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Involvement of β_3-Adrenoceptor in Altered β-Adrenergic Response in Senescent Heart

Role of Nitric Oxide Synthase 1–derived Nitric Oxide

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Background: **In senescent heart, β-adrenergic response is altered in parallel with β_1- and β_2-adrenoceptor down-regulation. A negative inotropic effect of β_3-adrenoceptor could be involved. In this study, the authors tested the hypothesis that β_3-adrenoceptor plays a role in β-adrenergic dysfunction in senescent heart.**

Methods: **β-Adrenergic responses were investigated *in vivo* (echocardiography–dobutamine, electron paramagnetic resonance) and *in vitro* (isolated left ventricular papillary muscle, electron paramagnetic resonance) in young adult (3-month-old) and senescent (24-month-old) rats. Nitric oxide synthase (NOS) immunolabeling (confocal microscopy), nitric oxide production (electron paramagnetic resonance) and β-adrenoceptor Western blots were performed *in vitro*. Data are mean percentages of baseline ± SD.**

Results: **An impaired positive inotropic effect (isoproterenol) was confirmed in senescent hearts *in vivo* (117 ± 23 *vs.* 162 ± 16%; $P < 0.05$) and *in vitro* (127 ± 10 *vs.* 179 ± 15%; $P < 0.05$). In the young adult group, the positive inotropic effect was not significantly modified by the nonselective NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME; 183 ± 19%), the selective NOS1 inhibitor vinyl-L-*N*-5(1-imino-3-butenyl)-L-ornithine (L-VNIO; 172 ± 13%), or the selective NOS2 inhibitor 1400W (183 ± 19%). In the senescent group, in parallel with β_3-adrenoceptor up-regulation and increased nitric oxide production, the positive inotropic effect was partially restored by L-NAME (151 ± 8%; $P < 0.05$) and L-VNIO (149 ± 7%; $P < 0.05$) but not by 1400W (132 ± 11%; not significant). The positive inotropic effect induced by dibutyryl-cyclic adenosine monophosphate was decreased in the senescent group with the specific β_3-adrenoceptor agonist BRL 37344 (167 ± 10 *vs.* 142 ± 10%; $P < 0.05$). NOS1 and NOS2 were significantly up-regulated in the senescent rat.**

Conclusions: **In senescent cardiomyopathy, β_3-adrenoceptor overexpression plays an important role in the altered β-adrenergic response *via* induction of NOS1-nitric oxide.**

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

This article is accompanied by an Editorial View. Please see: Zaugg M, Schaub MC: β_3-Adrenergic receptor subtype signaling in senescent heart: Nitric oxide intoxication or "endogenous" β blockade for protection? ANESTHESIOLOGY 2008; 109:956–9.

IN senescent heart, among different age-related changes, such as contraction and relaxation dysfunction,[1,2] the cardiovascular effects of adrenoceptor stimulation are attenuated[3,4] even though plasma catecholamine concentration increases with age.[5]

In the heart, at least three types of β-adrenoceptor potentially modulate cardiac function. Stimulation of β_1- and β_2-adrenoceptors induces a positive inotropic effect resulting from the cyclic adenosine monophosphate production and protein kinase A activation, whereas β_3-adrenoceptor stimulation induces a negative inotropic effect mediated through a nitric oxide pathway.[6–9] Therefore, on one hand, nitric oxide–derived cyclic guanosine monophosphate induces the activation of phosphodiesterases, which increase the catabolism of the produced cyclic adenosine monophosphate. On the other hand, cyclic guanosine monophosphate activates protein kinase G, which decreases protein kinase A activity.[8] We have previously demonstrated that both down-regulation of β_1-adrenoceptor and up-regulation of β_3-adrenoceptor contribute to decrease the positive inotropic effect of β-adrenoceptor stimulation in diabetic cardiomyopathy.[7] Nitric oxide synthase 1 (NOS1) is the NOS isoform coupled to the β_3-adrenoceptor/caveolin-3 complex in the diabetic cardiomyocyte.[7] Nevertheless, the nature of the NOS isoform may be variable according to the type of cardiomyopathy.[7,10–13]

In senescent heart, both β_1- and β_2-adrenoceptors are down-regulated,[4] but the β_3-adrenoceptor has never previously been investigated. Specific inhibition of protein Gi coupled to β_2-adrenoceptor with pertussis toxin did not restore any contractility,[4] and adenylyl cyclase activity, NOS production, and protein kinase A activity are

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impaired.[3,14–17] We previously demonstrated that NOS1 activity is increased in senescent heart after myocardial infarction–induced heart failure.[11]

The aim of this study was to test the hypothesis that β_3-adrenoceptor is involved in the altered response of β-adrenergic stimulation in senescent heart.

Materials and Methods

Animals

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (A5550-01, Paris, France) and the recommendations of the Declaration of Helsinki. Therefore, these experiments were conducted in authorized laboratories and under the supervision of an authorized researcher for each institution (B.R., C.H., and R.A. for EA 3975, INSERM U689, and UMR-INSERM 771-CNRS 6214, respectively). Young adult (3 months) and senescent male Wistar rats (24 months) (Charles River, Saint Germain sur l'Arbresle, France), were used.

