Neurokinins Induce Relaxation of Human Pulmonary Vessels Through Stimulation of Endothelial NK_1 Receptors

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Abstract: The effects of neurokinins and neurokinin receptor selective agonists have been investigated on human intralobar pulmonary vessels. Substance P (SP) and [$Sar^9Met(O_2)^{11}$]SP, a selective NK_1 receptor agonist, induced concentration-dependent relaxation of pulmonary vessels precontracted with phenylephrine. The mean negative log (M) EC_{50} values for SP and [$Sar^9Met(O_2)^{11}$]SP were 8.6 and 8.9, respectively, on arterial preparations and 8.9 and 8.6, respectively, on venous preparations. Relaxations to [$Sar^9Met(O_2)^{11}$]SP were abolished by the NK_1 receptor antagonist SR140333. The relaxations to a second application of [$Sar^9Met(O_2)^{11}$]SP were markedly reduced, suggesting a rapid desensitization of the NK_1 receptor. Such desensitization was not observed with acetylcholine. The selective NK_2 receptor agonist, [Nle^{10}]NKA, and the selective NK_3 receptor agonist, [$MePhe^7$]NKB, caused neither contractions nor relaxations of pulmonary vessels. The NK_1 receptor-mediated relaxations were abolished by removing the endothelium or by a combination of N^G-nitro-L-arginine and indomethacin, whereas each compound exerted a partial inhibitory effect. Similar results were observed with acetylcholine. Positive immunostaining for NK_1 receptors was only found in the endothelium. Reverse transcription-polymerase chain reaction detected messenger RNA for NK_1 receptors without any detection of messenger RNA for NK_2 or NK_3 receptors. In conclusion, human pulmonary arteries and veins express endothelial NK_1 receptors that mediate relaxation through a combination of cyclooxygenase and nitric oxide activities and are subjected to rapid tachyphylaxis.

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Pulmonary circulation is regulated by various hormonal, humoral, and neural factors, including neurokinins (1). Sensory nerves containing neurokinins, such as substance P (SP) and neurokinin A (NKA), have been found to supply the human intrapulmonary vasculature (2). Neurokinin receptors are classified into three main types, referred to as NK_1, NK_2, and NK_3, for which SP, NKA, and neurokinin B (NKB) are the preferred endogenous agonists, respectively, although considerable cross-reactivity occurs (3). SP has recently been reported to cause relaxation of human isolated pulmonary arteries through stimulation of NK_1 receptors (4,5) and by the release of nitric oxide and prostaglandins by the endothelium (5). In rabbit pulmonary arteries, SP not only evokes endothelium-dependent relaxation but also causes endothelium-dependent contraction via activation of NK_1 receptors and thromboxane A_2 production at low concentrations (6,7) and endothelium-independent contraction via NK_2 receptors at higher concentrations (6). Therefore, in pulmonary arteries, neurokinins can act through activation of different receptors not only located on the endothelium and induce responses other than an endothelium-dependent relaxation. The first aim of the current study was to investigate further the function, location, and expression of the different neurokinin receptors in human isolated pulmonary arteries.

Major differences in receptor expression and function can occur between arteries and veins from the same tissue. Recent functional studies have shown differences in the receptor subtypes involved in the responses to prostanoids (8), 5-hydroxytryptamine (9), acetylcholine (10), or cysteinyl-leukotrienes (11) between human isolated pulmonary arteries and veins. The effects of neurokinins have not been reported in human pulmonary vein. Therefore, the second aim of the current study was to investigate the function, location, and expression of the different neurokinin receptors in human isolated pulmonary veins.

Since the selective NK_1 receptor agonist, [$Sar^9Met(O_2)^{11}$]SP, was found to induce an endothelium-dependent relaxation in human pulmonary arteries and veins, the third aim of the current study was to determine and to compare the involvement of nitric oxide synthase and cyclooxygenase in NK_1 receptor-mediated relaxation in pulmonary vessels. Furthermore, acetylcholine (ACh) induces an endothelium-dependent relaxation in human pulmonary arteries (10,12–14) and veins (10). The mechanisms underlying the relaxant response to ACh have been previously described to be cyclooxygenase and NO-synthase pathway dependent in human pulmonary arteries (12,14,15) but have not been reported in pulmonary veins. We have also determined the involvement of nitric oxide synthase and cyclooxygenase in ACh-mediated relaxation in human pulmonary arteries and especially in pulmonary veins to further characterize the SP-mediated relaxation by providing comparative pharmacologic information with a classic endothelium-dependent relaxant.

METHODS

Human lung tissue was obtained from patients (42 men and 2 women) who had undergone lobectomy or pneumectomy for removal of lung carcinoma. All patients were smokers or former smokers. The mean age was 63 ± 2 years. Pulmonary veins and arteries (2–5 mm internal diameter) were carefully removed from the macroscopically normal regions of the diseased lung and dissected free from adjoining connective tissue and lung parenchyma. Pulmonary vessels were placed either in cooled (4°C) Krebs-Hepes medium for organ bath studies or quickly frozen in liquid nitrogen and stored at −80°C for immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). On reaching the pharmacology laboratory, tissues in Krebs solution were immediately mounted in organ baths for experimentation. All the tissues were used for functional experiments within 1.5 to 4 hour of surgery.

Functional Experiments

Vessels were cut as rings (5–6 mm in length). Experiments were performed on 173 arterial preparations from 32 patients and 142 venous preparations from 25 patients. The rings were then set up in 10-ml organ baths containing Krebs solution (composition in m*M*: NaCl, 118; KCl, 5.4; $CaCl_2$, 2.5; $MgSO_4$, 0.6; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11.7; pH 7.4), continuously gassed with 5% CO_2 in O_2, and maintained at 37°C. They were suspended on wires; the lower wire was fixed to a micrometer (Mitutoyo, Japan), and the upper wire was attached to an isometric force displacement transducer (UF-1, Pioden). Changes in force were recorded on two-channel recorders (Linseis E200, Polylabo, France). An optimal load (1.5–2 g), which ensured maximal responses, was applied to each ring (8,12). Subsequently, preparations were allowed to equilibrate for 45 minutes with bath fluid changes every 15 minutes. Each experiment began by contracting the vessel rings twice to maximal tension with KCl, 90 m*M*. This initial maximal contraction was performed to stabilize the preparations and ensure reproducible contractile responsiveness. The preparations were then washed with two successive bath fluid changes and allowed to equilibrate for 45 minutes during which the Krebs solution was changed every 15 minutes. Since human pulmonary vessels are known to have little or no inherent tone, the relaxant effect of neurokinin receptor agonists was studied in preparations contracted with phenylephrine (30 μ*M*). After the contraction had stabilized (approximately 10 minutes), concentration–response curves were generated for SP, [$Sar^9Met(O_2)^{11}$]SP, [Nle^{10}]NKA (4–10), and [$MePhe^7$]NKB. The concentration of the different neurokinin agonists was increased by 1-log increments, each concentration added when the maximal effect was produced by the previous concentration, or every 5 minutes when no response occurred. The effect of a NK_1 selective antagonist (SR 140333, 0.1 μ*M*) was examined by adding the compound to the tissue bath 40 minutes before addition of NK receptor agonists (16). In some experiments, stimulation with the NK receptor agonists was performed in vessels at basal tone, i.e., the phenylephrine-induced precontraction was omitted.

