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Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial α_2-adrenoceptor activation

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1 To assess the involvement of endothelial α_2-adrenoceptors in the clonidine-induced vasodilatation, the mesenteric artery of Sprague Dawley rats was cannulated and perfused with Tyrode solution (2 ml min^{-1}). We measured perfusion pressure, nitric oxide (NO) in the perfusate using chemiluminescence, and tissue cyclic GMP by RIA.

2 In phenylephrine-precontracted mesenteries, clonidine elicited concentration-dependent vasodilatations associated to a rise in luminal NO. One hundred nM rauwolscine or 100 μM L$^{\omega}$-nitro-L-arginine antagonized the clonidine-induced vasodilatation. Guanabenz, guanfacine, and oxymetazoline mimicked the clonidine-induced vasorelaxation.

3 In non-contracted mesenteries, 100 nM clonidine elicited a maximal rise of NO (123 ± 13 pmol); associated to a peak in tissue cyclic GMP. Endothelium removal, L$^{\omega}$-nitro-L-arginine, or rauwolscine ablated the rise in NO. One hundred nM aminoclonidine, guanfacine, guanabenz, UK14,304 and oxymetazoline mimicked the clonidine-induced surge of NO. Ten μM ODQ obliterated the clonidine-induced vasorelaxation and the associated tissue cyclic GMP accumulation; 10–100 nM sildenafil increased tissue cyclic GMP accumulation without altering the clonidine-induced NO release.

4 α_2-Adrenergic blockers antagonized the clonidine-induced rise in NO. Consistent with a preferential α_{2D}-adrenoceptor activation, the K_Bs for yohimbine, rauwolscine, phentolamine, WB-4101, and prazosin were: 6.8, 24, 19, 165, and 1489 nM, respectively.

5 Rat pretreatment with 100 mg kg^{-1} 6-hydroxydopamine reduced 95% tissue noradrenaline and 60% neuropeptide Y. In these preparations, 100 nM clonidine elicited a rise of 91.9 ± 15.5 pmol NO. Perfusion with 1 μM guanethidine or 1 μM guanethidine plus 1 μM atropine did not modify the NO surge evoked by 100 nM clonidine.

6 Clonidine and congeners activate endothelial α_{2D}-adrenoceptors coupled to the L-arginine pathway, suggesting that the antihypertensive action of clonidine involves an endothelial vasorelaxation mediated by NO release, in addition to presynaptic mechanisms.
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Abbreviations: L-NNA, N$^{\omega}$-nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one; RIA, radio immunoanalysis

Introduction

Endothelial cells produce vasoconstrictor and vasodilator factors that participate in the local control of blood flow. Nitric oxide (NO) is a principal vasodilator released by endothelial cells. NO is generated from the N^{G} guanidino group of L-arginine in a Ca^{2+}-calmodulin dependent reaction catalyzed by NO synthase, which is expressed constitutively in endothelial cells. Once produced, NO diffuses to the underlying smooth muscle cell layer, activating soluble guanylyl cyclase. The resulting increase in intracellular cyclic GMP induces vasorelaxation (Moncada *et al*., 1991) following nucleotide-dependent protein kinase activity. Functionally the L-arginine pathway is a main mechanism of endothelium-smooth muscle signalling; several physiologically relevant compounds and drugs modify the vascular tone through the activation of this cascade (Lucas *et al*., 2000; Carvajal *et al*., 2000).

In the rat arterial mesenteric bed perfused at a constant flow rate, an increase in perfusion pressure elicited by either electrical stimulation of the perivascular sympathetic nerves or by exogenous norepinephrine causes a release of NO (Boric *et al*., 1999). This effect was attributed to an increase in shear stress, a mechanism known to stimulate NO production (Kuo *et al*., 1991). However, a possible direct contribution of endothelial adrenoceptors was not ruled out. This mechanism is worth considering since endothelial α_2-adrenoceptors could play a physiological role in the control of vascular smooth muscle tone. In fact, norepinephrine and

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imidazolines with selective α_2-adrenergic properties relax precontracted canine and pig vessels through an endothelium-dependent mechanism (Miller & Vanhoutte, 1985; Angus *et al*., 1986; Vanhoutte & Miller 1989). Furthermore, Bockman *et al*. (1996) demonstrated that the relaxation induced by norepinephrine or UK14,304 in rings of the rat superior mesenteric artery is mediated by the activation of endothelial α_{2D}-adrenoceptors.

The relevance of the endothelial α_2-adrenoceptors is curtailed by their apparent lack of physiological and therapeutic significance, although clonidine and related compounds are recognized as clinically efficacious antihypertensive drugs (Oates, 1996). The main mechanism that explains clonidine's hypotensive effect is a reduction in central sympathetic outflow, an effect mediated by activation of central α_2-adrenoceptors as was proposed almost 20 years ago by Langer *et al*. (1980). Clonidine therefore, reduces peripheral resistance by decreasing efferent sympathetic neuronal firing and by reducing also the release of norepinephrine from vascular neuroeffector junctions. However, two recent studies demonstrate that the vasodilator action of clonidine in rats is reduced following NO synthase inhibition (Yatomi *et al*., 1998; Soares de Moura, 2000). In addition UK14,304 elicited a concentration-dependent vasorelaxation, which suggests that clonidine may similarly activate the L-arginine pathway (Bockman *et al*., 1993; 1996). These studies raised the hypothesis that endothelial α_2-adrenoceptors may be involved in the antihypertensive action of clonidine and structurally related analogues.

To evaluate this working hypothesis, the arterial mesenteric bed of the rat was chosen. This preparation is suited to perform controlled time course experiments because it allows the simultaneous determination of vasodilatation, the luminally accessible release of NO and the tissue accumulation of cyclic GMP (Boric *et al*., 1999; Poblete *et al*., 2000). Due to the *in-vitro* nature of this preparation, it is also particularly suited to investigate the involvement of non-neuronal mechanisms in the clonidine and related structural analogues induced vasorelaxation. Furthermore, a battery of α-adrenoceptor blockers would assist in the tentative identification of the alpha-adrenoceptor subtype and the intracellular signalling pathway activated by clonidine to induced vasorelaxation. The present results demonstrate that clonidine and structurally related analogues relax arterial mesenteric vessels eliciting a concentration-dependent release of endothelial NO and cyclic GMP production, which seems to occur through α_{2D}-adrenoceptor activation, highlighting a role of the endothelium in the antihypertensive action of clonidine.

Methods

Perfusion of the arterial mesenteric bed

Rats were anaesthetized with 40 mg kg^{-1} sodium pentobarbitone i.p.. The abdomen was opened by a midline incision. The superior mesenteric artery was cannulated and perfused at 2 ml min^{-1} with a Tyrode solution at 37°C equilibrated with 95% O_2-5% CO_2 (Donoso *et al*., 1996; Boric *et al*., 1999). The arterial mesenteric bed was isolated from the intestinal wall, as detailed by McGregor (1965), and was transferred to a perfusion chamber. A pressure transducer was connected at the entrance of the mesenteric artery to monitor changes in the perfusion pressure in a recording polygraph. All drugs were dissolved in the buffer solutions.

