

# Altered Contractile Response due to Increased $\beta_3$ -Adrenoceptor Stimulation in Diabetic Cardiomyopathy

## The Role of Nitric Oxide Synthase 1-derived Nitric Oxide

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**Background:** In the diabetic heart, the positive inotropic response to  $\beta$ -adrenoceptor stimulation is altered and  $\beta_1$  and  $\beta_2$  adrenoceptors are down-regulated, whereas  $\beta_3$  adrenoceptor is up-regulated. In heart failure,  $\beta_3$ -adrenoceptor stimulation induces a negative inotropic effect that results from endothelial nitric oxide synthase (NOS3)-derived nitric oxide production. The objective of our study was to investigate the role of  $\beta_3$ -adrenoceptor in diabetic cardiomyopathy.

**Methods:**  $\beta$ -Adrenergic responses were investigated *in vivo* (dobutamine echocardiography) and *in vitro* (left ventricular papillary muscle) in healthy and streptozotocin-induced diabetic rats. The effect of  $\beta_3$ -adrenoceptor inhibition on the inotropic response was studied *in vitro*. Immunoblots and NOS activities were performed in heart homogenates (electron paramagnetic resonance) and isolated cardiomyocytes. Data are mean percentage of baseline  $\pm$  SD.

**Results:** The impaired positive inotropic effect was confirmed in diabetes both *in vivo* ( $121 \pm 15\%$  vs.  $160 \pm 16\%$ ;  $P < 0.05$ ) and *in vitro* ( $112 \pm 5\%$  vs.  $179 \pm 15\%$ ;  $P < 0.05$ ). In healthy rat, the positive inotropic effect was not significantly modified in presence of  $\beta_3$ -adrenoceptor antagonist ( $174 \pm 20\%$ ), nonselective NOS inhibitor (*N*<sup>G</sup>-nitro-L-arginine methylester [L-NAME];  $183 \pm 19\%$ ), or selective NOS1 inhibitor (vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine [L-VNIO];  $172 \pm 13\%$ ). In diabetes, in parallel with the increase in  $\beta_3$ -adrenoceptor protein expression, the positive inotropic effect was partially restored by  $\beta_3$ -adrenocep-

tor antagonist ( $137 \pm 8\%$ ;  $P < 0.05$ ), L-NAME ( $133 \pm 11\%$ ;  $P < 0.05$ ), or L-VNIO ( $130 \pm 13\%$ ;  $P < 0.05$ ). Nitric oxide was exclusively produced by NOS1 within diabetic cardiomyocytes. NOS2 and NOS3 proteins were undetectable.

**Conclusions:**  $\beta_3$ -Adrenoceptor is involved in altered positive inotropic response to  $\beta$ -adrenoceptor stimulation in diabetic cardiomyopathy. This effect is mediated by NOS1-derived nitric oxide in diabetic cardiomyocyte.

DIABETES mellitus affects 170 million individuals worldwide today and is a growing public health problem. The World Health Organization estimates that there will be 300 million diabetic patients in 2025, which will represent 5.4% of the world population.<sup>1</sup> For the anesthesiologist, the proportion of diabetic patients undergoing surgery increases daily while both diabetes and the variability of blood glucose concentration are known to enhance the perioperative morbidity and mortality.<sup>2-4</sup> Diabetes is associated with a specific cardiomyopathy in 60% of well-controlled diabetic type II patients, independently of the coronary artery and valvular diseases, and hypertension.<sup>5</sup> Cardiac filling pressures are raised in 28% of them,<sup>6</sup> which are susceptible to rapid increase leading to congestive heart failure during the perioperative period. Diabetic cardiomyopathy results from a variety of alterations involving the sarcoplasmic reticulum,<sup>7</sup> calcium channels and intracellular calcium metabolism,<sup>8-10</sup> sodium-calcium exchange,<sup>11</sup> mitochondria,<sup>12</sup> and contractile proteins.<sup>13</sup> These anomalies lead to inotropic and lusitropic alterations, which are mainly responsible for slowing of contraction and relaxation velocities without significant change in the developed muscular tension.<sup>14</sup>

In the heart, at least three types of  $\beta$  adrenoceptors potentially modulate cardiac function. Stimulation of  $\beta_1$  and  $\beta_2$  adrenoceptors induces a positive inotropic effect, whereas  $\beta_3$ -adrenoceptor stimulation induces a negative inotropic effect,<sup>15</sup> involving the inhibitory  $G_i$  protein<sup>15</sup> and results from the production of nitric oxide by nitric oxide synthase (NOS) leading to an increase in intracellular cyclic guanosine monophosphate level.<sup>16</sup> During heart failure, the  $\beta_1$  adrenoceptor is down-regulated, whereas the  $\beta_3$  adrenoceptor and inhibitory G protein are up-regulated.<sup>17</sup> The functional loss of catecholamine positive control of cardiac contractility may result from the shift between the  $\beta_1$ -adrenoceptor-mediated positive pathway and  $\beta_3$ -adrenoceptor-mediated negative inotropic pathway.<sup>17</sup>

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In diabetic cardiomyopathy, an increase in sympathetic drive may represent an important mechanism for maintaining cardiac output in perioperative period or in intensive care units. Nevertheless, the positive inotropic response to  $\beta$ -adrenoceptor stimulation is markedly altered,<sup>18,19</sup> and the reasons for this  $\beta$ -adrenoceptor pathway dysfunction remain unknown. The distribution of  $\beta$  adrenoceptors is modified in diabetes and involves a down-regulation of both  $\beta_1$  and  $\beta_2$  adrenoceptors,<sup>20,21</sup> a decreased expression of inhibitory G protein,<sup>20</sup> and an up-regulation of  $\beta_3$  adrenoceptor.<sup>22</sup> It has been shown that NOS inhibition can restore the positive inotropic effect of  $\beta$ -adrenoceptor stimulation,<sup>19</sup> but the direct involvement of  $\beta_3$  adrenoceptor is still unknown in this disease.