Endothelial dependence of [$Sar^9Met(O_2)^{11}$]SP-induced relaxation was assessed in experiments in which the endothelium of one of a pair of adjacent pulmonary arterial or venous rings was removed. Endothelium was mechanically removed by inserting a smooth-edged arm of a dissecting forceps into the lumen of the vessel ring and gently rolling the moistened preparation between the surface of a forefinger and the forceps for about 10 seconds without undue stretching. The second ring of the pair, in which the endothelium was left intact, served as the control. Cumulative concentration–response curves for [$Sar^9Met(O_2)^{11}$]SP were generated in endothelial-denuded and -intact preparations as detailed above. Endothelium removal was confirmed by the loss of the relaxation response to acetylcholine (100 μ*M*) in phenylephrine-contracted rings assessed at the end of the experimental protocol.

Determination of the involvement of nitric oxide and prostanoids in the vascular relaxation produced by [$Sar^9Met(O_2)^{11}$]SP in human pulmonary vessels was achieved by examining the effect of the nitric oxide synthase inhibitor (N^G-nitro-L-arginine, L-NOARG) and the cyclooxygenase inhibitor (indomethacin) on the relaxation response to this agonist. In these experiments, L-NOARG (100 μ*M*), indomethacin (10 μ*M*), or a combination of the

two was added to tissue baths 50 minutes before [$Sar^9Met(O_2)^{11}$]SP addition. At 40 minutes, the vascular tissues were contracted with phenylephrine (30 μ*M*); after the contraction reached a steady state, cumulative concentration–response curves were generated for the agonist as described above. Control tissues received the vehicle instead of L-NOARG or indomethacin. Neither indomethacin nor L-NOARG had a significant effect on the magnitude of the contraction induced by phenylephrine. In some experiments conducted in similar conditions, concentration–response curves to acetylcholine (0.1 n*M*–1 m*M*) were also performed as controls. In all experimental protocols, only one cumulative concentration–effect curve was obtained for each ring preparation, except where otherwise stated (tachyphylaxis studies).

Immunohistochemistry

Cryostat sections (5 μm) of pulmonary arterial or venous segments were immunostained with antibodies to NK_1 and NK_2 receptors through the streptavidin-biotin-complex/peroxidase method. The slides were fixed for 10 minutes with fresh acetone at room temperature. After rehydrating the slides in phosphate buffered saline (PBS) for 5 minutes, nonspecific binding was blocked by incubating the slides for 10 minutes in blocked serum (Clinisciences, Trappes, France). The sections were then incubated overnight at 4°C with the primary antibody. The antibody to NK_1 receptors was a rabbit polyclonal antibody (1:1500 dilution, Sigma, St Quentin Fallavier, France) developed against the C-terminal sequence (residues 393–407) of the NK_1 receptor, which is highly conserved in human, guinea pig, and mouse NK_1 receptors but different from the corresponding sequences of the human NK_2 and NK_3 receptors. Negative controls were produced by omitting the primary antibody and by substituting the primary antibody with PBS.

To detect NK_2 receptors, a mouse monoclonal antibody to the human NK_2 receptor, kindly provided by Dr. P. Geppetti (University of Ferrara, Italy), was used (1:100 dilution). A description and characterization of this antibody has been reported (17). Negative controls were produced by preabsorbing the antibody with the immunogenic peptide diluted at 2.5×10^{-5} *M* in a 1:100 dilution of the antibody and incubating for at least 4 hours before applying the antibody to tissue. Omitting the primary antibody produced further negative controls. After washing in PBS, the sections stained for NK_1 or NK_2 receptor were incubated for 30 minutes with multilink biotinylated anti-IgG (Biogenex, Chevilly Larue, France). All sections were then washed and incubated for 30 minutes with streptavidin-biotin complex reagent (Biogenex, Chevilly Larue, France).

Immunoreactivity was visualized with amino-3-ethyl-9-carbazol (AEC). Sections were dehydrated and mounted in a hydrophobic mounting medium (glycergel, Dako, Montrouge, France).

Reverse Transcription-Polymerase Chain Reaction

These reactions were performed as previously described (18). Total RNA of human pulmonary artery or vein was isolated according to Chomczynski and Sacchi (19). Residual genomic DNA was removed by incubating the RNA samples with RNase-free, FPLC pure DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random hexamers according to the manufacturer's instructions (First-strand cDNA synthesis kit, Amersham Pharmacia Biotech). The resulting cDNA samples were amplified by PCR using a DNA thermal cycler (MJ Research, Watertown, MA, U.S.A.) and the following specific primer pairs: 1) NK_1 receptor, forward 5′-CGCTACCAYGAGCAAGTCTC-3′ and reverse 5′-CCTGTCRTTGAGGCARCAGT-3′, based on the published sequence of the human NK_1 receptor (20), giving a PCR product of 231 base pairs (bp); 2) NK_2 receptor, forward 5′-GCCCTACCACCTC-TACTTCATCC-3′ and reverse 5′-AGCAAACCATAC-CCAAACCA-3′, based on the sequence of the human NK_2 receptor gene (21) and designed to amplify a PCR product of 375 bp; 3) NK_3 receptor, forward 5′-TCCTKGTRAACCTGGCTTTC-3′ and reverse 5′-TGTTGYTTGGGACCTTCTGG-3′, based on the human NK_3 receptor DNA sequence (22), giving a PCR product of 364 bp. Amplification of the human β-actin gene transcript was used to control the efficiency of RT-PCR among the samples. Sequences of forward and reverse primers for β-actin were 5′-TCCCTG-GAGAAGAGCTACGA-3′ and 5′-ATCTGCTGGAAG-GTGGACAG-3′, respectively. The expected size of the PCR product was 362 bp. PCR mixes contained 0.2 μ*M* primers, 1.5 U of Taq polymerase (Amersham Pharmacia Biotech), the buffer supplied, 2.5 m*M* $MgCl_2$, 200 μ*M* dNTPs and cDNA in 25 μl. After 2 minutes at 94°C, the parameters used for PCR amplification were 10 seconds at 94°C; 20 seconds at 60°C, and 30 seconds at 72°C. Cycle numbers were 36 for tachykinin receptors and 24 for β-actin. A higher number of cycles (n = 43) was used in some experiments to amplify the cDNAs of tachykinin receptors.