NO release and vasorelaxation

We evaluated whether the clonidine and related structural analogues-induced vasorelaxation is mediated through the activation of the L-arginine-NO-cyclic GMP cascade. α_2-Adrenergic agonists were always perfused for 1 min; other endothelium-dependent vasodilators were used to compare drug efficacy and were also applied for 1 min. Separate protocols were performed to assess the vasodilatation and to quantify the luminal release of NO and cyclic GMP since precontraction is required to assess the vasodilatation and this procedure elicits a rise in basal luminal NO (Boric *et al*., 1999). The two types of protocols and their specific aims were: (a) To assess the vasodilatation, mesenteries were precontracted with either 10 μM phenylephrine or 40 mM KCl. Once the perfusion pressure reached a stable value, generally within 2 min, drugs were added to the perfusion media. Mesenteries were next perfused with buffer containing phenylephrine or KCl for an additional 5 min and thereafter with drug-free buffer. In only some of these protocols, perfusate aliquots were collected every minute to determine luminally accessible NO. Vasodilatation was quantified by measuring the drug-induced decrease in perfusion pressure, or the percentual decrease of the contractile tone; (b) To quantify more accurately the changes in the luminally accessible NO and cyclic GMP elicited by clonidine, separate protocols were performed in non-contracted mesenteries. The perfusate was collected every minute in test tubes before, during and after drug application.

Clonidine concentration-response curves To study the vasodilatation, mesenteries were pre-contracted with phenylephrine and perfused with 1, 10, 100 or 1000 nM clonidine. The clonidine vasodilatation was next assessed in separate mesenteries previously perfused for 30 min with either 100 nM rauwolscine, or with 100 μM L-NNA.

Separate protocols performed in non-contracted preparations examined the concentration dependence of clonidine on luminal accessible NO. In a set of these protocols, we examined in the same samples the luminal release of NO and cyclic GMP ($n=4$).

Removal of endothelial cells with saponin To assess the participation of the endothelium in the clonidine-induced vasodilatation and luminal NO release, mesenteries were first challenged with 100 nM clonidine and then 30 min after a 55 s perfusion with 0.1% saponin (Peredo & Enero, 1993). Control protocols evaluated the reproducibility of the total outflow of NO following two successive applications of 100 nM clonidine, spaced 60 min apart.

Blockade of NO synthase To assess whether the clonidine-evoked vasodilatation and the NO released is sensitive to NO synthase blockade, a series of mesenteries were challenged with 100 nM clonidine and next, 45 min after perfusion with 100 μM L-NNA ($n=4$). The same control protocols as above, assessed that two consecutive challenges of clonidine evoked

a similar rise in NO released. Previous studies had determined that the inhibitor does not interfere with NO determinations (Boric *et al*., 1999).

Clonidine structurally related α_2-adrenoceptor agonists and rauwolscine antagonism In separate mesenteries, we investigated the efficacy of 100 nM oxymetazoline, aminoclonidine, xylozine, guanabenz, guanfacine and UK14,304. Agmatine, the decarboxylated product of arginine and a putative endogenous ligand of imidazoline receptors, was also tested. Parallel experiments evaluated whether a 30 min treatment with 100 nM rauwolscine antagonized the NO production elicited by these adrenergic agonists.

Blockade of the clonidine-induced NO production by α_2-adrenoceptor antagonists To classify the pharmacological nature of the α_2-adrenoceptor subtype, yohimbine, rauwolscine, phentolamine, WB 4101, and prazosin were used as blocking agents. Mesenteries were treated for 30 min with (nM): yohimbine 30–1000, rauwolscine 10–1000, phentolamine 100, WB 4101 10–3000, and prazosin 100–3000. The concentration ratio-1 was derived comparing the half-effective clonidine concentration (EC_{50}) required to increase the luminal accessible NO in the absence and in the presence of each antagonist concentration. Schild plots provided pA_2 and the slope (Arunlakshana & Schild, 1959). K_B values were derived from the expression $pA_2 = -\log K_B$ (nM). Four separate rats were used for each clonidine concentration; at least four clonidine concentrations were used per antagonist concentration. In the studies with yohimbine, phentolamine and prazosin, the pA_2 was derived from the simpler expression: $pA_2 = -\log \text{antagonist} + [\log EC_{50} \text{ ratio } -1]$.

Time course of intracellular cyclic GMP production, effect of ODQ and sildenafil The time course of cyclic GMP tissue accumulation was determined in a series of separate mesenteries that were processed before and 0.5, 1, 3, and 6 min after 100 nM clonidine application ($n=4-7$). The nucleotide was extracted to quantify tissue cyclic GMP by RIA.

To further assess the involvement of the L-arginine–NO pathway, we investigated whether blockade of soluble guanylyl cyclase with 10 μM ODQ reduced the clonidine vasodilatation and the corresponding tissue rise in cyclic GMP. For this purpose, a set of phenylephrine-precontracted mesenteries was perfused with 100 nM clonidine before and after a 20 min treatment with 10 μM ODQ. In a separate set of non-contracted mesenteries treated with 10 μM ODQ, we examined the tissue content of cyclic GMP before and 3 min after perfusion with 100 nM clonidine ($n=4$). Likewise, in a separate group of rats ($n=5-6$) we assessed the effect of a 25 min tissue treatment with 10 nM sildenafil, a selective inhibitor of phosphodiesterase V. As with ODQ, these protocols examined the accumulation of tissue cyclic GMP in mesenteries perfused with drug-free buffer and 100 nM clonidine.

Other vasodilators dependent on the L-arginine–NO pathway To study the physiological relevance of α_2-adrenoceptor drugs, a set of experiments was performed with 100 nM acetylcholine or 100 nM bradykinin. In addition, we evaluated the activity of 100 nM L (−) epinephrine, as a putative endogenous ligand of these endothelial α_2-adrenoceptors. To confirm the dependence of the endothelium in the NO surge, a series of mesenteries were treated with saponin as detailed above ($n=4$, each).

Acute chemical sympathectomy To discard the influence of sympathetic nerve terminals in the clonidine-induced release of NO, a group of four rats was pretreated with 100 mg kg^{-1} 6-hydroxydopamine i.v. Forty-eight hours later, the mesenteries were perfused as usual and challenged with 100 nM clonidine perfused for 1 min to measure the luminally accessible NO released. Tissues were next homogenized to extract and quantify noradrenaline and neuropeptide Y. Results are compared to those of a subgroup of saline-treated controls. An additional protocol examined the influence of a 30 min perfusion with 1 μM guanethidine or 1 μM guanethidine plus 1 μM atropine (Brock & Cunane, 1988) on the 1 min 100 nM clonidine evoked NO release.

