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PRECLINICAL RESEARCH

Characterization of Beta$_3$-Adrenoceptors in Human Internal Mammary Artery and Putative Involvement in Coronary Artery Bypass Management

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OBJECTIVES The aim of the present study was to analyze whether beta$_3$-adrenoceptors (β_3-ARs) were effectively present and functional in the human internal mammary artery (IMA).

BACKGROUND The beta$_1$- and beta$_2$-adrenoceptors classically mediate the relaxant effects of catecholamines in the vessels. In vitro and in vivo studies performed in various animal species described vasodilating effects due to activation of a third beta-ARs subtype (β_3).

METHODS Reverse transcription-polymerase chain reaction analysis, Western blot experiments, and pharmacological studies were carried out in human IMA samples harvested from 27 patients undergoing coronary bypass surgery.

RESULTS The β_3-ARs messenger ribonucleic acid and protein were detected in intact IMA, but were absent in endothelium-free samples. This finding was confirmed by immunohistochemical experiments. In organ baths, a β_3-AR agonist, SR 58611A, induced an endothelium-dependent relaxation of phenylephrine-precontracted IMA rings. This vasodilation was not modified by β_1/β_2-AR antagonists, but was greatly altered in the presence of L-748,337, a selective human β_3-AR antagonist. Moreover, the inhibition of nitric oxide (NO) synthases abolished the β_3-adrenergic vasodilation, suggesting the involvement of a NO-signaling pathway.

CONCLUSIONS Those results demonstrated the presence of β_3-ARs in the endothelial layer of human IMA. The present work highlights the role of β_3-ARs in vasomotor control of IMA and opens new fields of investigation in coronary bypass graft management, heart failure, and hypertension. (J Am Coll Cardiol 2005;46:351–9) © 2005 by the American College of Cardiology Foundation

The sympathetic nervous system is the central network of the neurohumoral regulation of vascular tone. Stimulation of beta-adrenoceptors (β-ARs) leads to a relaxation of the vascular smooth muscle, thereby controlling the blood flow distribution in different organs. Since the classification established by Lands et al. (1), β-ARs have been pharmacologically classified into β_1 and β_2-AR subtypes. Although both β_1-and β_2-ARs are involved in vasodilation, the role of each subtype varies depending on species and vascular beds (2). In cerebral and coronary arteries, β_1-ARs predominate in contrast to the mesenteric, pulmonary, and muscular vessels, where β_2-ARs largely predominate (2). The role of endothelium in the vasodilating effect of β-AR agonists also varies with the species and the vascular bed. Thus, in humans, isoprenaline, a nonspecific β-AR agonist, induced a vasodilation in umbilical vein by stimulating endothelial β_2-ARs (3), whereas the relaxation of internal mammary artery (IMA) and saphenous vein was shown to be mediated via β_2-AR located on the smooth muscle (4).

During the 1980s, the classification of β-ARs was challenged because of atypical effect of β-AR agonists in some tissues such as heart, fat, and digestive tract. The receptor involved in non-β_1- and/or non-β_2-AR effects was first described as atypical β-AR. Later on, a new β-AR subtype, β_3, was cloned (5) and a low-affinity site for the β_1-AR identified (6). The participation of a third β-AR in vasorelaxation was suggested by in vitro and in vivo studies. Isoprenaline induced a vasodilation resistant to propranolol (a nonspecific β-AR antagonist) in rat carotid artery (7), aorta (8), pulmonary vessels (9), and in canine pulmonary artery (10). The use of preferential β_3-AR agonists confirmed these findings (7,8,11). Recently, an in vitro study in human coronary microarteries reported that a preferential β_3-AR agonist produced a vasodilation involving both nitric oxide (NO) and endothelial-derived hyperpolarizing factor pathway (12). In vivo studies have also suggested the presence of functional β_3-ARs in vessels, but their role was highly species-dependent. Thus, in conscious dogs, β_3-AR

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Abbreviations and Acronyms

Ach	= acetylcholine
CABG	= coronary artery bypass graft
IMA	= internal mammary artery
LIPE	= hormone-sensitive lipase
L-NMMA	= NG-monomethyl-L-arginine monoacetate
NO	= nitric oxide
PE	= phenylephrine
RT-PCR	= reverse transcription-polymerase chain reaction
α-AR	= alpha-adrenoceptor
β-AR	= beta-adrenoceptor

agonists induced a peripheral vasodilation, primarily in skin and adipose tissues (13,14); β_3-AR stimulation was shown to be less important in rats than in dogs, and essentially absent in nonhuman primates (15). In humans, the β_3-AR played a weaker role in the control of blood flow in adipose tissue than β_1- and β_2-AR (16). There is also a great variability of the signaling pathways according to the species and the vascular bed studied. A cAMP-dependent pathway seemed to be involved in the β_3-adrenergic effect in canine pulmonary artery (10) and rat portal vein myocytes (17). In rat thoracic aorta, the stimulation of endothelial β_3-ARs activated an endothelial NO synthase leading to an increase of intracellular cGMP (11) and an activation of several potassium channels (BK_{Ca}, K_{ATP}, K_v) of the vascular smooth muscle cells (18).

