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Research Article

The Endothelial Dysfunction Could Be a Cause of Heart Failure with Preserved Ejection Fraction Development in a Rat Model

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50% of patients with heart failure have a preserved ejection fraction (HFpEF). Numerous studies have investigated the pathophysiological mechanisms of HFpEF and have shown that endothelial dysfunction plays an important role in HFpEF. Yet no studies answered whether endothelial dysfunction could be the cause or is the consequence of HFpEF. Recently, we have shown that the endothelial overexpression of human β_3-adrenoreceptor (Tgβ_3) in rats leads to the slow development of diastolic dysfunction over ageing. The aim of the study is to decipher the involvement of endothelial dysfunction in the HFpEF development. For that, we investigated endothelial and cardiac function in 15-, 30-, and 45-week-old wild-type (WT) and Tgβ_3 rats. The aortic expression of $^{\bullet}$NO synthase (NOS) isoforms was evaluated by Western blot. Finally, electron paramagnetic resonance measurements were performed on aortas to evaluate $^{\bullet}$NO and $O_2^{\bullet-}$ production. Vascular reactivity was altered as early as 15 weeks of age in response to isoproterenol in Tgβ_3 aortas and mesenteric arteries. NOS1 (neuronal NOS) expression was higher in the Tgβ_3 aorta at 30 and 45 weeks of age (30 weeks: WT: 1.00 ± 0.21; Tgβ_3: 6.08 ± 2.30; 45 weeks: WT: 1.00 ± 0.12; Tgβ_3: 1.55 ± 0.17; $p < 0.05$). Interestingly, the endothelial NOS (NOS3) monomer form is increased in Tgβ_3 rats at 45 weeks of age (ratio NOS3 dimer/NOS3 monomer; WT: 1.00 ± 0.37; Tgβ_3: 0.13 ± 0.05; $p < 0.05$). Aortic $^{\bullet}$NO production was increased by NOS2 (inducible NOS) at 15 weeks of age in Tgβ_3 rats (+52% vs. WT). Aortic $O_2^{\bullet-}$ production was increased in Tgβ_3 rats at 30 and 45 weeks of age (+75% and+76%, respectively, vs. WT, $p < 0.05$). We have shown that endothelial dysfunction and oxidative stress are present as early as 15 weeks of age and therefore conclude that endothelial dysfunction could be a cause of HFpEF development.

1. Introduction

Over the past decade, cardiovascular diseases have been one of the leading causes of death worldwide [1]. Among these diseases, heart failure (HF) affects 2-3% of the world's population [2]. There are several forms of HF, including HF with preserved ejection fraction (HFpEF) which has no efficient treatment at present day. This type of HF is characterized by a diastolic dysfunction without major alteration of ejection fraction. The main clinical characteristics of patients with HFpEF are advanced age and female sex [3]. However, the mechanisms leading to HFpEF are not completely understood so far. Few years ago, Paulus and Tschöpe put forward the idea that endothelial function could be the common trigger of all HFpEF etiologies. More specifically, the nitric oxide ($^{\bullet}$NO) pathway was pointed out [4]. Indeed, the $^{\bullet}$NO is a major player in the maintenance of cardiovascular function. The $^{\bullet}$NO is mainly produced by the endothelial cells *via* the activation of the $^{\bullet}$NO synthase (NOS) which exists under three isoforms: the neuronal NOS (nNOS or

NOS1), the inducible NOS (iNOS or NOS2), and the endothelial NOS (eNOS or NOS3). In physiological conditions, most of the $^{\bullet}$NO production in the vessels and the heart is mediated by NOS3 [5]. Paracrine action of $^{\bullet}$NO, produced by endothelial cells, induces a relaxation of smooth muscle cells in the vessels. At the cardiac level, endothelial cells represent 20% of the cellular population and the $^{\bullet}$NO plays a role in cardiac contractility regulation [6]. The $^{\bullet}$NO production is partly mediated by the activation of β-adrenoceptors (β-AR) and more specifically the β_3-adrenoceptor (β_3-AR) through the activation of NOS3 or NOS1 [7, 8]. β_3-AR activation, expressed in cardiomyocytes and endothelial cells, induced $^{\bullet}$NO release [9]. Considering the putative link between HFpEF and endothelial dysfunction and the link between β_3-AR and $^{\bullet}$NO signaling, the purpose of the study is to evaluate the link between endothelial dysfunction and HFpEF development. An animal model that overexpresses the human β_3-AR (Tgβ_3) on endothelial cells has been developed. Previously, we have shown that Tgβ_3 animals overproduced $^{\bullet}$NO at the cardiac level and develop a diastolic dysfunction at 45 weeks of age, characteristic of HFpEF [10]. The aim of this study is to understand if endothelial dysfunction could be the cause of HFpEF.

2. Methods

2.1. Experimental Animals. All animal experimental protocols were approved by the Pays de la Loire Ethical Committee and were performed in accordance with the French law on animal welfare, EU Directive 2010/63/EU for animal experiments, the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 2011), and the 1964 Declaration of Helsinki and its later amendments, and all the animals were housed according to standard living conditions. All animals used in the study are male Sprague-Dawley rats. Tgβ_3 rats which overexpress human β_3-AR in endothelial cells and their control (WT) were used at 15, 30, and 45 weeks of age and fed *ad libitum* with a soy-free diet (Envigo, #2914C, Huntingdon, United Kingdom) as previously described [10]. Then, animals were euthanized, and the thoracic aorta and superior branch of mesenteric arteries were harvested in order to perform vascular reactivity (thoracic aorta and mesenteric arteries), electronic paramagnetic resonance (EPR) quantification of $^{\bullet}$NO and $O_2^{\bullet-}$ (thoracic aorta), and Western blot protein analysis (thoracic aorta) as described below.

2.2. Echocardiography. Echocardiography was performed on anesthetized rats (O_2/isoflurane mixture 1.5% with flow rate of 1 L/min) at 15, 30, and 45 weeks of age, using a Vingmed-General Electric ultrasound system (VIVID 7, Horten, Norway) equipped with a 10 MHz imaging probe and offline cine loop analysis software (Echopac TVI, GE-Vingmed Ultrasound) on the Therassay platform of Nantes, as previously described [10].

2.3. Vascular Reactivity for Pharmacological Studies. Rats were anesthetized with an O_2/isoflurane mixture (induction: 5% isoflurane, flow rate 1 L/min; maintenance: 2% isoflurane, flow rate 0.5 L/min). The thoracic aorta and mesenteric arteries were carefully excised and cleared of fat and connective tissue. Vascular tensions were recorded as previously described [11, 12]. Briefly, aortic and mesenteric rings were mounted on a multichannel isometric myograph (Danish Myo Technology) (95% O_2 and 5% CO_2) Krebs-Henseleit bicarbonate solution (millimolar concentrations: NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, KCl 4.5, glucose 11, and $CaCl_2$ 1.5) at 37°C [13]. To verify the endothelium integrity, rings were precontracted with phenylephrine (1 μM, Sigma-Aldrich, P6126) and then exposed to a single concentration of acetylcholine (10 μM, Sigma-Aldrich, A6625). The tension was recorded on precontracted rings with phenylephrine (1 μM). The vasodilation response to cumulative concentrations of isoproterenol, a nonselective β-AR agonist (1 nM to 100 μM, Sigma-Aldrich, I6504), and of CL 316 243, a β_3-AR agonist (1 nM to 100 μM, Tocris, 1499), was evaluated. The vasodilation response to isoproterenol (1 nM to 100 μM) was repeated after 30 min of vessel incubation with NOS inhibitors prior to the phenylephrine precontraction: L-NIO, a NOS3 inhibitor (10 μM, N^5-(1-iminoethyl)-L-ornithine dihydrochloride, Tocris, 0546); vinyl-L-VNIO, a NOS1 inhibitor (10 μM, N^5-(1-imino-3-butenyl)-L-ornithine, L-VNIO, Enzo Life Sciences, ALX-270-216-M005); 1400W, a NOS2 inhibitor (10 μM, *N*-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride, Tocris, 1415); and L-NMMA, a nonselective NOS inhibitor (30 μM, NG-monomethyl-L-arginine acetate, Tocris, 0771) were evaluated. A chamber wire myograph was connected to a digital data recorder (MacLab/4e; AD Instruments), and recordings were analyzed using LabChart v7 software (AD Instruments).

2.4. Electronic Paramagnetic Resonance (EPR). EPR measurements were performed on aortas harvested from rats at 15, 30, and 45 weeks of age, as previously described [14]. Briefly, aortas were incubated 45 min at 37°C in a Krebs-HEPES colloid solution containing Na-diethyldithiocarbamate trihydrate (DETC—Sigma-Aldrich) mixed to $FeSO_4$-$7H_2O$ to form Fe^{2+}-$(DETC)_2$ as spin trap for $^{\bullet}$NO detection in an electromagnetic field. Five conditions were tested: without inhibitors, with L-NMMA (10 μM, negative control), with L-NIO (10 μM) and L-VNIO (10 μM), with L-NIO (10 μM) and 1400W (10 μM), and with L-VNIO (10 μM) and 1400W (10 μM). For $O_2^{\bullet-}$ detection, aortas were incubated 45 min at 37°C in a Krebs-HEPES solution containing 1-hydroxy-3methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH, 500 μM, Noxygen) as spin probe, deferoxamine (25 μM, Sigma-Aldrich) as an iron chelator, and DETC (5 μM) to minimize CMH autooxidation, with or without L-NMMA (10 μM). Then, each sample was snap-frozen in liquid nitrogen and analyzed in a Dewar flask at 77°K using an EPR Miniscope MS5000 (Freiberg Instruments, Germany). The instrument settings were as follows: microwave power of 10 mW; 1 mT or 0.400 mT of amplitude modulation for $^{\bullet}$NO and $O_2^{\bullet-}$, respectively; 100 kHz modulation frequency; sweep time of 150 s; and 3 scans for $^{\bullet}$NO measurements or 60 s and 3 scans for $O_2^{\bullet-}$ spectra. Signals were quantified by measuring and analyzing the total amplitude of the peaks of the spectra obtained, using the ESRS-Studio

software (Freiberg Instruments, Germany) and expressed in arbitrary units (A.U.) and normalized to dry weight of the sample.

2.5. Western Blot. Total proteins were extracted from aorta powder as previously described [15]. The amount of protein used for Western blot analysis was 25 μg. Western blots were performed with a migration in 4-15% polyacrylamide stain-free gels in order to evaluate the expression of the NOS1 (#4231S, Cell Signaling, Danvers, USA), NOS2 (AB5382, Millipore, USA), NOS3 (610296, BD Biosciences, San Diego, USA), and phosphorylated Ser1177 NOS3 (p-NOS3; #9571, Cell Signaling, Danvers, USA) (Table 1). Total NOS1, NOS2, and NOS3 expression was expressed as a ratio with stain-free quantification of total proteins. Phosphorylated Ser1177 NOS3 and NOS3 dimers were expressed as a ratio with a NOS3 monomer.

2.6. Statistical Analysis. Data were presented as mean ± SEM of n different rats. For the comparisons involving two groups, animal's significances were defined using the Mann-Whitney test. For vascular studies, a two-way ANOVA for repeated measures was used with the Bonferroni posttest. For electronic paramagnetic resonance studies, the Kruskal-Wallis test was used followed by an uncorrected Dunn's test. A value of $p < 0.05$ was considered significant. All statistical calculations and graphs were performed using GraphPad Prism software (version 8.00).

3. Results

3.1. β_3-AR Overexpression in the Long Term Induces Diastolic Dysfunction. HFpEF is characterized by a diastolic dysfunction without major alteration of ejection fraction. In order to validate that our rat develops a diastolic dysfunction as described in Dhot et al. [10], we performed echocardiographic analyses. As shown in Figure 1, heart rate and ejection fraction were similar in both WT and Tgβ_3 rats at the same age (Figure 1(a)). The E wave was not changed between WT and Tgβ_3 rats (Figure 1(b)). The A wave was decreased in the Tgβ_3 group compared to the WT group at 30 and 45 weeks of age (30 weeks of age: WT: 1.02 ± 0.03; Tgβ_3: 0.79 ± 0.05; 45 weeks of age: WT: 1.02 ± 0.03; Tgβ_3: 0.86 ± 0.04; $p < 0.05$) leading to a significant increase in the E/A ratio in the Tgβ_3 group (30 weeks of age: WT: 1.09 ± 0.04; Tgβ_3: 1.26 ± 0.06; 45 weeks of age: WT: 1.15 ± 0.01; Tgβ_3: 1.33 ± 0.04; $p < 0.05$) (Figure 1(b)). These data validated that our model develops a diastolic dysfunction throughout ageing.

3.2. β_3-AR Overexpression in the Long Term Induces Vasodilation Alteration. Concentration-dependent vasodilation to isoproterenol, a nonselective β-AR agonist, was significantly reduced in aortic rings from Tgβ_3 rats at the age of 15 weeks (Emax; WT: 94.5 ± 2.5; Tgβ_3: 74.1 ± 7.5; $p < 0.05$), 30 weeks (pD_2; WT: 6.73 ± 0.30; Tgβ_3: 5.49 ± 0.11; $p < 0.05$), and 45 weeks (pD_2; WT: 6.56 ± 0.21; Tgβ_3: 5.82 ± 0.15; $p < 0.05$) (Figure 2(a) and Table 2(a)). In mesenteric rings, concentration-dependent vasodilation to isoproterenol was significantly reduced in Tgβ_3 rats at 30 weeks of age (Emax; WT: 100.7 ± 4.6; Tgβ_3: 73.5 ± 10.4; $p < 0.05$) and at 45 weeks of age (pD_2; WT: 7.21 ± 0.15; Tgβ_3: 6.17 ± 0.17; $p < 0.05$) (Figure 2(b) and Table 2(b)). β3-AR overexpression seems to alter vasodilation of aortas and mesenteric arteries through the β-AR signaling pathway over ageing.

CL 316 243, a β_3-AR agonist, produced a concentration-dependent vasodilation in both WT and Tgβ_3 vessels. This vasodilation was similar between the two groups of rats at all evaluated ages in both aortic and mesenteric arteries (Figures 2(c) and 2(d) and Tables 2(a) and 2(b)). Interestingly, the difference in vasodilation in response to isoproterenol between the WT and Tgβ_3 groups did not seem to be associated with the β3-AR despite its overexpression in Tgβ_3 rats.

To evaluate the potential involvement of the $^{\bullet}$NO production through NO synthase (NOS) activity on the observed vascular dysfunction in Tgβ_3 rats, vasodilation induced by isoproterenol has been evaluated in the presence of L-NMMA, a nonselective NOS inhibitor. In these conditions, the vasodilation was similar between WT and Tgβ_3 at 15, 30, and 45 weeks of age on both thoracic aortic rings (Figure 2(e) and Table 2(a)) and mesenteric artery rings (Figure 2(f) and Table 2(b)). These results indicate that the altered vasodilation observed in Tgβ_3 rats at 30 and 45 weeks seems to be due to NOS activity and $^{\bullet}$NO production. In particular, L-NMMA blunted the vascular relaxation in mesenteric arteries in both WT and Tgβ_3 rats at 45 weeks, suggesting a central role of $^{\bullet}$NO in relaxation in resistance arteries at this age. Subsequently, we were interested in the involvement of different NOS isoforms in the vasodilation in order to highlight the link between β3-AR overexpression and $^{\bullet}$NO signaling.