Analytical techniques

Quantification of NO by chemiluminescence The technique was described previously by Boric *et al*. (1999). In essence, mesenteric perfusion samples were collected in test tubes and immediately sealed with Parafilm. All buffers and drug solutions were prepared in freshly obtained triple-distilled water. The sample content of NO was quantified using a Sievers 280 NO analyzer within an hour after concluding the experiment. The reaction chamber of the equipment was filled with 8 ml of glacial acetic acid containing 100 mg of potassium iodide to reduce the sample nitrites to NO. A 50-μl perfusate sample was injected into the reaction chamber, and a stream of nitrogen carried the resulting NO to a cell in which the chemiluminescence generated by the NO-ozone reaction was detected by a photomultiplier. Calibration of the equipment was performed with 10–1000 nM sodium nitrite. The sensitivity of the equipment allows for a detection threshold of 0.5–1 pmol NO (10–20 pmol ml^{-1}). Background buffer readings were subtracted to determine mesentery NO release. Results are expressed either as the time course of luminally accessible NO (pmol ml^{-1}), or as the integrated NO recovered above basal values (pmol NO).

cyclic GMP determinations The nucleotide was quantified with a RIA for acetylated cyclic GMP as detailed by Boric *et al*. (1999). As the radioactive tracer we used 2′-O-succiniyl-guanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester. This compound was labelled locally with ^{125}I. The mesenteric perfusion samples were concentrated by passage through C-18 Sep-Pak columns (Merck), eluted with 2 ml methanol, evaporated and resuspended in 1 ml of RIA buffer for cyclic GMP determinations. Tissue cyclic GMP was extracted following homogenization of the whole mesentery in 3 ml 10% trichloroacetic acid and centrifuged 30 min at 3000 r.p.m. (4°C). The aqueous phase was extracted 4-times with 4 vols of ethyl ether each time. The samples were dried on a speed-vac and stored at −20°C for less than a week until the RIA was performed. Results are expressed as pmol per g of wet tissue.

Tissue noradrenaline and neuropeptide Y determinations Noradrenaline and neuropeptide Y were measured from a same biological sample. The whole mesentery was homogenized in

a mixture of 1N HCl plus 100 μM EDTA and 0.01% sodium metabisulfite and heated at 90°C for 5-min to inactivate proteinases. Following centrifugation at 2500 r.p.m. for 30 min, 0.5 ml tissue supernatant was neutralized with 2 ml of a mixture of 0.2 M dibasic sodium phosphate, 50 mM Na_2CO_3, and 100 μM EDTA before application to Sep-Pak columns (Hunter *et al*., 1992). Noradrenaline was eluted with a mobile phase according to Donoso *et al*. (1997) and neuropeptide Y with methanol-trifluoroacetic acid. Samples were dried and prepared for quantification. Noradrenaline plus an internal standard were separated and quantified by HPLC, using the Merck L-6200 A Intelligent pump and the Metrohm 656 electrochemical detector coupled to a Merck chromato integrator. Neuropeptide Y was quantified by RIA (Donoso *et al*., 1997).

Animal and drug sources

Male Sprague-Dawley rats (250–300 g) bred in the faculty Animal Reproduction Laboratories were used throughout. We carefully followed the Helsinki guidelines on research involving laboratory animals. Protocols complied with the American Pharmacological Society norms as well as our local guides of Animal Care Committees.

Saponin, acetylcholine chloride, guanethidine and atropine sulphate, phenylephrine, prazosin and yohimbine hydrochlorides, N^{ω}-nitro-L(−)-arginine (L-NNA), ODQ (1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1 one) 6-hydroxydopamine hydrobromide and bradykinin acetate were purchased from Sigma Chemicals (Saint Louis, MO, U.S.A.). UK14,304 (5-bromo-N-(4,5 dihydro-1H-imidazol-2-yl)-6-quinoxalinamine tartrate) and sildenafil citrate were kindly provided by Pfizer Central Laboratories, (U.K.) while phentolamine methanosulfonate was graciously supplied by Ciba Pharmaceuticals. Clonidine, aminoclonidine, oxymetazoline, WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane) and xylozine hydrochlorides, guanabenz acetate, guanfacine and agmatine sulphates, and rauwolscine hydrochloride were purchased from RBI (Natick, MA, U.S.A.). All reagents used to prepare buffer solutions were analytical grade and purchased from Merck Chemicals, Darmstad, Germany.

Data analysis

All experiments including the time course protocols were analysed using two-way ANOVA. Paired or unpaired Student's *t*-test and regression analysis were used to compare differences between experimental groups. Dunnett's tables for multiple comparisons with a common control were used when appropriate. Significance was set at a probability of $P<0.05$.

Results

Clonidine-induced vasodilatation of resistance arteries

In phenylephrine precontracted mesenteries clonidine elicited a concentration-dependent vasorelaxation. One hundred nM clonidine dilated 19.2±1.3 mmHg (Figure 1, $n=15$), corresponding to 44±4% of the precontractile tension (33.2±3.4 mmHg ($n=23$), Figure 2). The clonidine-induced vasodilatation was antagonized in a competitive fashion by

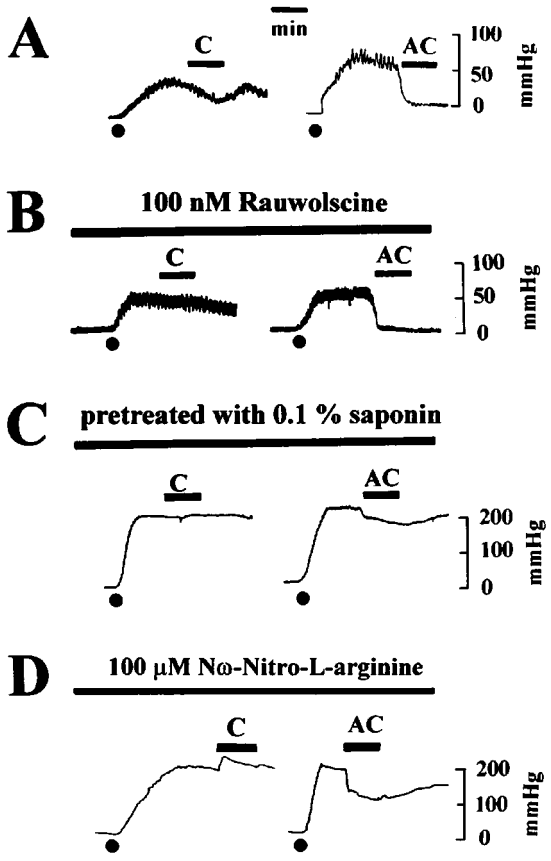

Figure 1 Involvement of α_2-adrenoceptors, the endothelial cell layer and NO synthase in the vasodilatation in the clonidine and acetylcholine-induced vasodilatation. Representative recordings illustrate the vasodilatation induced by a 1 min perfusion with 100 nM clonidine (C), and 10 μM acetylcholine (AC) in 10 μM phenylephrine-precontracted mesenteries (dots). (A) Control preparation. (B) the same mesentery shown in A was perfused for 30-min with 100 nM rauwolscine. (C) Shedding of the endothelial cell layer after perfusion for 55 s with 0.1% saponin, and (D) 30-min after perfusion with 100 μM N^{ω}-nitro-L-arginine. All preparations were challenged with clonidine and a few minutes later with acetylcholine as depicted in A. Note that in the the latter two cases, the contraction elicited by phenylephrine was notably increased.

100 nM rauwolscine (Figures 1B and 2, upper panel). Upon endothelium removal, 100 nM clonidine dilated only 14.6±2.5% ($n=10$, $P<0.05$, Figure 1C); the clonidine concentration-response was almost identical to that shown after L-NNA treatment. Blockade of NO synthase with L-NNA significantly shifted the clonidine concentration-response curve to the right, revealing a reduction of 40–60% ($P<0.05$, Figure 2, lower panel). In a few experiments, clonidine elicited a transient vasomotor response (Figure 1D). As a control, in the same mesenteries 10 μM acetylcholine evoked a rauwolscine-resistant relaxation, which was markedly attenuated after endothelial removal and/or NO synthase blockade (Figure 1).