Echocardiography-Dobutamine

Echocardiography was performed on anesthetized rats (1–2% isoflurane) using a General Electric Vivid 7 instrument (Aulnay-sous-Bois, France) equipped with an 8- to 14-MHz linear transducer. Data were transferred on-line to a computer for analysis (EchoPAC PC version 2.0.x; General Electric). Left ventricular diameter was measured in the parasternal long-axis and short-axis views in M mode. Left ventricular ejection fraction and left ventricular fractional shortening were measured using a modified version of Simpson monoplane analysis.[7,18] Left ventricular diastolic parameters were derived from pulsed-wave spectral Doppler mitral flow and from pulsed-wave spectral mitral tissue Doppler imaging from apical view, with the sample volume paced at the lateral corner of the mitral annulus as reported previously.[7,18,19] Left ventricular systolic function was evaluated using the left ventricular diameter, left ventricular ejection fraction, and left ventricular fractional shortening. Left ventricular diastolic function was investigated using peak velocity of early (E) and late (A) filling waves and E/A ratio.[7,18,19] In addition, isovolumic relaxation time and deceleration time of the E wave were derived from pulsed-wave mitral Doppler spectra and the peak early diastolic velocity Ea from pulsed-wave spectral mitral tissue Doppler imaging.[18,19] The left ventricular end-diastolic pressure was measured using the E/Ea ratio.[7,18,19] Dobutamine (4 μg/kg) was administrated intraperitoneally, and measurements were performed when the increase in heart rate was stabilized.[7,18]

Isolated Left Ventricular Papillary Muscle

Papillary muscle mechanics were studied in Krebs-Henseleit bicarbonate buffer solution as described previously.[1,2,7,18,20] After brief anesthesia with sodium pentobarbital, the heart was quickly removed. The whole heart and the left ventricle were dissected and weighed, and the left ventricular papillary muscles were carefully excised and suspended vertically in a 200-ml jacketed reservoir with Krebs-Henseleit bicarbonate buffer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.1 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 4.5 mM glucose) and maintained at 29°C with a thermostatic water circulator. Preparations were field stimulated at 12 pulses/min with 5-ms rectangular wave pulses set just above threshold. The bathing solution was bubbled with 95% O_2 and 5% CO_2, resulting in a pH of 7.40. After a 60-min stabilization period with the initial muscle length at the apex of the length–active isometric tension curve, papillary muscles recovered their optimal mechanical performance. The extracellular concentration of Ca^{2+} was decreased from 2.5 to 0.5 mM because rat myocardial contractility is nearly maximal at 2.5 mM.[18,20,21] Conventional mechanical parameters at the initial muscle length at the apex of the length–active isometric tension curve were calculated from three twitches. The first twitch was isotonic and was loaded with the preload corresponding to the initial muscle length at the apex of the length–active isometric tension curve. The second twitch was abruptly clamped to zero load just after the electrical stimulus with a critical damping. The third twitch was fully isometric at the initial muscle length at the apex of the length–active isometric tension curve (L_{max}). We determined the maximum unloaded shortening velocity using the zero-load technique, and time to peak shortening of the twitch with preload only. In addition, the maximum isometric active force normalized per cross-sectional area and the time to peak force were recorded from the isometric twitch. At the end of the study, the muscle cross-sectional area was calculated from the length and weight of papillary muscle, assuming a density of 1.

β-Adrenoceptor stimulation was induced by cumulative concentrations of isoproterenol (10^{-8} to 10^{-4} M), a nonselective β-adrenoceptor agonist, in the presence of phentolamine (10^{-6} M), a specific α_1-adrenoceptor antagonist.[7,18]

To assess the role of the β_3-adrenoceptor, we studied additional groups exposed to cumulative concentrations of BRL 37344 (10^{-8} to 10^{-5} M),[22] a specific β_3-adrenoceptor agonist, in the presence of nadolol (10^{-5} M), a specific β_1- and β_2-adrenoceptor antagonist.[22] The effect of β_3-adrenoceptor stimulation on the cyclic adenosine monophosphate resulting from the β_1- and β_2-adrenoceptor stimulation was studied using dibutyryl-cyclic adenosine monophosphate (5.10^{-4} M), a fat-soluble and diffusible analog of cyclic adenosine monophosphate resistant to hydrolysis in the intracellular involvement,[7,18] in the presence of nadolol (10^{-5} M) and in the presence or not in the presence of BRL 37344 (10^{-5} M).

To assess the NOS isoform involved in the β_3-adrenoceptor pathway, we studied additional groups exposed to N^G-nitro-L-arginine methylester (L-NAME; 10^{-5} M), an unspecific NOS inhibitor; to vinyl-L-N-5(1-imino-3-butenyl)-L-ornithine (L-VNIO; 10^{-4} M), a specific NOS1 inhibitor; or to 1400W (10^{-4} M), a specific NOS2 inhibitor, as reported previously.[7,22]

The total volume of added drugs did not exceed 2% of the bath volume. All drugs were purchased from Sigma Chemical (L'Isle d'Abeau Chesne, France), except L-VNIO, which was purchased from Coger (Paris, France), and BRL 37344, which was purchased from Tocris Biosciences (Bristol, United Kingdom).