3.3. At 15 Weeks of Age, Endothelial Function Is Not Altered by Endothelial β_3-AR Overexpression. As mentioned before, in our Tgβ_3 model, the diastolic dysfunction appeared at 30 weeks [10]. We investigated whether rats developed endothelial dysfunction over the time through the NOS expression and activity evaluation.

3.3.1. The Vasodilation Is Predominantly Mediated by NOS1 in Tgβ_3 Rats at 15 Weeks. At 15 weeks of age, vasodilation in response to isoproterenol was only altered in aortic rings from WT and Tgβ_3 rats with a decrease in maximal effect in the aorta of the Tgβ_3 group and a significant reduced response to isoproterenol stimulation (Figure 2(a) and Table 2(a)). To decipher the implication of each NOS isoform, several NOS inhibitors: L-NIO, L-VNIO, and 1400W which inhibit NOS3, NOS1, and NOS2, respectively, have been used.

In the presence of L-NIO, the maximal effect was reduced in the aortic rings from WT rats (WT: 60.0 ± 12.1; WT+L-NIO: 52.6 ± 7.80; $p < 0.05$) (Figure 3(a) and Table 3(a)). NOS3 inhibition using L-NIO had no significant impact on aorta and mesenteric artery reactivity from Tgβ_3 rats, suggesting reduced implication of $^{\bullet}$NO production from NOS3 in vascular relaxation of 15-week-old Tgβ_3 rats compared to WT (Figures 3(a) and 3(b) and Tables 3(a) and 3(b)). In

Table 1: Antibodies used for Western blotting experiments.

Protein of interest	Primary antibody			Secondary antibody		
	Reference	Species	Dilution	Reference	Species	Dilution
NOS1	Cell Signaling (4231)	Rabbit	1/500*	Cell Signaling (7074)	Goat	1/10000*
NOS2	Millipore (AB5382)	Rabbit	1/2000*			
NOS3	BD Biosciences (610296)	Mouse	1/1000*	Cell Signaling (7076)	Horse	
p-NOS3	Cell Signaling (9571)	Rabbit	1/1000*	Cell Signaling (7074)	Goat	

*Dilution carried out in 5% milk.

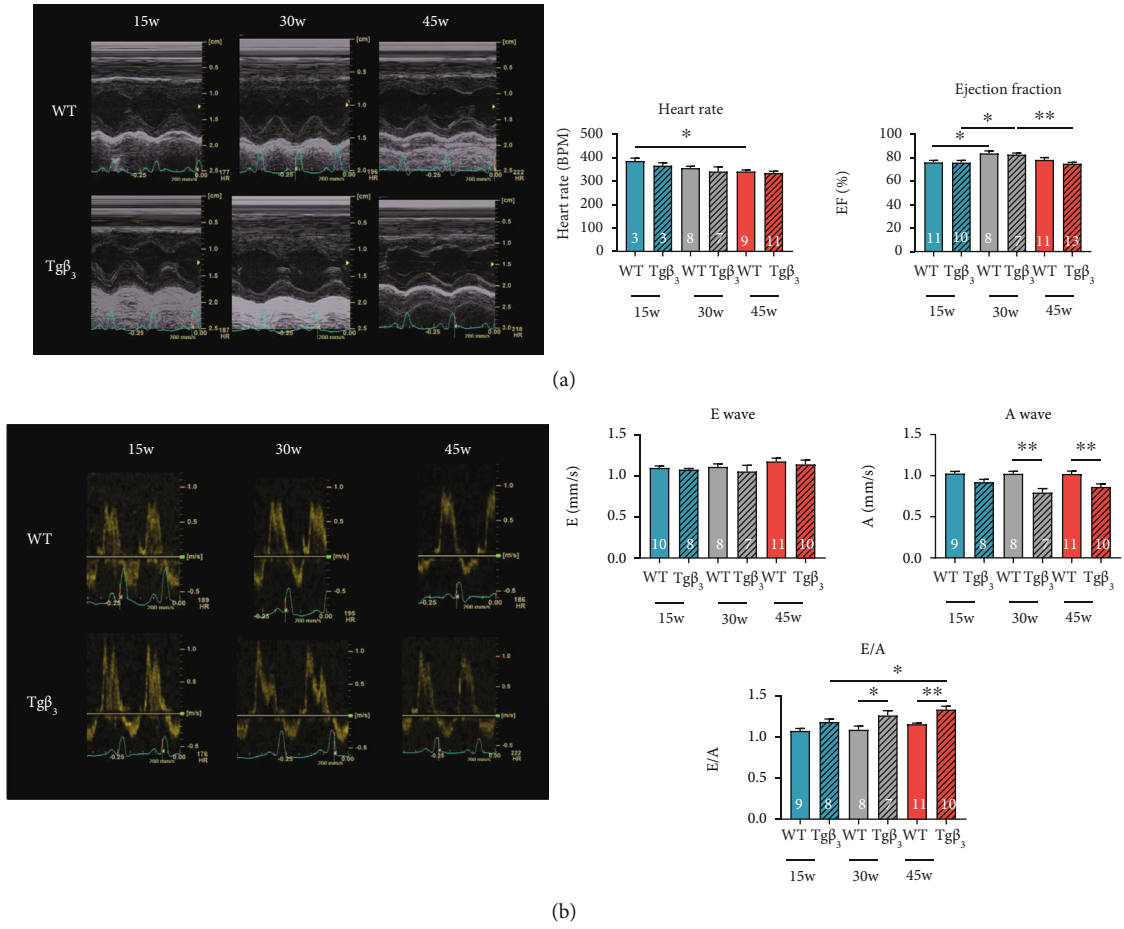
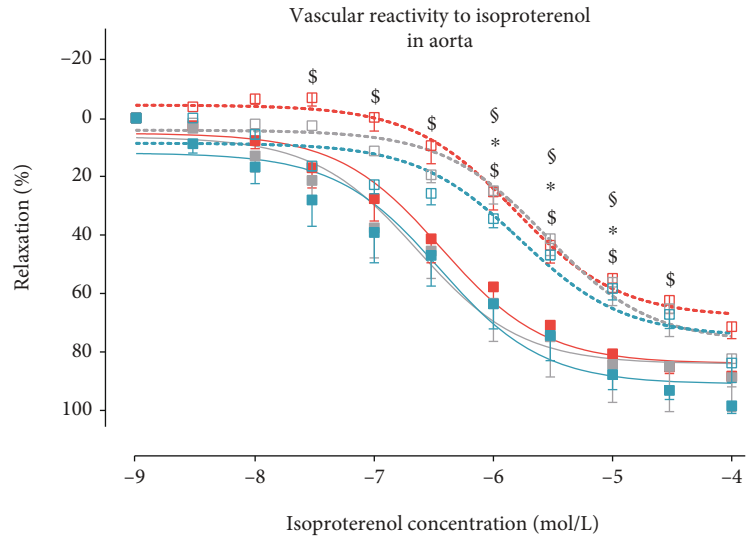


Figure 1: Evaluation of cardiac function in WT and Tgβ_3 rats at 15, 30, and 45 weeks of age. Evolution of systolic function (a) and diastolic function (b) in WT and Tgβ_3 rats at 15, 30, and 45 weeks of age. Values represent mean ± SEM. $n = 3$-13. $^*p < 0.05$.

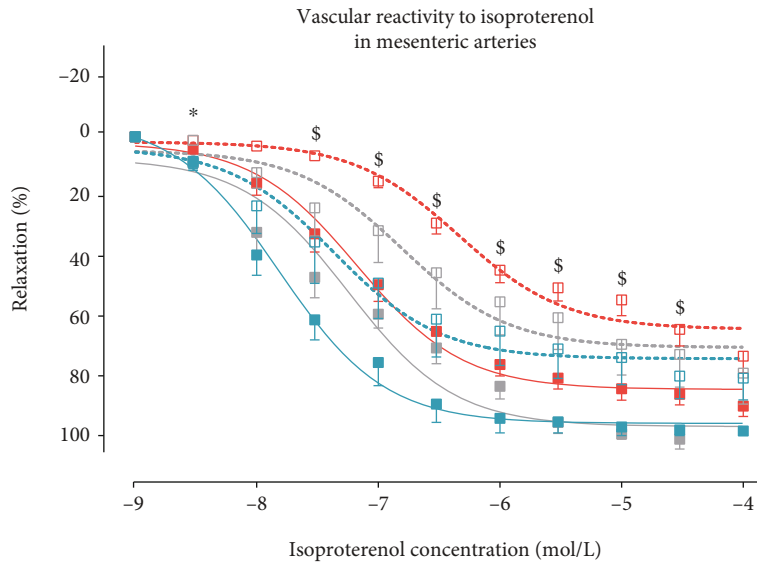
contrast, concentration-dependent vasodilation to isoproterenol in the presence of L-VNIO, a NOS1 inhibitor, was significantly decreased in the aortic rings of Tgβ_3 rats (Figure 3(c)), while the difference was not significant on mesenteric arteries (Figure 3(d)). In the presence of 1400W, a NOS2 inhibitor, the vasodilation in response to isoproterenol was not modified on both aortic and mesenteric rings of the two groups of rats (Figures 3(e) and 3(f) and Tables 3(a) and 3(b)). Taken together, these results suggest that vasodilation was predominantly mediated by $^{\bullet}$NO produced by NOS1 in the aorta from Tgβ_3 rats at 15 weeks of age.

3.3.2. NOS2 Expression Is Increased in Tgβ_3 Rats at 15 Weeks. The aortic ratio of NOS3 dimer/monomer and p-NOS3/NOS3 monomer protein expression levels and NOS1 expression was not significantly modified at 15 weeks of age (Figures 4(a), 4(b), and 4(d)). The overexpression of β_3-AR induced a significant 2-fold increase in NOS2 protein expression in the aorta (WT: 1.00 ± 0.14; Tgβ_3: 2.06 ± 0.31; $p < 0.05$) (Figure 4(c)).

3.3.3. $^{\bullet}$NO and $O_2^{\bullet-}$ Production Is Unchanged in the Tgβ_3 Rats at 15 Weeks. $^{\bullet}$NO production remained stable on

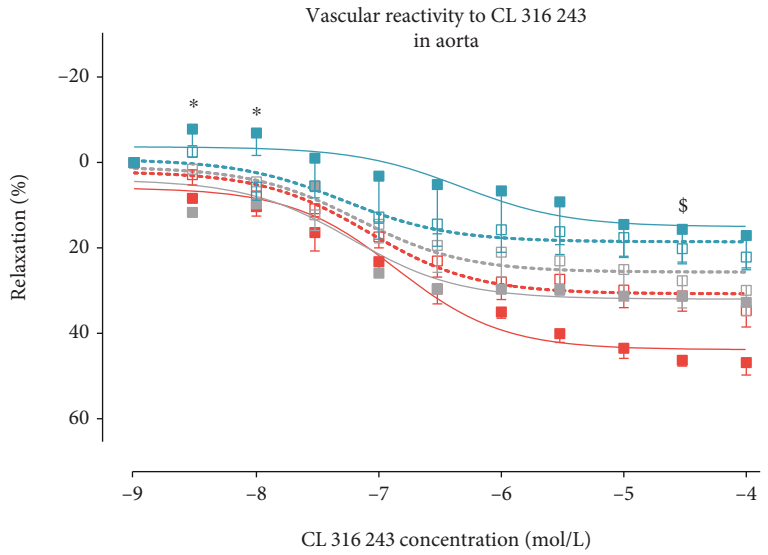


(a)

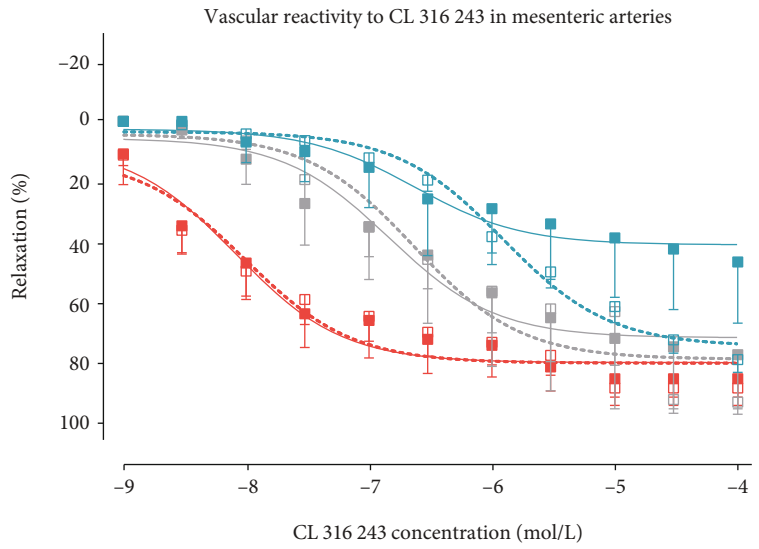


(b)

Figure 2: Continued.



(c)



(d)

Figure 2: Continued.