Serial half dilutions of cDNA were amplified at the indicated number of cycles for the three tachykinin receptors and β-actin to ensure analysis of products in the linear range of amplification. The PCR products were separated by gel electrophoresis, stained with ethidium bromide, and visualized and photographed with UV transilluminator

(Spectronics Corp., NY, U.S.A.). The band intensities were scanned by densitometry using a video documentation system and the image analysis software Intelligent Quantifier (BioImage Systems Corp., Ann Arbor, MI, U.S.A.). Messenger RNA levels for the three tachykinin receptors and β-actin were analyzed on each tissue, with each RT-PCR assay performed in triplicate. The identity of each PCR product was established by DNA sequence analysis, as previously described (18).

Expression of the Results and Statistical Analysis

All numerical data are expressed as the arithmetic mean ± SEM. The pD_2 values were determined for each cumulative concentration–response curve as the negative logarithm of the molar EC_{50} value (the concentration of agonist inducing a relaxation that was 50% of the maximal relaxation produced by agonist). The relaxations produced by the NK_1 agonists or acetylcholine were expressed as a percent inhibition of phenylephrine-induced contraction. E_{max} represents the maximal effect observed during cumulative concentration–response curves or for 1 μ*M* of NK agonists. Differences between concentration–response curves were tested using analysis of variance (ANOVA) for repeated measures followed by Bonferroni-Dunn *t* test if required. Probability values (p) for ANOVA for repeated measures correspond to concentration–treatment interactions. Differences between means were determined using Student *t* test; $p < 0.05$ was considered significant.

Drugs and Solutions for Functional Studies

SP, [Sar^{9}Met(O$_2$)^{11}]SP, NKA, [Nle^{10}]NKA (4–10), and [MePhe^7]NKB were obtained from BACHEM (Voisins-le-Bretonneux, France). SR 140333 was kindly provided by Dr X. Emonds-Alt (Sanofi-Synthelabo, Montpellier, France). Phenylephrine, acetylcholine, indomethacin, and L-NOARG were obtained from Sigma (St Quentin Fallavier, France). Indomethacin was dissolved in $NaHCO_3$ (0.5 *M*). Stock solutions of SR 140333 (10 m*M*) and NK-receptor agonists (1 m*M*) were prepared in ethanol and water, respectively. They were diluted to final concentration in Kreb's buffer solution. Phenylephrine, ACh, and L-NOARG were dissolved in distilled water.

RESULTS

Vascular Muscle Responses

On basal tone, neither NK_1 agonists (SP, [Sar^{9}Met(O$_2$)^{11}]SP), NK_2 agonists (NKA, [Nle^{10}]NKA (4–10), nor the NK_3 agonist ([MePhe^7]NKB), applied in cumulative 1-log increments (up to 1 μ*M*), induced contraction of the vessel rings (n = 2–6 for each peptide in arteries and veins) even after endothelium removal (n = 2 for each agonist). In arterial and venous preparations contracted with phenylephrine (30 μ*M*), SP and [Sar^{9}Met(O$_2$)^{11}]SP induced concentration-dependent relaxations (Fig. 1), whereas neither the NK_2 selective agonist nor the NK_3 selective agonist caused relaxations at concentrations up to 1 μ*M* (n = 3–4 for these two agonists). The potency and efficacy of SP and [Sar^{9}Met(O$_2$)^{11}]SP were similar in the two preparations (Table 1, Fig. 1). Neither in artery nor in vein segments denuded of their endothelium did [Sar^{9}Met(O$_2$)^{11}]SP induce relaxation (Table 1, Fig. 1). In endothelium-removed arterial preparations, ACh (0.1 m*M*) induced a contraction over the phenylephrine con-

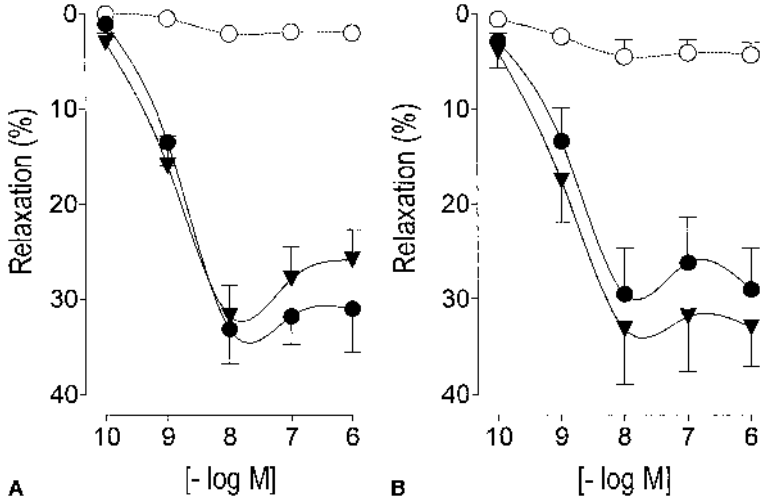

FIG. 1. Concentration–response curves for substance P and [Sar^{9}Met(O$_2$)^{11}]SP in causing relaxation of human pulmonary arteries (**A**) or human pulmonary veins (**B**) with intact endothelium (SP, ▼; n = 15–18; [Sar^{9}Met(O$_2$)^{11}]SP, ●; n = 25–37) and with denuded endothelium ([Sar^{9}Met(O$_2$)^{11}]SP, ○; n = 4–8). The relaxation is expressed as a percentage of the precontraction induced by phenylephrine (30 μ*M*). Values are expressed as means, and vertical lines show SEM.

TABLE 1. E_{max} and pD_2 calculated from concentration-response curves obtained after cumulative addition of substance P (SP) and [$Sar^9Met(O_2)^{11}$]SP

Treatment	Pulmonary arteries			Pulmonary veins		
	n/p	E_{max} (%)	pD_2	n/p	E_{max} (%)	pD_2
Endothelium-intact						
SP	18/10	32 ± 3	8.6 ± 0.1	15/6	37 ± 6	8.9 ± 0.2
[$Sar^9Met(O_2)^{11}$]SP	37/27	33 ± 3	8.9 ± 0.1	25/15	32 ± 5	8.6 ± 0.2
Endothelium-denuded						
[$Sar^9Met(O_2)^{11}$]SP	8/4	2 ± 1*	NC	4/2	4 ± 3*	NC

Values calculated after cumulative addition of substance P (SP) and [$Sar^9Met(O_2)^{11}$]SP in human pulmonary arteries and veins precontracted with phenylephrine.

E_{max} is expressed as percentage of precontraction induced by phenylephrine (30 μ*M*); pD_2 values are negative $\log_{10}$ of the concentration required to achieve half maximum relaxation.