Nitrite Oxide Spin Trapping and Electronic Paramagnetic Resonance Studies

Detection of nitric oxide production was performed both *in vivo* and *in vitro* using the technique with Fe^{2+} diethyldithiocarbamate (Sigma Chemical) as the spin trap as previously described.[23] To measure nitrite oxide production *in vivo*, Fe^{2+} diethyldithiocarbamate was injected intraperitoneally (400 mg/kg) with or without injection of intraperitoneal dobutamine and $FeSO_4$-$7H_2O$ (40 mg/kg) and citrate (200 mg/kg) by subcutaneous injection on the neck of adult or senescent rats. After 30 min, rats were killed to harvest the left ventricle from the heart to measure nitric oxide. In another set of experiments, *in vitro*, small pieces of left ventricular myocardium from adult and senescent rats were placed in 24-well clusters filled with 250 μl Krebs solution containing phentolamine (10^{-6} M) with or without isoproterenol (10^{-6} M) in presence or not in the presence of NOS inhibitors: L-NAME (100 μM), 1400W (100 μM), or L-VNIO (100 μM). The left ventricular myocardium was treated with 250 μl of the colloid Fe(Fe^{2+} diethyldithiocarbamate)$_2$ and incubated at 37°C for 1 h. All nitric oxide measures were performed on a tabletop x-band spectrometer miniscope (Magnettech, Berlin, Germany). Recordings were made at 77°K using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 60 s of sweep time, and 10 scans.

Staining and Imaging by Confocal Microscopy

Staining and imaging of NOS1 and NOS2 were investigated by confocal microscopy. Pieces of left ventricular myocardium were frozen and cut into 7-μm sections. Fixed sections were incubated (2 h at room temperature) in a blocking buffer (5% nonfat dry milk in phosphate-buffered saline). Tissue sections were then incubated overnight (4°C) with monoclonal murine anti-NOS2 (1:100; Transduction Laboratories, Heidelberg, Germany) or anti-NOS1 (1:100; Transduction Laboratories) antibodies. Three washes were followed by incubation (1 h, 37°C) with secondary murine and rabbit, respectively, fluorescent Alexa fluor-488-labeled antibody (1:100; Invitrogen Molecular Probes, Leiden, The Netherlands). Slides were examined with an Olympus light microscope Fluoview FU 300 Laser Scanning Confocal Imaging System (Olympus, Paris, France) equipped with an argon ion laser (EM 488 nm). Pictures were taken with a ×10 objective (water immersion). The laser was adjusted in the green fluorescent mode. Z series were collected in 1-μm steps, and final images were obtained after stacking.

Western Blot Studies

Western Blots were performed on left ventricular homogenates with specific antibodies to measure protein expression of β_1- and β_3-adrenoceptors, as described previously.[7,18] Briefly, cardiomyocytes were homogenized in Triton X-100 buffer (1% Triton X-100 with 50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 0.2 mM orthovanadate, 0.1 mM leupeptin, and 0.001 mM aprotinin) for 1 h at 4°C. After centrifuging at 15,000*g* for 15 min at 4°C, supernatant protein concentrations were measured using the BCA protein assay kit (Perbio Science, Brebières, France). Proteins were prepared as previously described,[7,18] and 50 μg protein per lane was immunoblotted using anti–β_1-adrenoceptor (1:1,000; Affinity Bioreagents, Saint Quentin en Yvelines, France) and goat polyclonal anti–β_3-adrenoceptor (1:1,000; Santa Cruz Biotechnology, Le Perray en Yvelines, France). All the Western blot experiments were quantified using normalization, including a standardization of the different gels by loading a reference sample on every gel and checking that a similar total amount of protein was loaded by measurement of total protein level present on the membrane colored by S-Ponceau. The S-Ponceau staining enabled us to verify that equal amounts of protein were loaded. Accordingly, all of the results were normalized with a link (actin) and the amounts of protein transferred on the membrane. A control by performing a Western blot using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was performed and validated that there was no variation in protein gel loading in our hands.

Statistical Analysis

Data are expressed as mean ± SD. The maximum effect and the concentration that results in 50% of maximum effect were determined as described previously.[1,2,7,18,20,24] Comparison of two means was performed using the paired Student *t* test. Comparison of several means was performed using one-way or two-way analysis of variance, when appropriate. Repeated-measures analysis of variance was used when required, and the *post hoc* test used was the Newman–Keuls test. All *P* values were two-tailed, and a *P* value less than 0.05 was considered significant. Statistical analysis was performed using NCSS 2007 software (Statistical Solutions Ltd., Cork, Ireland).

Results

We studied 49 young adult and 60 senescent rats. For assessment of mechanical variables with isolated left ventricular papillary muscles and protein expression with Western blot, we investigated 31 young adult and 42 senescent hearts. This difference was explained by the fact that it was more difficult to remove and obtain stable preparation in senescent rats. Nitric oxide assessment, staining, and imaging were performed *in vivo* in 12 rats of each age. Nitric oxide assessment was performed *in vitro* in 6 rats of each age.

Senescent rats had significantly higher body weight (551 ± 130 *vs.* 328 ± 35 g; $P < 0.05$) and heart weight (1,078 ± 185 *vs.* 664 ± 72 mg; $P < 0.05$) than young adult rats. Nevertheless, the heart weight–to–body weight ratio (2.1 ± 0.8 *vs.* 2.0 ± 0.2 mg/g; not significant [NS]) and left ventricular weight–to–body weight ratio (1.62 ± 0.52 *vs.* 1.63 ± 0.21 mg/g; NS) were not significantly different between young adult and senescent rats.

