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Effects of isoproterenol treatment for 7 days on inflammatory mediators in the rat aorta

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Davel AP, Fukuda LE, Sá LL, Munhoz CD, Scavone C, Sanz-Rosa D, Cachofeiro V, Lahera V, Rossoni LV. Effects of isoproterenol treatment for 7 days on inflammatory mediators in the rat aorta. *Am J Physiol Heart Circ Physiol* 295: H211–H219, 2008. First published May 16, 2008; doi:10.1152/ajpheart.00581.2007.—The aim of the present study was to evaluate the effect of overstimulation of β-adrenoceptors on vascular inflammatory mediators. Wistar rats were treated with the β-adrenoceptor agonist isoproterenol (0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ sc) or vehicle (control) for 7 days. At the end of treatment, the right carotid artery was catheterized for arterial and left ventricular (LV) hemodynamic evaluation. Isoproterenol treatment increased LV weight but did not change hemodynamic parameters. Aortic mRNA and protein expression were quantified by real-time RT-PCR and Western blot analysis, respectively. Isoproterenol enhanced aortic mRNA and protein expression of IL-1β (124% and 125%) and IL-6 (231% and 40%) compared with controls but did not change TNF-α expression. The nuclear-to-cytoplasmatic protein expression ration of the NF-κB p65 subunit was increased by isoproterenol treatment (51%); in addition, it reduced the cytoplasmatic expression of IκB-α (52%) in aortas. An electrophoretic mobility shift assay was performed using the aorta, and increased NF-κB DNA binding (31%) was observed in isoproterenol-treated rats compared with controls ($P < 0.05$). Isoproterenol treatment increased phenylephrine-induced contraction in aortic rigs ($P < 0.05$), which was significantly reduced by superoxide dismutase (150 U/ml) and sodium salicylate (5 mM). Cotreatment with thalidomide (150 $mg \cdot kg^{-1} \cdot day^{-1}$ for 7 days) also reduced hyperreactivity to phenylephrine induced by isoproterenol. In conclusion, overstimulation of β-adrenoceptors increased proinflammatory cytokines and upregulated NF-κB in the rat aorta. Moreover, local oxidative stress and the proinflammatory state seem to play key roles in the altered vascular reactivity of the rat aorta induced by chronic β-adrenergic stimulation.

β-adrenoceptor; blood vessels; cytokines; nuclear factor-κB

INCREASED SYMPATHETIC TONE resulting in sustained stimulation of adrenoceptors has been associated with cardiovascular diseases such as essential hypertension, cardiac hypertrophy, and heart failure (5, 20).

Persistent β-adrenergic stimulation with isoproterenol is associated with deleterious myocardial effects, including ventricular hypertrophy (36, 41), increased ventricular collagen content (3), and a reduced inotropic response to isoproterenol (10, 23, 42). In blood vessels, we recently demonstrated that 7-day isoproterenol treatment increases the vasoconstrictor response to the α-adrenergic receptor agonist phenylephrine and to the 5-HT agonist serotonin in the rat aorta (17). The observed changes in the vascular reactivity were related to an altered endothelial modulation of the response to phenylephrine, mostly due to increased superoxide anion generation and the consequent reduced bioavailability of nitric oxide (NO) induced by isoproterenol treatment (17).

Reduced availability of NO due to impairment in its synthesis and/or to enhanced degradation by superoxide anion has been implicated as a major cause of endothelial dysfunction in numerous cardiovascular diseases (2, 6, 19). In addition, an increase of production of cytokines and other mediators of inflammation has been associated with endothelial dysfunction in atherosclerosis (38), hypertension (39), and heart failure (1). Importantly, a previous study (33) has shown that chronic β-adrenergic stimulation is a stimulus for the myocardial expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, unaccompanied by any significant change in plasma cytokines levels. Despite evidence implicating a role for β-adrenergic activation as a stimulus for myocardial cytokine expression, there is no clear evidence on whether β-adrenergic stimulation could alter vascular cytokine synthesis.

The NF-κB family is a family of transcription factors, and their subunits form homo- and heterodimers; the most prominent one is the p65/p50 heterodimer (18). The activation of NF-κB in vascular cells can regulate genes implicated in vascular function as well as cyclooxygenase (COX)-2, inducible NO synthase (iNOS), IL-1β, IL-6, E-selectin, and VCAM-1 (18). In vascular cells, inflammatory stimuli (e.g., TNF-α, IL-1β, and oxidative stress) ultimately activate NF-κB (34). In addition, Chandresakar et al. (9) have shown that chronic β-adrenergic stimulation induced by isoproterenol treatment activates myocardial NF-κB in the mouse. These data led us to hypothesize that vascular oxidative stress induced by isoproterenol treatment can activate NF-κB and consequently increase proinflammatory cytokine release contributing to altered vascular function.