Data shown as mean ± SEM of n/p (number of preparations/number of patients) experiments.

Comparisons were performed using the Student *t* test. $*P < 0.05$ for control values in the same tissue.

E_{max}, maximal relaxation; NC, not calculated; pD_2, potency.

traction (mean increase = 26 ± 8%, n = 12), whereas in adjacent endothelium-intact arterial rings, the same concentration of ACh induced a relaxation (81 ± 5%). The relaxation induced by ACh (0.1 m*M*) in endothelium-intact venous rings (78 ± 7%, n = 4) was eliminated by endothelium removal in adjacent rings (6 ± 3%); however, in contrast with the arterial preparations, no contraction over the phenylephrine contraction was observed. The values of maximum responses to the NK_1-agonists in endothelium-intact preparations showed large interindividual variability in pulmonary arteries (range, 6–69%; n = 53) and veins (range, 6–82%; n = 40) and were not correlated to the responses induced by acetylcholine (data not shown).

Since the neurokinin agonists did not induce contraction of pulmonary vessels, the U-shaped concentration–response curves obtained after cumulative addition of SP and [$Sar^9Met(O_2)^{11}$]SP in some preparations (arterial, 46%; venous, 39%) suggested a rapid desensitization of NK_1 agonist-induced relaxations. In these preparations, SP and [$Sar^9Met(O_2)^{11}$]SP (10^{-10}–10^{-9} *M*) induced an initial phase of concentration-dependent relaxation, and for higher concentrations, the relaxation became transient; the addition of a consecutive incremental dose of agonists did not prevent the preparations to recontract progressively. This desensitization was confirmed in arterial and venous preparations by the much weaker response to a second cumulative addition of [$Sar^9Met(O_2)^{11}$]SP (Fig. 2) performed after an extensive washing of the preparation with Krebs solution, a reequilibration period of 45 minutes with bath fluid changes every 15 minutes, and after recontraction with phenylephrine. To better characterize this desensitization to [$Sar^9Met(O_2)^{11}$]SP-induced relaxation, the initial response to a maximal concentration (1 μ*M*) of [$Sar^9Met(O_2)^{11}$]SP was compared, in arterial and venous preparations, with the response to a second addition of the peptide performed after a washing of the preparations and a 2-hour reequilibration period. Similar to the results obtained with the two consecutive cumulative additions of [$Sar^9Met(O_2)^{11}$]SP, the second relaxations in response to the single concentration of the NK_1 agonist were much weaker than the first responses (Table 2). In contrast, the responses to a first and a second application of ACh (100 μ*M*), performed in the same experimental conditions, were similar, attesting that the endothelial-mediated pathways of relaxation were not altered (Table 2). In pulmonary artery and vein, the relaxation to [$Sar^9Met(O_2)^{11}$]SP (1 μ*M*) was markedly inhibited in the presence of the selective and potent NK_1 receptor antagonist SR 140333 (0.1 μ*M*), whereas the relaxations to ACh were not altered (Table 2).

Addition of L-NOARG (100 μ*M*) or indomethacin (10 μ*M*) significantly inhibited the [$Sar^9Met(O_2)^{11}$]SP concentration-dependent relaxation curves in pulmonary arteries and veins (Fig. 3, Table 3). The effect of L-NOARG and indomethacin were additive such that the combination of the two compounds nearly abolished the [$Sar^9Met(O_2)^{11}$]SP-induced relaxations in both vascular tissues (n = 2–3; Fig. 3). In human pulmonary artery, the same concentrations of L-NOARG or indomethacin significantly reduced the relaxation induced by ACh (Fig. 4, Table 4). In pulmonary veins, nitric oxide synthase inhibition also significantly attenuated the response to ACh. Qualitatively similar effects of cyclooxygenase inhibition were obtained against responses to ACh, although this did not reach statistical significance (Table 4).

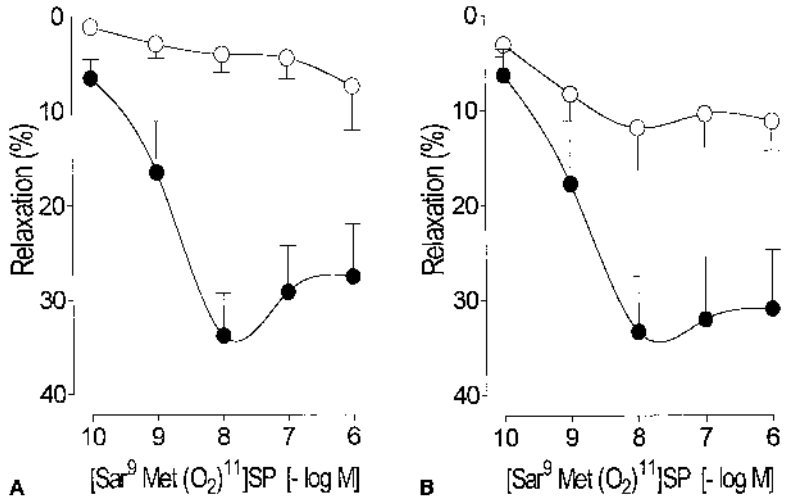

FIG. 2. Concentration–response curves for [Sar^{9}Met(O$_2$)^{11}]SP in causing relaxation of human pulmonary arteries (**A**) or human pulmonary veins (**B**) with intact endothelium. The initial curve (●) and the second curve (○) subsequent to a 45-minute incubation with Krebs solution and recontraction with phenylephrine are presented. The relaxation is expressed as a percentage of the precontraction induced by phenylephrine (30 μ*M*). Values are expressed as means, and vertical lines show SEM for 8 to 10 experiments.

Immunohistochemical Studies

In five different preparations of pulmonary arteries and veins, positive immunostaining for NK_1 receptors was only found in endothelium (Fig. 5). No immunostaining for NK_2 receptors was found in endothelium or in smooth muscle layer of pulmonary arteries and veins.