To further examine the clonidine-induced relaxation in mesenteries contracted by a non-receptor mediated mechanism, we next examined the clonidine-induced vasorelaxation in mesenteries precontracted with 40 mM KCl, which raised the perfusion pressure in 42.5±1.1 mmHg ($n=11$). In these tissues, 100 nM clonidine also elicited a significant relaxation

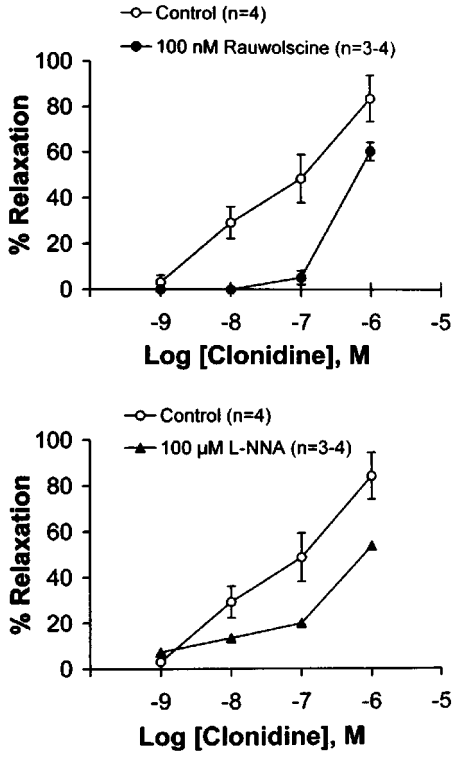

Figure 2 Clonidine-induced concentration-dependent vasodilatatation; displacement of the curves by α_2-adrenoceptor or NO synthase blockage. Clonidine concentration-response protocols were performed in 10 μM phenylephrine-precontracted mesenteries. One set of protocols served as controls, while parallel mesenteries were pretreated for 30 min with either 100 nM rauwolscine ($n=3-4$), or with N^{ω}-nitro-L-arginine (L-NNA, $n=3-4$). Clonidine was perfused for 1 min. Symbols represent mean values; bars s.e.mean ANOVA analysis indicated rauwolscine and L-NNA caused significant rightward displacements ($P<0.05$) of the clonidine concentration-response curve.

(14.3±1.5%, $n=6$, $P<0.05$). As observed in the phenylephrine precontracted mesenteries, 100 nM rauwolscine obliterated the clonidine-evoked relaxation (2.6±1.5%, $n=4$, $P<0.001$) without altering the acetylcholine-induced vasorelaxation (data not shown). In a separate two mesenteries, a clonidine concentration-response curve was performed applying 10, 100 and 1000 nM clonidine. The corresponding vasodilatations were: 9.2±0.8, 15.7±1.6, and 18.4±1.6%, respectively.

Guanfacine and oxymetazoline also vasorelaxed phenylephrine precontracted mesenteries. Guanfacine 0.1 and 1 μM caused a vasorelaxation of 38.8±9.7 and 50±7.1% ($n=2-4$), respectively. Likewise, 0.1 and 1 μM oxymetazoline elicited a vasorelaxation of 33.3±0.5 and 52.5±15.9%, respectively ($n=2-4$). In addition, 0.1 μM guanabenz elicited a 50±10% relaxation ($n=2$). The 1 μM guanfacine or oxymetazoline-induced relaxation was reduced by 100 nM rauwolscine to 12.5±3% and 10±3.5%, respectively ($n=2$).

NO production in precontracted mesenteries

Small day to day fluctuations in basal luminal NO were observed; averaging 85.5±2.6 pmol ml^{-1} ($n=82$). Mesenteries precontracted with phenylephrine or KCl caused a significant net increase in luminally accessible NO (133.7±22.9, $n=4$ and 125.1±35.8 pmol, $n=4$, respectively).

In phenylephrine precontracted mesenteries, 100 nM clonidine raised luminal NO over the basal, reaching a net increase of 240.8±61.8 pmol ($P<0.05$, $n=4$). Prolonging the clonidine application from 1 to 6 min, caused a similar rise in luminal NO (182.3±64.3 pmol, $n=4$). In KCl precontracted mesenteries the net release of luminal NO elicited by 100 nM clonidine did not reach significance (96.4±34.4 pmol, $n=4$).

Luminal outflow of NO and cyclic GMP in non-contracted mesenteries

To avoid eventual interference derived from the precontraction procedure we further characterized the clonidine-evoked luminal NO peak in a series of non-contracted protocols.

Clonidine concentration-response curve, time course of NO and cyclic GMP outflow The threshold clonidine concentration that evoked a surge in luminal NO was 1 nM; larger concentrations caused a proportional increase in NO reaching its maximum at 100 nM (Figure 3). The effect was rapid and transient, luminally accessible NO peaked during drug application and faded after a min (Figure 4). The magnitude of the NO peak was 123±12.8 pmol ($n=20$) above basal values. In agreement with the vasorelaxation, after an initial

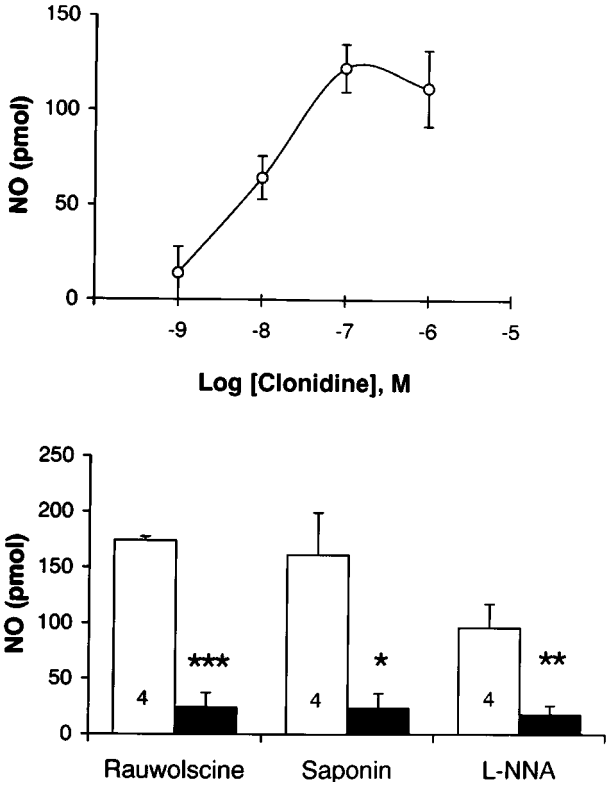

Figure 3 Clonidine-evoked concentration-dependent rises in luminal NO; blockade by rauwolscine, endothelium shedding and NO synthase inhibition. Upper panel: A 1-min clonidine perfusion evoked a concentration-dependent rise in luminally accessible NO. Lower panel: The rise in the luminal NO evoked by 100 nM clonidine was blunted by 100 nM rauwolscine, the shedding of the endothelial cell layer elicited by a 55 s perfusion with 0.1% saponin or 100 μM N^{ω}-nitro-L-arginine (L-NNA). Columns indicate the mean average; bars the s.e.mean. NO is expressed as the integral net release. *$P<0.05$; **$P<0.01$ and ***$P<0.001$ vs control (paired t-test).

