ($0.25 \text{ nmol kg}^{-1}$), IRL 1620 (0.4 nmol kg^{-1}) or U 46619 ($0.57 \text{ nmol kg}^{-1}$) in the anaesthetized guinea-pig

Treatment	MAP (mmHg)						
	-3 min	0 min	0.5 min	1 min	2 min	5 min	10 min
L-NAME	62.7 ± 0.4	63.0 ± 0.7	$68.3 \pm 0.3^*$	$78.3 \pm 5.6^*$	$90.3 \pm 1.6^*$	$92.3 \pm 1.7^*$	$94.8 \pm 2.2^*$
ET-1	60.7 ± 1.2	63.3 ± 1.6	$85.7 \pm 3.7^*$	78.0 ± 5.9	70.0 ± 6.5	63.4 ± 4.9	59.5 ± 1.9
Indomethacin + ET-1	62.4 ± 4.4	65.4 ± 4.7	$75.6 \pm 4.3^*$	$78.2 \pm 3.7^*$	$78.3 \pm 3.2^*$	$79.0 \pm 4.1^*$	$74.9 \pm 5.5^*$
L-NAME + ET-1	62.6 ± 2.3	$88.2 \pm 5.0^\dagger$	95.5 ± 4.8	76.6 ± 9.0	$50.4 \pm 11.0^*$	$48.3 \pm 8.3^*$	$52.4 \pm 8.0^*$
Indomethacin + L-NAME + ET-1	66.2 ± 3.9	$92.5 \pm 1.8^\dagger$	$97.8 \pm 0.7^*$	$97.8 \pm 0.3^*$	$97.5 \pm 0.6^*$	$98.2 \pm 1.5^*$	$97.5 \pm 1.5^*$
BQ-788 + L-NAME + ET-1	60.1 ± 3.5	$85.5 \pm 2.7^\dagger$	$92.5 \pm 2.7^*$	$96.5 \pm 2.7^*$	$98.4 \pm 2.9^*$	$102.2 \pm 3.4^*$	$102.7 \pm 3.2^*$
IRL 1620	57.8 ± 2.1	56.2 ± 1.7	$66.3 \pm 1.9^*$	63.9 ± 4.3	57.6 ± 5.2	54.7 ± 5.6	55.9 ± 2.8
L-NAME + IRL 1620	59.5 ± 3.3	$93.5 \pm 2.5^\dagger$	106.1 ± 3.4	102.8 ± 1.7	84.5 ± 8.2	$59.2 \pm 11.6^*$	77.6 ± 6.7
Indomethacin + L-NAME + IRL 1620	59.6 ± 3.7	$94.2 \pm 3.6^\dagger$	100.8 ± 2.2	96.5 ± 1.4	92.5 ± 1.4	93.9 ± 3.3	95.8 ± 3.2
U 46619	56.0 ± 1.8	55.0 ± 1.4	$67.5 \pm 3.0^*$	$66.8 \pm 2.8^*$	$63.4 \pm 2.6^*$	60.0 ± 3.7	59.5 ± 3.0
L-NAME + U 46619	59.3 ± 3.1	$88.8 \pm 1.5^\dagger$	89.5 ± 0.7	$96.1 \pm 1.8^*$	$95.2 \pm 0.5^*$	$91.7 \pm 1.6^*$	90.4 ± 2.6

Each value represents the mean $\pm$ s.e. mean of at least five experiments. † , $P < 0.05$ when compared at time point -3. *, $P < 0.05$ when compared to MAP at time point 0.

Figure 5 Effect of L-NAME on ET-1 ($0.25 \text{ nmol kg}^{-1}$) (a), IRL 1620 (0.4 nmol kg^{-1}) (b) or BK (1 nmol kg^{-1}) (c) evoked increase of pulmonary insufflation pressure (PIP) in the anaesthetized guinea-pig. Agonists were injected i.v. alone or 3 min after L-NAME administration (5 mg kg^{-1} , i.v.). The arrows indicate the time of administration of the peptides. Each value represents the mean $\pm$ s.e. mean of at least five experiments. *, $P < 0.05$ when compared with the variation of PIP induced without L-NAME at each time point.