Therefore, the aim of this study was to investigate whether chronic β-adrenergic stimulation with isoproterenol in the rat aorta is associated with changes in the vascular synthesis of TNF-α, IL-1β, and IL-6. In addition, as NF-κB is a convergent signal of proinflammatory cytokines and oxidative stress signaling pathways, we also investigated the protein expression of its inhibitor IκB-α and nuclear expression and activity of NF-κB as well its role in the alterations of vascular function induced by 7-day isoproterenol treatment in the rat aorta.

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MATERIALS AND METHODS

Animals. Experiments were performed in male Wistar rats (12–14 wk old, $n = 30$) obtained from colonies and maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Complutense de Madrid and at the Biomedical Sciences Institute of the University of São Paulo. Rats were housed at a constant room temperature and light cycle (12:12-h light-dark cycle) with free access to standard rat chow and tap water. Rats were randomly divided into two groups: *1*) isoproterenol-treated rats (0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ sc, once daily, suspended in 0.1 ml of soy bean oil); and *2*) control rats, which received only the vehicle (0.1 ml sc). Isoproterenol or vehicle was administered for 7 days.

At the end of the treatment period, animals were anesthetized for measurements of hemodynamic parameters. Animals were killed by decapitation, the heart was removed for ventricular weight measurement, and the aorta was isolated for vascular function experiments, mRNA quantification, protein expression, and NF-κB activity analysis. Except for vascular reactivity experiments, the aorta was kept frozen (−80°C) until the day of experiments. For real-time RT-PCR performance, isolation and manipulation of the aorta were always performed under sterile conditions (RNase free). An additional group of rats was cotreated orally with thalidomide (150 $mg \cdot kg^{-1} \cdot day^{-1}$ po) along with a subcutaneous injection of isoproterenol (0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ sc) for 7 days to evaluate the possible role of NF-κB activity on the vascular reactivity alterations induced by isoproterenol treatment.

All experimental procedures were approved by the Animal Care and Use Committee of Universidad Complutense, according to guidelines for the ethical care of experimental animals of the European Union, and by the Animal Care and Use Committee of the Biomedical Institute of São Paulo University, according to the Brazilian Societies of Experimental Biology.

Arterial and left ventricular hemodynamic parameters. At the end of treatment, control and isoproterenol-treated rats were anesthetized with a ketamine-xylazine mixture (90 and 5 mg/kg ip), and a polyethylene catheter (PE-50, 8 cm, filled with heparinized saline) was introduced through the right carotid artery into the left ventricle (LV). During this procedure, arterial blood pressure, LV systolic pressure (LVSP), and LV end-diastolic pressure (LVEDP) were obtained by a pressure transducer (Transpac IV-Abbbot, Critical Care Systems, Nashua, NH) and recorded continuously (Mc Lab 8E, AD Instruments). The positive and negative first derivatives of LV pressure (dP/dt_{max} and dP/dt_{min}, respectively) and heart rate were also determined and recorded (Mc Lab 8E, AD Instruments). The LV catheter was then pulled out, and arterial blood pressure was measured. The maintenance of diastolic blood pressure at proper values was the guarantee that the aortic valve was not damaged during the procedure.

After arterial and LV pressure recordings, animals were killed by decapitation, and the heart was isolated and weighed. The LV weight was normalized to body weight (in mg/g). This ratio was used as an index of LV hypertrophy.

RNA isolation. Frozen rat aortas were pulverized in liquid nitrogen and homogenized together with 1 ml of Tri Reagent (Sigma). RNA isolation was performed according to the Chomczynski method (13). RNA was quantified by optical density (OD) measurements at 260 nm using a BioPhotometer (Eppendorf). RNA was frozen at −20°C until used.

RT for cDNA synthesis. Total RNA (5 μg) was taken to perform RT. It was previously heated with 2 μM random hexamers at 70°C for 5 min and quickly chilled on ice. Subsequently, a mixture of RNase inhibitor (0.7 units), 25 mM Tris·HCl (pH 8.3), 37 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, dNTPs (0.4 mM each), and 2.5 units Moloney murine leukemia virus reverse transcriptase enzyme was added, and samples were incubated at 37°C for 60 min followed by heating at 95°C for 10 min and chilling on ice. The mixture was then completed with DNase-free water until a final volume of 50 μl.

Real-time RT-PCR. cDNA (2 μl) was taken for a real-time RT-PCR using Premix Ex Taq (Takara Bio). Each RT-PCR consisted of 2 μl cDNA, 12.5 μl Premix Ex Taq buffer (TaKaRaEx Taq HS enzyme, dNTP mixture, and Mg^{2+}), 400 nM of each specific forward and reverse primer, and 300 nM TaqMan probe in a total reaction volume of 25 μl. Reactions were carried out on a fluorescence temperature cypler (SmartCycler, Cepheid, Sunnyvale, CA).