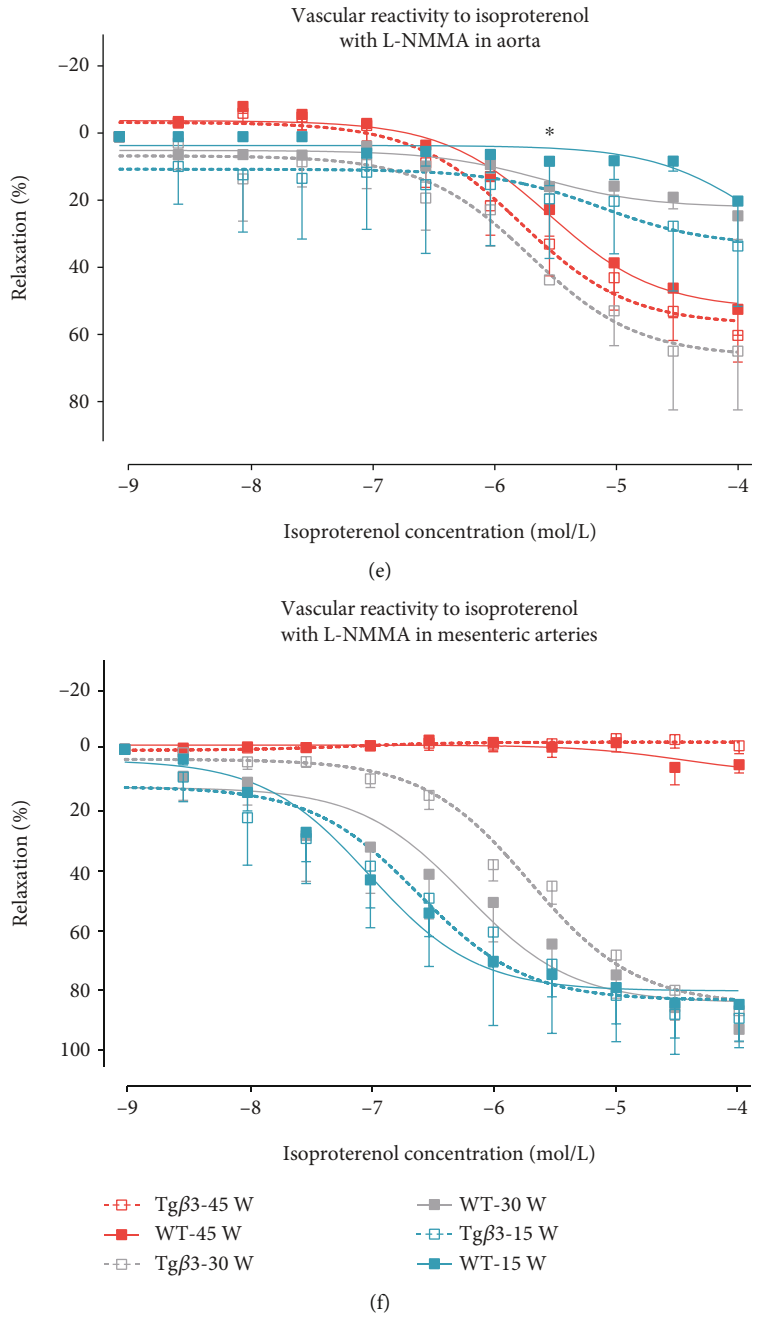


Figure 2: Vasodilation in WT and Tgβ_3 rats at 15, 30, and 45 weeks of age. Concentration-relaxation curves to isoproterenol, a nonselective β-AR agonist, on thoracic aortic rings (a) and mesenteric artery rings (b). Concentration-relaxation curves to CL 316 243, a β_3-AR agonist, on thoracic aortic rings (c) and mesenteric artery rings (d). Concentration-relaxation curves to isoproterenol, a nonselective β-AR agonist, in the presence of L-NMMA, a nonselective NOS inhibitor, on thoracic aortic rings (e) and mesenteric arteries rings (f). Results were expressed as the percentage of relaxation from the maximal contraction induced by phenylephrine. Values represent mean ± SEM. n = 2-12. $^{\$}p < 0.05$: WT—15 weeks versus Tg$\beta_3$—15 weeks; $^{*}p < 0.05$: WT—30 weeks versus Tgβ_3—30 weeks; $^{\$}p < 0.05$: WT—45 weeks versus Tg$\beta_3$—45 weeks.

thoracic aortas between WT and transgenic rats. With L-NMMA, the $^{\bullet}$NO production was almost abolished as expected, confirming that NOS-dependent $^{\bullet}$NO production (negative control). The NOS2-dependent $^{\bullet}$NO production was not significantly increased in the Tgβ_3 rats (+51%; p = 0.09). The NOS1- and NOS3-dependent $^{\bullet}$NO production was not modified between the two groups (Figure 4(e)). The generation of $O_2^{\bullet-}$ has been also evaluated under baseline conditions and in the presence of L-NMMA, and no changes on $O_2^{\bullet-}$ production with or without L-NMMA were reported between the two groups of rats at this age (Figure 4(f)).

3.4. β_3-AR Overexpression Induced Endothelial Dysfunction at 30 Weeks. We investigated whether endothelial dysfunction is worsened at 30 weeks of age in Tgβ_3 rats.

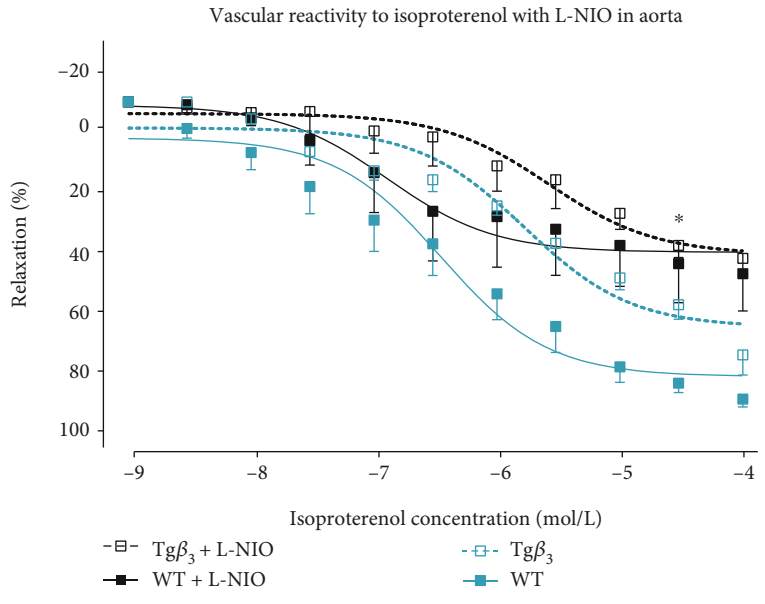
Table 2: Efficiencies and potencies of isoproterenol with or without the NOS inhibitor and of CL 316 243 on thoracic aortic arteries (a) and mesenteric arteries (b) from WT and Tgβ_3 rats at 15, 30, and 45 weeks old.

(a)

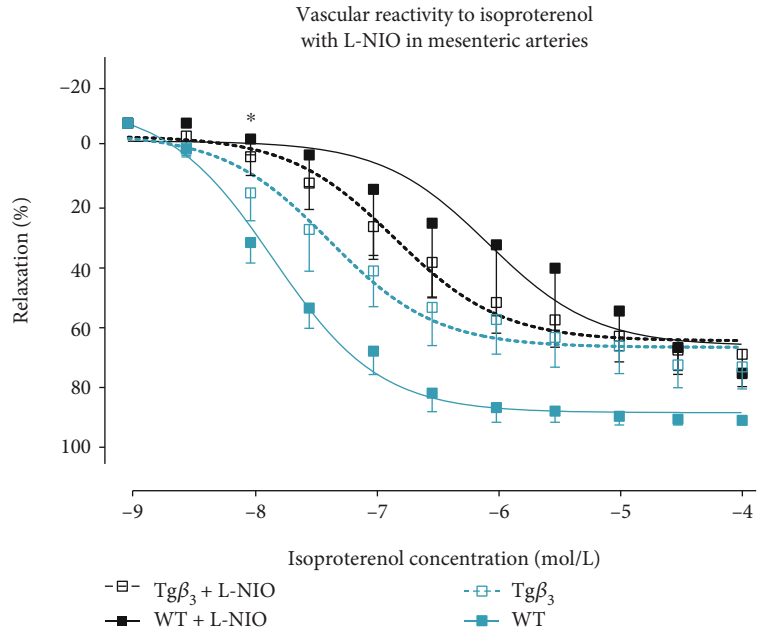
Group		Thoracic aortic arteries—WT			Thoracic aortic arteries—Tgβ_3			Statistics WT vs. Tgβ_3	
		n	pD_2	Emax	n	pD_2	Emax	p pD_2	p Emax
Isoproterenol	15 w	7	6.53 ± 0.33	94.5 ± 2.5	5	5.93 ± 0.20	74.1 ± 7.5	0.239	0.015
	30 w	4	6.73 ± 0.30	85.6 ± 14.3	7	5.49 ± 0.11	79.0 ± 9.5	0.002	0.245
	45 w	11	6.56 ± 0.21	85.9 ± 2.2	10	5.82 ± 0.15	69.2 ± 3.5	0.023	0.016
Isoproterenol+L-NMMA	15 w	3	5.18 ± 0.80	15.0 ± 5.52	2	6.79 ± 2.07	25.7 ± 9.43	0.408	0.679
	30 w	2	5.62 ± 0.11	19.9 ± 5.40	3	5.79 ± 0.34	63.4 ± 18.5	0.981	0.080
	45 w	9	5.46 ± 0.24	51.9 ± 7.27	8	5.62 ± 0.22	56.0 ± 6.55	0.697	0.760
Isoproterenol+L-NIO	15 w	7	6.06 ± 0.45	60.0 ± 12.1	4	5.87 ± 0.43	52.6 ± 7.80	0.756	0.491
	30 w	3	5.25 ± 0.14	57.0 ± 22.8	6	5.27 ± 0.27	42.1 ± 14.3	0.537	0.647
	45 w	8	4.93 ± 0.11	33.0 ± 4.42	8	5.30 ± 0.18	45.9 ± 5.90	0.212	0.278
Isoproterenol+L-VNIO	15 w	4	6.79 ± 0.638	61.9 ± 16.0	5	5.63 ± 0.512	47.9 ± 21.6	0.118	0.449
	30 w	4	5.65 ± 0.63	35.25 ± 11.9	4	6.02 ± 0.59	18.6 ± 8.6	0.572	0.530
	45 w	5	4.97 ± 0.14	33.6 ± 6.15	5	5.54 ± 0.15	42.3 ± 2.84	0.186	0.702
Isoproterenol+1400W	15 w	4	6.51 ± 0.700	83.2 ± 7.23	4	6.32 ± 0.656	65.7 ± 8.77	0.905	0.151
	30 w	4	6.09 ± 0.41	45.58 ± 13.7	7	5.26 ± 0.32	63.1 ± 12.6	0.213	0.296
	45 w	7	5.43 ± 0.46	58.1 ± 9.44	8	5.35 ± 0.13	53.0 ± 7.52	0.896	0.589
CL 316 243	15 w	3	6.16 ± 0.67	17.1 ± 6.9	4	6.75 ± 0.74	22.8 ± 2.5	0.197	0.533
	30 w	2	7.22 ± 0.00	32.0 ± 0.0	3	6.87 ± 0.45	26.3 ± 5.0	0.785	0.442
	45 w	4	6.94 ± 0.20	43.7 ± 1.9	9	7.06 ± 0.09	30.9 ± 3.8	0.646	0.044

(b)

Group		Mesenteric arteries—WT			Mesenteric arteries—Tgβ_3			Statistics WT vs. Tgβ_3	
		n	pD_2	Emax	n	pD_2	Emax	p pD_2	p Emax
Isoproterenol	15 w	4	7.78 ± 0.17	96.4 ± 3.0	8	7.15 ± 0.32	78.0 ± 8.5	0.112	0.151
	30 w	8	7.03 ± 0.20	100.7 ± 4.6	8	6.67 ± 0.28	73.5 ± 10.4	0.338	0.022
	45 w	12	7.21 ± 0.15	85.7 ± 3.4	12	6.17 ± 0.17	68.2 ± 5.5	0.001	0.061
Isoproterenol+L-NMMA	15 w	4	6.67 ± 0.49	83.8 ± 14.2	6	6.82 ± 0.53	87.5 ± 8.25	0.877	0.920
	30 w	7	6.37 ± 0.50	92.1 ± 4.60	6	5.72 ± 0.15	86.1 ± 7.93	0.392	0.815
	45 w	9	6.33 ± 0.47	5.07 ± 4.53	8	6.38 ± 0.47	−4.03 ± 2.60	0.918	0.533
Isoproterenol+L-NIO	15 w	3	6.04 ± 0.78	86.1 ± 1.28	9	6.89 ± 0.30	75.4 ± 8.08	0.300	0.646
	30 w	5	5.91 ± 0.31	76.9 ± 4.70	4	6.66 ± 0.38	86.8 ± 5.66	0.195	0.344
	45 w	9	5.83 ± 0.29	35.1 ± 5.91	9	5.65 ± 0.36	64.0 ± 7.98	0.818	0.033
Isoproterenol+L-VNIO	15 w	6	7.08 ± 0.52	84.1 ± 8.64	6	6.24 ± 0.45	75.8 ± 9.94	0.221	0.744
	30 w	3	6.29 ± 0.27	71.5 ± 4.59	6	6.78 ± 0.54	75.1 ± 8.33	0.491	0.564
	45 w	8	6.41 ± 0.30	49.6 ± 8.63	8	6.82 ± 0.43	22.79 ± 7.38	0.392	0.171
Isoproterenol+1400W	15 w	4	7.34 ± 0.25	96.4 ± 2.02	7	6.49 ± 0.31	81.8 ± 8.00	0.082	0.515
	30 w	8	6.64 ± 0.30	84.0 ± 10.9	6	6.43 ± 0.44	92.3 ± 3.23	0.759	0.789
	45 w	8	6.32 ± 0.40	46.52 ± 10.8	8	6.27 ± 0.29	52.7 ± 13.8	0.781	0.325
CL 316 243	15 w	5	5.98 ± 0.61	48.4 ± 19.5	6	5.90 ± 0.14	75.7 ± 5	0.654	0.856
	30 w	3	6.53 ± 0.64	75.2 ± 16.8	4	6.52 ± 0.65	93.2 ± 14.5	0.968	0.568
	45 w	3	7.96 ± 0.33	81.1 ± 7.4	7	7.68 ± 0.42	84.5 ± 5.8	0.731	0.737



(a)



(b)

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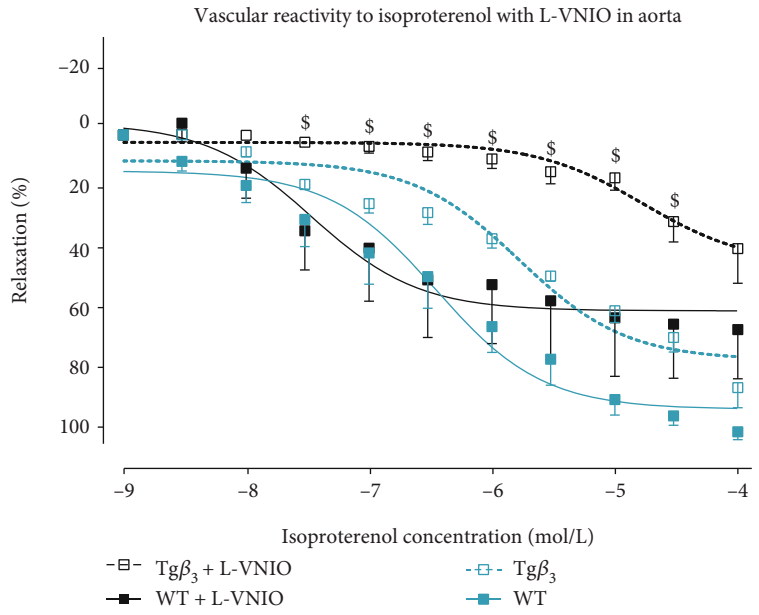

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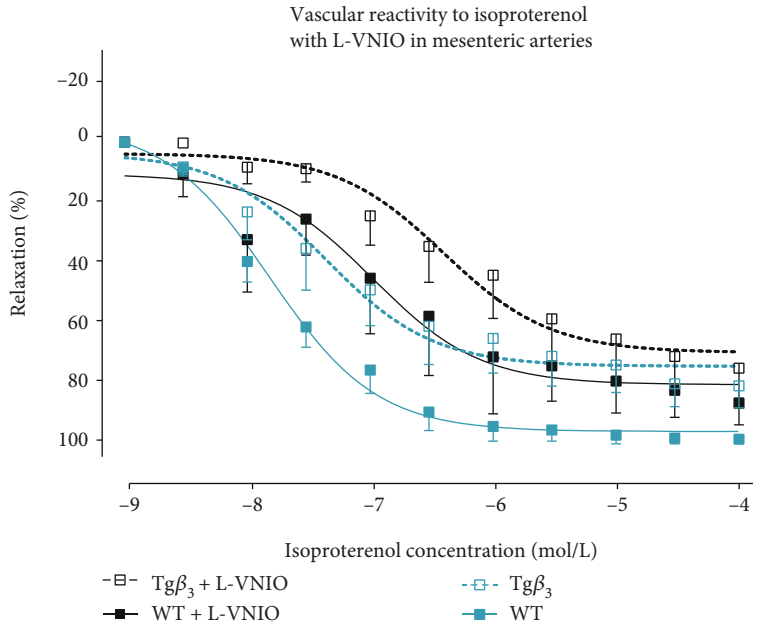