PIP or MAP (results not shown). Indomethacin (INDO) fully suppressed the increases in PIP stimulated by ET-1 ($0.25 \text{ nmol kg}^{-1}$), IRL 1620 (0.4 nmol kg^{-1}) or BK (1 nmol kg^{-1}) in either untreated (ET-1: 7.6 ± 1.6 ; + INDO: 0, IRL-1620: 6.1 ± 1.7 ; + INDO: 0, BK: 5.2 ± 1.1 ; + INDO: 0 mmHg, $n = 5$, $P < 0.05$ when compared to control in absence of indomethacin) or L-NAME-treated guinea-pigs. (ET-1: 20.2 ± 2.6 ; + INDO: 0, IRL-1620: 17.5 ± 2.1 ; + INDO: 0, BK: 13.1 ± 2.7 ; + INDO: 0 mmHg, $n = 5$, $P < 0.01$ when compared to control in absence of indomethacin). Moreover, though the cyclo-oxygenase blocker did not change the magnitude of the peak ET-1-induced MAP increase in untreated guinea-pigs, it prolonged the peptide's hypertensive effect substantially (Table 1). Importantly, indomethacin did not modify the hypertensive response to L-NAME administration, yet the typical hypotensive response to subsequent ET-1 injection was abolished and replaced by a discrete additional increase in MAP. Almost identical alterations in MAP

responses were observed when IRL 1620 (Table 1) or BK (i.e. this peptide also induced a small but prolonged hypertensive effect) were injected instead of ET-1 in indomethacin and L-NAME pretreated animals.

An administration of L-arginine (100 mg kg^{-1}), 5 min prior to treatment with L-NAME co-administered with L-arginine (5 and 100 mg kg^{-1} , respectively) in the anaesthetized and ventilated guinea-pig, fully reversed the L-NAME-dependent potentiation of ET-1- ($0.25 \text{ nmol kg}^{-1}$), IRL 1620- (0.4 nmol kg^{-1}) or BK- (1 nmol kg^{-1}) induced increases in PIP (Figure 7), and significantly blunted the hypertensive response to L-NAME as well as the hypotension induced by subsequent ET-1 injection. The points chosen to study the above mentioned three effects, were set in the ED_{50} range, as interpolated from the dose-response curves for each agonist in presence of L-NAME, as illustrated in Figure 6.

On the other hand, pretreatment with the selective ET_B receptor antagonist BQ-788 (0.25 mg kg^{-1}), 2 min prior to L-NAME administration, markedly blunted the PIP responses to ET-1 (ET-1: 20.2 ± 2.6 ; + BQ-788: $1.5 \pm 0.1 \text{ mmHg}$, $n = 5$, $P < 0.01$) as well as its depressor effect on MAP and actually unmasked a pronounced and long-lasting hypertensive response to the peptide (Table 1).

Discussion

Our results demonstrate that endogenous NO potentially downregulates the ability of ET-1 to induce three ET_B receptor-mediated effects, namely the release of TxA_2 from the perfused lung, as well as eicosanoid-dependent bronchoconstriction and hypotension *in vivo* in the guinea-pig. Infusion with the NO-synthase blocker L-NAME selectively enhanced the TxA_2 release triggered by either ET-1 or the selective ET_B receptor agonist IRL 1620 in the guinea-pig perfused lung, without modifying their respective capacity to enhance prostacyclin output. In fact, IRL 1620 does not trigger prostacyclin release in the guinea-pig perfused lung (D'Orléans-Juste *et al.*, 1994). This modulatory influence of NO on ET-1 actions in the pulmonary system appears to be largely restricted to the production of TxA_2 . Furthermore, the finding that L-NAME also affected the eicosanoid-releasing effect of BK in a similar fashion indicates that the inhibitory influence of NO is not restricted to TxA_2 release stimulated *via* ET_B receptors.

As previously reported (D'Orléans-Juste *et al.*, 1994), the guinea-pig perfused lung displays a marked tachyphylaxis as far as ET-1-induced release of prostacyclin is concerned. In addition, L-NAME delayed the ET-1- induced release of the above-mentioned prostanoid. This state of event may not allow us to interpret accurately the potentiating properties of NO-synthase inhibition of the ET-1 induced release of prostacyclin. Similar phenomena occur with regards to the release of TxA_2 stimulated by two consecutive infusions of BK (tachyphylaxis and delayed response in L-NAME-treated lungs). It is however of interest that L-NAME still significantly potentiated the TxA_2 release induced by BK without enhancing the generation of prostacyclin by the same agonist in the perfused lungs. Furthermore, as discussed below, L-NAME also significantly potentiated the dose-dependent increases in PIP triggered by BK *in vivo*.