Contractile Responses to β-Adrenergic Stimulation

In vivo, the baseline echocardiographic characteristics were compared with young adult (n = 12) and senescent (n = 9) rats. The heart rate was not significantly different between young adult and senescent rats (340 ± 14 *vs.* 342 ± 11 beats/min, respectively; NS). Systolic function was preserved in senescent rats, as shown by the lack of a significant difference between young adult and senescent rats in left ventricular ejection fraction (86 ± 5 *vs.* 90 ± 5%; NS) and left ventricular fraction shortening (54 ± 5 *vs.* 56 ± 8%; NS). In contrast, diastolic function was altered in the senescent group, as shown by the significant prolongation of isovolumic relaxation time (22 ± 1 *vs.* 30 ± 4 ms; $P < 0.05$), the impairment in deceleration time of E (44 ± 7 *vs.* 32 ± 6 ms; $P < 0.05$), and the increased value of the E/A ratio (1.2 ± 0.1 *vs.* 2.7 ± 1.1; $P < 0.05$). Assessment of A, E/A ratio, and isovolumic relaxation time was technically impossible for two young adult rats. Left ventricular end-diastolic pressure was enhanced in comparison with the young adult rats, as shown by the increased E/Ea ratio (13.1 ± 2.5 *vs.* 17.7 ± 4.3; $P < 0.05$). *In vitro*, using left ventricular papillary muscle (n = 56 from young adult and n = 56 senescent hearts), the inotropic properties were significantly altered in the senescent group under both low-load conditions (maximum unloaded shortening velocity, 3.21 ± 0.39 *vs.* 2.60 ± 0.60 L_{max}/s; $P < 0.05$) and high-load conditions (maximum isometric active force normalized per cross-sectional area, 60 ± 17 *vs.* 51 ± 18 mN/mm^2; $P < 0.05$) in comparison with the young adult group. The contraction time was increased in senescent rats as shown by the prolongation of both time to peak shortening (175 ± 16 *vs.* 206 ± 24 ms; $P < 0.05$) and time to peak force (150 ± 15 *vs.* 177 ± 25 ms; $P < 0.05$).

Table 1. Inotropic Effect of β-Adrenoceptor Stimulation (4 μg/kg Dobutamine) in Young Adult and Senescent Rats *In Vivo* Using Echocardiography

Echocardiographic Parameter	Young Adult Rats, n = 12	Senescent Rats, n = 9
HR	112 ± 4*	103 ± 3*†
LVEF	116 ± 7*	105 ± 7*†
LVFS	162 ± 16*	117 ± 23*†

Data are percentage of baseline value, expressed as mean ± SD.

* $P < 0.05$ *vs.* baseline value. † $P < 0.05$ *vs.* adult rats.

HR = heart rate; LVEF = left ventricular ejection fraction; LVFS = left ventricular shortening fraction.

β-Adrenoceptor stimulation induced a marked positive inotropic effect in young adult rats *in vivo* (table 1) and *in vitro* (table 2 and fig. 1A). This positive inotropic effect was markedly diminished both *in vivo* (table 1) and *in vitro* (table 2 and fig. 1B) in senescent rats.

In vitro, L-NAME, L-VNIO, and 1400W *per se* did not significantly modify the maximum isometric active force normalized per cross-sectional area in young adult or senescent groups (data not shown). With L-NAME, L-VNIO, or 1400W, the positive inotropic effect of β-adrenoceptor stimulation was not significantly modified in young adult rats (table 2 and fig. 1A). In senescent rats, both L-NAME and L-VNIO partially restored the positive inotropic effect of β-adrenoceptor stimulation (table 2 and fig. 1B). In contrast, this positive inotropic effect was not significantly modified by 1400W (table 2 and fig. 1B).

Table 2. Effects of L-NAME, L-VNIO, and 1400W on the Inotropic Response to β-Adrenoceptor Stimulation in Young Adult and Senescent Rats

	Young Adult Rats, n = 8		Senescent Rats, n = 8	
	V_{max}	AF	V_{max}	AF
Eff_{max}, %				
Control	182 ± 11*	179 ± 15*	127 ± 11*†	127 ± 10*†
L-NAME	183 ± 10*	183 ± 19*	166 ± 11*‡	151 ± 8*†‡
L-VNIO	185 ± 12*	172 ± 13*	168 ± 10*‡	149 ± 7*†‡
1400W	190 ± 23*	183 ± 19*	136 ± 8*†	132 ± 11*†
C_{50}, μM				
Control	0.11 ± 0.06	0.16 ± 0.12	0.06 ± 0.06	0.36 ± 0.27
L-NAME	0.07 ± 0.09	0.08 ± 0.11	0.28 ± 0.28	0.61 ± 0.93†
L-VNIO	0.10 ± 0.09	0.42 ± 0.26	0.08 ± 0.08	0.09 ± 0.09
1400W	0.10 ± 0.08	0.28 ± 0.23	0.38 ± 0.53	0.10 ± 0.14

Data are expressed as mean ± SD.

* $P < 0.05$ *vs.* baseline value. † $P < 0.05$ *vs.* adult rats. ‡ $P < 0.05$ *vs.* control group.

C_{50} = concentration of isoproterenol that results in 50% of Eff_{max}; AF = active force per cross-sectional area; Eff_{max} = maximum effect in percentage of baseline value; L-NAME = N^G-nitro-L-arginine methylester; L-VNIO = vinyl-L-*N*-5(1-imino-3-butenyl)-L-ornithine; V_{max} = maximum unloaded shortening velocity.