Therefore, the aim of this experimental study was to test the hypothesis that  $\beta_3$ -adrenoceptor stimulation could decrease the magnitude of the positive inotropic effect of  $\beta$ -adrenergic stimulation within the diabetic cardiomyocyte and to determine the NOS isoform involved.

## Materials and Methods

Care of the animals used conformed to the published *Guiding Principles in the Care and Use of Animals*,<sup>23</sup> and the study was performed under the supervision of authorized researchers in authorized laboratories in accordance with the regulations issued by the French Ministry of Agriculture.

### Animals

Six-week-old male Wistar rats (Charles River, Saint Germain sur l'Arbresle, France) were divided into two groups, a healthy group and a diabetic group. Diabetes mellitus was induced using streptozotocin (65 mg/kg; Sigma Chemical, L'Isle d'Abeau Chesnes, France), which was injected intravenously. Diabetic rats were studied 4 weeks later, as previously reported.<sup>18</sup> All animals had continuous access to rat chow and were given water *ad libitum*. Transcutaneous determination of blood glucose level (Glucotrend; Boehringer, Mannheim, Germany) was performed to ensure that the rats became diabetic (*i.e.*, a blood glucose level > 35 mM). At the moment of euthanasia, blood samples were withdrawn from diabetic and control rats and were centrifuged at 5,000g for 15 min. The plasma fraction was collected and stored at  $-20^\circ\text{C}$  for further determination of glucose and bicarbonate concentration (Cobas Integra 400; Roche Diagnostic, Mannheim, Germany).

### Transthoracic Echocardiography

Echocardiography was performed on anesthetized rats (1–2% isoflurane) using a General Electric Vivid 7 instrument (Aulnay-sous-Bois, France) equipped with a 8- to

14-MHz linear transducer. Data were transferred online to a computer for analysis (EchoPAC PC version 2.0.x; General Electric, Velizy, Paris). Left ventricular diameter was measured in the parasternal long-axis and short-axis views in M mode. Left ventricular ejection fraction, using a modified version of Simpson's monoplane analysis, and left ventricular shortening fractions were measured.<sup>24</sup> Left ventricular diastolic parameters were derived from pulsed-wave spectral Doppler mitral flow (peak velocity of early E, late filling waves A, isovolumic relaxation time) and from pulsed-wave spectral mitral tissue Doppler imaging from apical view (peak early diastolic velocity, Ea), with the sample volume paced at the lateral corner of the mitral annulus as reported previously.<sup>25</sup> The E/A ratio was used to evaluate the diastolic function associated the E/Ea ratio, which is used to measure the left ventricular end-diastolic pressure independently of load conditions.<sup>25</sup> Dobutamine (4  $\mu\text{g}/\text{kg}$ ) was administered intraperitoneally,<sup>26</sup> and measures were performed when the increase in heart rate was stabilized.

### Isolated Left Ventricular Papillary Muscle

After brief anesthesia with pentobarbital sodium, the heart was quickly removed. The whole heart and the left ventricular 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  $\text{MgSO}_4$ , 1.1 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 2.5 mM  $\text{CaCl}_2$ , and 4.5 mM glucose) and maintained at  $29^\circ\text{C}$  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%  $\text{O}_2$  and 5%  $\text{CO}_2$ , resulting in a pH of 7.40. After a 60-min stabilization period with the initial muscle length at the apex of length-active isometric tension curve ( $L_{\text{max}}$ ), papillary muscles recovered their optimal mechanical performance. The extracellular concentration of  $\text{Ca}^{2+}$  was decreased from 2.5 mM to 0.5 mM because rat myocardial contractility is nearly maximal at 2.5 mM.<sup>18,27</sup> Conventional mechanical parameters at  $L_{\text{max}}$  were calculated from three twitches. The first twitch was isotonic and was loaded with the preload corresponding to  $L_{\text{max}}$ . 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  $L_{\text{max}}$ . We determined the maximum unloaded shortening velocity ( $V_{\text{max}}$ ) 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.

$\beta$ -Adrenoceptor stimulation was induced with cumulative concentrations of isoproterenol ( $10^{-8}$  to  $10^{-4}$  M), a nonselective  $\beta$ -adrenoceptor agonist, in the presence of phentolamine ( $10^{-6}$  M).

To assess the role of the  $\beta_3$  adrenoceptor, we studied additional groups exposed to *S*-cyanopindolol hemifumarate salt ( $10^{-12}$  M), a  $\beta_3$ -adrenoceptor antagonist previously studied in the rat,<sup>28</sup> or to *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME;  $10^{-5}$  M), an unspecific NOS inhibitor, or to vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO;  $10^{-4}$  M), a specific NOS1 inhibitor.<sup>29</sup> The combination of *S*-cyanopindolol and L-NAME was also tested. We studied additional experimental groups exposed to forskolin ( $5 \times 10^{-5}$  M), which directly activates adenylyl cyclase, or dibutyryl cyclic adenosine monophosphate (dB-cAMP;  $5 \times 10^{-4}$  M), a fat-soluble and diffusible analog of cyclic adenosine monophosphate resistant to hydrolysis in the intracellular involvement, as previously reported.<sup>30</sup> 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).

#### *Isolated Cardiomyocyte*

Single ventricular cardiomyocytes were enzymatically (1 mg/ml collagenase, Worthington type II) isolated from 10-week-old control or diabetic rats ( $n = 5$  per group), as previously described.<sup>31</sup> Isolated cells were then snap-frozen and used for NOS activity assay and Western blot experiments.