The IMA is the preferred bypass graft for myocardial revascularization owing to superior graft patency and increased long-term survival compared with saphenous vein grafts (19,20). However, the IMA has a greater tendency to spasm leading to a perioperative increase in morbidity and mortality. The vasoconstriction can be evoked by several stimuli such as mechanical trauma, nerve stimulation, and vasoconstrictor substances. Circulating sympathomimetic substances are considered as possible spasmogenic agents (21). The knowledge of the IMA pharmacology and physiology is, therefore, essential to treat and to prevent the vasospasm. In this regard, several projects have been carried out on the IMA, in order to analyze alpha-adrenoceptors (α-AR) physiology (subtypes, function, regulation by age) (22,23). By contrast, although β-blockers are widely used in the treatment of ischemic cardiopathy, only few works evaluated the presence and the possible role of the different β-AR subtypes. Molenaar et al. (4) reported the predominant role of β_2-ARs in the isoproterenol induced-vasodilation in the IMA. Consequently, the aim of the present study was to characterize by molecular, biochemical, and pharmacological approaches the presence of β_3-AR in the human IMA. Then we discuss possible functional roles of β_3-ARs, effectively present in endothelial layer of the IMA, in physiological and/or pathophysiological conditions. Finally, clinical implications of our study are pointed out.

METHODS

Tissue origin. Human IMA samples were harvested from 27 patients undergoing coronary bypass surgery. There were 25 men and 2 women with an age of 68.2 ± 7.5 years, body weight 75.7 ± 10.3 kg (mean ± SD). All suffered from coronaropathy (New York Heart Association functional class [angina pectoris] II to III) but without apparent heart failure. Their clinical characteristics are detailed in Table 1. The discarded distal IMA segments proximal to the bifurcation were collected during the operation and immediately placed in Tyrode solution composed as follows (mM): NaCl, 130; KCl, 5.6; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 11; HEPES, 8; pH was adjusted to 7.4. The samples were transferred to the laboratory within 4 h.

RNA preparations. Total RNAs were extracted from human IMA without adventice by a modification of the acid guanidium-thiocyanate-phenol-chloroform method, as previously described by Chomczynski and Sacchi (24). Briefly, the vessels were homogenized with an ultra-turrax homogenizer in Trizol solution (Gibco BRL, Villiers-Le-Bel, France). After centrifugation and precipitation by addition of isopropanol, the RNA pellets were washed with 75% ethanol and resuspended in RNAse-free water. The concentrations were determined spectrophotometrically at 260 nm, and the integrity of the RNA was verified on 0.8% Tris-acetate EDTA agarose gels.

Reverse transcription-polymerase chain reaction (RT-PCR) experiments. The oligonucleotides were purchased from Genosis (Cambridgeshire, United Kingdom). Sequences of the different primers are reported in Table 2. First-strand synthesis of cDNA from total RNAs was carried out using moloney murine leukemia virus reverse transcriptase (Gibco BRL) with oligo(dT) (Sigma, L'Isle d'Abeau Chesnes, France). The single strand cDNAs were subsequently amplified by polymerase chain reaction using Taq DNA polymerase (Pharmacia, Saclay, France). Each polymerase chain reaction experiment included a negative control consisting of a reverse transcriptase reaction containing no added enzyme and positive controls correspond-

Table 1. Clinical Characteristics of Patients

Age (yrs)	68.2 ± 7.5
Gender ratio (M/F)	(25/2)
Body weight (kg)	75.7 ± 10.3
Associated pathologies	
Aortic valvular stenosis	2
Mitral valvular insufficiency	2
Hypercholesterolemia	16
Type II diabetes mellitus	9
Systemic hypertension	7
Drug regimens	
Beta-blockers	21
Calcium-blockers	11
Nitrates	13
ACE inhibitors	9
Diuretics	8
Statins	13

ACE = angiotensin-converting enzyme.

Table 2. Oligonucleotides Used as Primers for PCR Reaction

Name	Length	Strand	Sequence	Number of Cycles	Amplification Size
α_{1A}-AR-S	23	Forward	5′ TGATTTCAAGCCCTCTGAAACAG 3′	40	224 pb
α_{1A}-AR-AS	23	Reverse	5′ ATGTCCTTGTGTTGCCCTTCCAC 3′		
β_1-AR-S	17	Forward	5′ GCTGCAGACGCTCACCA 3′	40	212 pb
β_1-AR-AS	17	Reverse	5′ GCGAGGTAGCGGTCCAG 3′		
β_2-AR-S	20	Forward	5′ CACAGCCATTGCCAAGTTCG 3′	40	287 pb
β_2-AR-AS	21	Reverse	5′ CGGGCCTTATTCTTGGTCAGC 3′		
β_3-AR-S	17	Forward	5′ ACCTGGCTGTGACCAAC 3′	40	122 pb
β_3-AR-AS	20	Reverse	5′ ACTGGCTCATGATGGGCGC 3′		
LIPE-S	17	Forward	5′ GCTCAACTCCTTCCTGG 3′	40	459 pb
LIPE-AS	19	Reverse	5′ AAGGGGTTCTTGACTATGG 3′		

PCR = polymerase chain reaction.