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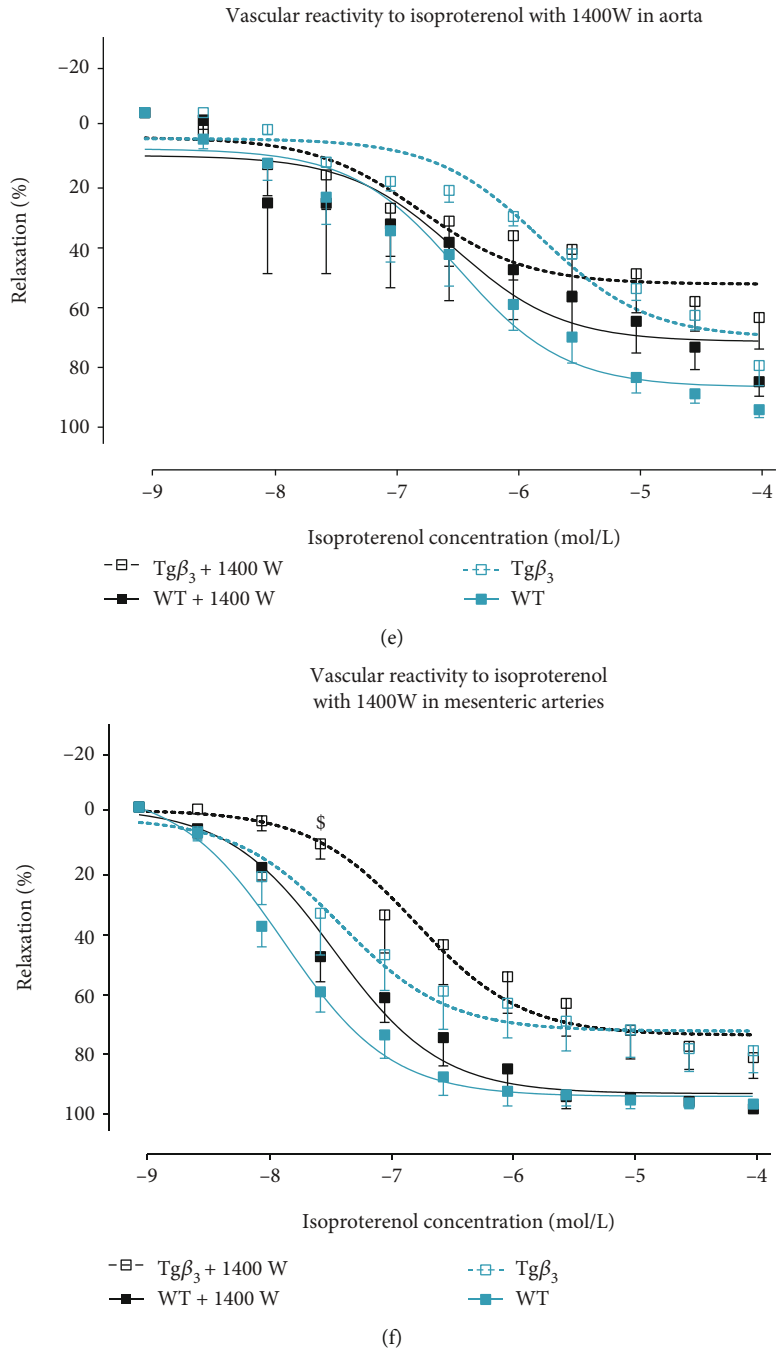


Figure 3: Evaluation of function of NOS in WT and Tgβ_3 rats at 15 weeks of age. Concentration-relaxation curves to isoproterenol, a nonselective β-AR agonist, on thoracic aortic rings in the presence or absence of a selective NOS inhibitor: L-NIO (NOS3 inhibitor) (a), 1400W (NOS2 inhibitor) (b), and L-VNIO (NOS1 inhibitor) (c). The same experiments were done on mesenteric artery rings with L-NIO (d), 1400W (e), and L-VNIO (f). Results were expressed as the percentage of relaxation from the maximal contraction induced by phenylephrine. Values represent mean ± SEM. $n = 3$-9. $^*p < 0.05$: WT versus WT+inhibitor; $^\$p < 0.05$: Tg$\beta_3$ versus Tgβ_3+inhibitor.

3.4.1. NOS1 Modulates the Vasodilatation in the Tgβ_3 Group. At 30 weeks of age, vasodilation in response to isoproterenol was reduced in aortic rings of Tgβ_3 rats associated with a decreased pD_2 (WT: 6.73 ± 0.30; Tgβ_3: 5.49 ± 0.11; $p < 0.05$) (Figure 2(a) and Table 2(a)).

In the presence of L-NIO, the pD_2 was reduced in the aortic rings (WT: 6.73 ± 0.30; WT+L-NIO: 5.25 ± 0.14; $p < 0.05$) and mesenteric arteries (WT: 7.03 ± 0.20; WT+L-NIO: 5.91 ± 0.31; $p < 0.05$) from WT rats (Figures 5(a) and 5(b) and Tables 3(a) and 3(b)) suggesting the implication of $^\bullet$NO from NOS3.

Vasodilation in response to isoproterenol in the presence of the NOS1 inhibitor (L-VNIO) was blunted with a reduction in maximal effect in the aortic rings from both WT rats (WT: 85.6 ± 14.3; WT+L-NIO: 35.25 ± 11.19; $p < 0.05$) and Tgβ_3 rats (Tgβ_3: 79 ± 9.5; Tgβ_3+L-NIO: 18.6 ± 8.6; $p < 0.05$) and a

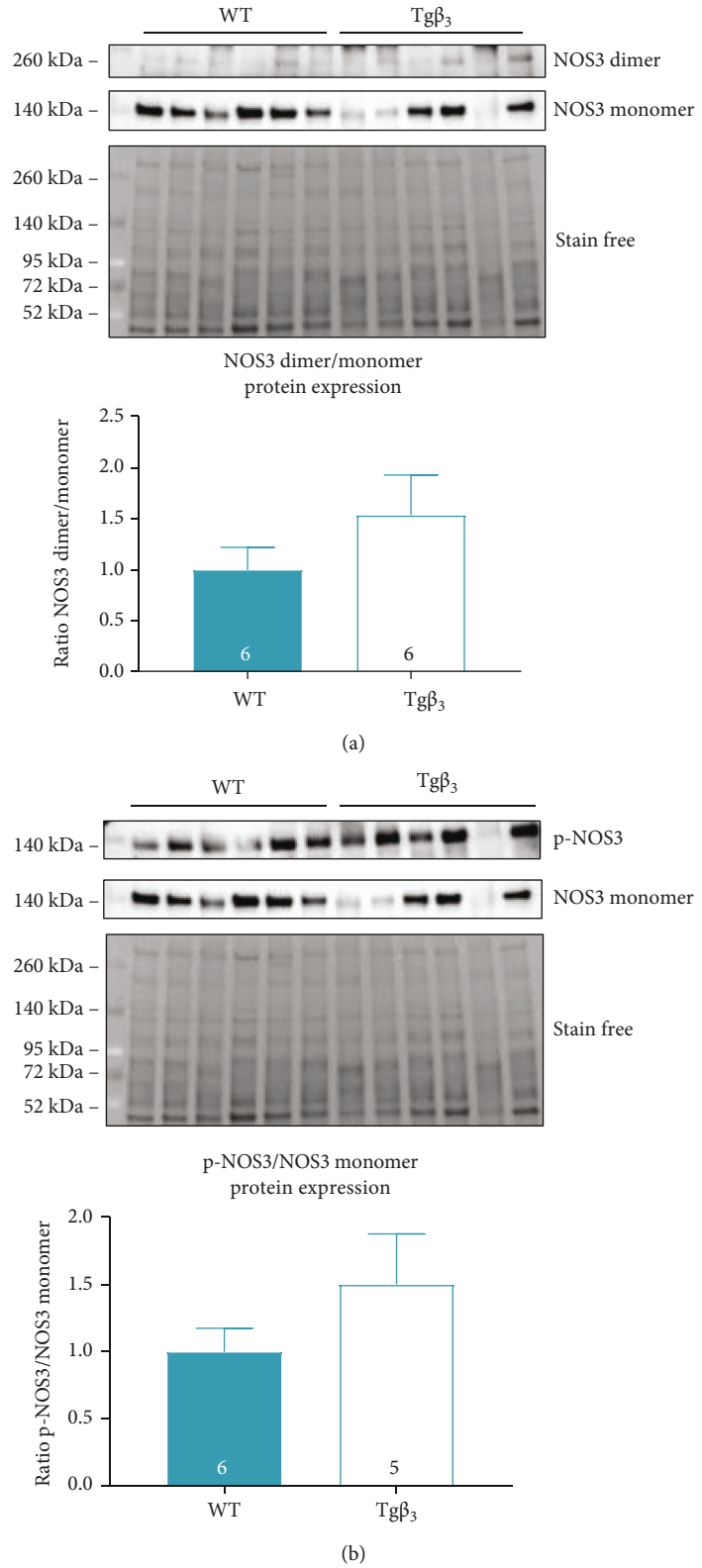
Table 3: Statistical comparison of the efficiencies and potencies of isoproterenol with or without the NOS inhibitor and of CL 316 243 on thoracic aortic arteries (a) and mesenteric arteries (b) from WT and Tgβ_3 rats at 15, 30, and 45 weeks old.

(a)

Group		Thoracic aortic arteries—WT			Thoracic aortic arteries—Tgβ_3			WT vs. WT+isoproterenol		Tgβ_3 vs. Tgβ_3+isoproterenol	
		n	pD_2	Emax	n	pD_2	Emax	p pD_2	p Emax	p pD_2	p Emax
Isoproterenol	15 w	7	6.53 ± 0.33	94.5 ± 2.5	5	5.93 ± 0.20	74.1 ± 7.5				
	30 w	4	6.73 ± 0.30	85.6 ± 14.3	7	5.49 ± 0.11	79.0 ± 9.5				
	45 w	11	6.56 ± 0.21	85.9 ± 2.2	10	5.82 ± 0.15	69.2 ± 3.5				
Isoproterenol+L-NIO	15 w	7	6.06 ± 0.45	60.0 ± 12.1	4	5.87 ± 0.43	52.6 ± 7.80	0.430	0.013	0.939	0.322
	30 w	3	5.25 ± 0.14	57.0 ± 22.8	6	5.27 ± 0.27	42.1 ± 14.3	0.006	0.083	0.661	0.080
	45 w	8	4.93 ± 0.11	33.0 ± 4.42	8	5.30 ± 0.18	45.9 ± 5.90	<0.0001	<0.0001	0.134	0.088
Isoproterenol+L-VNIO	15 w	4	6.79 ± 0.638	61.9 ± 16.0	5	5.63 ± 0.512	47.9 ± 21.6	0.620	0.125	0.799	0.414
	30 w	4	5.65 ± 0.63	35.25 ± 11.9	4	6.02 ± 0.59	18.6 ± 8.6	0.090	0.024	0.346	0.019
	45 w	5	4.97 ± 0.14	33.6 ± 6.15	5	5.54 ± 0.15	42.3 ± 2.84	<0.0001	<0.0001	0.377	0.068
Isoproterenol+1400W	15 w	4	6.51 ± 0.700	83.2 ± 7.23	4	6.32 ± 0.656	65.7 ± 8.77	0.665	0.259	0.588	0.623
	30 w	4	6.09 ± 0.41	45.58 ± 13.7	7	5.26 ± 0.32	63.1 ± 12.6	0.191	0.019	0.902	0.323
	45 w	7	5.43 ± 0.46	58.1 ± 9.44	8	5.35 ± 0.13	53.0 ± 7.52	0.002	0.002	0.105	0.265

(b)

Group		Mesenteric arteries—WT			Mesenteric arteries—Tgβ_3			WT vs. WT+isoproterenol		Tgβ_3 vs. Tgβ_3+isoproterenol	
		n	pD_2	Emax	n	pD_2	Emax	p pD_2	p Emax	p pD_2	p Emax
Isoproterenol	15 w	4	7.78 ± 0.17	96.4 ± 3.0	8	7.15 ± 0.32	78.0 ± 8.5				
	30 w	8	7.03 ± 0.20	100.7 ± 4.6	8	6.67 ± 0.28	73.5 ± 10.4				
	45 w	12	7.21 ± 0.15	85.7 ± 3.4	12	6.17 ± 0.17	68.2 ± 5.5				
Isoproterenol+L-NIO	15 w	3	6.04 ± 0.78	86.1 ± 1.28	9	6.89 ± 0.30	75.4 ± 8.08	0.024	0.431	0.555	0.776
	30 w	5	5.91 ± 0.31	76.9 ± 4.70	4	6.66 ± 0.38	86.8 ± 5.66	0.014	0.003	0.978	0.627
	45 w	9	5.83 ± 0.29	35.1 ± 5.91	9	5.65 ± 0.36	64.0 ± 7.98	0.0004	<0.0001	0.408	0.640
Isoproterenol+L-VNIO	15 w	6	7.08 ± 0.52	84.1 ± 8.64	6	6.24 ± 0.45	75.8 ± 9.94	0.290	0.361	0.127	0.844
	30w	3	6.29 ± 0.27	71.5 ± 4.59	6	6.78 ± 0.54	75.1 ± 8.33	0.195	0.013	0.850	0.777
	45 w	8	6.41 ± 0.30	49.6 ± 8.63	8	6.82 ± 0.43	22.79 ± 7.38	0.032	0.003	0.079	0.004
Isoproterenol+1400W	15 w	4	7.34 ± 0.25	96.4 ± 2.02	7	6.49 ± 0.31	81.8 ± 8.00	0.404	0.980	0.125	0.780
	30 w	8	6.64 ± 0.30	84.0 ± 10.9	6	6.43 ± 0.44	92.3 ± 3.23	0.378	0.418	0.680	0.307
	45 w	8	6.32 ± 0.40	46.52 ± 10.8	8	6.27 ± 0.29	52.7 ± 13.8	0.013	0.002	0.679	0.773