#### *NOS Activity, Immunoblotting, and Coimmunoprecipitation Assays*

The nitric oxide content was first assayed on left ventricular homogenates, as described previously<sup>32</sup> after formation of the paramagnetic adduct, Fe(II)nitric oxide(dithyldithiocarbamate [DETC])<sub>2</sub>, detectable by electron paramagnetic resonance spectrometry in samples treated (for 30 min at 37°C) with 0.5 mM Fe(II)(DETC)<sub>2</sub> complex as colloid.<sup>33</sup> Tissues were frozen in liquid nitrogen until electron paramagnetic resonance spectrometry measurements. Electron paramagnetic resonance spectrometry spectra were recorded on an MS100 spectrometer (Magnettech GmbH, Berlin, Germany) under the following conditions: temperature, 77°K; microwave frequency, 9.34 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; and time constant, 100 ms. After electron paramagnetic resonance spectrometry measurements, the tissue samples were dried and weighted. The relative (II) nitric oxide(DETC)<sub>2</sub> concentrations ( $A/W_{ds}$ ) were determined dividing the third component amplitude (A) of the three-line electron paramagnetic resonance spectrometry signal by the weight of the dried sample ( $W_{ds}$ ).

Nitric oxide synthase activity was also measured on isolated cardiac myocytes by the conversion of (<sup>3</sup>H)-L-arginine to (<sup>3</sup>H)-L-citrulline,<sup>29</sup> using a commercial kit (nitric oxide synthase assay kit; Calbiochem, Darmstadt, Germany). Assays for specific NOS1 and NOS2 activities were performed in the presence of L-VNIO ( $10^{-4}$  M) and specific NOS2 inhibitor (1,400 W, 0.1  $\mu$ M).<sup>29</sup>

Western blots and coimmunoprecipitation were performed with specific antibodies to measure protein expression of  $\beta_1$  and  $\beta_3$  adrenoceptors, NOS1, NOS2, NOS3, and caveolin 3. Because the most of the positive inotropic effect of  $\beta$ -adrenergic stimulation results from the  $\beta_1$  adrenoceptor, whereas  $\beta_2$  adrenoceptor had a insignificant role in positive inotropic effect in the diabetic cardiomyopathy,<sup>22</sup> Western blots were performed especially to quantify the  $\beta_1$ - and  $\beta_3$ -adrenoceptor protein expression. 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  $\beta$ -glycerophosphate, 0.2 mM orthovanadate, 0.1 mM leupeptin, and 0.001 mM aprotinin) for 1 h at 4°C. After centrifuging at 15,000g for 15 min at 4°C, supernatant protein concentrations were measured using the BCA protein assay kit (Pierce, Perbio Sciences, Brebières, France). For  $\beta$ -adrenoceptors, proteins were prepared, as previously described.<sup>34</sup> Fifty micrograms protein per lane was immunoblotted using rabbit polyclonal anti-NOS1 (1:500; Affinity Bioreagents, Saint Quentin en Yvelines, France), anti-NOS2 (1:1,000; Transduction Laboratories, San Jose, CA),<sup>29</sup> anti- $\beta_1$  adrenoceptor (1:1,000; Affinity Bioreagents), and goat polyclonal anti- $\beta_3$  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 the results were normalized with a link (actin) and the amounts of protein transferred on the membrane, and the relation between NOS1 and NOS3 was based on these corrected values. A control by performing a Western blot using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was performed and validated that there was no variation on protein gel loading in our hands.

For coimmunoprecipitation assays, cardiomyocytes lysates (150  $\mu$ g protein) were incubated overnight at 4°C with either a monoclonal antibody for Cav-3, polyclonal antibody for NOS3 or NOS1 (Santa Cruz Biotechnology) at a final concentration of 4  $\mu$ g/ml in 400  $\mu$ l intraperitoneal buffer (1% Triton X-100, and 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM orthovanadate, and 0.2 mM PMSF). Immune complexes

were precipitated for an additional 3 h by the addition of anti-mouse immunoglobulin G-conjugated agarose (50  $\mu$ l) or protein A-conjugated agarose (15  $\mu$ l), centrifuged, washed three times, and then boiled in Laemmli loading buffer. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with a monoclonal antibody for NOS3 or NOS1 (1:1,000; Santa Cruz Biotechnology), a monoclonal antibody for caveolin 3 (1:1,000; Transduction Laboratories), respectively.

### Statistical Analysis

Data are expressed as mean  $\pm$  SD. Comparison of two means was performed using the Student *t* test or Mann-Whitney test for nongaussian distribution of the variables. Comparison of several means was performed using one-way analysis of variance and 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 2001 software (Statistical Solutions Ltd., Cork, Ireland). Concentration-response curves were determined by fitting the data to the Hill sigmoid pharmacological model according to the following equation:

$$\text{Eff}_o = \text{Eff}_{\text{max}} \cdot (1 + (C_{50} \cdot C^{-1})^n)^{-1},$$

in which  $\text{Eff}_o$  is the observed effect,  $\text{Eff}_{\text{max}}$  is the maximum effect,  $C_{50}$  is the concentration that results in 50% of  $\text{Eff}_{\text{max}}$ , and *C* is the studied concentration and *n* the Hill coefficient.<sup>18</sup> Iterative nonlinear regression curve fitting was used to obtain the best fit (Matlab 1.2c software; The MathWorks, South Natick, MA).

## Results

We studied 52 healthy and 64 diabetic rats. No data were missing for the analyses in this study. Diabetic rats had significantly lower body weight (218  $\pm$  31 *vs.* 348  $\pm$  43 g; *P* < 0.05) and heart weight (472  $\pm$  75 *vs.* 715  $\pm$  76 mg; *P* < 0.05) than healthy rats, without significant difference in the heart weight to body weight ratio (2.2  $\pm$  0.2 *vs.* 2.1  $\pm$  0.2 mg/g; not significant [NS]). Blood glucose levels were four times higher in diabetic rats than in healthy rats (45  $\pm$  6 *vs.* 10  $\pm$  1 mM; *P* < 0.05). Serum bicarbonate levels were slightly higher in diabetic rats (25  $\pm$  4 *vs.* 24  $\pm$  4 mM; *P* < 0.05), showing that ketoacidosis did not occur.