In a Smart Cycler under annealing conditions, the TaqMan probe specifically hybridizes to template DNA. However, the fluorescence substance at the 5′-end of oligonucleotides is suppressed by a quencher at the 3′-end. In the extension step, the 5′- to 3′-exonuclease activity of *Taq* DNA polymerase degrades the TaqMan probe hybridized to a template. Accordingly, the TaqMan probe is released from the suppression with a quencher, resulting in the emission of fluorescence. By measuring the fluorescence intensity, the amount of amplified product can be monitored. Primer and probe sequences used in real-time RT-PCR experiments are shown in Table 1.

Parameters included the initial activation of hotStart Taq DNA polymerase (TaKaRaEx Taq HS) at 95°C for 10 s followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. Results were calculated as percentages of control rats using the $\Delta\Delta C_T$ method (28) (where C_T is the threshold cycle) with GAPDH as the control gene.

Protein extracts. Nuclear and cytoplasmatic protein extracts of aortas were prepared as previously described (11). Briefly, aortic tissue was pulverized in liquid nitrogen and homogenized in ice-cold *buffer A* [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml antipain] using a Dounce homogenizer. After 10 min of incubation on ice, samples were centrifuged at 4°C for 10 min at 850 *g*. Supernatants were discarded, and pellets were resuspended in ice-cold *buffer A* containing 0.1% Triton-X. After 10 min of incubation on ice, samples were centrifuged as described above. The supernatants represent the cytoplasmatic protein extract from samples, and they were removed and stored at −80°C for posterior analysis. Pellets were washed in ice-cold *buffer A* and centrifuged again (850 *g* for 10 min at 4°C). Nuclear pellets were resuspended in ice-cold *buffer B* [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1.0 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml antipain]. After 30 min of incubation on ice, the suspension was centrifuged for 15 min at 16,000 *g* at 4°C. The resulting supernatants containing nuclear proteins were stored at −80°C.

For total protein extraction, aortas from control and isoproterenol-treated rats were homogeneized in cold RIPA lysis buffer (Upstate,

Table 1. *Primer and probe sequences*

	Probe	Forward Primer	Reverse Primer
IL-1β	5′-FAM-TGACTCGTGGGATGATGACGACCTG-BHQ1-3′	5′-TCTTCGAGGCACAAGGCA-3′	5′-CAGAGGTCCAGGTCCTGGAA-3′
IL-6	5′-FAM-ATGTTGTTGACAGCCACTGCCTTCCC-BHQ1-3′	5′-CAGCTATGAAGTTTCTCTCCGCA-3′	5′-CAGAATTGCCATTGCACAACTC-3′
TNF-α	5′-FAM-TGGCCCAGACCCTCACACTCAGATCA-TAMRA-3′	5′-GGTGATCGGTCCCAACAAGGA-3′	5′-CACGCTGGCTCAGCCACTC-3′
GAPDH	5′-Texas red-ATCACGCCACAGCTTTCCAGAGGG-BHQ2-3′	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′

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Temecula, CA) containing PMSF (1 mM) and Na_3VO_4 (1 mM). After centrifugation (1,500 *g* for 20 min at 4°C), supernatants were isolated and stored at −80°C.

Protein concentration was determined using Bio-Rad protein reagent.

Western blot analysis. Nuclear (30 μg), cytoplasmatic (40 μg), and total (100 μg) protein extracts of the aorta were electrophoretically separated by 10% or 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes overnight at 4°C using a Mini Trans-Blot Cell system (Bio-Rad) containing 25 mM Tris, 190 mM glycine, 20% methanol, and 0.05% SDS.

After blockade of nonspecific sites with 5% nonfat dry milk, membranes containing nuclear or cytoplasmatic samples were incubated overnight at 4°C with the primary antibody raised against NF-κB p65 subunit (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); membranes containing cytoplasmatic proteins were incubated with anti-IκB-α antibody (1:1,000, Santa Cruz Biotechnology). Membranes containing total protein extract were incubated with the following primary antibodies: anti-IL-1β (1:1,500, BioLegend), anti-TNF-α (1:500, BioLegend), and anti-IL-6 (1:1,000, Abcam). After being washed (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20), membranes were incubated with peroxidase-conjugated anti-rabbit IgG antibody (1:1,500, Bio-Rad) for the NF-κB p65 subunit, IκB-α, and IL-6 and with anti-Armenian hamster IgG (1:3,000, Jackson ImmunoResearch) for IL-1β and TNF-α. Membranes were thoroughly washed, and immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminescence system (ECLPlus, Amersham) and subjected to autoradiography (Hyperfilm ECL, Amersham). Signals on the immunoblot were quantified with Scion Image software. In the same membrane, α-actin protein expression was determined using a monoclonal anti-α-actin antibody (1: 3,000 dilution, Sigma), and its content was used as an internal control for the experiments.