RT-PCR Studies

Polymerase chain reaction amplification of equal amounts of cDNA from human pulmonary arteries and veins, as determined from the amplification of the β-actin sequence, revealed the presence of a single transcript, corresponding to the expected product size encoding the tachykinin NK_1 receptor (231 bp; Fig. 6). The identity of the amplified fragment was confirmed by DNA sequence analysis. The level of NK_1 receptor mRNA was very low (n = 3 arteries and 4 veins) or undetectable (n = 4 arteries and 3 veins). Moreover, a correlation was observed between the relative level of NK_1 receptor mRNA and the magnitude of the functional response to [Sar^{9}Met(O$_2$)^{11}]SP. Hence, relative higher NK_1 receptor mRNA levels were observed in those tissues in which the selective NK_1 agonist elicited the higher relaxant response (25–35%). In the same way, NK_1 receptor mRNA was undetectable in those

TABLE 2. Relaxations induced by [Sar^{9}Met(O$_2$)^{11}]SP and acetylcholine

	Pulmonary arteries			Pulmonary veins		
Treatment	**n/p**	**[Sar^{9}Met(O$_2$)^{11}]**	**Ach**	**n/p**	**[Sar^{9}Met(O$_2$)^{11}]**	**Ach**
Tachyphylaxis						
First addition	4/2	55 ± 13	82 ± 12	4/2	46 ± 6	69 ± 9
Second addition	4/2	2 ± 1*	87 ± 4	4/2	17 ± 5*	75 ± 4
NK_1 receptor blockade						
Control	6/3	36 ± 8	83 ± 5	6/3	28 ± 6	73 ± 4
SR140633 (0.1 μ*M*)	6/3	4 ± 1*	79 ± 16	6/3	6 ± 3*	76 ± 5

Values shows relaxations induced bySar^{9}Met(O$_2$)^{11}]SP (1μ*M*) and by acetylcholine (Ach, 100 μ*M*) in human pulmonary arteries and veins precontracted with phenylephrine. Relaxations are expressed as percentage of precontraction induced by phenylephrine (30 μ*M*).

Data shown as mean ± SEM of n/p (number of preparations/number of patients) experiments.

Comparisons were performed using the Student *t* test. *$P < 0.01$ for control values.

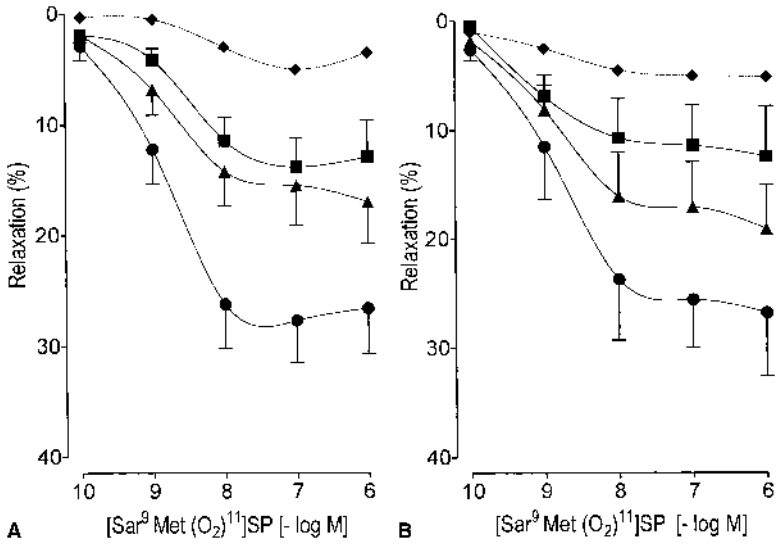

FIG. 3. Concentration–response curves for [Sar^{9}Met(O$_2$)^{11}]SP in causing relaxation of human pulmonary arteries (**A**) or human pulmonary veins (**B**) precontracted with phenylephrine: control (●), in the presence of indomethacin (▲, 10 μ*M*), L-NOARG (■, 100 μ*M*), or a combination of both inhibitors at these concentrations (◆). The relaxation is expressed as a percentage of the precontraction induced by phenylephrine (30 μ*M*). Values are expressed as means, and vertical lines show SEM for the number of experiments shown in Table 3. For the combination of inhibitors, values are only expressed as mean of two or three experiments.

tissues in which [Sar^{9}Met(O$_2$)^{11}]SP induced a small relaxant response (6–15%). However, the NK$_1$ receptor mRNA transcript could be observed in all tissues assayed after amplification of cDNA for 43 cycles. Conversely, the mRNA encoding the product expected for the NK$_2$ receptor (375 bp) or the NK$_3$ receptor (364 bp) was undetectable, even after amplification of a high amount of cDNA for 43 cycles. As a positive control, tachykinin receptors mRNA expression was also studied in human bronchi and rat uteri. NK$_1$ receptor and NK$_2$ receptor mRNA, but not the NK$_3$ receptor transcript, could be observed in human bronchi (n = 4) assayed after amplification of cDNA for 36 cycles (data not shown). The primers for the NK$_3$ receptor were designed to amplify a region common to the rat and human receptor and permitted to amplify the NK$_3$ receptor transcript in the rat uterus (data not shown).

No PCR product was detectable when the samples were amplified without the RT step, suggesting that geno-

TABLE 3. E_{max} and pD_2 calculated from concentration-response curves obtained after cumulative addition of [Sar^{9}Met(O$_2$)^{11}]SP

	Pulmonary arteries			Pulmonary veins		
Treatment	**n/p**	**E_{max} (%)**	**pD_2**	**n/p**	**E_{max} (%)**	**pD_2**
Control	17/14	28 ± 4	8.8 ± 0.2	14/10	27 ± 6	8.7 ± 0.3
L-NOARG	15/12*	15 ± 4†	8.4 ± 0.1†	11/10*	12 ± 3†	8.3 ± 0.2
Indomethacin	12/8*	18 ± 6	8.5 ± 0.2	13/10*	19 ± 5	8.4 ± 0.3

Values calculated after cumulative addition [Sar^{9}Met(O$_2$)^{11}]SP in human endothelial intact pulmonary arteries and veins precontracted with phenylephrine in the absence (control) or presence of the NO-synthase inhibitor (L-NOARG, 100 μ*M*) or of the cyclo-oxygenase inhibitor (indomethacin, 10 μ*M*).

The E_{max} is expressed as percentage of precontraction induced by phenylephrine (30 μ*M*); pD_2 values are negative $\log_{10}$ of the concentration required to achieve half maximum relaxation.

Data shown as mean ± SEM of n/p (number of preparations/number of patients) experiments. For each preparation, comparisons versus control were performed using ANOVA for repeated measures (*$P < 0.05$) or the Student *t* test (†$P < 0.05$).

E_{max}, maximal relaxation; pD_2, potency.