ing to genomic DNA or fat cDNA. After initial heating of samples at 94°C for 5 min, each cycle of amplification consisted of 1 min at 92°C, 1 min at annealing temperature appropriate for the primers used, and 1 min at 72°C. Annealing temperatures were 56°C for all primers. After 40 cycles of amplification, the polymerase chain reaction products were separated through a 2% TAE agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Western blotting experiments. The expression of the β_3-AR was examined with a monoclonal antibody raised against the human isoform of the receptor (25). Human IMAs were cut in rings. For some rings, the endothelium was removed by gently rubbing the intimal surface with a fine pair of small forceps. Protein fractions (100 μg) with or without endothelium were denatured with 5% β-mercaptoethanol in Laemmli's sample buffer and electrophoresed on 10% polyacrylamide/SDS gels and transferred onto a hybond C super membrane (Amersham, Saclay, France) using an electroblotting apparatus (Biorad, Marnes La Coquette, France). The protein amount was checked by staining with Coomassie. Nonspecific binding was blocked by incubating membranes in nonfat dry milk 5% in TBS. Then they were incubated in milk with 1% TBS alone or with the primary antiserum, washed in TBST (TBS containing 0.1% Tween 20), and hybridized with the secondary antibody anti-IgG peroxydase conjugate (Sigma). The membranes were washed with TBS, and antibody complexes were revealed by the enhanced chemiluminescence detection procedure (Amersham).

Immunohistochemistry analysis. Fixation and tissue processing protocols were performed as described previously (18). After freezing in 2-methyl butane solution (−50°C), 10-μmol/l sections of human IMA were cut on cryostat microtome and stored at −20°C until using. The expression of the β_3-AR was examined with the same antibody used for the Western blotting experiments. The sections were incubated with the primary antibody or with buffer alone as control of specificity for the human β_3-AR antiserum (human β_3-AR Ab), washed, and incubated with the secondary antibody. After the secondary hybridization with an anti-rat IgG peroxidase conjugated developed in rabbit (Sigma), the antibody complexes were revealed for sensitive detection of the enzymatic activity with peroxydase substrate kit AEC (SK4200, Vector, AbCys SA, Paris, France).

Pharmacological studies. The vessels were carefully cleared of fat and connective tissue and cut into 4-mm-long rings. In some rings, the endothelium was removed by gentle rubbing of the intimal surface with a fine pair of small forceps. They were suspended on stainless steel wires in a 10-ml organ bath containing Krebs solution composed as follows (mmol/l): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; EDTA (ethylenediaminetetraacetic acid), 0.016; glucose, 11.1; and $CaCl_2$, 2.5; pH 7.4. Bath temperature was maintained at 37°C, and the Krebs solution was continuously oxygenated with a 95% O_2, 5% CO_2 gas mixture. Isometric tension was recorded by a force displacement transducer (IT2, EMKA Technologies, Paris, France) and displayed on a computer (IOX software, EMKA Technologies). Data were analyzed using Datanalyst software (EMKA Technologies). After a 30-min equilibration period, rings were progressively stretched to a resting tension of 2.5 g (this resting tension was determined in preliminary studies to obtain steady and reproducible contractions). The rings were then challenged twice with KCl (90 mmol/l) with a 15-min interval to ensure that responses were reproducible. After a further 30-min equilibration period, rings were precontracted to 2.5 g at least with 1 μmol/l phenylephrine (PE), and functional endothelium was checked by the presence of at least 50% relaxation with 1 μmol/l acetylcholine (Ach). In denuded vascular rings, endothelium removal was confirmed by the absence of Ach-induced relaxation. Arterial rings were contracted again with 1 μmol/l PE after another 30-min equilibration period. A cumulative concentration-response curve to SR 58611A (a preferential β_3-AR agonist) was then constructed. In some experiments, rings were equilibrated in Krebs containing 10 μmol/l nadolol (a β_1- and β_2-AR antagonist), 3 μmol/l L-748,337 (a β_3-AR antagonist), or 100 μmol/l N^G-monomethyl-L-arginine monoacetate (L-NMMA; a NO synthase inhibitor), for 30 min before PE application. Control rings were only treated by solvent during this period. Relaxation produced by each