BRL 37344 did not induce any significant inotropic effect in the presence of nadolol, a selective β_1- and β_2-adrenoceptor antagonist, in young adult (101 ± 5%; NS) or senescent rats (98 ± 7%; NS). The positive ino-

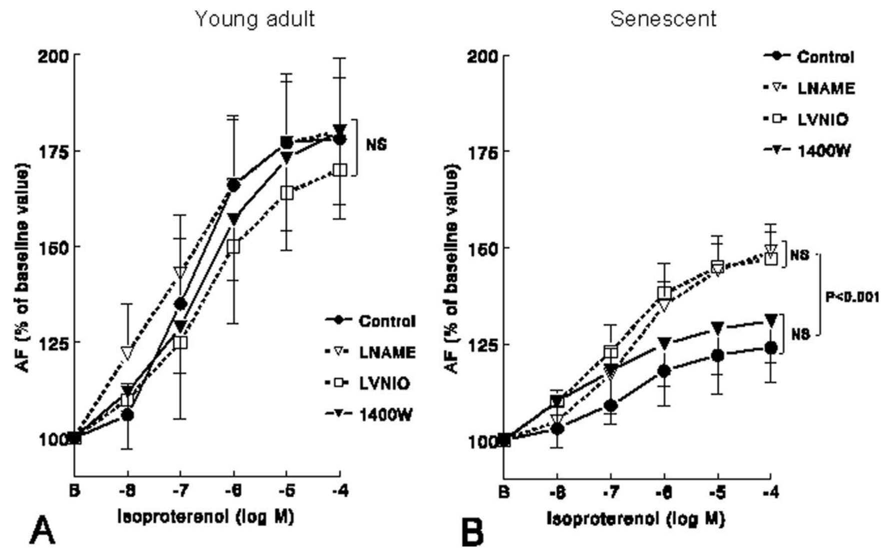

Fig. 1. Inotropic response to β-adrenoceptor stimulation (isoproterenol) in young adult (*A*) and senescent rats (*B*), under high load. AF = isometric active force normalized per cross-sectional area; L-NAME = N^G-nitro-L-arginine methylester, nonspecific nitric oxide synthase (NOS) inhibitor; L-VNIO = vinyl-L-*N*-5(1-imino-3-butenyl)-L-ornithine, specific NOS1 inhibitor; 1400W = specific NOS2 inhibitor; NS = not significant. Data are mean percentage of baseline value ± SD (n = 8 in each group). The *P* values refer to the comparison of the maximum effect on active force per cross-sectional area reported in table 2. $^*P < 0.05$ *versus* control group.

tropic effect of dibutyryl-cyclic adenosine monophosphate was not significantly different in young adult and senescent rats (fig. 2). BRL 37344 significantly decreased the positive inotropic effect of dibutyryl-cyclic adenosine monophosphate in senescent rats but not in young adult rats (fig. 2).

Expression of β-Adrenoceptor Subtypes

In agreement with the functional changes observed in the papillary muscle experiments, we found that protein expression of β_1-adrenoceptor was reduced by 33% in senescent hearts compared with young adult hearts (fig. 3). In contrast, β_3-adrenoceptor protein expression was significantly increased in senescent hearts compared with young adult hearts (fig. 3).

Increased Nitric Oxide Production in β-Adrenoceptor Stimulation

In young adult hearts, the β-adrenoceptor stimulation did not induce any nitric oxide production *in vivo* (fig. 4) or *in vitro* (fig. 5A). *In vitro*, L-NAME abolished the nitric oxide production without any significant influence of L-VNIO or 1400W in comparison with the control group (fig. 5A). Nitric oxide production was significantly decreased by L-VNIO in comparison with the isoproterenol group (fig. 5A).

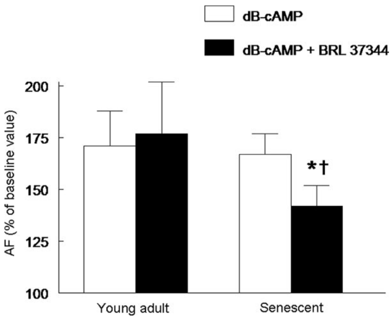

Fig. 2. Comparison of the positive inotropic effects of dibutyryl cyclic adenosine monophosphate (dB-cAMP; 5×10^{-4} M) with or without specific inhibitor or β_3-adrenoceptor (BRL 37344; 10^{-5} M) in left ventricular papillary muscles from young adult and senescent rats, under high load. AF = isometric active force normalized per cross-sectional area. Data are mean percentage of baseline value ± SD (n = 8 in each group). $^*P < 0.05$ *versus* young adult rats. † $P < 0.05$ *versus* dB-cAMP.

In senescent hearts, however, nitric oxide production significantly increased with β-adrenoceptor stimulation, both *in vivo* (fig. 4) and *in vitro* (fig. 5B). *In vitro*, L-NAME abolished nitric oxide production. Both L-VNIO and 1400W decreased around 50% of global nitric oxide production (fig. 5B).

Staining and Imaging of NOS1 and NOS2 by Confocal Microscopy

Both NOS1 and NOS2 protein immunoreactivities were significantly increased in senescent left ventricular myocardium in comparison with young adult rats (fig. 6).

Discussion

In the current study, we confirmed that the positive inotropic effect of β-adrenoceptor stimulation was altered *in vivo* and *in vitro* in senescent rat heart. Both *in vivo* and *in vitro*, we provide evidence for involvement of the β_3-adrenoceptor in the decreased positive inotropic effect of β-adrenoceptor stimulation in senescent heart, in parallel to down-regulation of β_1-adrenoceptor and up-regulation of β_3-adrenoceptor protein expressions. *In vitro*, NOS1 seems to be the functional isoform involved in the β_3-adrenoceptor pathway. These findings suggest that the β_3-adrenoceptor plays an important role in the β-adrenergic dysfunction associated with senescent heart.