### Contractile Responses to $\beta$ -Adrenergic Stimulation

Using echocardiography, we measured *in vivo* the baseline characteristics in healthy and diabetic rats. Systolic function was preserved in diabetic rats as shown by the normal values of ejection fraction (92  $\pm$  4% *vs.* 89  $\pm$  3%; *P* < 0.05) and shortening fraction (58  $\pm$  7% *vs.* 55  $\pm$  4%; NS). The heart rate was not significantly different

**Table 1. Inotropic Response to  $\beta$ -Adrenoceptor Stimulation (4  $\mu$ g/kg Dobutamine) in Healthy and Diabetic Rats *In Vivo* Using Echocardiography**

	Healthy Rats (n = 10)	Diabetic Rats (n = 8)
Heart rate, % baseline value	109 $\pm$ 6*	105 $\pm$ 7*
Ejection fraction, % baseline value	112 $\pm$ 3*	104 $\pm$ 1*†
Shortening fraction, % baseline value	160 $\pm$ 16*	121 $\pm$ 15*†

Values are mean  $\pm$  SD.

\* *P* < 0.05 *vs.* baseline value. † *P* < 0.05 *vs.* healthy rats.

between healthy and diabetic rats (338  $\pm$  36% *vs.* 318  $\pm$  26%, respectively; NS). In contrast, diastolic function was altered in the diabetic group as shown by the prolongation of isovolumic relaxation time (29  $\pm$  8 *vs.* 22  $\pm$  1 ms; *P* < 0.05) and the increased value E/A (2.6  $\pm$  0.9 *vs.* 1.3  $\pm$  0.2; *P* < 0.05). Left ventricular end-diastolic pressure was increased as shown by the E/Ea ratio (16  $\pm$  4 *vs.* 12  $\pm$  3; *P* < 0.05). These results were confirmed *in vitro* using 56 left ventricular papillary muscles from healthy rats and 64 from diabetic rats. Maximum unloaded shortening velocity was significantly decreased in diabetic rats (2.73  $\pm$  0.36 *vs.* 3.24  $\pm$  0.33  $L_{\text{max}}$ /s; *P* < 0.05), whereas active force was not (64  $\pm$  28 *vs.* 62  $\pm$  18 mN/mm<sup>2</sup>; NS). Prolongation of the duration of contraction was observed in diabetic rats as shown by the prolongation of time to peak shortening (212  $\pm$  24 *vs.* 173  $\pm$  13 ms; *P* < 0.05) and time to peak force (186  $\pm$  25 *vs.* 149  $\pm$  16 ms; *P* < 0.05).

$\beta$ -Adrenoceptor stimulation induced a marked positive inotropic effect in healthy rats both *in vivo* (table 1) and *in vitro* (table 2 and fig. 1). This positive inotropic effect was markedly diminished in diabetic rats *in vivo* (table 1) and *in vitro* (table 2 and fig. 1).

*In vitro*, *S*-cyanopindolol *per se* did not significantly modify active force in healthy (100  $\pm$  5% of baseline; NS) and diabetic (100  $\pm$  5% of baseline; NS) rats. In the presence of the  $\beta_3$ -adrenoceptor antagonist *S*-cyanopindolol, the positive inotropic effect of  $\beta$ -adrenoceptor stimulation was not significantly modified in healthy rats. In contrast, *S*-cyanopindolol partially restored the positive inotropic effect of  $\beta$ -adrenoceptor stimulation in diabetic rats (table 2 and fig. 1).

*L*-NAME *per se* did not significantly modify active force in healthy (100  $\pm$  5% of baseline; NS) and diabetic (100  $\pm$  5% of baseline; NS) rats. In the presence of *L*-NAME, a nonselective NOS inhibitor, the positive inotropic effect of  $\beta$ -adrenoceptor stimulation was not significantly modified in healthy rats. In contrast, *L*-NAME partially restored the positive inotropic effect of  $\beta$ -adrenoceptor stimulation in diabetic rats (table 2 and fig. 1). No further improvement was observed with the association of *S*-cyanopindolol and *L*-NAME (table 2).

*L*-NIO *per se* did not significantly modify isometric active force normalized per cross-sectional area in healthy (100  $\pm$  1% of baseline; NS) and diabetic (100

**Table 2. Effects of S-Cyanopindolol, L-NAME, and Both S-Cyanopindolol and L-NAME and L-VNIO on the Inotropic Responses to  $\beta$ -Adrenoceptor Stimulation (Isoproterenol) in Healthy and Diabetic Rats (n = 8 Papillary Muscles in Each Group)**

	Healthy Rats		Diabetic Rats	
	$V_{max}$	AF	$V_{max}$	AF
Eff <sub>max</sub> , %				
Control	182 ± 11*	179 ± 15*	128 ± 7*†	112 ± 5*†
S-cyanopindolol	178 ± 7*	174 ± 20*	161 ± 12*†‡	137 ± 8*†‡
L-NAME	183 ± 10*	183 ± 19*	166 ± 12*†‡	133 ± 11*†‡
S-cyanopindolol and L-NAME	189 ± 18*	181 ± 21*	156 ± 15*†‡	133 ± 13*†‡
L-VNIO	185 ± 12*	172 ± 13*	158 ± 19*†‡	130 ± 13*†‡
C <sub>50</sub> , $\mu$ M				
Control	0.11 ± 0.06	0.16 ± 0.12	0.07 ± 0.06	0.07 ± 0.05
S-cyanopindolol	0.08 ± 0.08	0.10 ± 0.07	0.14 ± 0.11	0.29 ± 0.26
L-NAME	0.07 ± 0.09	0.08 ± 0.11	0.16 ± 0.11†	0.45 ± 0.83
S-cyanopindolol and L-NAME	0.07 ± 0.04	0.14 ± 0.08	0.06 ± 0.05	0.11 ± 0.12
L-VNIO	0.10 ± 0.09	0.42 ± 0.26	0.09 ± 0.06	0.10 ± 0.14

Values are mean ± SD.

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

AF = active force per cross-sectional area; C<sub>50</sub> = concentration of isoproterenol that results in 50% of Eff<sub>max</sub>; Eff<sub>max</sub> = maximum effect in percentage of baseline value; L-NAME = N<sup>G</sup>-nitro-L-arginine methylester; L-VNIO = vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine; V<sub>max</sub> = maximum unloaded shortening velocity.