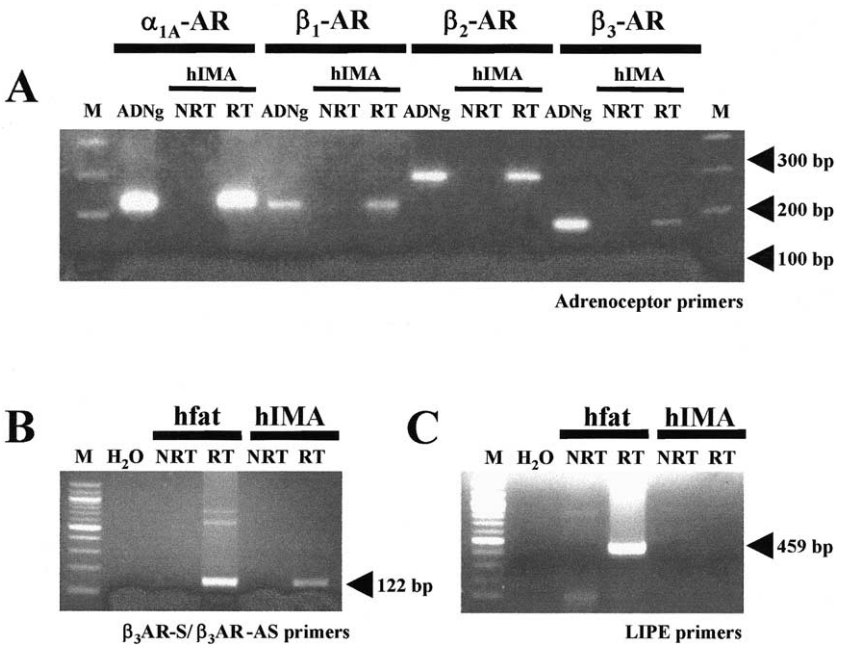

Figure 1. Detection by reverse transcription (RT) polymerase chain reaction of human α_{1A}-adrenoceptors (α_{1A}-ARs), β_1-adrenoceptors (β_1-AR), β_2-ARs, and β_3-ARs, and hormone-sensitive lipase (LIPE) gene transcripts in human internal mammary artery (IMA) and white adipose tissue. Amplified cDNA fragments were separated on a 2% agarose gel and visualized by staining with ethidium bromide. Sizes of the polymerase chain reaction products are indicated on the **middle of the panels**. This experiment is representative of three investigations performed on three vessels obtained from different patients. ADNg = genomic DNA; AR-AS = reverse strand; AR-S = forward strand; hfat = human fat; M = 100 pb ladder (Biolabs, New England); NRT = none RT.

concentration of the β_3-AR agonist was measured after a steady-state was reached. As SR 58611A induced long-lasting relaxation, spontaneous time-dependent relaxation was taken into account by systematic subtraction of the corresponding spontaneous relaxation obtained in a control vascular ring taken from the same IMA segments. Values were expressed as the percentage change in the maximal tension of the vessel rings after addition of 1 μmol/l PE.

Data and statistical analysis. Results were expressed as the mean ± SEM of n experiments. The resting tension, the contractile response to 1 μmol/l PE, and the Ach-induced relaxation (up to 50%) obtained in the different protocols were compared using a Student t test. The statistical significance of a drug effect was assessed using a one-way analysis of variance (ANOVA) followed by a Dunnett's test. Comparison of the different concentration-response curves was performed by a two-way ANOVA (concentration, treatment) with repeated measures completed when appropriate by a Bonferroni t test. A p value <0.05 was considered statistically significant.

Drugs. L-PE hydrochloride, Ach chloride, nadolol, and L-NMMA were obtained from Sigma. SR 58611A, [(RS)-N-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2 hydroethanamide hydrochloride], and L-748,337, (S)-N-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]amino] ethyl]phenyl]benzenesulfonamide were generous gifts from Sanofi-Synthélabo (Montpellier, France) and Merck (Rahway, New Jersey), respectively. All drugs were prepared as stock solution in distilled water, with the exception of nadolol, which was dissolved in hydrochloric acid before being neutralized to pH 7.4 with NaOH 1N and L-748,337, which was dissolved in dimethylsulfoxide (Sigma). The final concentration of the solvent in the organ bath was less than 0.1% $v.v^{-1}$ and was checked to produce no effect on the tissue response.

RESULTS

Detection of β_3-AR mRNA in IMA. The main purpose of this study was to compare semiquantitatively the amount of the different adrenoceptor subtypes (α_{1A}-, β_1-, β_2-, and β_3-ARs) in the same preparation of first-strand synthesis of cDNA from IMA. The present finding demonstrates that the α_{1A}- and β_2-ARs were strongly expressed in IMA, while β_1- and β_3-ARs were expressed at low level in this vessel (Fig. 1A). Furthermore, the RT-PCR analysis indicated that expected amplified products of 122 base pairs (bp) with the human β_3-AR primer couple could be identified by ethidium bromide staining for the amplified samples of genomic DNA (positive control) and for human IMA RNAs (Figs. 1A and 1B). As expected, no signal was observed when the RNA samples were not reverse transcripted into cDNA and directly used for the different polymerase chain reaction experiments. As this receptor is present in the adipose tissues, hormone-sensitive lipase (LIPE) marker was tested to check a fat contamination in our preparations. The RT-PCR analysis with the LIPE primer couple indicated an expected polymerase chain reaction product of 459 bp for amplified samples of white

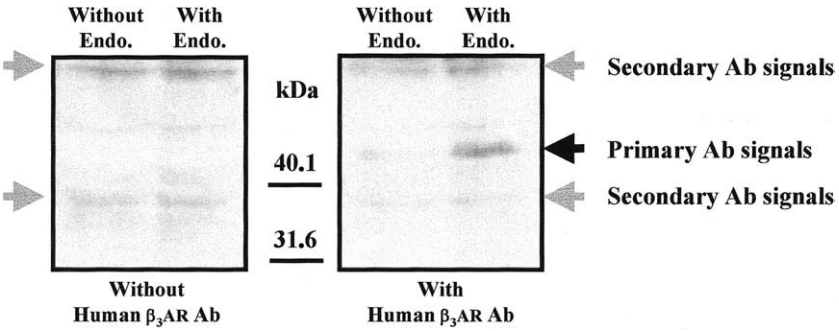

Figure 2. Western blot analysis of human beta$_3$-adrenoceptor (β_3-AR) expression in internal mammary artery with or without endothelium. The membrane was incubated without **(left panel)** or with antihuman β_3-AR antibody (Ab) **(right panel)** and revealed by chemiluminescence detection procedure. This experiment is representative of five investigations performed on five vessels obtained from different patients. With endo. = with endothelium; Without endo. = without endothelium.

adipose tissue, but not for the IMA preparation (Fig. 1C). Under these conditions, the mRNAs encoding for human β_3-AR were still present (Figs. 1A and 1B).