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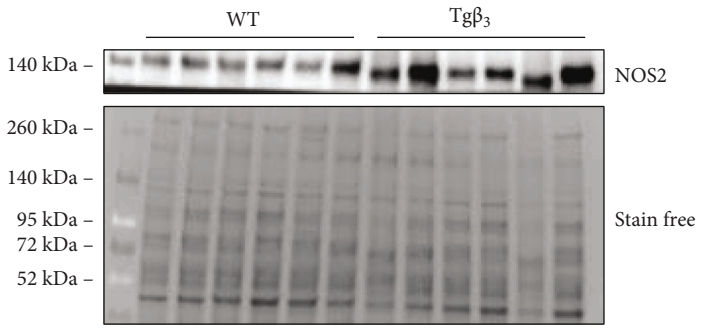
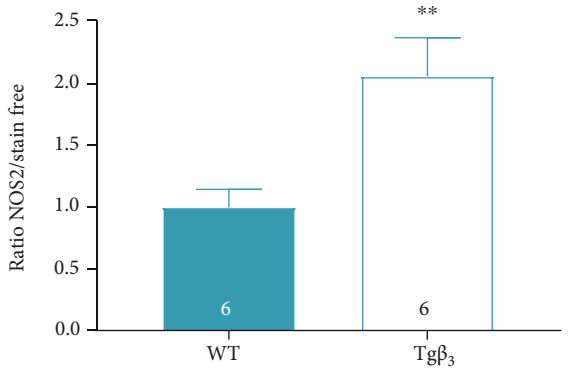
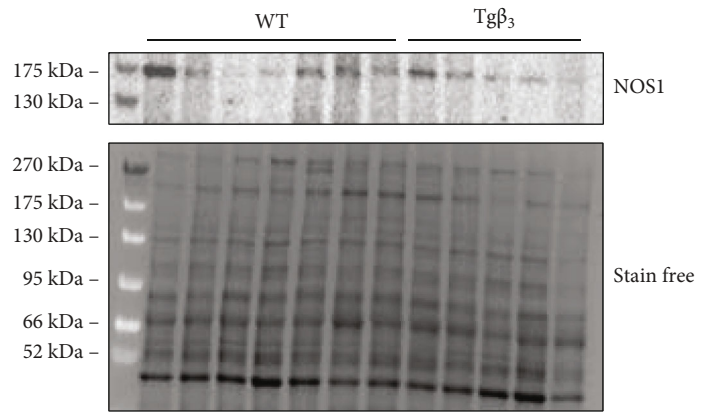


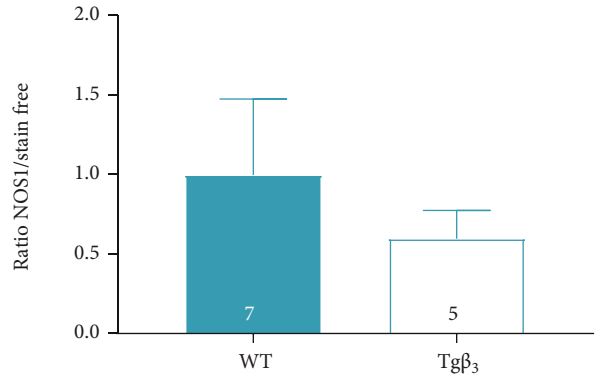
NOS2 protein expression



(c)



NOS1 protein expression



(d)

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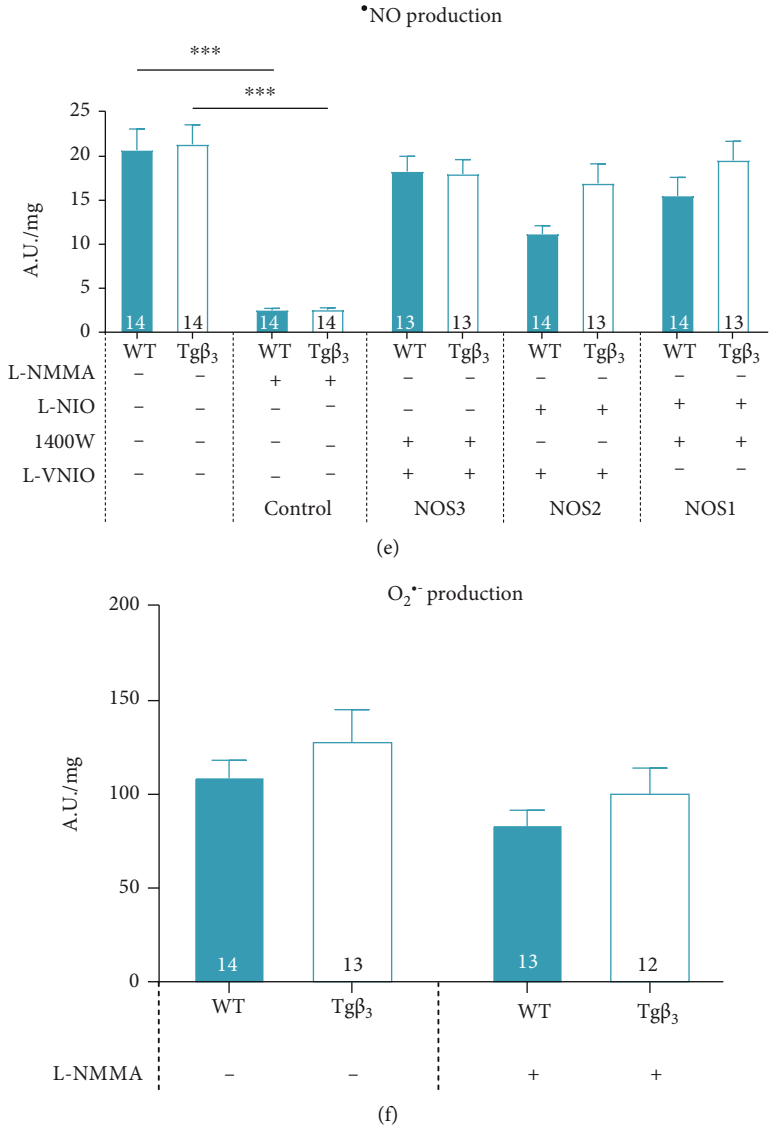


Figure 4: Expression and activity production of NOS in WT and Tgβ_3 rats at 15 weeks of age. Effects of the β_3-AR overexpression in Tgβ_3 rats on aortic protein expression of the NOS3 dimer/monomer ratio (a), p-NOS3/NOS3 monomer (b), NOS2 (c), and NOS1 (d). Evaluation of $^{\bullet}$NO production on thoracic aortic rings with or without an inhibitor (L-NMMA, L-NIO, 1400W, and L-VNIO) (e) and $O_2^{\bullet-}$ production with or without L-NMMA from WT and Tgβ_3 rats (f). Data are expressed as mean ± SEM. $n = 5$-14. $^{**}p < 0.01$, $^{***}p < 0.001$.

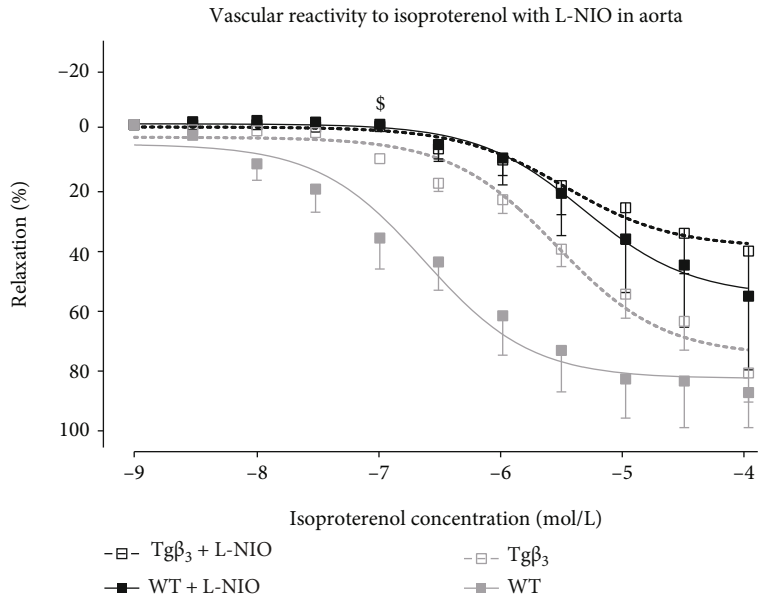
decrease in the maximal effect in the mesenteric arteries from WT rats (WT: 100.7 ± 4.6; WT+L-NIO: 71.5 ± 4.59; $p < 0.05$) (Figures 5(c) and 5(d) and Tables 3(a) and 3(b)). These data suggest an implication of $^{\bullet}$NO production from NOS1.

In the presence of 1400W, the vasodilation in response to isoproterenol was modified on the aorta from WT rats with reduced maximal effect (WT: 85.6 ± 14.3; WT+1400W: 45.58 ± 13.7; $p < 0.05$) (Figure 5(e) and Table 3(a)) with no impact on mesenteric arteries (Figure 5(f) and Table 3(b)). At 30 weeks of age, the vasodilation induced by isoproterenol seems to involve all NOS isoforms in WT rats while only NOS1 seems to be involved in the vasodilation of vessels from Tgβ_3 rats.

3.4.2. β_3-AR Overexpression Is Associated with an Increase in NOS1 Expression. The aortic ratio of the NOS3 dimer/monomer and p-NOS3/NOS3 monomer and NOS2 protein expression levels were not significantly modified between the two groups at 30 weeks (Figures 6(a), 6(b), and 6(c)). The β_3-AR overexpression induces a significant 6-fold increase in NOS1 protein expression in the aorta (Figure 6(d)) which can explain the decrease in vasodilation in vessels from Tgβ_3 with the NOS1 inhibitor.

3.4.3. Oxidative Stress Is Increased in Tgβ3 Rats at 30 Weeks. At 30 weeks of age, $^{\bullet}$NO production tends to increase in Tgβ_3 rats (+28%). In the presence of L-NMMA, the production of $^{\bullet}$NO is significantly reduced in WT and Tgβ_3 rats. NOS2-dependent $^{\bullet}$NO production is increased in the Tgβ_3 group (+70% vs. WT; $p < 0.05$) (Figure 6(e)).

The $O_2^{\bullet-}$ production was significantly increased in the Tgβ_3 group under baseline conditions (+75% vs. WT; $p < 0.05$) while no change is shown with L-NMMA (Figure 6(f)).



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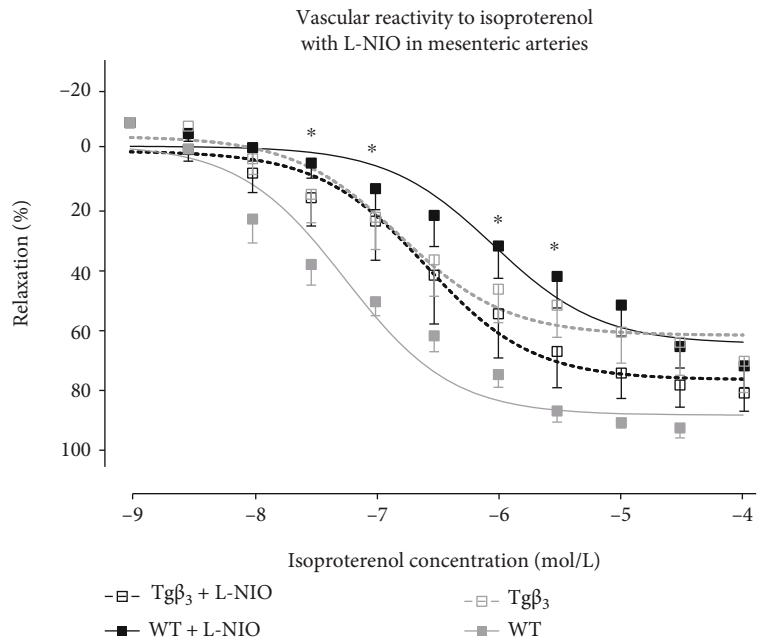

(b)

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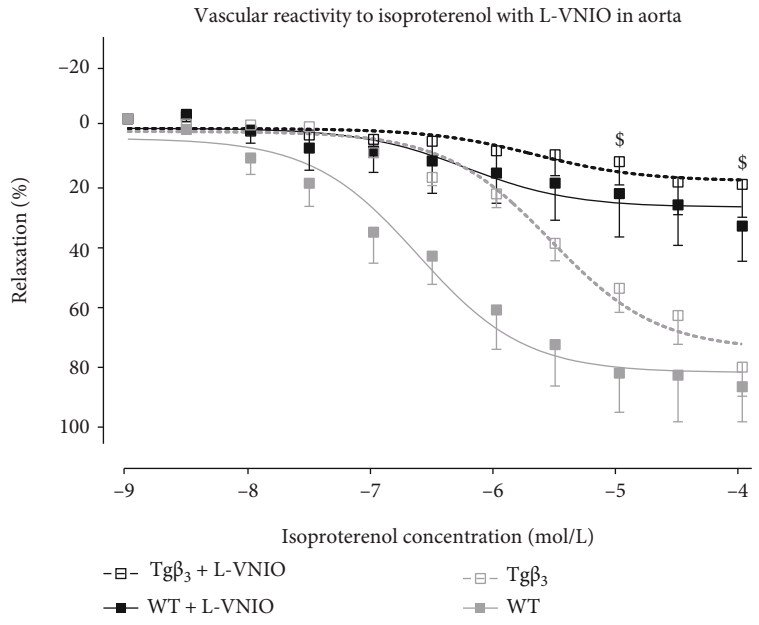

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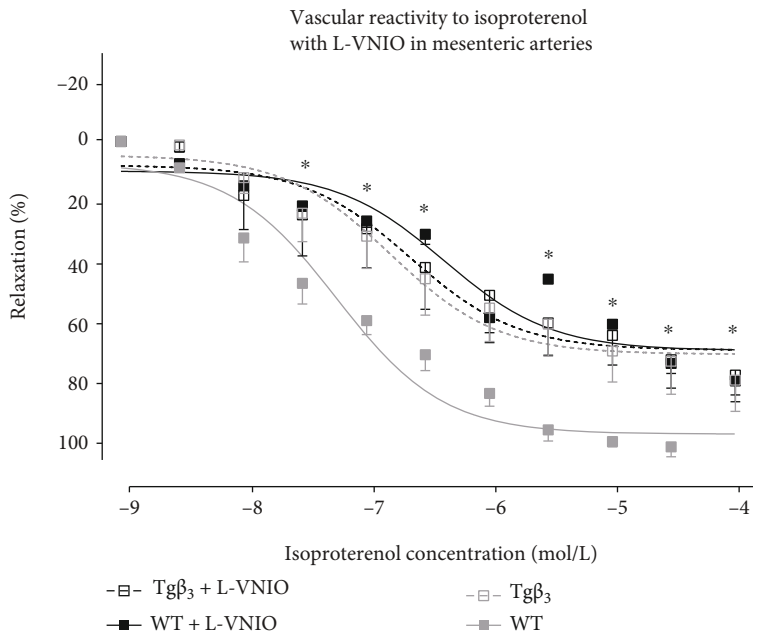