The bronchoconstrictor effects of intravenously injected ET-1 *in vivo* in the guinea-pig also seem to be mediated importantly, perhaps even exclusively, *via* an ET_B receptor-mediated eicosanoid mechanism. Firstly, they are mimicked by IRL 1620. Secondly, they are abolished following treatment

Figure 6 Dose-response curves of endothelin-1 (ET-1) (a), IRL 1620 (b), BK (c) and U 46619 (d) of evoked increase of pulmonary insufflation pressure (PIP) in the absence or presence of L-NAME (5 mg kg⁻¹) in the anaesthetized guinea-pig. Each value represents the mean $\pm$ s.e. mean of at least four experiments. *, $P < 0.05$ when compared with the variation of PIP without L-NAME at each dose.

with either a thromboxane A₂ blocker (L-670,596) (Noguchi *et al.*, 1993), the selective ET_B receptor antagonist BQ-788 (present study) or the cyclo-oxygenase inhibitor indomethacin (Payne & Whittle, 1988; this study). The findings that L-NAME potentiated and prolonged the increases in PIP induced by ET-1 and IRL-1620 (and BK) correlate remarkably well with the results obtained in the perfused lung experiments. The fact that in L-NAME-treated animals the PIP responses to all three peptides were again fully blocked by indomethacin, strongly suggests that selective inhibition by NO of the TxA₂ release stimulated by these agonists (as evidenced in the perfused lung experiments) also plays a major role in limiting their effects on airway resistance *in vivo*. This hypothesis is reinforced by the finding that L-NAME failed to potentiate PIP responses induced by the TxA₂-mimetic compound U 46619, which was also found to be devoid of eicosanoid-releasing properties in the perfused lung. Another noteworthy aspect is that L-NAME did not affect basal TxA₂ release in the perfused lung or PIP *in vitro*, indicating that NO truly modulates the action of eicosanoid-releasing substances in the pulmonary system.

In contrast to its well known sustained hypertensive effect in the rat (Yanagisawa *et al.*, 1988; de Nucci *et al.*, 1988), ET-1 induced only a transitory increase in MAP in the anaesthetized guinea-pig, confirming previous reports (Gratton *et al.*, 1995; Noguchi *et al.*, 1993; Pons *et al.*, 1991a). The current study now reveals that the transient nature of

this effect of ET-1 in the later species is due to ET_B receptor-mediated generation of vasodilator prostanoids which limit its pressor action, because indomethacin treatment prolonged markedly the duration of the elevation of MAP induced by the peptide. Moreover, treatment with L-NAME unveiled pronounced hypotensive actions of ET-1 and IRL 1620, as well as potentiated the already normally hypotensive responses triggered by BK. Since L-NAME, if anything, actually enhanced the pressor response to U 46619, the shift in the profile of activity of ET-1 on MAP afforded by the NO synthase blocker cannot be ascribed solely to the substantial pressor response it causes. In other words, the profile change in ET-1 action is not simply because L-NAME enhances the window for reduction of MAP induced by hypotensive agents. This view is further substantiated by the finding that indomethacin not only abolished the hypotension induced by ET-1 or IRL-1620 in L-NAME-treated animals, but unmasked as well a sustained hypertensive effect of ET-1 which was additive to the elevation of MAP induced by the NO synthase blocker. On the other hand, the findings that IRL 1620 also caused hypotension in L-NAME-treated guinea-pigs, and that MAP responses of these animals to ET-1 were changed by BQ-788 injection in a manner similar to that seen with indomethacin, are strong indications that ET_B receptors are critically involved in mediating the prostanoid-dependent hypotensive effects of both agonists. Our results also suggest that this

Figure 7 Maximal increase of pulmonary insufflation pressure evoked by an i.v. injection of ET-1 ($0.25 \text{ nmol kg}^{-1}$), IRL 1620 (0.4 nmol kg^{-1}) or BK (1 nmol kg^{-1}) in absence or presence of L-NAME alone (5 mg kg^{-1}) or co-administered with L-arginine (100 mg kg^{-1}) in the anaesthetized guinea-pig. Each value represents the mean $\pm$ s.e. mean of at least five experiments. *, $P < 0.05$ when compared with the variation of PIP without L-NAME.

particular pathway is substantially limited by endogenous NO.