Western blotting experiments. Western blot probed with an antibody directed against human β_3-AR showed a band of roughly 55 kDa with a strong intensity for the total protein fraction of human IMA and at the limit of detection for the sample where the endothelium was removed (Fig. 2). As expected, no reactivity was observed in the absence of the human β_3-AR serum. The protein amount between the different conditions, with or without endothelium, could be checked by the staining only due to the secondary antibody peroxydase conjugate complexes that were revealed by the detection procedure for the incubation without the human β_3-AR serum (Fig. 2).

Immunohistochemistry analysis. The serum antibody raised against the human β_3-AR was used for an immunohistochemical analysis. The pattern of human β_3-AR immunoreactivity was compared with the profile obtained without primary antiserum. The human β_3-AR Ab highly stained cells from the endothelial layer of IMA (Figs. 3Bb, 3Dd, and 3Ff), and a light and diffuse signal was only observed in the smooth muscle layer. Furthermore, the incubation without the primary antibody totally abolished the staining observed in the endothelial layer but did not modify the staining obtained in the smooth muscle layer of IMA (Figs. 3Aa, 3Cc, and 3Ee).

Pharmacological study. All IMA groups of the present study presented no significant difference in contractile responses to 1 μmol/l PE and Ach-induced relaxations. SR 58611A (0.1 to 30 μmol/l) induced a concentration-dependent relaxation of IMA rings contracted with 1 μmol/l PE (Fig. 4). The relaxing effect reached its maximal level after 10 to 15 min of exposure to SR 58611A (Fig. 4A). Because of the long-lasting relaxation induced by β_3-AR agonists, the spontaneous time-dependent relaxation had to be evaluated in control rings for each protocol. The maximum spontaneous relaxation was measured to be 14.5 ± 5.7% during SR 58611A experiments (n = 6). After substraction of the spontaneous relaxation, the vasorelaxation obtained in the presence of 30 μmol/l of SR 58611A was weaker (Emax: 35.1 ± 5.9%, n = 6) than the one induced by a submaximal concentration (1 μmol/l) of Ach (Emax: 79.8 ± 2.3%, n = 50) (Fig. 4A). In order to confirm that SR 58611A activated effectively β_3-ARs, additional experiments were performed in the presence of either nadolol, a specific β_1- and β_2-AR antagonist, or L-748,337, a specific human β_3-AR antagonist. The concentration-response curve to SR 58611A was not modified by a 30-min

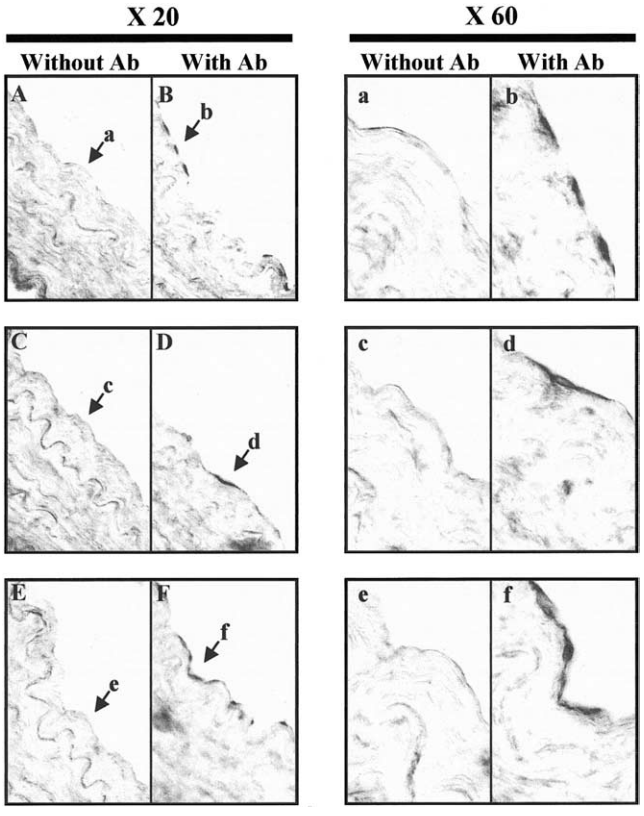

Figure 3. Immunohistochemistry analysis of human beta$_3$-adrenoceptors (hβ_3-AR) expression in internal mammary artery. Adjacent 10-μm thick sections were incubated without **(A, C, E, a, c, and e)** or with anti-hβ_3-AR antibody (Ab) **(B, D, F, b, d, and f)** revealed by peroxydase-conjugated second antiserum. This experiment is representative of three investigations performed on three vessels obtained from different patients. **Black arrowhead** shows interesting areas at low magnitude (micrographs ×20) that were further observed at high magnitude (micrographs ×60).