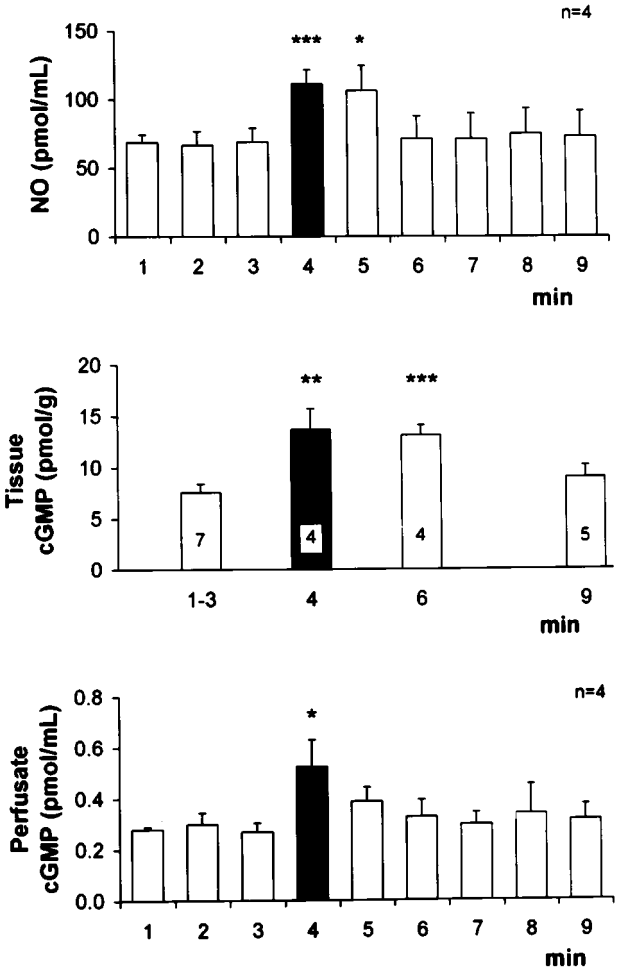

Figure 4 Rise in luminal accessible NO is mirrored by a correspondent rise in tissue cyclic GMP. In a set of mesenteries, 1 min perfusion with 100 nM clonidine (dark columns) induced a transient peak of luminally accessible NO (upper panel) and a correspondent surge of tissue cyclic GMP (middle panel). The cyclic nucleotide levels also rose in the perfusate, reflecting the rise in tissue nucleotide (lower panel). Luminal NO and cyclic GMP were determined in the same samples of the perfusate ($n=4$). Columns indicate the mean values; bars the s.e.mean. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$ as compared to the average value prior to the clonidine application ($n=4$). The numbers inside columns shown in the middle panel depict the times the experiment was repeated in separate mesenteries.

100 nM clonidine application, rauwolscine, endothelium removal or blockade of NO synthase inhibited the NO surge induced by a second drug addition (Figure 3, lower panel). In controls, two successive 100 nM clonidine applications, spaced 60 min apart, caused a similar rise in NO production (124 vs 110 pmol).

Consistent with the surge in luminal NO, 100 nM clonidine significantly increased tissue cyclic GMP content. The rise in tissue cyclic GMP production peaked during the 1 min clonidine application and remained significantly elevated for at least 3 min; basal values were attained 6 min later (Figure 4). We did not find a significant rise in tissue cyclic GMP within the first 30 s of clonidine perfusion. We observed a corresponding rise in the luminally accessible cyclic GMP, which represents about 2–5% of the tissue cyclic GMP content during the whole protocol (Figure 4). The net luminal increase was 248 ± 61 fmol cyclic GMP ($P<0.05$, $n=4$), doubling the basal cyclic GMP. The time course of tissue cyclic GMP production and the luminally accessible cyclic GMP extrusion mirrored the rise in NO (Figure 4, $n=4$).

Inhibition of soluble guanylyl cyclase and phosphodiesterase V Blockade of soluble guanylyl cyclase with 3–10 μM ODQ abolished the clonidine vasodilatation and consequently, the rise in tissue cyclic GMP elicited by 100 nM clonidine. Furthermore, ODQ reduced the corresponding basal tissue cyclic GMP content (Figure 5). As with L-NNA, in mesenteries treated with 3–10 μM ODQ, we observed a significant rise in the perfusion pressure elicited by 10 μM phenylephrine (upper panel Figure 5). While in these mesenteries the 100 nM clonidine-induced vasorelaxation was abolished ($n=10$), the 1 μM acetylcholine-evoked relaxation was only halved (Figure 5 and Table 1). ODQ treatment did not alter the basal luminally accessible NO (108.5 ± 6.7 vs 99.3 ± 5.5 pmol after ODQ treatment), nor the clonidine or acetylcholine-evoked rise in NO (Table 1). In mesenteries perfused with 100 nM clonidine, ODQ annulled the clonidine-evoked surge of tissue cyclic GMP (Figure 5) as well as the 1 μM acetylcholine-evoked rise in cyclic GMP production (Table 1).

In contrast, sildenafil, a phosphodiesterase V inhibitor, increased basal tissue and the clonidine-stimulated cyclic GMP, although the latter did not reach significance (Figure 5).

α_2-adrenoceptor agonists Structurally related α_2-adrenergic agonists mimicked the clonidine-induced rise in luminal NO. Clonidine 100 nM, oxymetazoline, and guanfacine were equally active while aminoclonidine, UK14,304, and guanabenz released slightly less NO. Xylozine 100 nM and agmatine resulted inactive (Table 2). The NO surge elicited by these drugs was consistent with their vasodilatory action. Compatible with α_2-adrenoceptor activation, 100 nM rauwolscine reduced 80–90% the rise in luminal NO evoked by these drugs (Table 2).

α_2-adrenoceptor antagonism A series of α_2-adrenoceptor antagonists were tested to classify the α_2-adrenoceptor subtype activated by clonidine. The antagonists shifted to the right, in a parallel fashion, the clonidine-evoked concentration-response curves of NO production. The rauwolscine-evoked rightward shifts of the clonidine-concentration response curves are illustrated in Figure 6, while the parallel shifts elicited by WB 4101 are shown in Figure 7. The Schild plots of each antagonist are shown as an insert in each figure; their correlation coefficients were 0.93 and 0.99, respectively. The rauwolscine, yohimbine, phentolamine, WB 4101 and prazosin pA_2 and their corresponding K_B values (nM) are listed in Table 3; the rank order of potency of these antagonists is: yohimbine > phentolamine $\cong$ rauwolscine > WB 4101 > prazosin.

In a paired series of protocols, the 100 nM clonidine-induced rise in NO production was blocked in a concentration-dependent fashion by rauwolscine, yohimbine, and prazosin; the concentration-response curves generated were parallel. The concentration of antagonist required to reduce 50% (IC_{50}) the effect of 100 nM clonidine was 34.1, 140 and 2404 nM for rauwolscine, yohimbine and prazosin, respectively.