± 2% of baseline; NS) rats. In the presence of L-VNIO, a selective NOS1 inhibitor, the positive inotropic effect of  $\beta$ -adrenoceptor stimulation was not significantly modified in healthy rats. In contrast, L-VNIO partially restored the positive inotropic effect of  $\beta$ -adrenoceptor stimulation in diabetic rats (table 2 and fig. 1).

Figure 2 shows the comparison of the stimulation of  $\beta$ -adrenoceptors by isoproterenol, the stimulation of adenylyl cyclase by forskolin, or direct stimulation by dB-cAMP. The magnitude of restoration of active force obtained with S-cyanopindolol (78 ± 10% of the mean response in healthy rats) was not significantly different from those of forskolin (76 ± 16% of the mean response in healthy rats) or of dB-cAMP (83 ± 18% of the mean response in healthy rats). Finally, S-cyanopindolol did

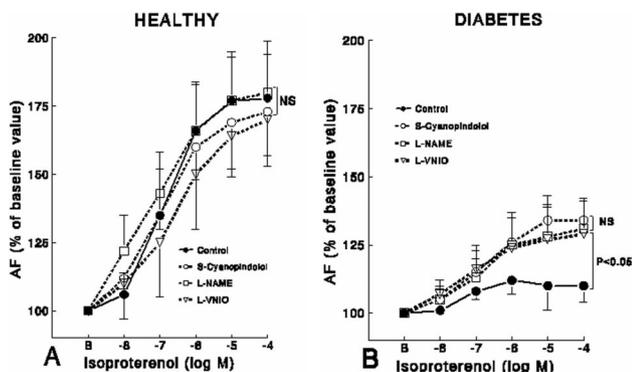
not significantly modify the positive inotropic effect of forskolin in diabetic rats (fig. 3).

#### Expression of $\beta$ -Adrenoceptor Subtypes

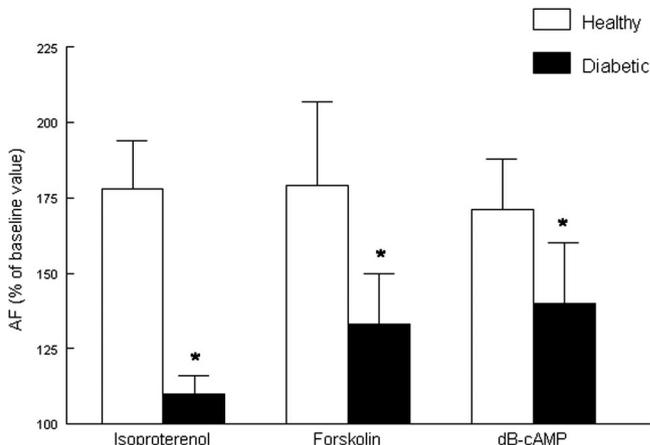
In agreement with the functional changes observed in the papillary muscle experiments, we found that the abundance of protein for  $\beta_1$  adrenoceptor was reduced by 33% in diabetic hearts compared with levels measured in healthy hearts (fig. 4). In contrast, compared with levels observed in healthy hearts,  $\beta_3$  adrenoceptors were significantly increased in diabetic hearts (fig. 4).

#### Potential Role of NOS-increased Nitric Oxide Production in $\beta_3$ -Adrenoceptor Effect

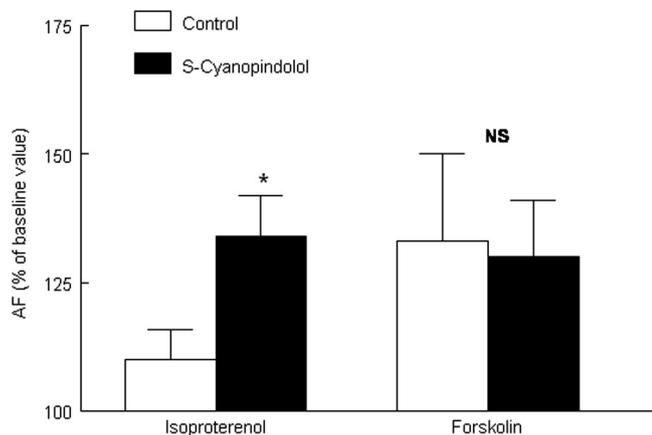
In failing heart, previous studies have demonstrated that the contractile effect of  $\beta_3$ -adrenoceptor agonist is



**Fig. 1. Inotropic response to  $\beta$ -adrenoceptor stimulation in healthy (A) and diabetic (B) rats, under high load. AF = isometric active force normalized per cross-sectional area; NS = not significant. S-cyanopindolol ( $\beta_3$ -adrenoceptor antagonist), N<sup>G</sup>-nitro-L-arginine methylester (L-NAME; nonspecific nitric oxide synthase [NOS] inhibitor) and vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO; specific NOS1 inhibitor) markedly restored the positive inotropic of  $\beta$ -adrenoceptor stimulation in diabetic rats as compared with healthy rats. Data are mean percentage of baseline value ± SD (n = 8 in each group).**



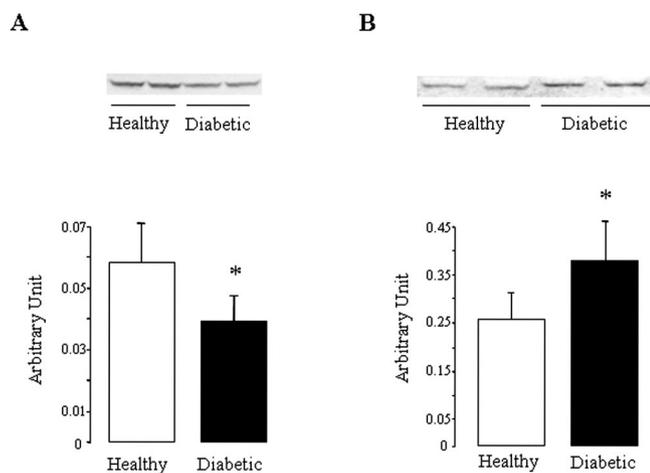
**Fig. 2. Comparison of the positive inotropic effects of isoproterenol, dibutyryl cyclic adenosine monophosphate (dB-cAMP;  $5 \times 10^{-4}$  M), and forskolin ( $5 \times 10^{-5}$  M), in left ventricular papillary muscles from healthy and diabetic rats, under high load. AF = isometric active force normalized per cross-sectional area. Data are mean percentage of baseline value ± SD (n = 8 in each group). \*  $P < 0.05$  versus healthy group.**