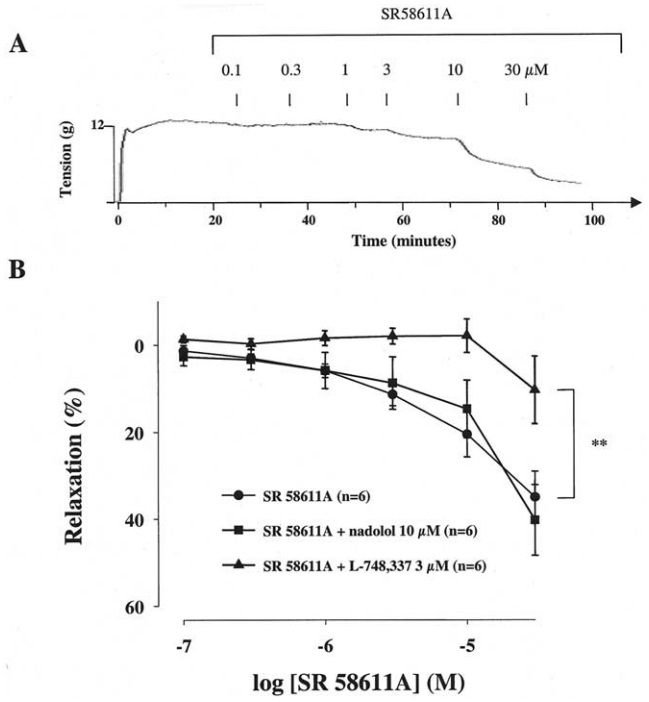

Figure 4. Effect of beta$_3$-adrenoceptor (β_3-AR) agonists in human internal mammary arteries (IMA). **(A)** Typical records of relaxant effect of SR 58611A, a preferential β_3-AR agonist, and acetylcholine (1 μmol/l) in human IMA rings. **(B)** Concentration-response curves to SR 58611A performed in the absence or in the presence of 10 μmol/l nadolol, an β_1 and β_2-AR antagonist, and 3 μmol/l L-748,337, a human β_3-AR antagonist, in human IMA rings constricted with 1 μmol/l phenylephrine. The **mean curves** resulting from subtraction of the spontaneous relaxation of control vessels are shown. Results are expressed as percentage of relaxation from the contraction level induced by phenylephrine. Each point is the mean value of six experiments, and **error bars** represent SEM. $^*p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from response to SR 58611A alone.

pretreatment with 10 μmol/l nadolol (Emax = 40.3 ± 8.1%, n = 6) (Fig. 4B), but was markedly reduced in the presence of 3 μmol/l L-748,337 (two-way ANOVA: $p < 0.001$; Emax = 10.5 ± 7.7%, n = 6; Fig. 4B). The concentration-response of SR 58611A was significantly blunted after endothelium removal (two-way ANOVA: $p < 0.01$; Emax = 6.8 ± 6.3, n = 5, Fig. 5). On the basis of this result, the involvement of the NO pathway in the SR 58611A-induced relaxation was investigated by incubating IMA rings with 100 μmol/l L-NMMA. In such conditions, the relaxant effect of SR 58611A was abolished (two-way ANOVA $p < 0.001$). The Emax value obtained at 30 μmol/l SR 58611A was 4.3 ± 6.9% ($n = 6$; $p < 0.05$ vs. 30 μmol/l SR 58611A alone) (Fig. 5).

DISCUSSION

By complementary approaches of molecular biology, biochemistry, and pharmacology, we have shown the presence of functional endothelial β_3-ARs to be responsible for a vasodilation involving the NO pathway in the human IMA.

The β_3-AR transcripts were clearly detected in human IMA preparations by RT-PCR. Their expression was similar to that of β_1-ARs, but lower than β_2-ARs. As β_3-ARs are also present in adipose tissues, LIPE marker was used to check for fat contamination; LIPE is highly expressed in white adipose tissue and steroidogenic tissues, with lower amounts expressed in cardiac and skeletal muscles, macrophages, and islets of Langerhans (26). In our conditions, no specific products corresponding to LIPE were detected with reverse transcription performed in IMA samples. Thus, these results indicated that the expression of the β_3-AR in IMA was not due to the presence of adipocytes and/or macrophages. In addition, the present work showed that Western blotting pattern of human monoclonal β_3-AR antibody revealed a strong labeling for the total protein fraction of IMA. The labeling was almost undetectable in samples where the endothelium was removed. Thus, expression of β_3-AR was mainly located in endothelial layer and almost absent in smooth muscle cells. In the same way, immunohistochemical analysis performed in intact IMA demonstrated a strong staining in the endothelial layer with human β_3-AR antibody (that did not appear when the primary antiserum was not incubated). By contrast, the absence of staining observed in the smooth muscle layer suggested the lack or a very low level (under the limit of detection of the procedure used) of β_3-AR expression in smooth muscle cells. In a recent study using the same monoclonal antibody, an endothelial localization of β_3-AR was also reported in human coronary arteries (12). Conversely, in human gastrointestinal arteries, immunohistochemistry with an antipeptide polyclonal antibody revealed a β_3-AR expression localized in smooth muscle cells, but not in the endothelial layer (27). Thus, localization of