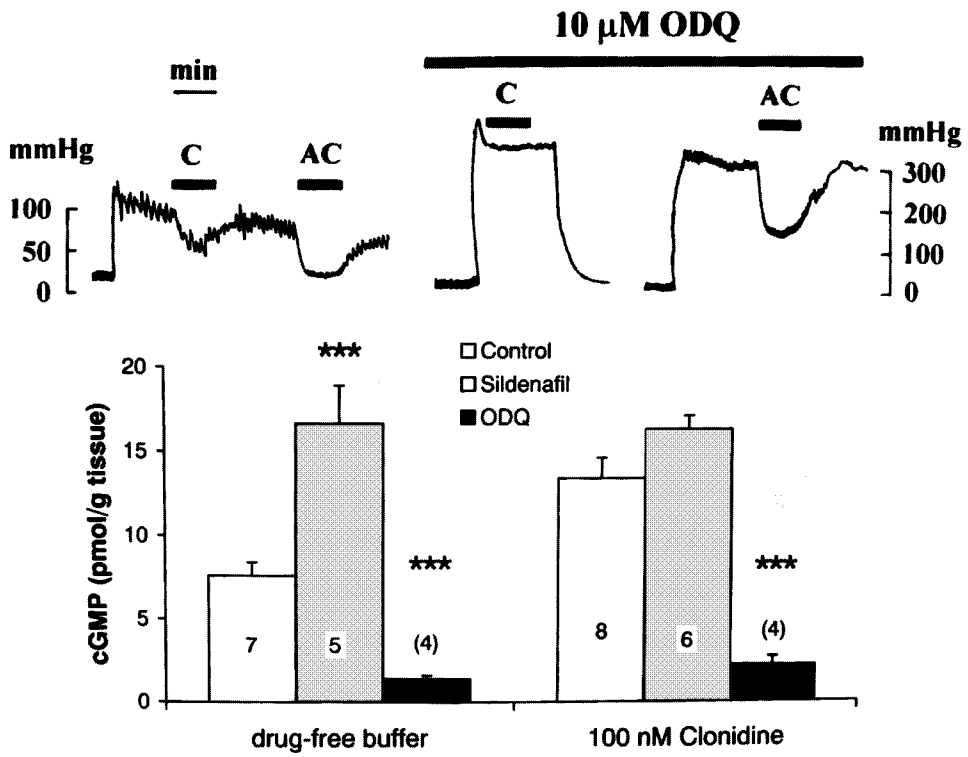

Figure 5 ODQ blunted the rise in cyclic GMP elicited by clonidine while sildenafil increased tissue cyclic GMP. Upper panel: Representative tracing shows the clonidine (C) and acetylcholine (AC) induced vasodilatation before and 20 min after treatment with 10 μM ODQ. Lower panel: Tissue content of cyclic GMP was processed 3 min after a 1 min 100 nM clonidine in two sets of protocols. In the first, the basal content of tissue cyclic GMP was assessed in mesenteries perfused with drug-free buffer (control), or buffer plus 10 nM sildenafil or buffer plus 10 μM ODQ. In the second set, the same determinations were performed after clonidine challenge. Columns indicate the mean value; bars, the s.e.mean. Numbers inside each column indicate the time each determination was performed. ***$P<0.001$ as compared with the controls.

Table 1 Relaxation, nitric oxide (NO) release and tissue cyclic GMP production evoked by perfusion of the rat arterial mesenteric bed with 1 μM acetylcholine

	Control	n	+100 μM *L-NN*	*n*	+3 μM *ODQ*[a]	n
Dilatation (%)	90.6±3.9	7	58.7±5.9*	6	56.2±2.1*	4
NO (pmol)	202.4±37.6	12	17.3±7.0***	4	158±15.9	4
cyclic GMP (pmol g^{-1})	18.5±2.5	12	0.4±0.2***	4	0.1±0.0***	4

[a]L-NNA refers to N^{ω}-nitro-L-arginine, used to block NO synthase activity. [b]ODQ, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one, an inhibitor of soluble guanylyl cyclase. *$P<0.05$; ***$P<0.001$ as compared to its control.

Table 2 NO production elicited by a 1 min perfusion with clonidine and several structurally related α_2-adrenergic agonists; blockade by rauwolscine

α_2-*agonist* (100 nM)	*pmol NO (mean±s.e.mean)* *Control*	n	+100 nM *Rauwolscine*	n
Clonidine	196.8±21.4	4	12.0±6.6**	4
Aminoclonidine	147.9±9.9	5	27.2±14.3***	4
Guanabenz	132.2±12.0	5	11.8±7.4***	4
Oxymetazoline	238.1±39.4	4	210.±9.2**	4
UK-14304	147±29	4	28.3±20	4
Xylazine	16.5±7.3	4	17.2±9.8	4
Agmatine	15.2±5.0	3	N.D.	

N.D. = not determined. **$P<0.01$; ***$P<0.001$; with respect to matched-control mesenteries perfused with the agonist in the absence of 100 nM rauwolscine.

Comparison of the clonidine-elicited rise in NO with other vasodilators Acetylcholine 100 nM, bradykinin or L (−)epinephrine caused a rapid increase in luminal NO. The relative rank of activity based on the magnitude of NO production, is bradykinin > L (−)epinephrine > acetylcholine > clonidine (Table 4). As with clonidine, endothelial denudation reduced approx. 80% the rise in the luminally accessible NO evoked by these vasodilators (Table 4).

Acute chemical sympathectomy Pretreatment with 6-hydroxydopamine reduced by 95% the content of noradrenaline of the perivascular nerve endings (584.2±41.8 vs 33.7±4.5 pmol g^{-1} tissue, $n=4$, $P<0.001$) and 60% the content of neuropeptide Y (9.1±0.6 vs 3.6±0.9 pmolg^{-1} tissue, $n=4$, $P<0.01$). However, this treatment did not modify the surge

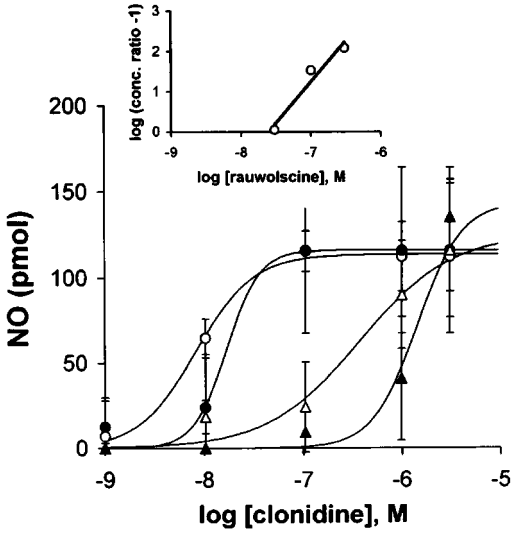

Figure 6 Rauwolscine blocks concentration-dependently the clonidine-induced rise in luminally accessible NO. Separate groups of four mesenteries were perfused with 30 (closed circles), 100 (open triangles), or 300 nM (closed triangles) rauwolscine to shift rightwards the clonidine-induced concentration-dependent increment in luminally accessible NO. Symbols indicate the mean value, bars the s.e.mean. Insert shows the Schild plot of this interaction; the pA_2 is 7.61.