In vivo, we have confirmed left ventricular diastolic dysfunction in senescent heart allowing a preserved left ventricular ejection fraction with both a "restrictive fill-

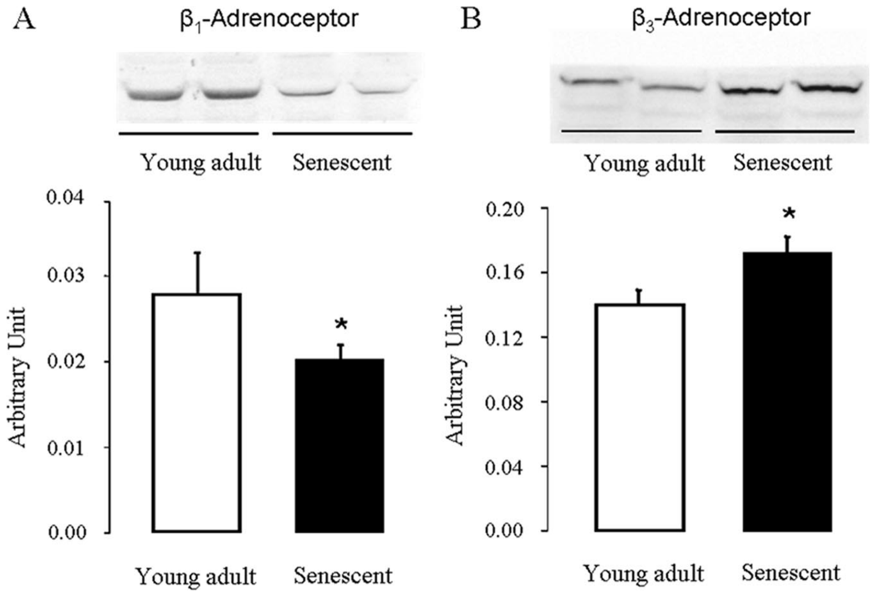

Fig. 3. Representative Western blot and densitometric data reflecting β_1-adrenoceptor proteins expression (*A*) and β_3-adrenoceptor (*B*) in senescent rats compared with young adult rats. Data are expressed as mean ± SD (n = 7 in each group). * $P < 0.05$ *versus* young adult group.

ing pattern" in pulsed-wave spectral Doppler mitral flow and increased left ventricular end-diastolic filling pressures.[2,25] *In vitro*, we have confirmed inotropic abnormalities involving the prolongation of the contraction velocities related to lower Ca^{2+} release from the sarcoplasmic reticulum,[25,26] slower cross-bridge cycling rate,[26] and decreased density of depolarizing potassium channels responsible for transient outward potassium current I_{to}.[27,28] The impairment in the maximum unloaded shortening velocity is attributable to a switch of the myosin heavy chain isoform with lower adenosine triphosphatase activity (β-MHC).[26] The decreased maximum isometric active force normalized per cross-sectional area results from the diminished myofibrillar content as a result of fibrosis and apoptosis.[2,25] The usual contrast between the normal systolic function *in vivo* and the myocardial inotropic abnormalities *in vitro* suggest the existence of neurohumoral compensatory mechanisms that may allow the heart to maintain a normal cardiac output.[25]

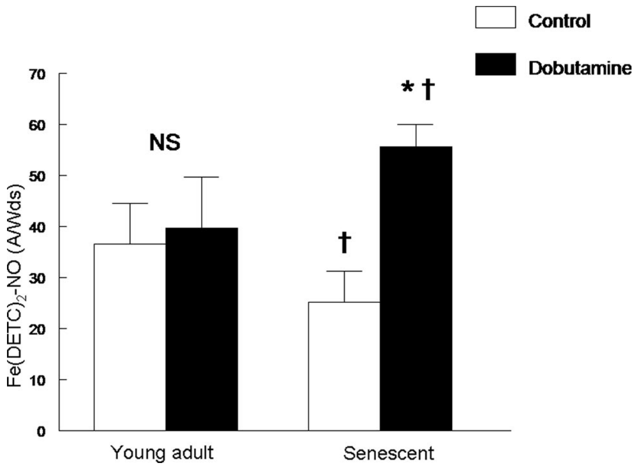

Fig. 4. Net nitric oxide (NO) level induced by β-adrenergic stimulation (4 μg/kg dobutamine), *in vivo*, in heart from young adult and senescent rats using Fe(diethyldithiocarbamate [DETC]) electron paramagnetic resonance. A/Wds = amplitude of the NO-Fe(DETC)$_2$ in unit/weight, *i.e.*, mg dried sample A/W(ds); NS = not significant. n = 6 in each group. * $P < 0.05$ *versus* control group. † $P < 0.05$ *versus* young adult rats.