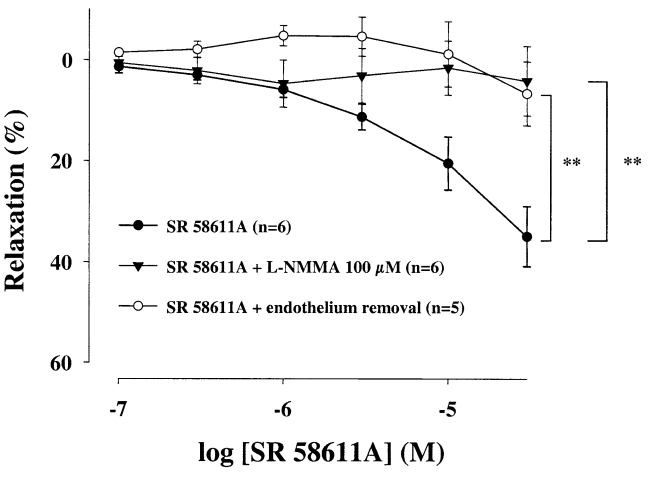

Figure 5. Effect of NG-monomethyl-L-arginine monoacetate (L-NMMA) treatment or endothelium removal on SR 58611A-induced vasodilation in human internal mammary artery rings constricted with 1 μm phenylephrine (PE). Concentration-response curves to SR 58611A were performed in the absence or in the presence of 100 μmol/l L-NMMA, an inhibitor of nitric oxide synthases, or after endothelium removal. The mean curves resulting from subtraction of the spontaneous relaxation of control vessels are shown. Results are expressed as percentage of relaxation from the contraction level induced by PE. Each **point** is the mean value of six experiments, and **error bars** represent SEM. $^{**}p < 0.01$ indicates significant differences from response to SR 58611A alone.

β_3-AR in vascular wall might be different according to the vascular beds. In other species, only two studies performed in rat vessels (portal vein and thoracic aorta) reported the expression of β_3-AR mRNA. These studies also reported a differential localization of β_3-AR according to vascular bed: smooth muscle cells in portal vein (17) and endothelial cells in thoracic aorta (18).

In the present work, the presence of β_3-AR in human IMA was also confirmed by a pharmacological approach. SR 58611A, a preferential β_3-AR agonist, induced a slow and weak relaxation. The relaxation induced by SR 58611A was not modified by a pretreatment with nadolol, a β_1- and β_2-AR antagonist, but was greatly reduced by L-748,337, a selective human β_3-AR antagonist (28). Albeit, it was impossible to determine the pD_2 values from the concentration-response curves of SR 58611A, it appeared that this compound produced an effect for relatively high concentrations suggesting a low potency for the β_3-AR agonists in human IMA. Of interest, similar slower kinetics were described for the relaxing effect of β_3-AR agonists in other conductive vessels (rat thoracic aorta [11]; rat carotid arteries [7]) as well as in the smooth muscle of the rat gastrointestinal tract (29,30). However, the potency of the β_3-AR agonists was lower in vessels than in the gastrointestinal tract. In resistive vessels like human coronary arteries, the potency of β_3-AR agonist was higher (12). It is still unclear why β_3-adrenoceptor agonists have poor potency for conductive vessel relaxation. In other tissues, like adipose tissues and the heart, the potency of β_3-AR agonists was higher than in vessels, around 10 to 100 nM. Moreover, concerning the signaling pathway involved in the β_3-AR-induced relaxation, endothelium removal strongly attenuated the vasodilating effect of the SR 58611A. In the same way, a pretreatment with L-NMMA, a NO-synthase inhibitor, almost abolished the SR 58611A-induced relaxation. These data strengthened the endothelial localization of β_3-AR in human IMA and suggested the involvement of the NO-synthase signaling pathway in the β_3-AR-induced vasorelaxation.