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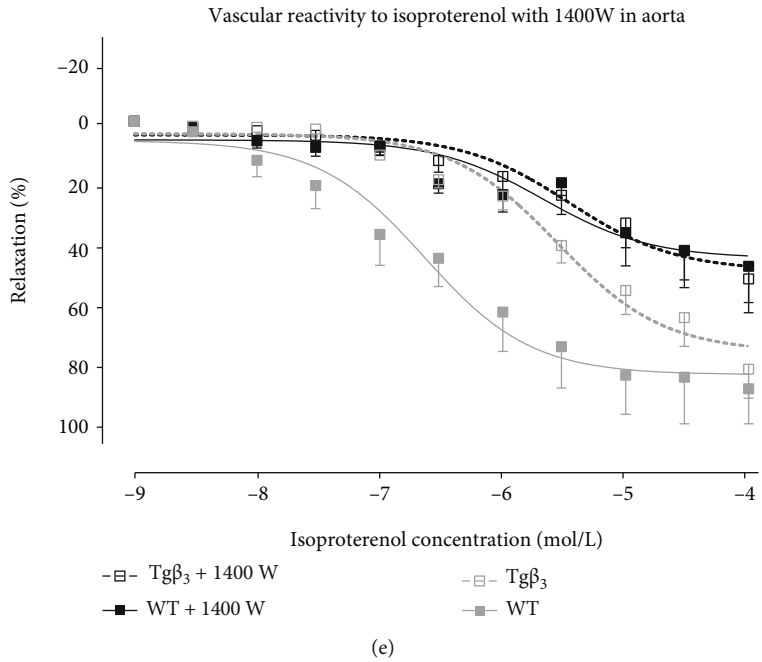
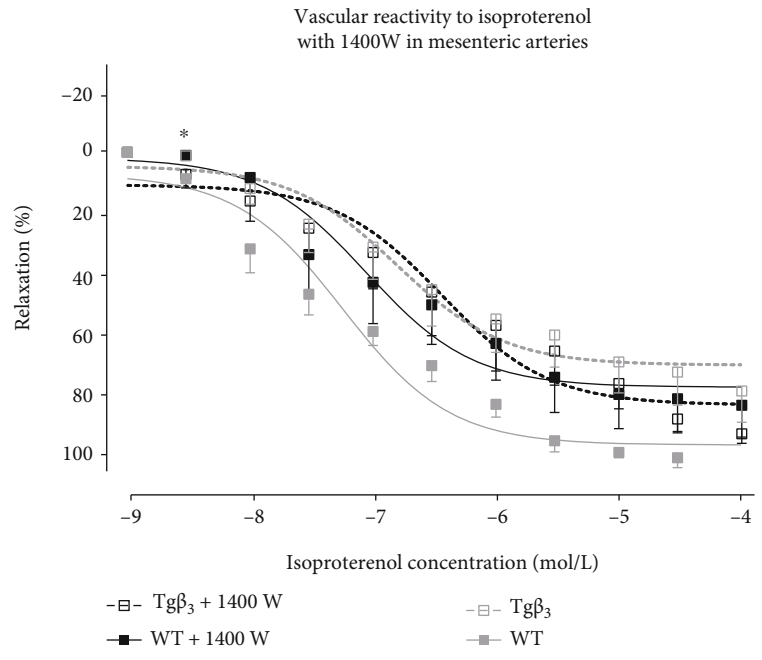


(e)

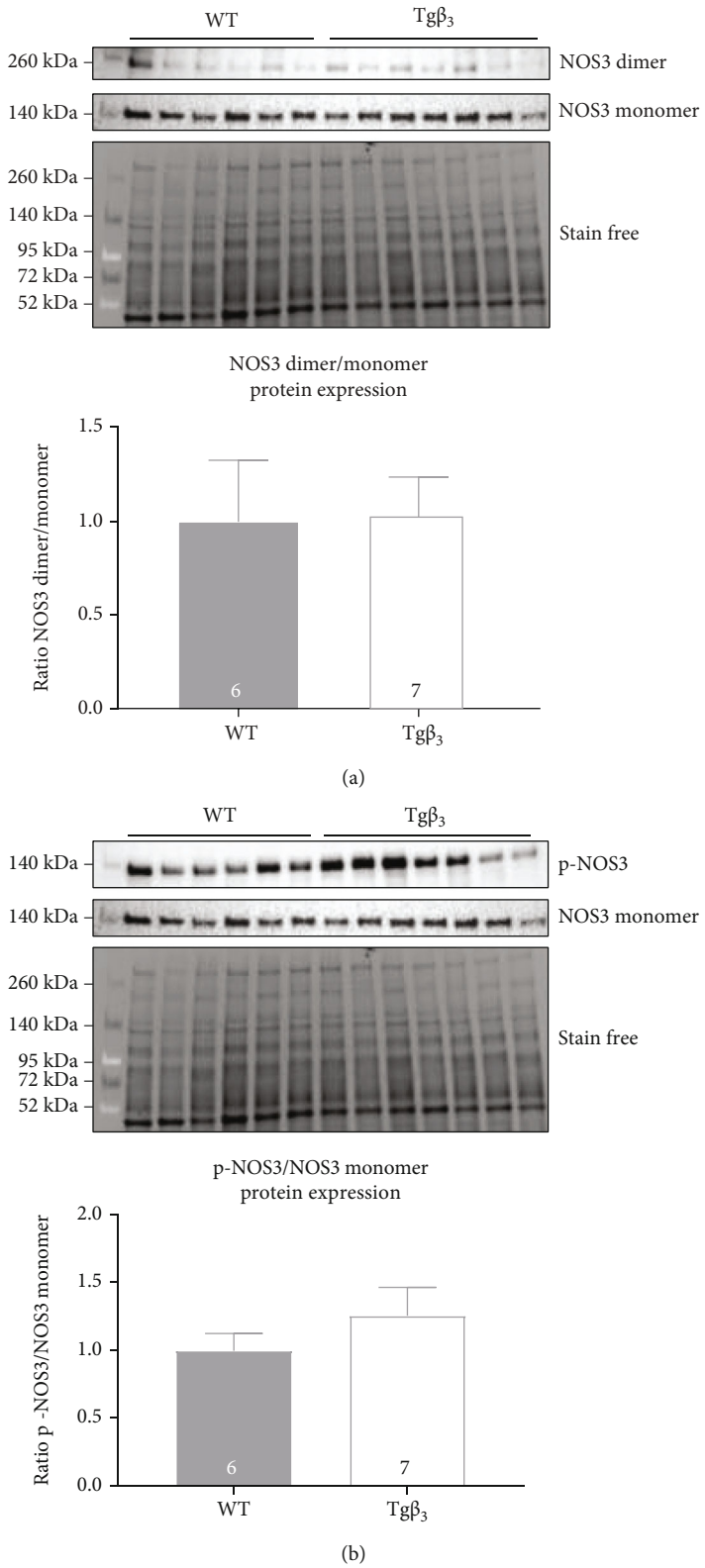


(f)

Figure 5: Evaluation of function of NOS in WT and Tgβ_3 rats at 30 weeks of age. Concentration-relaxation curves to isoproterenol, a nonselective β-AR agonist, on thoracic aortic rings in the presence or absence of a selective NOS inhibitor: L-NIO (NOS3 inhibitor) (a), 1400W (NOS2 inhibitor) (b), and L-VNIO (NOS1 inhibitor) (c). The same experiments were done on mesenteric artery rings with L-NIO (d), 1400W (e), and L-VNIO (f). Results were expressed as the percentage of relaxation from the maximal contraction induced by phenylephrine. Values represent mean ± SEM. $n = 3$-8. $^{*}p < 0.05$: WT versus WT+inhibitor; $^{\$}p < 0.05$: Tg$\beta_3$ versus Tgβ_3+inhibitor.

The results demonstrated that despite a similar expression of NOS2, NOS2-dependent $^{\bullet}$NO production is increased in the Tgβ_3 group, suggesting that its activity is enhanced in Tgβ_3 rats. Interestingly, NOS1 expression which is highly increased in Tgβ_3 rats compared to WT does not impact $^{\bullet}$NO production.

3.5. β_3-AR Overexpression Maintained the Endothelial Dysfunction at 45 Weeks of Age. At 30 weeks, results indicated an endothelial dysfunction associated with an alteration of the vasodilation. An increase in NOS1 expression could led to endothelial dysfunction and is associated with an increase in $O_2^{\bullet-}$ production. Thus, we investigated, at 45



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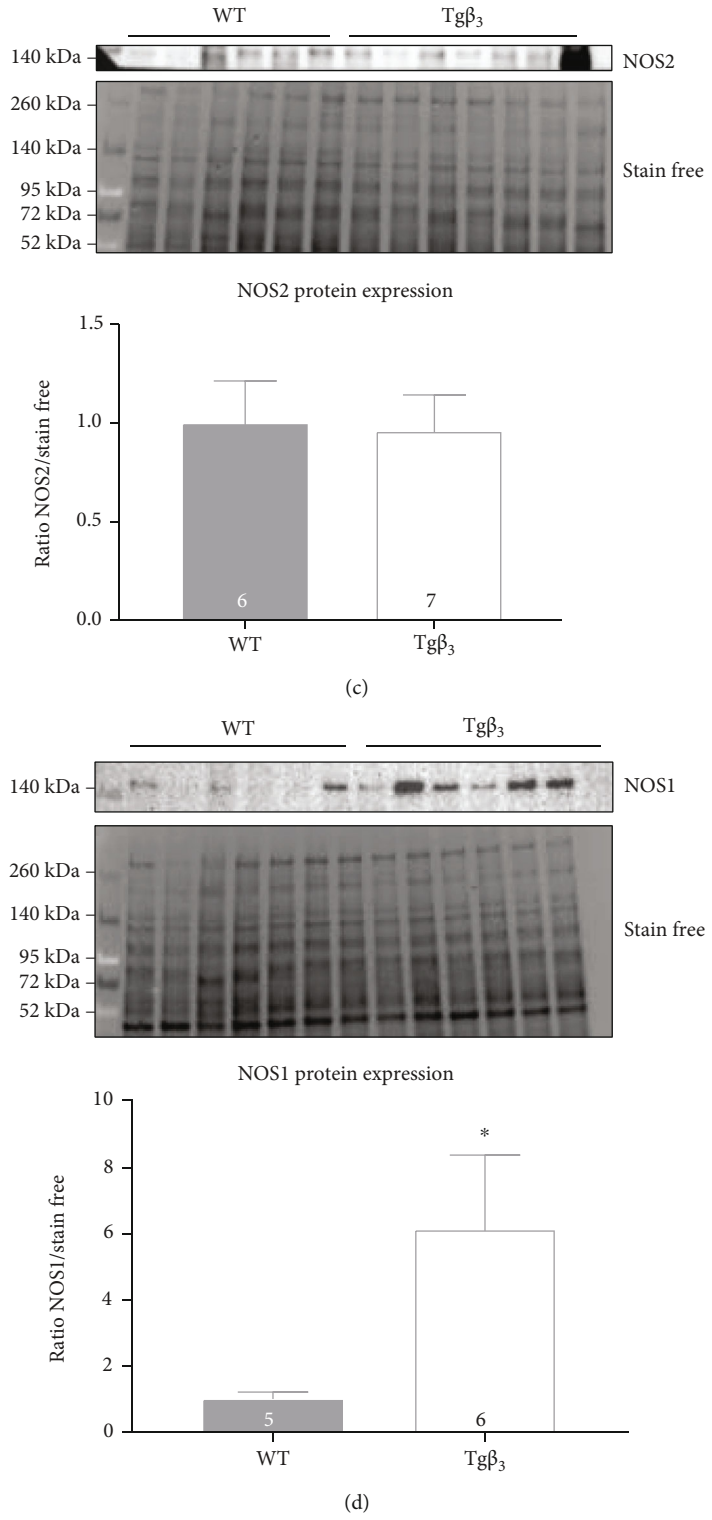

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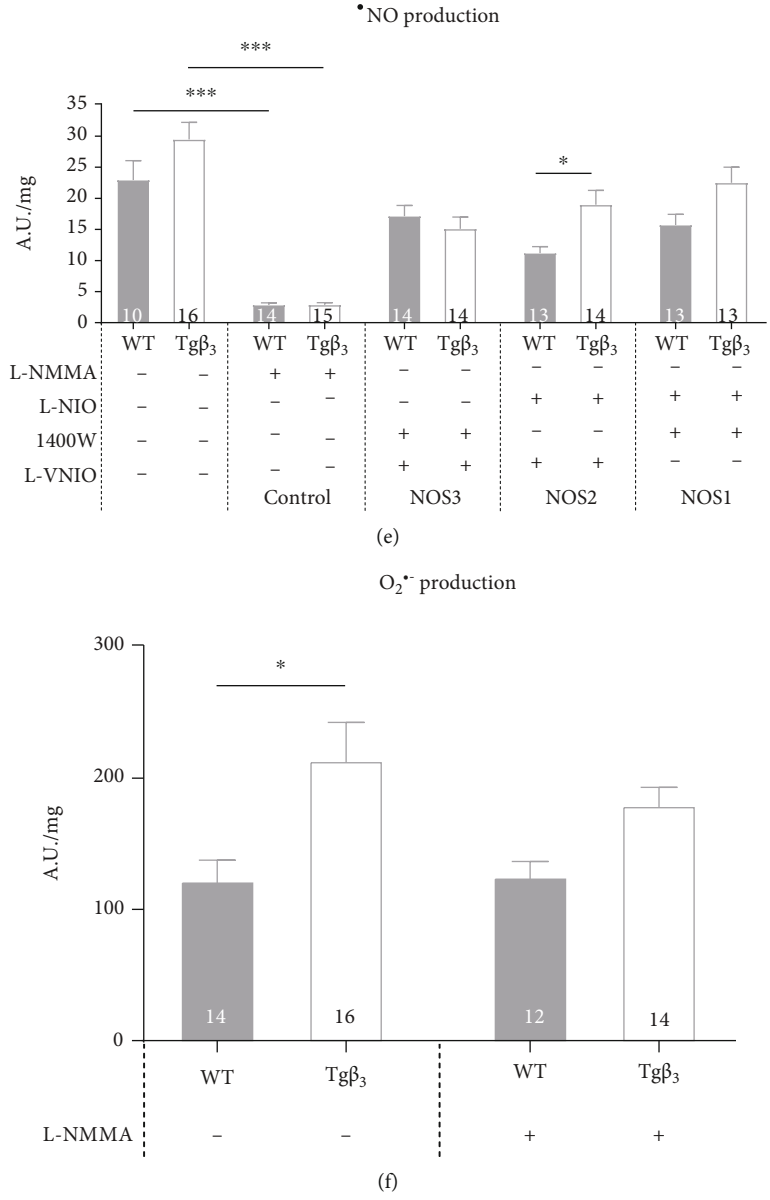
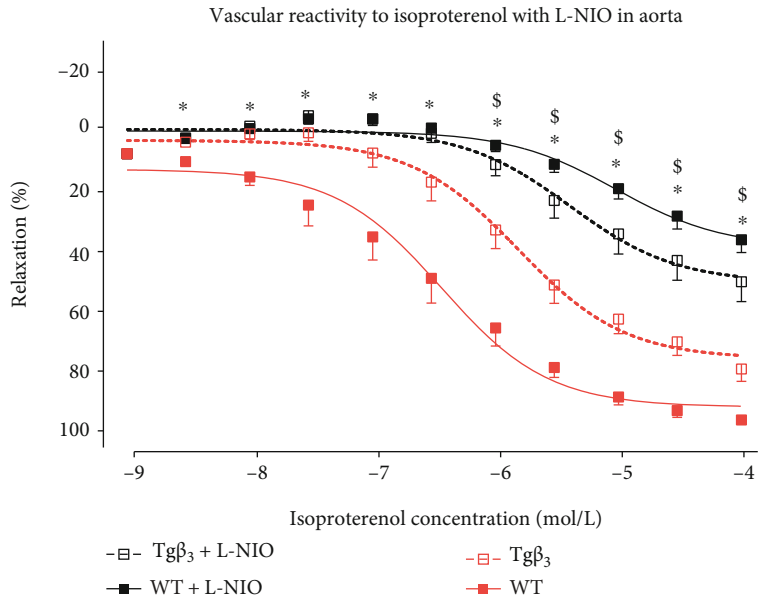


Figure 6: Expression and activity production of NOS in WT and Tgβ_3 rats at 30 weeks of age. Effects of the β_3-AR overexpression in Tgβ_3 rats on aortic protein expression of the NOS3 dimer/monomer ratio (a), p-NOS3/NOS3 monomer (b), NOS2 (c), and NOS1 (d). Evaluation of $^{\bullet}$NO production on thoracic aortic rings with or without an inhibitor (L-NMMA, L-NIO, L-VNIO, and 1400W) (e) and $O_2^{\bullet-}$ production with or without L-NMMA (f) from WT and Tgβ_3 rats. Data are expressed as mean ± SEM. $n = 5$-16. $^{*}p < 0.05$, $^{***}p < 0.001$.