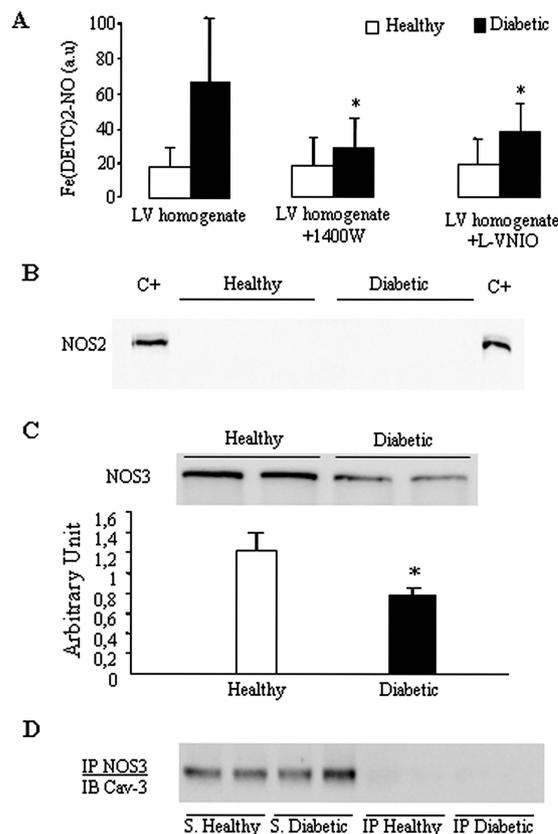
**Fig. 3.** Effect of *S*-cyanopindolol ( $10^{-12}$  M) on the positive inotropic effects of isoproterenol ( $10^{-4}$  M) and forskolin ( $5 \times 10^{-5}$  M) in left ventricular papillary muscles from diabetic rats, under high load. AF = isometric active force normalized per cross-sectional area; NS = not significant. Data are mean percentage of baseline value  $\pm$  SD ( $n = 8$  in each group). \*  $P < 0.05$  versus control diabetic group.

mediated, at least in part, through a stimulation of NOS3-induced nitric oxide production within the cardiomyocytes. To determine the potential relation between β<sub>3</sub>-adrenoceptor negative inotropic effect and nitric oxide, we first determined nitric oxide production in total left ventricular homogenates, using Fe-DETC electron paramagnetic resonance spectrometry (Magnettech GmbH). We observed that total nitric oxide production was not only significantly increased in diabetic rat hearts (fig. 5), but also that, using specific NOS isoform inhibitors, the main sources for the increase in cardiac nitric oxide production in left ventricular homogenates, containing endothelial cells, were NOS2 and NOS1.

In additional experiments, to assess more specifically the NOS isoform involved in this increased nitric oxide production within the cardiomyocyte exclusively, we performed both Western blot and coimmunoprecipitation

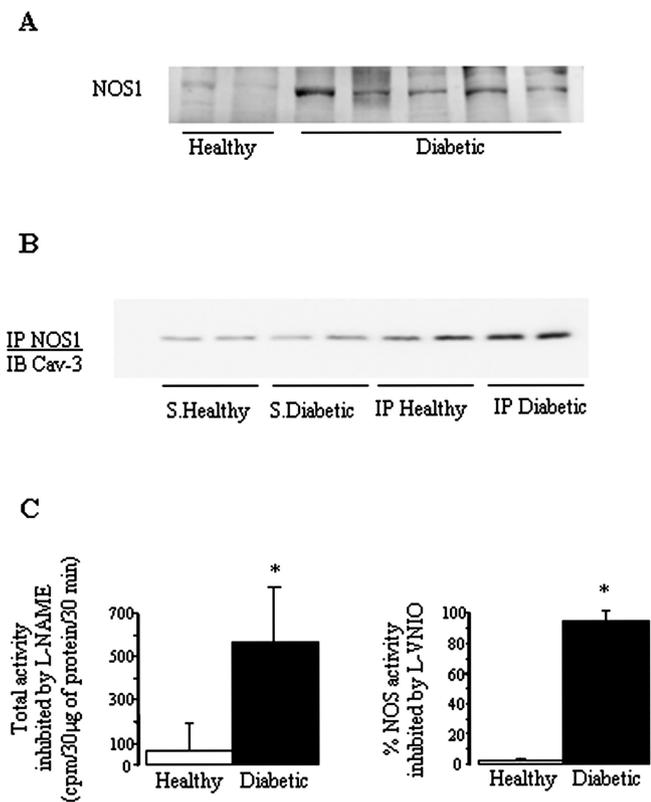


**Fig. 4.** Representative Western blot and densitometric data reflecting β<sub>1</sub>-adrenoceptor (A) and β<sub>3</sub>-adrenoceptor (B) expression in healthy and diabetic heart samples. Results are expressed as mean  $\pm$  SD. \*  $P < 0.05$  versus healthy group.