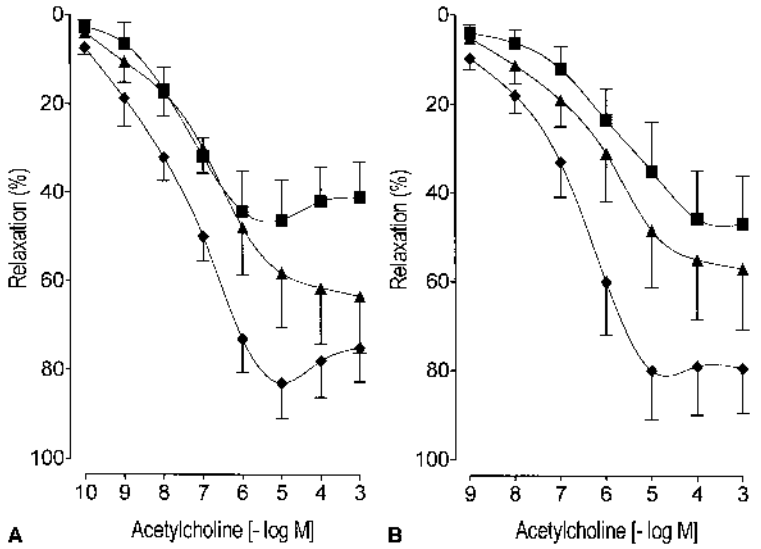

FIG. 4. Concentration–response curves for acetylcholine in causing relaxation of human pulmonary arteries (**A**) or human pulmonary veins (**B**) precontracted with phenylephrine: control (●), in the presence of indomethacin (▲, 10 μ*M*), and in the presence of L-NOARG (■, 100 μ*M*). The relaxation is expressed as a percentage of the precontraction induced by phenylephrine (30 μ*M*). Values are expressed as means, and vertical lines show SEM for the number of experiments shown in Table 4.

mic DNA contamination was eliminated by DNase treatment. Similarly, no products were detected when the RT-PCR steps were carried out with no added RNA, indicating that all reagents were free of target sequence contamination.

DISCUSSION

The present study demonstrates that SP and a selective NK_1-receptor agonist induce endothelium-dependent relaxations in human pulmonary arteries and veins. These results complete previous findings on the relaxant effects of SP on human pulmonary artery (5) and extend these observations to the human pulmonary veins, on which the effects of neurokinins had not been previously reported. The immunochemical and RT-PCR studies indicate for the first time that NK_1 receptor is the only neurokinin receptor subtype expressed in the proximal intralobar pulmonary vessels. The relaxations induced by SP and

TABLE 4. E_{max} and pD_2 calculated from concentration-response curves obtained after cumulative addition of acetylcholine in human endothelial intact pulmonary arteries and veins

	Pulmonary arteries			Pulmonary veins		
Treatment	**n/p**	**E_{max} (%)**	**pD_2**	**n/p**	**E_{max} (%)**	**pD_2**
Control	16/11	86 ± 8	7.4 ± 0.3	12/9	83 ± 9	6.4 ± 0.2
L-NOARG	8/6*	46 ± 8†	7.5 ± 0.4	8/7*	51 ± 10†	6.0 ± 0.2
Indomethacin	13/9*	63 ± 9	6.8 ± 0.3†	11/8	58 ± 8	5.9 ± 0.2

E_{max} and pD_2 values calculated from concentration-response curves obtained after cumulative addition of acetylcholine in human endothelial intact pulmonary arteries and veins precontracted with phenylephrine in the absence (control) or presence of the NO-synthase inhibitor (L-NOARG, 100 μ*M*) or of the cyclo-oxygenase inhibitor (indomethacin, 10 μ*M*).

E_{max} is expressed as percentage of precontraction induced by phenylephrine (30 μ*M*); pD_2 values are negative $\log_{10}$ of the concentration required to achieve half maximum relaxation. Data shown as mean ± SEM of n/p (number of preparations/number of patients) experiments.

For each preparation, comparisons versus control were performed using ANOVA for repeated measures (*$P < 0.05$) and the Student *t* test (†$P < 0.05$).

E_{max}, maximal relaxation; pD_2, potency.

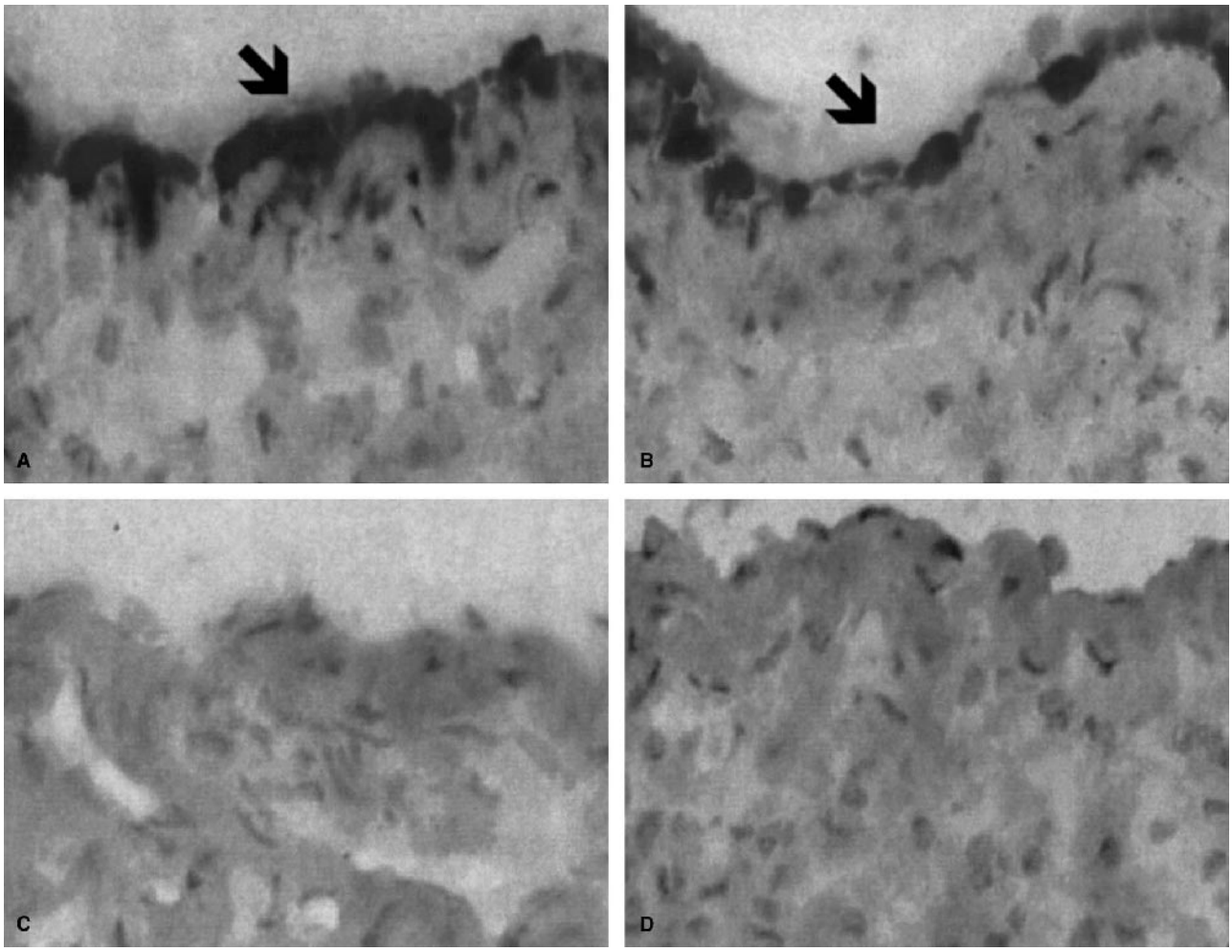

FIG. 5. Microphotographs showing NK_1 receptor immunostaining in the endothelium layer of human pulmonary artery (**A**) and human pulmonary vein (**B**). Negative controls (**C** and **D**) were performed by omission of the anti-NK_1-receptor antibody. Images of microscopic fields were at a magnification of $\times 250$.