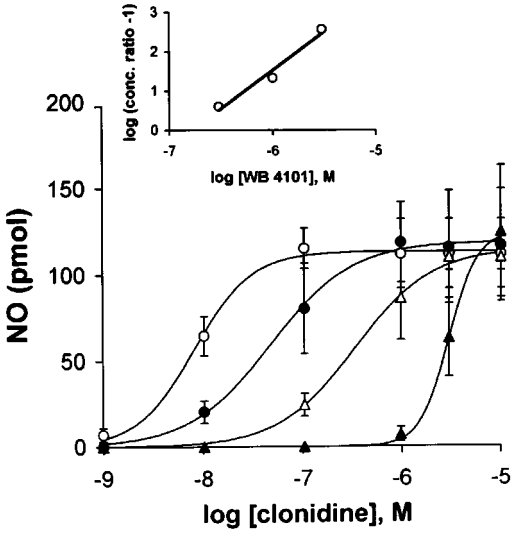

Figure 7 Concentration-dependent blockade of the clonidine induced rise in NO elicited by WB 4101. Concentration-dependent antagonism of the clonidine-induced concentration-dependent rise in luminally accessible NO elicited by 0.3 μM (closed circles), 1 μM (open triangles), or 3 μM (closed triangles) WB 4101 ($n=4$ each). Symbols represent the mean value; bars the s.e.mean. Insert shows the corresponding Schild plot; the pA_2 derived is 6.78.

of luminally accessible NO released elicited by a 1 min perfusion with 100 nM clonidine (98.4 ± 10.7 vs 91.9 ± 29.9 pmol NO, $n=4$). Likewise, perfusion with guanethidine or the combined perfusion of guanethidine plus atropine did not change the surge of NO elicited by a 1 min 100 nM clonidine perfusion. The rise in luminally accessible NO was 84.0 ± 11.2 and 111.3 ± 15.5, respectively as compared to 98.4 ± 10.7 pmol NO ($n=4$ per subgroup) obtained in parallel control groups ($n=4$, per rat subgroup).

Table 3 pA_2 and K_B values for several α_2-adrenergic antagonists

	pA_2 (mean ± s.e.mean)	K_B (nM) (mean ± s.e.mean)
Yohimbine	8.17 ± 0.03*	6.84 ± 0.44
Rauwolscine	7.61 ± 0.08	24.40 ± 2.34
Phentolamine	7.72 ± 0.01*	19.03 ± 0.51
WB 4101	6.78 ± 0.04	164.9 ± 12.9
Prazosin	6.06 ± 0.22*	1489.6 ± 547.4

The slope of the rauwolscine Schild plot was 2.05, and the slope of the WB 4101 was 1.96. *The pA_2 and the estimated K_B values for these antagonists was derived from the simple expression: $pA_2 = -\text{Log}\,[\text{antagonist}] + \text{Log}\,[EC_{50}\ \text{ratio-1}]$.

Table 4 The rise in luminal NO elicited by several physiological agonists requires an intact endothelium

	pmol NO (mean ± s.e.mean)			
Agonist (100 nM)	*With endothelium*	n	*Without endothelium*	n
Acetylcholine	174.2 ± 40.4	4	43.2 ± 12.8*	3
Bradykinin	274.0 ± 40.2	4	54.0 ± 10.2**	4
Clonidine	123.0 ± 12.8	20	23.2 ± 13.6**	4
L (−) Epinephrine	225.2 ± 16.8	6	46.2 ± 4.4***	4

Each agonist perfused for 1 min at 100 nM. A group of intact preparations served as controls (with endothelium), while separate mesenteries were denuded of the endothelial cell layer following a 55 s perfusion with 0.1% saponin, and are referred as preparations without endothelium. *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared to the matched-control.

Discussion

The present results substantiate that NO contributes to a more complete understanding of the vasorelaxant action of clonidine and structurally related agonists, highlighting a role of the endothelium in the antihypertensive action of clonidine. The present results demonstrate that clonidine and congeners relax the arterial mesenteric bed through the activation of endothelial α_2-adrenoceptors, tentatively belonging to the α_{2D}-adrenoceptor subtype. These receptors are coupled to the L-arginine pathway facilitating the synthesis of endothelial NO eliciting a consequent rise in cyclic GMP production. The clonidine-induced relaxation and the luminally accessible NO released occurred in the same range of concentrations likely evidencing a direct interdependence. We hypothesize therefore, that endothelial α_{2D}-adrenoceptors activate the L-arginine-NO-cyclic GMP cascade as a mechanism of endothelium-smooth muscle signalling.

Although the present observations cannot account for the systemic antihypertensive action of clonidine and related drugs, we interpret the present observations as a mechanism that complements the classical stimulation of central and peripheral presynaptic α_2-adrenoceptors (Sattler & van Zwieten, 1967; Langer *et al.*, 1980; Tibirica *et al.*, 1991; Langer, 1997). Our results discard the influence of perivas-

cular sympathetic nerve terminals in the clonidine-induced vasodilatation. The blockade of sympathetic transmitter release by guanethidine (Brock & Cunane, 1988) and by 6-hydroxydopamine treatment further allows the ruling out of a neuronal mechanism regulating transmitter's release from the perivascular nerve terminals. Altogether, the full significance of the present observations is highlighted by the report of Soares de Moura & Leao (1997) and confirmed by Yatomi *et al*. (1998), suggesting that the antihypertensive action of clonidine might involve endothelial NO production. Furthermore, Soares de Moura *et al*. (2000) extended his initial observation to include rilmenidine, showing that the hypotensive action of this novel α_2-adrenoceptor agonist is also NO-dependent.

The clonidine-evoked NO production is transient in spite of continued drug application. Clonidine applied for 1 min elicited a similar rise in NO production as that evoked by 6 min of drug perfusions, revealing that the production of NO is not necessarily proportional to the duration of drug application. This observation validates our choice of applying the agonists for 1 min. Apparently our observation is not a peculiarity of the α_2-adrenoceptor since Figueroa *et al*. (1998) found a similar result with the bradykinin-induced NO production. Furthermore, the acetylcholine and purinergic-induced NO release showed a similar time course (Buvinic & Huidobro-Toro, 2000; 2001), denoting the transient nature of the endothelial NO surge elicited by ligand-activated receptors in the rat mesenteric bed. Although the mechanism of this desensitization remains unknown, several explications may be adduced. Among others, we suggest a partial depletion of intracellular calcium or a negative feedback of NO synthase exerted by cyclic GMP.

The clonidine-induced vasorelaxation is linked to NO production through α_2-adrenergic occupation. This conclusion is supported by the use of a variety of selective drug congeners and a battery of antagonists. The vasodilatation and the associated NO surge observed with several agonists are effectively blocked by rauwolscine. Furthermore, the most active compounds have guanidino and/or imidazol moieties, while xylazine was essentially inactive, likely because it lacks these chemical groupings. These findings are consistent with known structural requirements for α_2-adrenergic receptor activation (Hoffman & Lefkowitz, 1996). Likewise, agmatine, decarboxylated arginine, was inactive, allowing us to discard the eventual participation of imidazoline receptors (Tibirica *et al*., 1991; Hieble & Ruffolo, 1992).