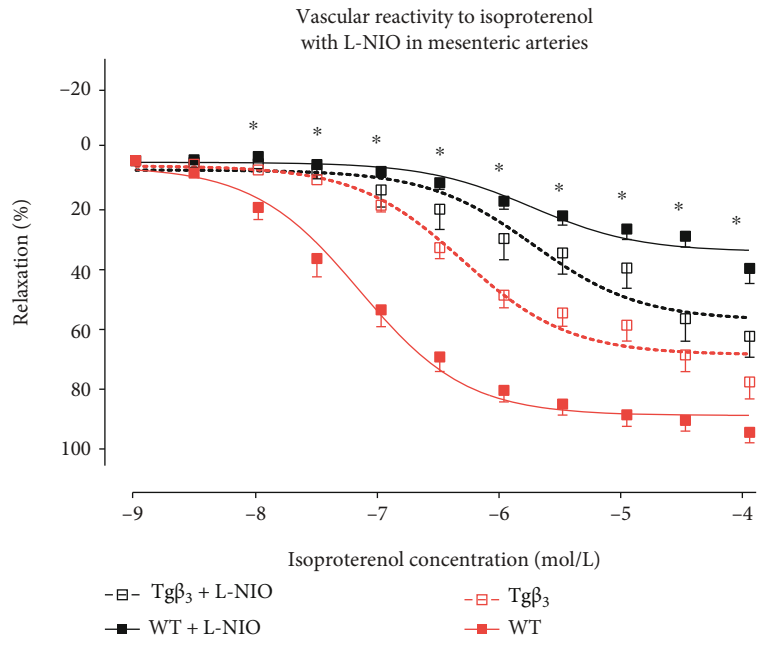
weeks of age, whether endothelial dysfunction was more severe compared to 30 weeks of age.

3.5.1. NOS1 and NOS3 Modulate the Vasodilation in the Tgβ_3 Group. At 45 weeks of age, vasodilation in response to isoproterenol was significantly reduced in Tgβ_3 rats compared to WT in both aortic and mesenteric arteries (Figures 2(a) and 2(b) and Tables 2(a) and 2(b)). In the presence of L-NIO, 1400W, or L-VNIO, vasodilation was significantly reduced on aortic and mesenteric artery rings from WT rats (Figures 7(a)–7(f) and Tables 3(a) and 3(b)). In Tgβ_3 rats, vasodilation was significantly reduced on aortic rings in the presence of L-NIO, while L-VNIO reduced the vasodilation in both aortic and mesenteric rings from Tgβ_3 rats (Figures 7(a), 7(c), and 7(d)). Taken together, these results demonstrated that the vasodilation in Tgβ_3 rats was altered compared to that in WT rats and seems to depend on NOS1 and NOS3 isoforms.

3.5.2. NOS3 Is Uncoupled in Tgβ_3 Rats. In Tgβ_3 rats, the aortic NOS3 dimer/NOS3 monomer protein expression ratio and the aortic p-NOS3/NOS3 monomer protein expression ratio were significantly reduced (-87% and -63%, respectively; $p < 0.05$) indicating a potential NOS3 uncoupling (Figures 8(a) and 8(b)). No modification was reported for NOS2 protein expression (Figure 8(c)). The aortic NOS1 protein expression was significantly increased at 45 weeks of age on Tgβ_3 rats (+57%; $p < 0.05$) (Figure 8(d)).

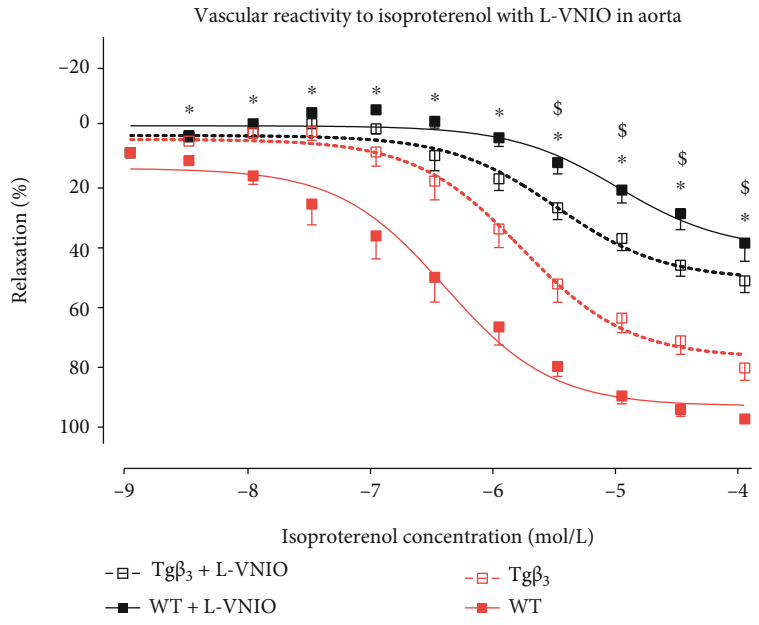


(a)



(b)

Figure 7: Continued.



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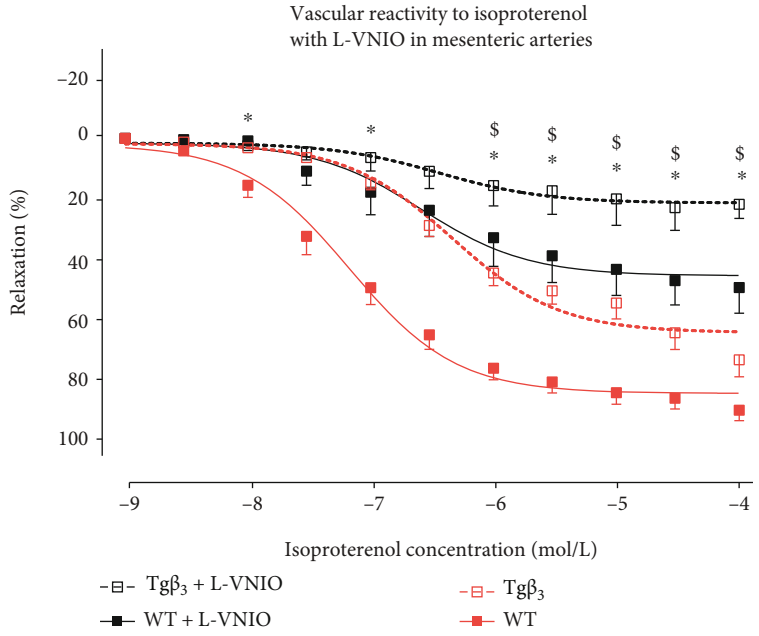

(d)

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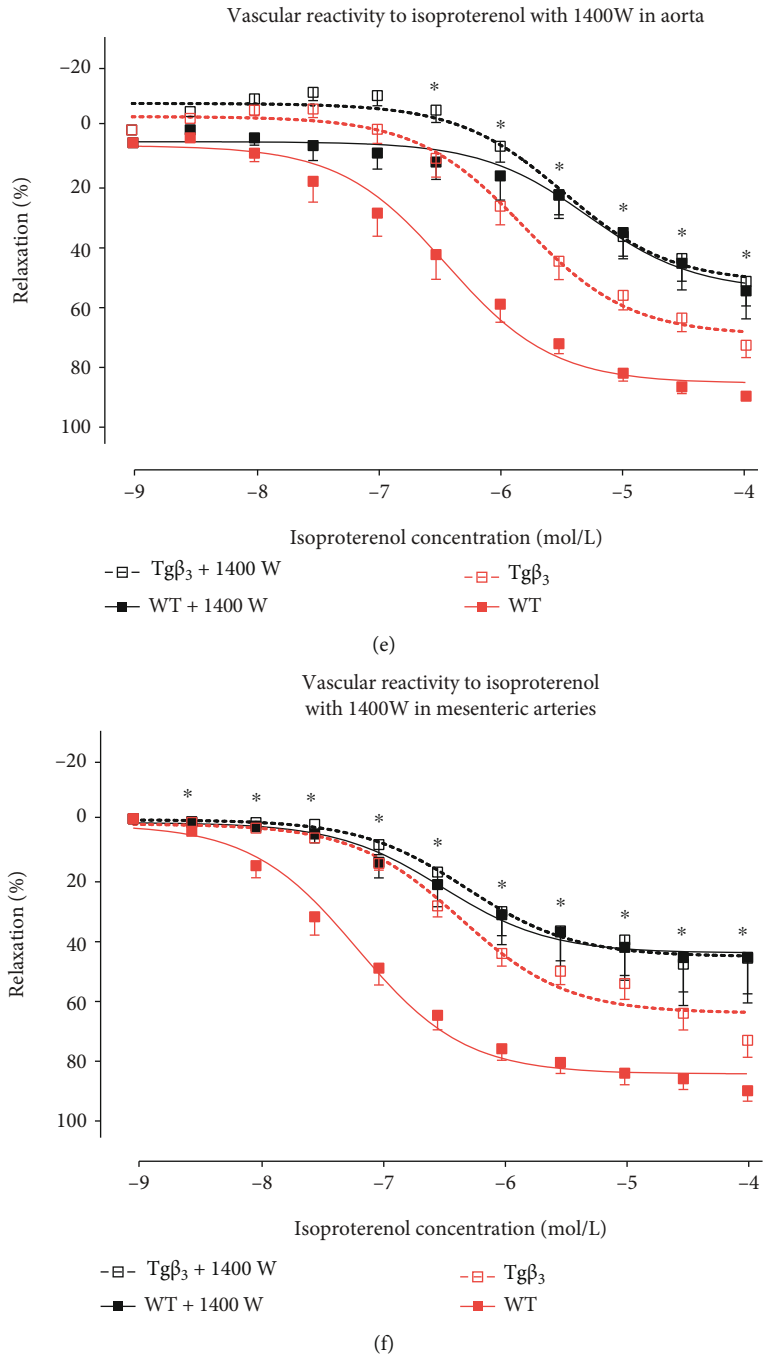

Figure 7: Evaluation of function of NOS in WT and Tgβ_3 rats at 45 weeks of age. Concentration-relaxation curves to isoproterenol, a nonselective β-AR agonist, on thoracic aortic rings in the presence or absence of a selective NOS inhibitor: L-NIO (NOS3 inhibitor) (a), 1400W (NOS2 inhibitor) (b), and L-VNIO (NOS1 inhibitor) (c). The same experiments were done on mesenteric artery rings with L-NIO (d), 1400W (e), and L-VNIO (f). Results were expressed as the percentage of relaxation from the maximal contraction induced by phenylephrine. Values represent mean ± SEM. $n = 5$-12. $^*p < 0.05$: WT versus WT+inhibitor; $^\$p < 0.05$: Tg$\beta_3$ versus Tgβ_3+inhibitor.

3.5.3. $O_2^{\bullet-}$ Production Is Increased in Tgβ_3 Rats. The production of $^\bullet$NO tends to increase in the aorta in Tgβ_3 rats under baseline conditions (+84% vs. WT) (Figure 8(e)). Addition of L-NMMA reduces $^\bullet$NO production in the two groups, indicating that the $^\bullet$NO production from Tgβ_3 and WT rats is mainly due to the NOS. The NOS1-dependent $^\bullet$NO production tends to increase in the Tgβ_3 rats (+90% vs. WT; $p = 0.052$) (Figure 8(e)). The production of $O_2^{\bullet-}$ was significantly increased in Tgβ_3 rats (+76% vs. WT; $p < 0.05$). L-NMMA treatment normalized the production with the absence of significant difference between the two groups, suggesting the involvement of NOS in $O_2^{\bullet-}$ production from Tgβ_3 rats (Figure 8(f)). Results obtained at 45 weeks of age confirm the previous results obtained at 30 weeks of age that Tgβ_3 rats present an endothelial dysfunction.

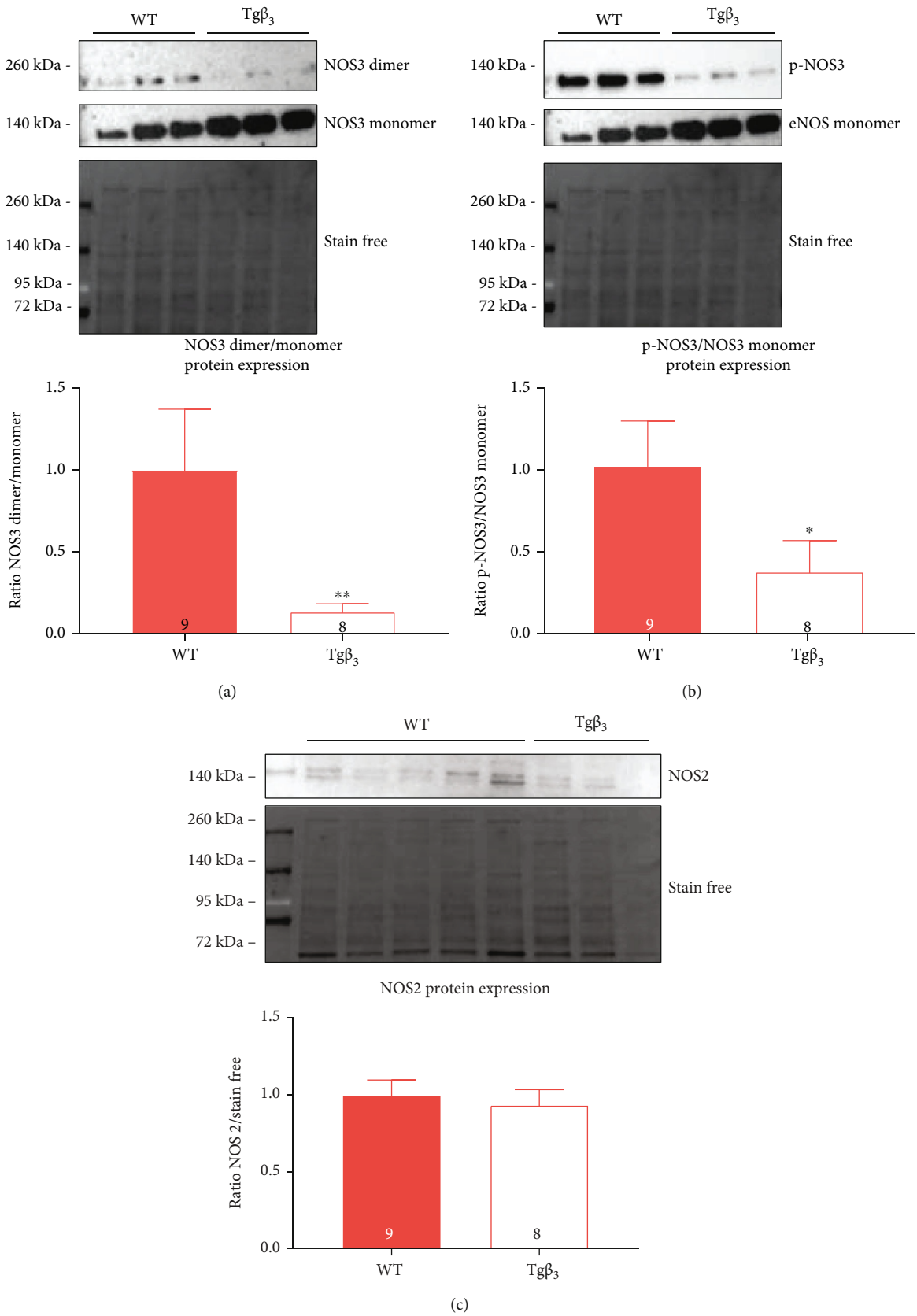

Figure 8: Continued.