$[Sar^9Met(O_2)^{11}]$SP were similar in pulmonary arteries and veins. The mean maximum response to SP in pulmonary arteries appears weaker than the mean responses reported previously in the same preparation (4,5). This apparent difference may be explained by the use of pulmonary vessels from smokers with lung carcinoma (present study) instead of pulmonary arteries from healthy and younger organ donors (4,5). In this regard, it is interesting to note that endothelial dysfunction of pulmonary arteries has been reported in smokers with or without mild chronic obstructive pulmonary disease (13,23) and that impairment of endothelium-dependent arterial dilatation of systemic arteries has been shown in smokers (24). In contrast, NK_1-receptor mRNA expression is significantly increased in smokers (25), and expression of neurokinin receptors in human airways does not appear to be modified by cigarette smoking (17).

The endogenous neurokinins and the selective neurokinin receptor agonists did not induce contraction of human pulmonary vessels in presence or absence of endothelium. This result contrasts with the endothelium-dependent contraction mediated via NK_1 receptors and the endothelium-independent contractions mediated via NK_2 receptors in rabbit pulmonary artery (6,7) and indicates that the endogenous neurokinins are not involved in pulmonary vasoconstriction in humans.

In the current study, we also show that the relaxation induced by the NK_1 agonist was characterized by the rapid development of tachyphylaxis in pulmonary arteries and veins. The development of tachyphylaxis to the relaxation induced by SP has been previously reported in human coronary artery (26) and in guinea pig pulmonary artery (27). Furthermore, a rapid development of tolerance to SP infusion-induced venodilation in hand veins has been de-

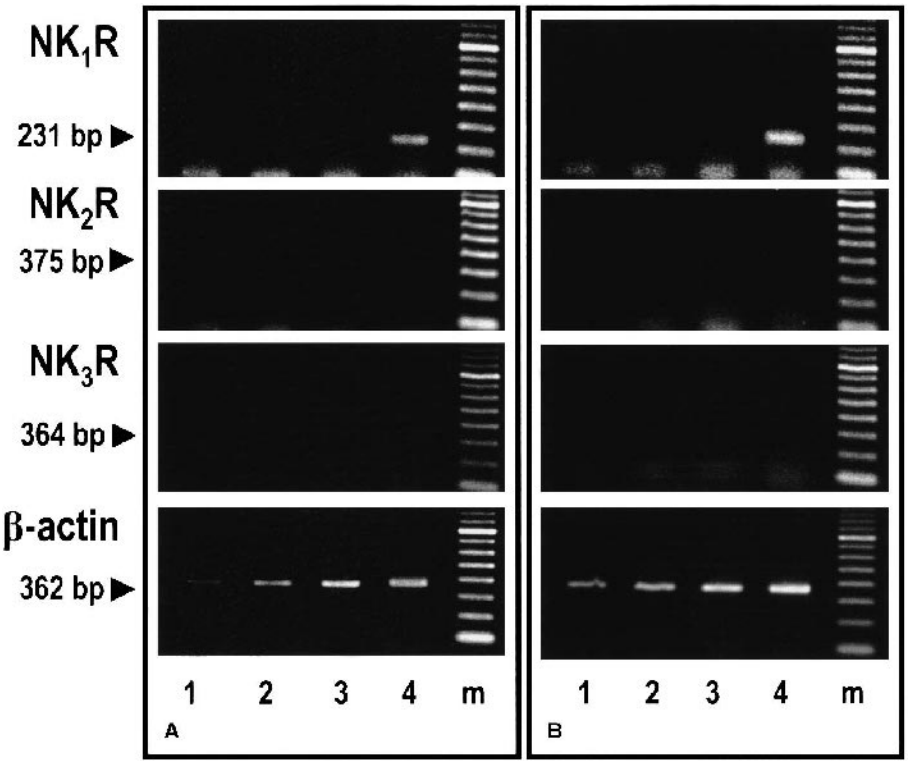

FIG. 6. Agarose gel showing products of reverse transcriptase-polymerase chain reaction (RT-PCR) assay for cDNA from (**A**) human pulmonary artery and (**B**) human pulmonary vein. After normalization to β-actin mRNA levels, serial half dilutions of cDNA were amplified for 25 (β-actin) or 36 cycles (tachykinin receptors) with specific oligonucleotide primers. A single and low abundant transcript corresponding to the size predicted for the NK_1 receptor (231 bp) was detected in approximately one half of the tissues assayed. The specific bands corresponding to tachykinin NK_2 and NK_3 receptors were undetectable. m = molecular size standards. Data are representative of typical results in different samples of pulmonary arteries (n = 7) and veins (n = 7).

scribed in seven of eight healthy volunteers (28). In contrast, the relaxation induced by two consecutive cumulative additions of SP did not differ in preparations of human omental arteries and veins (29) and human mesenteric arteries and veins (30), suggesting that desensitization of NK_1 agonist-induced response varies with the vessel type. The mechanisms of NK_1 receptor desensitization involve a SP-induced rapid (< 1 minute) and dose-dependent phosphorylation of serine and threonine residues by intracellular kinases (31). A common functional outcome of agonist-induced desensitization is a reduced coupling of the receptor to the signaling system and a potential sequestration of receptor in the cytoplasm. SP (10^{-10}–10^{-8} *M*) has been shown to induce rapid endocytosis (< 5 minutes) of the NK_1 receptor on enteric neurons of the guinea pig ileum. At 10^{-7} *M* SP, all the NK_1 receptors were in the cytoplasm, and none were detectable on the surface of the neurons (32). Recycling of internalized NK_1 receptors to the cell surface may contribute to resensitization of cellular responses to NK_1 agonists. In the enteric neurons, recycling of NK_1 receptors to the cell surface occurred within 30 minutes but required 60 to 240 minutes in transfected rat kidney cells (33). In our preparations of pulmonary vessels, the relaxation in response to $[Sar^9Met(O_2)^{11}]SP$ was not restored until 45 to 120 minutes after the initial exposure to the NK_1 agonist. Further experiments are clearly warranted on human pulmonary vessels to establish the mechanisms of desensitization and the possible occurrence of resensitization of NK_1 receptor-mediated responses. The rapid desensitization of NK_1 agonist-induced relaxations contrasts with the absence of desensitization of histamine-induced relaxations in human pulmonary arteries and veins (34) and of ACh-induced relaxations in human pulmonary arteries and veins (present study). Therefore, the rapid desensitization of NK_1 agonist-induced relaxation should be considered a limiting factor for the potential therapeutic use of SP to reverse the pulmonary endothelial dysfunction caused by congenital heart surgery (35). This desensitization process can also help to explain the impairment of SP-induced vasodilation in patients with primary pulmonary hypertension (36) since pulmonary hypertension is associated with an increase in lung SP levels at least in a rat model (37).