In senescent heart, the positive inotropic effect of β-adrenoceptor stimulation is altered both *in vivo* and *in vitro*.[3,4,29,30] In this context, dibutyryl-cyclic adenosine monophosphate induces comparable positive inotropic effects in young adult and senescent groups, suggesting that most of the abnormalities of the β-adrenergic pathway are located upstream of protein kinase A activation. While down-regulation of β_1- and β_2-adrenoceptors has been well established,[4] we have shown for the first time that the β_3-adrenoceptors are up-regulated in the left ventricular myocardium of senescent rats. The impairment in the inotropic effect induced by dibutyryl-cyclic adenosine monophosphate when BRL 37344 was used confirmed that the β_3-adrenoceptor pathway is involved in senescent heart. The lack of a negative inotropic effect induced by BRL 37344 when a specific β_1- and β_2-adrenoceptor antagonist (nadolol) was used could seem contradictory. In fact, nadolol inhibits the production of cyclic adenosine monophosphate induced by β_1- and β_2-adrenoceptor stimulation. In this context, the concentration of cyclic adenosine monophosphate might be insufficient to induce any β_3-adrenoceptor effect. On the other hand, when the cytosolic cyclic adenosine monophosphate concentration was restored using dibutyryl-cyclic adenosine monophosphate, the negative inotropic effect induced by BRL 37344 significantly decreased the positive inotropic effect induced by dibutyryl-cyclic adenosine monophosphate in senescent myocardium. As previously reported, nitric oxide production induced by the β-adrenoceptor stimulation is exclusively the fruit of the β_3-adrenoceptor pathway.[9]

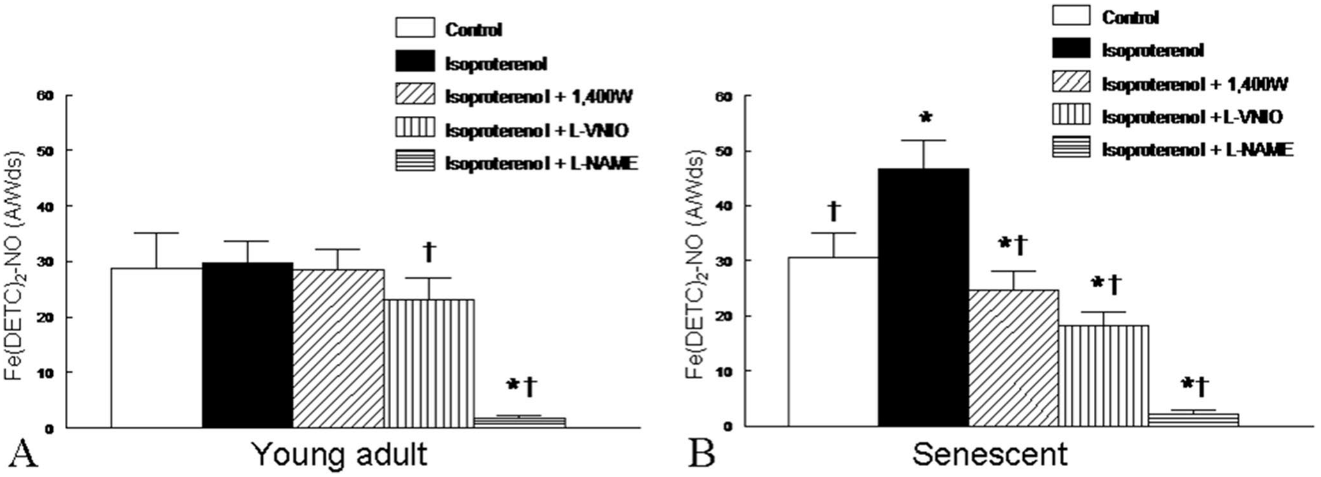

Fig. 5. Net nitric oxide (NO) level induced by *β*-adrenergic stimulation (isoproterenol), *in vitro*, in left ventricular myocardium from young adult (*A*) and senescent rats (*B*) using Fe(diethyldithiocarbamate [DETC]) electron paramagnetic resonance. A/Wds = amplitude of the NO-Fe(DETC)$_2$ in unit/weight, *i.e.*, mg dried sample A/W(ds); L-NAME = N^G-nitro-L-arginine methylester, nonspecific nitric oxide synthase (NOS) inhibitor; L-VNIO = vinyl-L-*N*-5(1-imino-3-butenyl)-L-ornithine, specific NOS1 inhibitor; 1400W = specific NOS2 inhibitor. n = 6 in each group. * *P* < 0.05 *versus* control group. † *P* < 0.05 *versus* isoproterenol group.

Therefore, the increased nitric oxide production assessed both *in vivo* and *in vitro* with *β*-adrenoceptor stimulation supports the hypothesis that β_3-adrenoceptor is involved in *β*-adrenergic dysfunction. Moreover, the dibutyryl-cyclic adenosine monophosphate concentrations used in our study are thought to produce a maximal positive inotropic effect in adult rats.[7,20] Nevertheless, cytosolic cyclic adenosine monophosphate concentration in senescent myocardium is impaired because of both the decreased β_1-adrenoceptor–Gs protein coupling and altered activity of adenylyl cyclase.[3,15,16] Therefore, the magnitude of the negative inotropic effect of β_3-adrenoceptor stimulation could be potentially underestimated. In contrast and as demonstrated previously, the contribution of β_3-adrenoceptor in young adult rat myocardium is insignificant.[7]

In our recent study of ischemic heart failure in the senescent rat, we demonstrated that NOS1, coupled to sarcoplasmic caveolin-3, was up-regulated in comparison with the matched control group, whereas NOS3 expression was decreased.[11] In another study performed in diabetic cardiomyopathy, we reported that nitric oxide production induced by β_3-adrenoceptor stimulation was exclusively issued by NOS1 coupled to sarcoplasmic caveolin-3.[7] In this study, using electron paramagnetic resonance *in vivo* and *in vitro* [11] further provided evidence that nitric oxide production induced by *β*-adrenoceptor stimulation involves NOS1 and/or NOS2 in senescent rats. However, we could not be more specific in our conclusions because only around 50% of the nitric oxide production recorded was induced by β_3-adrenoceptor stimulation. The rest of nitric oxide was from other