In the same model of human IMA, a previous study suggested the coexistence of three distinct β-AR subtypes (31). Cyanopindolol, a partial β_3-AR agonist, and BRL 37344, a preferential β_3-AR agonist, induced vasorelaxant effects in endothelium-denuded IMA segments precontracted with PE. In denuded IMA rings, the isoproterenol-induced relaxation was partially inhibited by propranolol only in a minority group of IMA segments, whereas cyanopindolol-induced vasodilation was completely resistant to blockade by propranolol, a β-AR nonselective antagonist. However, that study was incomplete because β_1/β_2-AR antagonists, such as propranolol or nadolol and selective β_3-AR antagonists, were not tested on the BRL 37344-induced relaxation, nor the involvement of endothelium in the relaxant effect induced by β_3-AR stimulation. Similar results were obtained by He et al. (32) that showed that isoproterenol produced a weak relaxation (24 $\pm$ 5%). The vasodilation was only partially antagonized by propranolol (about 50%), suggesting an atypical β-adrenergic effect. These latter authors also showed that isoproterenol-induced relaxation was less important in IMA rings precontracted with potassium instead of U46619. In addition, the vasodilation was completely abolished in the presence of propranolol. Those results could be explained by our own previous works performed in rat thoracic aorta, which showed the involvement of several types of potassium channels in the β_3-AR-mediated relaxation (18). The study performed by He et al. (32) being old, it was not possible at this period to suspect a putative involvement of β_3-ARs in the observed effects. Indeed, β_3-AR was cloned in 1989 (5). All those data strengthened our results on the functional presence of β_3-ARs in the human IMA. However, in our study, the activation of β_3-ARs by an endogenous catecholamine was not investigated, which represents a limitation of the present study. In another human vascular bed, coronary microarteries, BRL37344 also produced a vasorelaxation that was not modified by nadolol, but was blunted in the presence of bupranolol, a nonspecific β-AR antagonist (12). In that vascular bed, functional β_3-ARs were also located in endothelial cells, and their stimulation activated both the NO and the endothelial-derived hyperpolarizing factor pathway (12).

The development of IMA grafting is the most remarkable achievement in coronary artery surgery in the past two decades. The knowledge of the pharmacology of the vascular grafts allows a new approach in the drug management of bypass operated patients. Given the presence of β_3-AR in microcoronary arteries and its relative resistance to homologous desensitization, the relaxing effect of β_3-AR agonists could be interesting in the management after coronary artery bypass graft (CABG) of ischemic patients who often present an increased adrenergic tone (32,33).

After coronary artery bypass, the vascular β-AR responses are markedly abnormal, due in part to the exposure of vessels to endogenous catecholamines in cerebral arteries (33,34) and pulmonary vascular bed (34,35), for instance. Several mechanisms were suggested to explain β-ARs dysfunction after coronary artery bypass: 1) uncoupling of β-ARs from the Gs-protein-adenylate cyclase complex (attributed to the rapid phosphorylation of the receptor; and/or 2) impairment of the adenylyl cyclase moiety (36,37). By contrast, β_3-ARs are only activated by high concentrations of catecholamines such as isoproterenol (38). Furthermore, β_3-ARs have been shown to be relatively resistant to desensitization (39). On one hand, the effect of β_3-AR stimulation could increase the graft and the coronary flow after CABG. On the other hand, the surprising negative inotropic effect induced by β_3-AR agonists in the human heart could reduce the myocardial oxygen consumption (40). It may result in an improvement of the heart oxygen supply/consumption ratio and, thus, accommodate for a reduced perfusion. In this context, the potential role of β_3-AR in perioperative period of CABG might be worth being explored.

The IMA malperfusion syndrome is a critical complication of CABG (1.9% and up to 20% after primary coronary bypass and reoperation, respectively) and can have devastating effects on the outcome of a cardiac operation (41). The etiology of the syndrome is multifactorial, but a spasm of the graft has been frequently suggested, and, thus, the treatment implicated the use of vasodilator and inotropic drugs (42). However, the vasoconstrictive effects of inotropic substances could reduce arterial graft flow and worsen the situation (43). By the way, when β-AR agonists were nevertheless used in the perioperative period, drugs were usually nonselective and presented α-AR properties (epinephrine, norepinephrine, dobutamine, isoprenaline). Furthermore, it was admitted that IMA exhibited moderate β_1/β_2-AR function, which implied that use of β-AR agonists was not appropriate to reverse vasospasm (44). Thus, new β-AR agonists with β_3-AR agonistic properties might help to maintain the CABG flow.

The routine use of beta-blockers has been considered for patients who undergo revascularization after acute myocardial infarction in order to reduce mortality (45). Although most patients are rendered asymptomatic after coronary artery bypass, there is evidence that blood flow through IMA grafts is inadequate for maximal exercise (46). In a large cohort of patients who underwent coronary artery bypass after myocardial infarction, beta-blockers reduced mortality (45). It was admitted that the use of a β-AR antagonist should not evoke IMA vasospasm (21). The development of a third generation of beta-blockers with vasodilating properties resulting from a β_3-AR agonistic effect (nebivolol, bucindolol) might constitute an interesting new way of investigation (47,48). In isolated human umbilical vein endothelial cells, nebivolol dose-dependently increased NO formation (49). This effect was inhibited by β_3-AR blockers, but not by β_1/β_2-AR blockers, suggesting that endothelial β_3-ARs mediated the increased NO production. Furthermore, in rat aorta, nebivolol-induced vasorelaxation seemed to result from the β_3-AR activation (47). All together, those data strengthened the importance of developing and/or selecting drugs possessing β_1-AR antagonist *and* β_3-AR agonist properties to manage a large number of cardiovascular pathologies (hypertension, heart failure).

In conclusion, the present findings highlighted the potential role of β_3-ARs in blood flow regulation. Many cardiovascular pathologies, such as systemic hypertension and heart failure, are characterized by a sustained activation of the sympathetic nervous system. According to their pharmacological properties, β_3-ARs could play a potential role in those diseases. Thus, the present study opens a new field of investigation concerning the involvement of β_3-ARs in human cardiovascular physiopathology and might contribute to the development of new β-AR agonists and/or antagonists.

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