In our experiments, removal of endothelium resulted in suppression of the relaxant response to [$Sar^9Met(O_2)^{11}$]SP. These data demonstrate the endothelium-dependent pathway of the response to NK_1 receptor activation in human pulmonary arteries and veins, and are consistent with the results obtained by Pedersen et al. (5) in human pulmonary arteries. The mechanisms underlying the endothelium-dependent relaxant responses to the NK_1 selective agonist in human pulmonary arteries and veins were NO-synthase and cyclooxygenase pathway dependent since each inhibitor alone (L-NOARG or indomethacin) merely attenuated the relaxation, whereas the combination of both inhibitors was able to practically abolish the [$Sar^9Met(O_2)^{11}$]SP-induced relaxation in pulmonary arteries and veins. These results are in agreement with a recent study conducted in human pulmonary arteries with SP and Ac-[Arg^6,Sar^9,$Met(O_2)^{11}$]SP (6–11), a NK_1 receptor selective agonist (5). The observations that, in pulmonary arteries [(5) and present study] and veins (present study), it is a combination of the activities of nitric oxide and cyclooxygenase that predominantly mediates the endothelium-dependent vascular relaxation evoked by NK_1-receptor stimulation contrast sharply with that found in other blood vessels, and serve to underline the heterogenous nature of the vascular endothelium. In human penile veins, the endothelium-dependent dilatation in response to SP is abolished in presence of the inhibitor of nitric oxide synthase L-NAME (38). In human omental arteries and veins, NO-synthase inhibition markedly diminishes the SP-induced relaxation, whereas cyclooxygenase inhibition did not affect relaxation (29). These results contrast with the findings in the human umbilical artery, where prostanoids, but not NO, mediate the SP-induced relaxation (39).

To further characterize the NK_1 agonist-mediated relaxation of human pulmonary vessels, we have also examined the effects of cyclooxygenase and NO-synthase inhibition on ACh-induced relaxation in our preparations of pulmonary arteries and veins. As previously reported, ACh produces endothelium-dependent relaxation in human pulmonary artery (10,12–14) and veins (10). The mechanisms underlying the relaxant response to ACh in human pulmonary artery have been previously described to be cyclooxygenase and NO-synthase pathway dependent (12,14,15). As shown in the current study for the NK_1-receptor selective agonist, each inhibitor alone (indomethacin or L-NOARG) had only small effects in comparison with the marked inhibition obtained with the combination of both inhibitors on the ACh-induced relaxation (12,14, 15). In most studies, cyclooxygenase inhibition alone had little or no effect on ACh-induced relaxation (14,15), whereas NO-synthase inhibition has been shown to partly inhibit (14,15) or almost abolish ACh-induced relaxation (13). Our results in the human pulmonary artery are consistent with the findings of Norel et al. (12) because each inhibitor alone significantly attenuated the ACh-induced relaxation. The effects of NO-synthase inhibition or cyclooxygenase inhibition on the relaxation induced by ACh in human pulmonary vein had not been previously reported. In the current study, each inhibitor alone partly attenuated relaxations to ACh in human pulmonary vein, and the magnitude of the inhibitory effects was similar to those observed in human pulmonary artery. Taken together, the aforementioned observations indicate that the relaxations induced by NK_1 agonists or by ACh are cyclooxygenase and NO-synthase pathway dependent in human pulmonary vessels.

The involvement of endothelial NK_1 receptor in the relaxation induced by SP and [$Sar^9Met(O_2)^{11}$]SP is supported by the marked inhibition of the relaxation in presence of the selective NK_1-receptor antagonist SR140333 in pulmonary arteries and veins. This finding completes the results obtained in human pulmonary arteries with the same antagonist (5) or with other NK_1-receptor antagonists (4,5) and extends these observations to the pulmonary veins. In addition, immunostaining with either a specific NK_1-receptor antibody or a specific NK_2-receptor antibody clearly shows the restricted presence of NK_1 receptors on the endothelium in pulmonary artery and vein. This result fully agrees with the functional experiments in pulmonary artery [(5), present study) and vein (present study) since 1) the relaxation induced by NK_1-receptor selective agonists was abolished after endothelium removal; 2) the NK_2-receptor selective agonists did not induce relaxation in endothelium-intact preparations; and 3) the NK_1- and NK_2-receptor selective agonists did not induce contractions either on preparations at basal tone or on those preconstricted with phenylephrine in presence or absence of endothelium.

The presence of mRNA transcripts specific for NK_1 receptors was detected in all vessels after amplification of cDNA for 43 cycles, but only in some of these vessels after 36 cycles. This result is consistent with the restricted presence of the transcript in the endothelium and also suggests interindividual variation in the level of mRNA expression for NK_1 receptors. This interindividual variation appears to be associated with functional differences in terms of maximal relaxation in response to NK_1 agonist since the greater relaxation was observed in the vessels for which the mRNA transcripts specific for NK_1 receptors were detected after amplification of cDNA for 36 cycles instead of 43 cycles. No mRNA transcripts specific for NK_2 receptors were detected in all vessels, a result consistent with the absence of immunostaining with the specific NK_2-receptor antibody. In addition, no mRNA transcripts specific for NK_3 receptors were detected in all vessels. Our failure to detect mRNA transcripts for NK_2 and NK_3 receptors is

unlikely to have been due to a technical problem since these two transcripts have been detected with this technique and the same specific primer pairs in the human bronchus and the rat uterus (18), respectively (data not shown). Moreover, the absence of detection of mRNA transcripts for NK_3 receptors is in line with the absence of functional response to NK_3-receptor selective agonists in the pulmonary artery [(5), present study) and vein (present study). The immunochemical and RT-PCR studies indicate for the first time that NK_1 receptor is the only neurokinin receptor subtype expressed in the proximal intralobar pulmonary vessels.

In conclusion, NK_1 receptors are located on the endothelium in human intralobar pulmonary arteries and veins, mediate relaxation through a combination of cyclooxygenase and nitric oxide activities, and are subjected to rapid tachyphylaxis. ACh also induced an endothelium-dependent relaxation through the cyclooxygenase and NO-synthase pathway in both pulmonary vessels. ACh was less potent but more effective than SP at relaxing the human pulmonary artery and vein, and the relaxation induced by ACh was not subjected to rapid tachyphylaxis.

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