The study with the antagonists was aimed at the tentative classification of the α-adrenoceptor involved. Based on our finding that $0.01-1\ \mu$M prazosin did not modify the clonidine-induced NO production, we conclude that clonidine must activate α_2-adrenoceptors. To define the receptor subtype, we performed matched experiments with several α_2-adrenergic blockers with varying affinities for the different α_2-adrenoceptor subtypes (Bylund & Ray-Prenger, 1989; Michel *et al*., 1989; Simonneaux *et al*., 1991; O'Rourke *et al*., 1994; Renouard *et al*., 1994). Results with prazosin allowed discarding a contribution of the α_1, α_{2B} and α_{2C}-adrenoceptor subtypes because the prazosin K_B is at least 40 fold larger than its reported K_i for these subtypes (Bockman *et al*., 1996). The differences between the relative affinities of rauwolscine and WB 4101 are consistent with their relative affinities for the α_{2D}-adrenoceptor, a result that favours our interpretation that clonidine and congeners interact with the α_{2D}-adrenoceptors in the rat arterial mesenteric bed. It is puzzling that the rauwolscine K_B is not smaller than that of yohimbine; however, the IC_{50} determinations reveal a larger value. Perhaps this minor discrepancy reveals the heterogeneous nature of the endothelial cells in this vascular bed. Notwithstanding, our pA_2 and K_B values are reasonably close to those reported by Bockman *et al*. (1996) who studied the UK14,304-evoked relaxation of the rat isolated superior mesenteric artery. The nature of our work is totally different from the latter study since the arterial mesenteric bed includes resistance vessels, a territory that may express receptor heterogeneity. The expression of vascular adrenergic receptors is markedly territory and species-dependent (Bockman *et al*., 1996). We cannot discard the possibility that the endothelial cell layer of this vascular bed contains more than one subtype of α_2-adrenoceptors. We are aware that the slope of our Schild plots is not unitary; therefore our conclusion that clonidine activates solely the α_{2D}-adrenoceptor subtype is tentative.

Physical removal of the endothelium essentially ablates the NO production induced by clonidine, demonstrating that the α_2-adrenoceptors are localized in endothelial cells. Furthermore, inhibition of NO synthase obliterates the clonidine-evoked increment of NO production. The peak of NO was concomitant to a rise in tissue cyclic GMP. However, the return of cyclic GMP to basal values was delayed as compared to the luminal NO. The determinations of cyclic GMP in the perfusate demonstrate a $2-5\%$ extrusion of this nucleotide from the tissue, following a time course that reflects the changes in intracellular cyclic GMP. These results support the notion that a fraction of intracellular cyclic GMP reaches the extracellular space, likely as a negative feed back control mechanism of the cyclic GMP activity, in agreement with several recent reports (Coulson, 1976; Ahlstrom & Lamberg-Allardt, 2000; Buvinic & Huidobro-Toro, 2000). Consistent with the activation of the L-arginine pathway, blockade of guanylyl cyclase ablates the increase in tissue cyclic GMP elicited by clonidine blocking thereby the vasodilatation. Moreover, sildenafil, a drug that selectively blocks phosphodiesterase V, raises control and clonidine-stimulated tissue levels of cyclic GMP. Likely, cyclic GMP, through nucleotide-dependent protein kinases, reduces intracellular free calcium resulting ultimately in smooth muscle relaxation (Carvajal *et al*., 2000).

Postjunctional, α_2-adrenoceptors are present in some vascular territories and have been linked to vasomotor responses of clonidine and related drugs (Hoffman & Lefkowitz, 1996). *In-vivo*, α_2-adrenoceptor agonists may increase total vascular resistance (Kobinger, 1978). In the rat tail artery, UK14,304 enhances responses to other vasoconstrictors, an effect that is blocked by calcium channel blockers (Xiao & Rand, 1989a,b), leading to the hypothesis that this receptor is coupled to the mobilization of calcium-stores by a G-protein linked mechanism (Hoffman & Lefkowitz, 1996). The present results show that in some mesenteries pre-incubated with $100\ \mu$M L-NNA, clonidine elicits a contractile response, suggesting that postjunctional α_2-adrenoceptors are also present in the rat arterial mesenteric bed. Confirming this interpretation, we have consistently recorded that clonidine contracts endothelium

denuded rings of the rat superior mesenteric artery (J.P. Huidobro-Toro and C. Meynard, unpublished results) although with less efficacy than phenylephrine or norepinephrine. Postjunctional α_2-adrenoceptors have not been properly characterized; they show marked species and territorial differences within vascular beds (Hoffman & Lefkowitz, 1996). The full significance and pharmacological opportunities of the endothelial and the postjunctional smooth muscle α_2-adrenoceptors warrants the further research.

With regard to the interrelationship between vasodilatation and NO production, we consistently observed that while 100 nM clonidine evoked the maximal NO production, there is near a 50% increase of vascular relaxation between 100 and 1000 nM clonidine. To better define this apparent inconsistency, avoiding an eventual interaction between clonidine and phenylephrine, a series of mesenteries were contracted with 40 mM KCl instead of phenylephrine. In these preparations, clonidine caused a concentration-dependent relaxation that was similar between 100 and 1000 nM, suggesting that the maximal clonidine vasorelaxation is achieved at about 100 nM clonidine. This finding allows us to hypothesize that clonidine in concentrations around 1 μM may displace phenylephrine from the α_1-adrenoceptor diminishing its vasomotor activity, rather than admitting another endothelium-dependent mechanism of vasorelaxation. This explanation can also account for the finding that the 1 μM clonidine vasodilatation is only modestly antagonized by L-NNA or rauwolscine (Figure 2). In further support of our interpretation, we noted after endothelium denudation that the clonidine concentration-response curve was similar to that attained with L-NNA (data not shown). This collection of data permits discarding the existence of an additional endothelium-derived vasodilatation mechanism distinct from NO favouring our interpretation that clonidine indirectly decreases α_1-adrenoceptor activity. In our protocols we never observed contractions elicited by clonidine discarding that in our experimental conditions clonidine acts as an α_1-adrenoceptor agonist, except in mesenteries pretreated with L-NNA.

In contrast to the 1–100 nM clonidine-evoked vasodilatation that was abolished by L-NNA or ODQ, the acetylcholine vasodilatation is relative resistant to the action of these drug inhibitors. Consistent with this partial blockade of the acetylcholine-induced vasodilatation, there is consensus that acetylcholine activates a distinct relaxation mechanism independent of NO, such as the endothelium-derived hyperpolarizing factor (Edwards *et al*., 1998; Brandes *et al*., 2000). Therefore it is plausible to assume that in this vascular bed, about 50% of the acetylcholine-evoked vasodilation can be attributed to the release of this factor.

Studies on the role of endothelial α_2-adrenoceptors in the physiology and pharmacology of the endothelial cell are scarce but emerging. This receptor may be involved in vascular diseases. In this context, rat mineralocorticoid hypertension is associated to an increased compensatory release of NO (Bockman *et al*., 1992). In our laboratory, Poblete *et al*. (1999) reported that clonidine induces a larger NO production in rats with renovascular hypertension than in matched normotensive controls. These findings support the notion that endothelial α_2-adrenoceptors, and particularly the tentative α_{2D}-adrenoceptor subtype, may play a role in the control of blood pressure, which may be of pathophysiological interest as outlined by Kable *et al*. (2000). Although Wilson (1991) did not observe evidences of increased peripheral α-adrenoceptor binding in renovascular hypertensive rats, the Wilson study did not consider detailed binding or autoradiography to endothelial α_2-adrenoceptor subtypes in the arterial mesenteric bed.

In summary, the present communication highlights a therapeutic potential of the endothelial α_2-adrenoceptors linked to NO synthase activity by demonstrating the role of the tentative α_{2D}-adrenoceptors in the vasorelaxation of the rat arterial mesenteric bed. The activation of endothelial α_{2D}-adrenoceptors in this territory facilitates the L-arginine pathway, an additional mechanism that may contribute to explain the antihypertensive action of clonidine and related agonists. This study strongly supports the notion that endothelial α_2-adrenoceptors are involved in the control of blood pressure in health and disease, offering opportunities of therapeutic relevance.

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