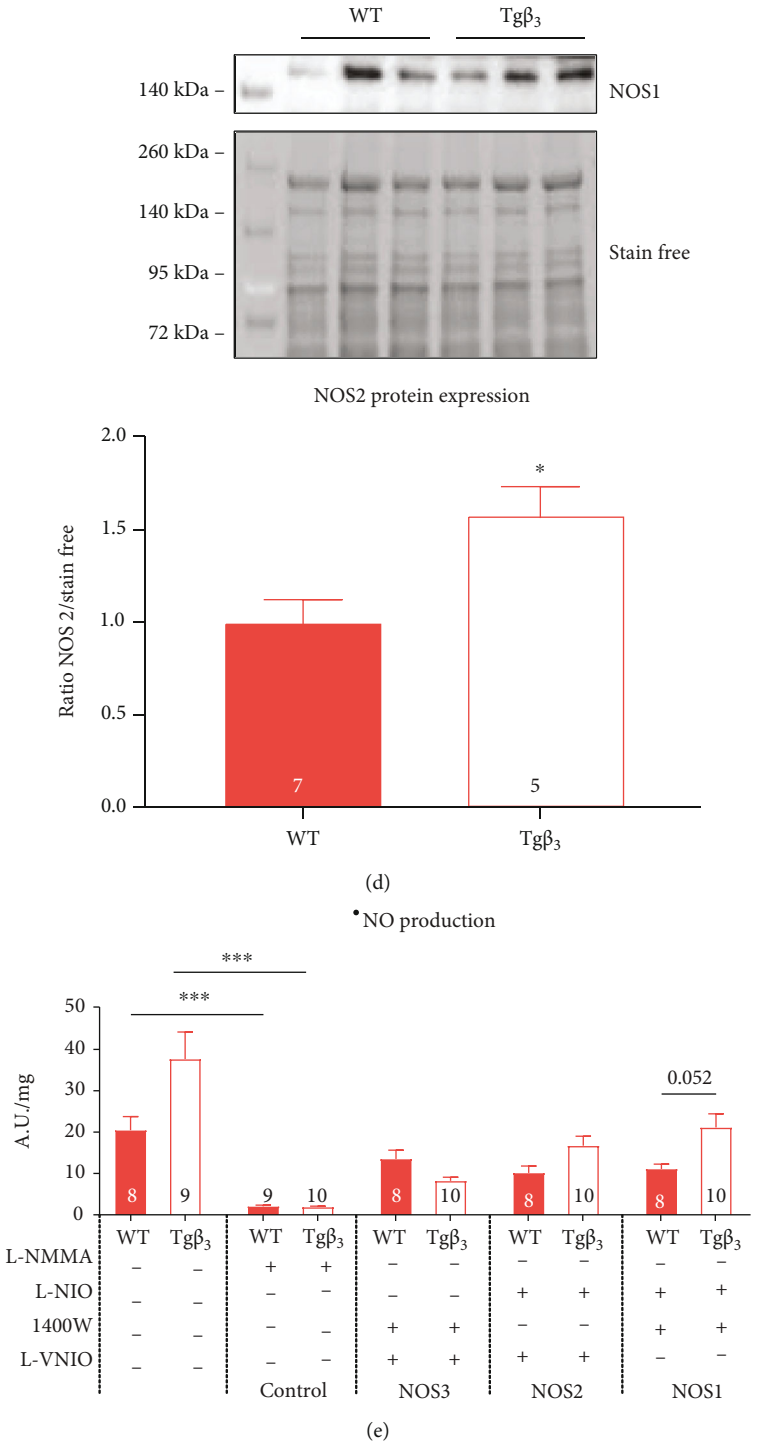

Figure 8: Continued.

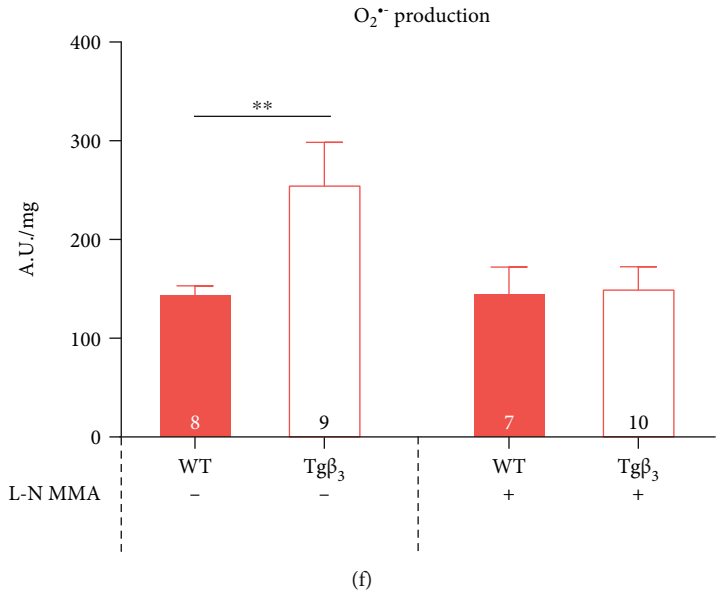

Figure 8: Expression and activity production of NOS in WT and Tgβ_3 rats at 45 weeks of age. Effects of the β_3-AR overexpression in Tgβ_3 rats on aortic protein expression of the NOS3 dimer/monomer ratio (a), p-NOS3/NOS3 monomer (b), NOS2 (c), and NOS1 (d). Evaluation of $^{\bullet}$NO production on thoracic aortic rings with or without an inhibitor (L-NMMA, L-NIO, L-VNIO, and 1400W) (e) and $O_2^{\bullet-}$ production with or without L-NMMA (f) from WT and Tgβ_3 rats. Data are expressed as mean ± SEM. $n = 5$-10. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

4. Discussion

The aim of this study was to decipher the link between endothelial dysfunction and diastolic dysfunction. We wanted to understand if endothelial dysfunction could be involved in HFpEF development. For that, we investigated cardiac and endothelial function in rats at 15, 30, and 45 weeks old of age. The major finding of this study was that β_3-AR overexpression led to endothelial dysfunction throughout ageing and that endothelium dysfunction appears prior to diastolic dysfunction.

4.1. β_3-AR Overexpression Is Associated with Endothelial Dysfunction. *Adrb3*, the gene coding for the β3 receptor, is the main subtype of the β adrenergic receptor expressed in the whole aorta in the rats. However, smooth muscle cells and endothelial cells are mainly expressing the gene coding for the β2 receptor (*Adrb2*) [16]. In the literature, β_3-AR has a low level of protein expression under physiological conditions, but β_3-AR expression is increased in heart failure [17], and many studies demonstrated a cardioprotective role of β_3-AR in pathophysiological conditions [18–22]. Also, specific β_3-AR overexpression on cardiomyocytes is associated with the cardioprotective effect [23]. In our study however, long-term endothelial overexpression of the β_3-AR was associated with deleterious effect such as the development of diastolic dysfunction and an alteration of the vascular function. Vascular reactivity, and specifically vasodilation in response to isoproterenol, was blunted as early as 15 weeks of age, before the apparition of the diastolic dysfunction, suggesting that endothelial dysfunction is a key process in the HFpEF development. Interestingly, altered vasodilation was first detected in the aorta at 15 weeks while it has been detected in both the aorta and mesenteric arteries at 30 weeks. This can be explained in particular by structural differences but also by differences in $^{\bullet}$NO production between elastic arteries such as the aorta and muscular arteries such as the mesenteric arteries [24].

4.2. Endothelial Dysfunction and HFpEF. Endothelial dysfunction has been suggested to be at the center of the HFpEF pathophysiology for about ten years [25–27], yet no study manages to demonstrate that endothelial dysfunction could be the *primum movens* of HFpEF development.

Endothelial dysfunction has been characterized in HFpEF by a decrease in the production of $^{\bullet}$NO, leading to a decrease in vasodilation, as a consequence of the NOS3 protein loss of function [25]. The decrease in $^{\bullet}$NO is explained by the decoupling of NOS3, which has been confirmed in patients with HFpEF or in animal models such as ZSF1 (Zucker fatty/spontaneously hypertensive heart failure F1) rats. NOS3 uncoupling leads to a shift of NOS3 from the dimer, which produces $^{\bullet}$NO, to the monomer, which generates $O_2^{\bullet-}$ [28]. This NOS3 uncoupling is also found in a diabetic HFpEF model [29]. In their study, the authors show a decrease in the activity of NOS3 due to uncoupling of this protein and thus a decrease in the production of $^{\bullet}$NO. Interestingly, Shibata et al. have shown that, by using NOS-knockout mice, the deletion of NOS causes diastolic dysfunction and cardiac hypertrophy [30]. These studies converge to demonstrate that during HFpEF development, the uncoupling of the NOS3 protein induces a decrease in $^{\bullet}$NO production, which could be at the origin of endothelial dysfunction.

NOS1 is largely expressed in the central nervous system, but its expression has also been found in smooth muscle cells and cardiomyocytes. Under physiological conditions, NOS1 expression in smooth muscle cells has been shown to be involved in vascular tone. Cau et al. described that NOS-dependent vasodilation is modified throughout ageing in human and animal models. In fact, NOS3 expression and activity are reduced with age, whereas NOS2 activity is increased and accompanied by peroxynitrite production [31]. These data validate our observations on the involvement of NOS isoforms in vasodilation throughout ageing, with a predominant involvement of NOS3 at 15 weeks of age and all NOS isoforms at 30 and 45 weeks of age. Interestingly, NOS1 has been shown to maintain some degree of vasodilation, when the predominant NOS3 becomes dysfunctional [32, 33] or absent in the knockout mouse model [34, 35]. In our Tgβ_3 rat model, we showed that NOS1 expression was increased at 30 and 45 weeks of age and that the NOS1 is the main isoform involved in the vasodilation. These data suggest that NOS1 could compensate for the dysfunction of NOS3 reported at 45 weeks of age.

4.3. Oxidative Stress: A Key Player in Endothelial and Diastolic Dysfunction. Oxidative stress in another mechanism recently described a potential trigger to the development of endothelial dysfunction. Indeed, it has been described that the H_2O_2 concentration in the myocardium of both HFpEF patients and ZSF1 rats is significantly elevated [25]. H_2O_2 results from the conversion of $O_2^{\bullet-}$ by superoxide dismutase (SOD), and the high $O_2^{\bullet-}$ concentrations in our model therefore suggest an increase in H_2O_2 concentration and SOD activity at the vascular and cardiac levels.

The endothelial dysfunction is linked to an increase in $^{\bullet}$NO production by NOS1. In the same time, NOS3 expression is decreased suggesting NOS3 uncoupling leading to $O_2^{\bullet-}$ production and oxidative stress as described earlier [9, 36]. Increase in oxidative stress is linked to the genesis of heart failure, but some studies suggested that β_3-AR activation inhibited oxidative stress and reactive oxygen production (ROS). Intriguingly, in our model, long-term endothelial β_3-AR overexpression did not protect against the ROS production, and the long-term overproduction of $^{\bullet}$NO is linked to endothelial dysfunction. Several hypotheses can explain this phenomenon. First, the increase in S-nitrosylation could explain the progressive decrease in cardiovascular function. Recently, Schiattarella et al. showed that elevated NOS2 activity leads to S-nitrosylation in multiple proteins and can impair their functions. Furthermore, the study highlighted, in human HFpEF hearts, an increase in NOS2 transcripts [27]. In our study, NOS2 expression was increased as early as 15 weeks of age in the Tgβ_3 group suggesting a disruption of protein function. However, nothing can be concluded since the expression of a protein and the enzymatic activity of the latter are not always linked. In the second hypothesis, the increase in the $^{\bullet}$NO bioavailability associated with an increase in ROS could lead to the production of peroxynitrite ($ONOO^-$). In our study, the trend to increase in $^{\bullet}$NO levels is associated with the overproduction of $O_2^{\bullet-}$ suggesting the production of $ONOO^-$. The production of $ONOO^-$ is increased when the NOS3 is uncoupled [37]. In our model, the decrease in $O_2^{\bullet-}$ production in the presence of L-NMMA suggested that the NOS uncoupling could be at the origin of a reduced vasodilation. More specifically, the uncoupled NOS3, reported in the Tgβ_3 rats, could be at the origin of the diastolic dysfunction. Oxidative stress and more particularly NOS impairment could have a key role in endothelial dysfunction and with ageing could lead to cardiac alteration.

5. Limits

The aim of the study is to better understand the role of endothelial dysfunction in the development of HFpEF. HFpEF is a disease that primarily affects women. In our model of human β3-adrenoreceptor overexpression and as discussed in our previous publication [10], only male rats develop heart failure with preserved ejection fraction. From our results, the involvement of the endothelium in the development of HFpEF could only be linked to the male phenotype.

6. Conclusion

Endothelial dysfunction appeared prior to cardiac dysfunction in our HFpEF rat model indicating a potential role of endothelial dysfunction in the development of HFpEF. We have demonstrated that alteration in the NOS function was a potential trigger of HFpEF development via an endothelial dysfunction. The increase in oxidative stress was characterized by an increase in $O_2^{\bullet-}$ which was the consequence of NOS deregulation in our model potentially via NOS3 uncoupling. Indeed, in many HFpEF models, the NOS3 protein is described as uncoupled, resulting in a decrease in the production of $^{\bullet}$NO and an increase in $O_2^{\bullet-}$, yet as a consequence of HFpEF and not as a cause. Our study provides evidence that endothelial dysfunction could be the trigger to develop HFpEF.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All animal experimental protocols were approved by the Pays de la Loire Ethical Committee and were performed in accordance with the French law on animal welfare, EU Directive 2010/63/EU for animal experiments, the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 2011), and the 1964 Declaration of Helsinki and its later amendments.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

All authors have reviewed and approved the manuscript. TD, TP, JD, CG, and BL were responsible for the conceptualization. MB, VA, AE, AT, DS, AP, ABL, CM, and VS were responsible for the investigation. TD, TP, JD, BR, MDW, CG, and BL wrote the original draft. TD, TP, JD, BR, and BL reviewed and edited the paper. VS, BR, MDW, CG, and BL were responsible for the resources and funding acquisition. Thomas Dupas and Thomas Pelé have contributed equally to this work and share the first authorship.

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