Although L-NAME enhanced eicosanoid-dependent ET_B receptor-mediated effects of intravenously injected ET-1 in both the pulmonary and cardiovascular systems, the particular eicosanoids involved in increasing PIP and reducing MAP in this condition are suggested to be distinct. As already discussed, the main eicosanoid mobilized by ET-1 in the lung is likely to be TxA₂, but this potent vasoconstrictor (Hamberg *et al.*, 1975) elevates MAP in the guinea-pig, as evidenced by our results with the TxA₂-mimetic U 46619 and those of Cui *et al.* (1997). Thus, although we have not demonstrated which is the hypotensive prostanoid(s) released *via* ET_B receptors in response to intravenous ET-1 in this species, the two most likely candidates would be prostacyclin and prostaglandin E₂. Either of these vasodilators could act to limit the peptide's pressor activity in the guinea-pig, as previously illustrated in the rat by de Nucci *et al.* (1988). However, prostacyclin release is not coupled to ET_B receptors (present study) and ET-1 is a poor stimulant of prostaglandin E₂ production in the guinea-pig perfused lungs (Botting & Vane, 1990). Based on the above mentioned premises we suggest that the generation of prostacyclin/prostaglandin E₂ and TxA₂ triggered by systemically administered ET-1 in this species occurs at different anatomical sites in the circulation, but NO effectively controls this action in both cases.

Conceivably, the hypotensive effect of ET-1 in L-NAME-treated animals could be ascribed to a sudden reduction in cardiac output, as the peptide can induce an indomethacin- and TxA₂ blocker-sensitive decrease in cardiac output in the guinea-pig heart and lung preparation (Del Basso & Argiolas, 1995). Furthermore, another TxA₂-mimetic, U 44069, reduces cardiac output in thoracotomized guinea-pigs (Dube *et al.*, 1995). However, this action was always associated with an increase in MAP, a result similar to that we obtained using U 46619 in the present study. These observations allow us to dissociate the hypotensive effect of ET-1 from a possible reduction in cardiac output by TxA₂ generated by the peptide.

The alterations caused by L-NAME of ET-1 induced eicosanoid release *in vitro* and PIP and MAP responses *in*

vivo appear to be related to specific inhibition of NO production, as they were all significantly attenuated by concomitant treatment with L-arginine. However, as L-NAME is a non-selective blocker of all NO synthase isoforms (Moore & Handy, 1997), it is not yet possible to pinpoint precisely which of these enzymes are responsible for the NO that normally limits ET_B receptor-mediated eicosanoid release, or which are the cells that express them. In the systemic circulation, eNOS in endothelial cells are an almost certain possibility, but iNOS in pulmonary macrophages, as well as eNOS in other blood borne cells and in airway epithelial cells may also participate importantly. Indeed, removal of the airway epithelium potentiates contractions induced by ET-1 in isolated guinea-pig trachea (Filep *et al.*, 1993).

Interestingly, the NO synthase blocker N^G-monomethyl-L-arginine increases prostaglandin E₂ and TxA₂ production in rat Kupffer cells, resident macrophages of the liver (Stadler *et al.*, 1993). Furthermore, a cross-talk between prostaglandin H synthase-2 (also known as cyclo-oxygenase-2) and NO has only recently been suggested by Gunther *et al.* (1997), in which NO radical may interfere with the activity of the cyclo-oxygenase pathway by nitrotyrosine formation. Also, Swierkosz *et al.* (1995) reported that large amounts of NO inhibit cyclo-oxygenase-2 expression and activity in murine macrophages incubated with bacterial endotoxin. All three of these studies were performed in conditions of upregulated iNOS and/or cyclo-oxygenase-2 activities. Since the experimental conditions adopted in the present study do not favour such an upregulation, we believe that we were dealing with the constitutive eNOS and cyclo-oxygenase-1 enzymes. Based on the later premise, we suggest that in the guinea-pig, NO would interfere with the metabolic pathway of the arachidonic acid cascade, thus acting as a potent modulator of the generation of vasodilatory and bronchoconstrictive eicosanoids stimulated by endothelins or kinins *in vivo*.

In a pathophysiological perspective, there are an increasing number of reports suggesting that variations in NO production, cyclo-oxygenase activity and ET-1 expression may be useful indicators of pulmonary dysfunction, such as in cystic fibrosis and primary pulmonary hypertension (Giaid *et al.*, 1993a,b; 1995; Kuitert *et al.*, 1996; Park *et al.*, 1997). Such considerations warrant further investigation of the possible cross-talk between these three vaso- and broncho-modulatory factors on a functional basis, as attempted in the present study.

In summary, our study demonstrates that endogenous NO limits ET-1-induced bronchoconstriction and pressor effects in the guinea-pig by downregulating the peptide's ability to induce ET_B receptor-mediated release of TxA₂ in the lungs and of vasodilatory prostanoids in the systemic circulation. Furthermore, it would seem that these eicosanoid-dependent actions of ET-1 in the pulmonary system and on systemic arterial resistance in this species are physiologically dissociated.

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