**Fig. 5.** (A) Net nitric oxide (NO) levels in left ventricle homogenates from healthy and diabetic rats using Fe(diethyldithiocarbamate [DETC]) electron paramagnetic resonance ( $n = 6$  in each group). L-VNIO = vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine. (B) Representative Western blot for NO synthase (NOS) 2 in isolated cardiomyocytes from healthy and diabetic rats. C+ corresponds to positive controls. (C) Representative Western blot for NOS3 left ventricle homogenates from healthy and diabetic rats. (D) Coimmunoprecipitation NOS3/Cav-3 from cardiomyocyte extracts of healthy and diabetic rats. IP = immunoprecipitate; S. = supernatant fraction. \*  $P < 0.05$  versus healthy group.

on isolated cardiomyocytes from healthy and diabetic rat hearts. We were unable to detect any signal for NOS2 protein expression in either group (fig. 5). NOS3 protein expression was significantly decreased in diabetic homogenate hearts (fig. 5). To determine the potential role of cardiomyocyte NOS1-derived nitric oxide or NOS3-derived nitric oxide in β<sub>3</sub>-adrenoceptor functional effect, NOS1 and NOS3 were immunoprecipitated with anti-caveolin-3 antibody (figs. 5 and 6). Although immunoblotting with monoclonal anti-caveolin-3 antibody revealed one band in the supernatant, no signal for NOS3 protein was immunoprecipitated in cardiomyocytes extracted from both groups. The same results were obtained when caveolin 3 was immunoprecipitated with polyclonal anti-NOS3 antibody. In contrast, Western blot analyses confirmed significantly greater expression of NOS1 protein in diabetic than in healthy cardiomyocytes homogenates (fig. 6). Immunoblotting with monoclonal anti-caveolin-3 antibody revealed interaction between NOS1 and caveolin 3 in cardiomyocytes extracts both in



**Fig. 6.** (A) Representative Western blot for nitric oxide synthase (NOS) 1 in isolated cardiomyocytes from healthy and diabetic rats. (B) Coimmunoprecipitation NOS1/Cav-3 from cardiomyocyte extracts of healthy and diabetic rats. IP = immunoprecipitate; S = supernatant fraction. (C) Bar graphs displaying total activity using  $N^G$ -nitro-L-arginine methylester (L-NAME; nonselective NOS inhibitor) and percentage NOS activity inhibited by vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO; NOS1 inhibitor) in isolated cardiomyocytes from healthy and diabetic rats. Results are expressed as mean  $\pm$  SD ( $n = 5$  in each group). \*  $P < 0.05$  versus healthy group.

healthy and in diabetic rats (fig. 6). To confirm the above results, we determined the specific activity of NOS1 in the same preparation of isolated cardiomyocytes by the conversion of ( $^3$ H)-L-arginine to ( $^3$ H)-L-citrulline, using the specific NOS1 inhibitor L-VNIO. With L-VNIO ( $10^{-4}$  M), more than 90% of total cardiomyocyte calcium-dependent NOS activity was found to be attributable to NOS1 in diabetic rats (fig. 6).

## Discussion

In the current study, we confirmed *in vivo* and *in vitro* the markedly decreased positive inotropic effect of  $\beta$ -adrenoceptor stimulation as previously reported *in vitro* in diabetic rats.<sup>13,15,18-20</sup> We observed that this effect was partially restored using a direct  $\beta_3$ -adrenoceptor antagonist, a nonselective NOS inhibitor, or a selective NOS1 inhibitor in diabetic heart and confirmed that  $\beta_3$  adrenoceptor was up-regulated and  $\beta_1$  adrenoceptor was down-regulated. Moreover, the increased nitric oxide production in diabetic cardiomyocyte was attribut-

able to the NOS1 activity. These results suggest that  $\beta_3$  adrenoceptor plays an important role in the  $\beta$ -adrenergic dysfunction associated with diabetic cardiomyopathy and that this effect is exclusively mediated by NOS1-derived nitric oxide.

As reported previously, we observed important alterations in the myocardium of diabetic rats using left ventricular papillary muscles.<sup>7,14,18-20</sup> In particular, we noted a decrease in  $V_{max}$  without change in active force, which was associated with a marked prolongation of the contraction phase. The decrease in  $V_{max}$  has been associated with an isomyosin shift from V1 ( $\alpha\alpha$  dimer) to V3 ( $\beta\beta$  dimer),<sup>35</sup> whereas the prolongation of contraction has been related to a slower cross-bridge cycling rate, a slower  $Ca^{2+}$  release from the sarcoplasmic reticulum, and an alteration of  $I_{to}$  potassium current.<sup>36</sup> Using echocardiography, we confirmed *in vivo* the diastolic dysfunction usually described in diabetic cardiomyopathy as shown by the prolongation of isovolumic relaxation time and the increases in E/A and E/Ea, which is independent of loading conditions.<sup>37</sup> Systolic function was still unchanged at this relatively short time of disease evolution (4 weeks of diabetes) in accordance with unchanged active force in papillary muscles.

The role of  $\beta_3$  adrenoceptor seems well established both in healthy and failing hearts.<sup>15-17,38-40</sup> In this specific context, it has been shown that stimulation of  $\beta_3$  adrenoceptors results in NOS3-derived nitric oxide which is at the beginning of cyclic guanosine monophosphate production.<sup>16,38,41</sup> Thereafter, cyclic guanosine monophosphate activates inhibitory G proteins, which decrease the dB-cAMP production resulting from  $\beta_1$ -adrenoceptor stimulation and thus induces a negative inotropic effect.<sup>15,16,39,41</sup> In our study, we confirmed the predominance of  $\beta_1$ -adrenoceptor protein expression in comparison with the  $\beta_3$ -adrenoceptor protein in healthy heart.