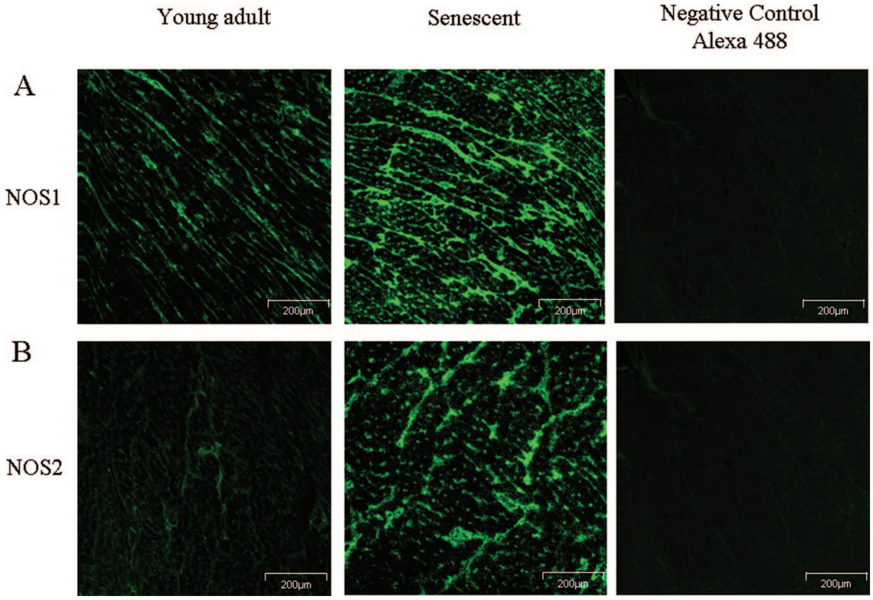

Fig. 6. Staining and imaging of both nitric oxide synthase (NOS) 1 (*A*) and NOS2 (*B*) from left ventricular myocardium in young adult and senescent rats by confocal microscopy. n = 3 in each group.

sources blocked by an unspecific NOS inhibitor (L-NAME). Using immunolabeling and confocal microscopy, both NOS1 and NOS2 were up-regulated in senescent myocardium in comparison with young adult myocardium. Nevertheless, *in vitro*, using left ventricular papillary muscles, the contribution of NOS1 seemed exclusive to the β_3-adrenoceptor pathway, whereas NOS2 inhibition did not significantly increase the positive inotropic effect of the β-adrenoceptor stimulation. Therefore, NOS2 contributed to the nitric oxide production but independently of the β_3-adrenoceptor pathway. Our findings support the idea that cardiac NOS1-derived nitric oxide is involved in the autocrine regulation of β-adrenergic (β_1- and β_3-adrenoceptor subtype) contractile responses in senescent heart and may explain, in part, the increased hemodynamic instability associated with the higher quantity of catecholamine during the perioperative period or in critical care during septic shock.[31–33] Therefore, these findings suggest that part of the marked altered response to the β-adrenergic stimulation could be corrected by the antagonism of the β_3-adrenoceptor pathway and could at least partly restore cardiac output by the inotropic effect induced. Further studies are needed to confirm these hypotheses in humans.

The following points should be considered when assessing the clinical relevance of our results. First, this study was performed in rat myocardium, which differs from human myocardium. Second, a part of this study conducted *in vitro* only dealt with intrinsic myocardial contractility. The inotropic effects observed on cardiac function using different adrenoceptor agonists or antagonists and different NOS inhibitors were independent of several *in vivo* factors, such as variations in cardiac loading, the autonomic nervous system, and compensatory mechanisms.[25] Nevertheless, the confluent results obtained using five different technologies are noteworthy. Third, despite the fact that the magnitudes of adenylate cyclase activity, peak calcium transients, and systolic cell shortening induced by dobutamine, a partial agonist of β-adrenoceptors, are known to be slightly less important than with isoproterenol, a full agonist of β-adrenoceptors,[34,35] we made the choice to use these two different nonselective β-adrenoceptor agonists *in vivo* and *in vitro*, respectively, because dobutamine is classically used *in vivo* for β-adrenoceptor stimulation in stress echocardiography[7,10,18] and isoproterenol is commonly used *in vitro* for β-adrenoceptor stimulation.[7,18,20,36] Anyway, the positive inotropic effect was significantly decreased *in vivo* with dobutamine as well as *in vitro* with isoproterenol. Fourth, part of the study was performed during halogenated anesthetic agent exposure, which is liable to interfere with the β-adrenergic stimulation in different kinds of cardiomyopathy.[20,37] However, our data obtained *in vivo* using a halogenated anesthetic agent is in agreement with those obtained *in vitro*. Fourth, inhibition of β_3-adrenoceptor could decrease its protective effect against arrhythmia in senescent heart as reported in acute myocardial infarction.[35] Further studies are needed to test this hypothesis.

In conclusion, in senescent heart, β_3-adrenoceptor plays an important role in the altered contractile response of β-adrenergic stimulation *via* induction of NOS1-nitric oxide.

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