Using left papillary muscles, the positive inotropic effect of  $\beta$ -adrenoceptor stimulation was not significantly enhanced by the  $\beta_3$ -adrenoceptor antagonist administration as previously reported.<sup>42</sup>

In diabetes, using dobutamine echocardiography, we confirmed *in vivo* the impaired positive inotropic effect of  $\beta$ -adrenoceptor stimulation previously reported in left ventricular papillary muscles.<sup>18</sup> We also confirmed the overexpression of  $\beta_3$ -adrenoceptor protein and the down-regulation of  $\beta_1$ -adrenoceptor protein, in accordance with a previous study.<sup>22</sup> Because of the collapsed positive inotropic effect of the  $\beta$ -adrenergic stimulation, we have chosen to use a  $\beta_3$ -adrenoceptor antagonist able to restore part of the positive inotropic effect rather than an agonist of  $\beta_3$  adrenoceptor because it would have been difficult to demonstrate a significant difference as compared with the nearly undetectable positive inotropic effect noted in diabetic cardiomyopathy. To test the hypothesis of  $\beta_3$ -adrenoceptor involvement, we have

used *S*-cyanopindolol, a specific antagonist of the  $\beta_3$  adrenoceptor in rat.<sup>28</sup> The positive inotropic effect of  $\beta$ -adrenoceptor stimulation was partially restored by *S*-cyanopindolol in diabetic left ventricular papillary muscle. In addition, the administration of L-NAME to inhibit nitric oxide produced by the stimulation of  $\beta_3$ -adrenoceptor was also able to restore the positive inotropic effect of  $\beta$ -adrenoceptor stimulation in diabetic rats in accordance with studies performed in heart failure.<sup>17</sup> The effects of *S*-cyanopindolol and L-NAME were comparable, and no further improvement was noted with their association. The magnitude of the maximum restoration induced by inhibition of  $\beta_3$  adrenoceptor could have been underestimated because we used a very low concentration of *S*-cyanopindolol. Nevertheless, it should be pointed out that higher concentrations could also partly antagonize  $\beta_1$  adrenoceptors because specificity is likely to be at least partly lost at high concentrations, as we observed in a preliminary study (data not shown). However, the fact that the association of *S*-cyanopindolol and L-NAME was not superior to each drug alone suggests that the correction reached its maximum. A complete restoration with  $\beta_3$ -adrenoceptor inhibition was not expected because diabetic cardiomyopathy results from a variety of alterations not only related to the adrenoceptor signaling pathway. In fact, through the direct activation of adenylyl cyclase by forskolin or the direct exposure to dB-cAMP, we observed that these alterations were also partly responsible for the decreased responses in diabetic rats. Our results suggest that most of the alterations responsible for the impairment in the  $\beta$  adrenoceptors and situated upward adenylyl cyclase activity are linked to  $\beta_3$  adrenoceptor. Indeed, the residual impairment after inhibition of  $\beta_3$  adrenoceptors was comparable to that observed with forskolin or dB-cAMP.

In the current study, we observed that in diabetes, as in heart failure,<sup>17,38</sup>  $\beta_3$  adrenoceptor plays an important role in the impairment of the inotropic response to  $\beta$ -adrenoceptor stimulation. In heart failure, previous studies have suggested that  $\beta_3$ -adrenoceptor agonists increase nitric oxide production either through direct activation of a constitutively expressed NOS, most likely NOS3. NOS2 and NOS3 were thought to be the sole isoform responsible for increased nitric oxide production within the cardiomyocyte of the failing heart.<sup>17,38,41</sup> More recently, identification of the NOS1 isoform in cardiomyocytes has markedly improved the understanding of the critical role of NOS1-derived nitric oxide in the control of myocardial contractility both in the normal and the failing heart. NOS1 has been found to be associated with the cardiac ryanodine receptor  $\text{Ca}^{2+}$  release channel in the cardiac sarcoplasmic reticulum, suggesting that cardiac NOS1-derived nitric oxide may modulate ion channels/transporters involved in myocardial  $\text{Ca}^{2+}$  cycling and contraction.<sup>29</sup> In diabetic cardiomyopathy, for the first time and in accordance with our previous

results regarding the role of NOS1-induced nitric oxide production in heart failure,<sup>29,43,44</sup> we have demonstrated that NOS1 protein expression was predominant and that nitric oxide production resulted quasi-exclusively from NOS1 within the isolated diabetic cardiomyocytes. The NOS3 isoform protein expression was found in homogenate heart of diabetic rats, probably within endothelial cells, but was not in the cardiomyocyte. NOS2 protein expression was undetectable in the same preparations.

Our data support the idea that cardiac NOS1-derived nitric oxide is involved in the autocrine regulation of  $\beta$ -adrenergic ( $\beta_1$ - and  $\beta_3$ -adrenoceptor subtypes) contractile responses in diabetic cardiomyopathy and may explain, in part, the increased perioperative cardiovascular lability and the more frequent perioperative need of vasoactive agents observed in diabetic patients.<sup>45</sup> Therefore, these findings suggest that part of the collapsed response to the  $\beta$ -adrenergic stimulation could be corrected by the antagonism of the  $\beta_3$ -adrenoceptor pathway and could at least partly restore cardiac output by the inotropic effect induced.

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, streptozotocin induces diabetes of type I, which is not the most frequently encountered clinical form. Nevertheless, streptozotocin-induced diabetic rat is widely used and is recognized as an appropriate animal model of diabetes, most of myocardial dysfunction being present after 4 weeks without any streptozotocin toxicity. Third, because L-NAME and L-VNIO restored, in part, the positive inotropic effect of  $\beta$ -adrenergic stimulation, we have chosen to use a specific antagonist of  $\beta_3$  adrenoceptor more than a specific agonist to compare their respective capacity to restore the positive inotropic effect. Whether a specific agonist of  $\beta_3$  adrenoceptor could be used, comparison between positive and negative inotropic magnitudes was less relevant. Fourth, a part of this study was performed during anesthetic agent exposure, which is susceptible to interfere with the  $\beta$ -adrenergic stimulation in diabetes as previously reported.<sup>18,46</sup> Last, the effects of insulin therapy were not studied. However, although insulin therapy may restore the diastolic dysfunction at the beginning of the disease,<sup>22</sup> apoptosis and fibrosis rapidly occur in diabetic heart,<sup>47</sup> and therefore, insulin therapy may not be sufficient to reverse all abnormalities involved in diabetic cardiomyopathy.

In conclusion, we observed that the  $\beta_3$  adrenoceptor plays an important role in the  $\beta$ -adrenergic dysfunction associated with diabetic cardiomyopathy and that  $\beta_3$ -adrenoceptor effect is mediated by NOS1-derived nitric oxide. The development of new therapeutic strategies based on these findings might be interesting for the treatment of shock or hemodynamic instability in diabetic patients.

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