EMSA for NF-κB. An EMSA for NF-κB was performed using the gel shift assay kit from Promega as previously described (37). [^{32}P]NF-κB double-stranded consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′, 20,000 counts/min) and nuclear extracts (10 μg) were used. DNA-protein complexes were separated by electrophoresis through a 6% nondenaturing acrylamide: bis-acrylamide (37.5:1) gel in 0.5× Tris-borate-EDTA for 2 h at 150 V. Gels were vacuum dried and analyzed by autoradiography. For competition experiments, NF-κB and transcription faction IID (5′-GCAGAGCATATAAGGTGAGGTAGGA-3′) unlabeled double-stranded consensus oligonucleotides were included in a 20-fold molar excess over the amount of [^{32}P]NF-κB probe to detect specific and nonspecific DNA-protein interactions, respectively.

Supershift assays using antibodies against different NF-κB subunits were also conducted according to the manufacturer's protocol (Santa Cruz Biotechnology) (data not shown). Autoradiographs were scanned (HP 1200 series), and the OD of the bands was quantified using Image J software (National Institutes of Health).

Vascular function. Vascular function was evaluated in aortic rings from control, isoproterenol-treated, and isoproterenol plus thalidomide-treated rats. Segments of the thoracic aorta (4 mm in length), free of fat and connective tissue, were mounted in an isolated tissue chamber containing Krebs-Henseleit solution [containing (in mM) 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 11 glucose, and 0.01 EDTA], gassed with 95% O_2 and 5% CO_2, and maintained resting tension of 1 g at 37°C at pH 7.4. Isometric tension was recorded using an isometric force transducer (Letica TRI 210) connected to an acquisition system (MP100, Biopac Systems).

After a 45-min equilibration period, aortic rings were exposed to 75 mM KCl to assess the maximal tension developed. Afterward, endothelial integrity was tested by ACh-induced relaxation (10 μM) in segments previously contracted with phenylephrine (∼10 μM). Relaxation of ≥80% was considered as demonstrative of the functional integrity of the endothelium. After a washout period, concentration-response curves to the α_1-adrenoceptor agonist phenylephrine (0.1 nM–30 μM) were obtained.

The role of superoxide anion and NF-κB activity in the vasoconstrictor response to phenylephrine was evaluated by the incubation of some aortic rings with the superoxide anion scavenger SOD (150 U/ml) or with sodium salicylate (NaSal; 5 mM). SOD was added 30 min and NaSal 60 min before the concentration-response curves to phenylephrine were assessed.

Vasoconstrictor responses to phenylephrine are expressed as percentages of the contraction produced by 75 mM KCl.

For each concentration-response curve, the maximum effect [maximal vasoconstrictor response (R_{max})] to phenylephrine was calculated using nonlinear regression analysis (GraphPad Prism software). To compare the effects of SOD and NaSal on the response to phenylephrine in aortic rings from control or isoproterenol-treated rats, some results were expressed as differences of areas under concentration-response curves (dAUC) in basal and experimental situations. AUCs were calculated from the individual concentration-response curves (GraphPad Prism software), and dAUCs were expressed as percentages of AUCs of corresponding basal values.

Statistical analysis and drugs. Results are expressed as means ± SE, and *n* represents the numbers of animals used in each experiment. Concentration-response curves to phenylephrine were analyzed by two-way ANOVA followed by the Bonferroni post hoc test. For specific two-means comparisons, an unpaired Student *t*-test was used. A value of $P < 0.05$ was considered significant.

(−/−)-Isoproterenol, ACh hydrochloride, phenylephrine hydrochloride, SOD (bovine erythrocyte), and NaSal were purchased from Sigma Chemical. [γ-^{32}P]ATP and poly dI-dC were from Amersham Biosciences. The gel shift assay system kit for NF-κB was from Promega, and the Bio-Rad protein assay kit was from Bio-Rad. Thalidomide was a generous gift from Dr. J. G. Mill (Federal University of Espírito Santo, Espírito Santo, Brazil).

RESULTS

Effects of isoproterenol treatment on hemodynamic parameters and ventricular weight. As shown in Table 2, treatment with isoproterenol for 7 days resulted in significant LV hypertrophy and did not affect body weights of the rats (control: 338 ± 6 g vs. isoproterenol: 345 ± 11 g, $P > 0.05$ by *t*-test). At the end of the treatment, no differences were observed in mean arterial pressure or heart rate between isoproterenol-treated and control anesthetized rats (Table 2). In addition, isoproterenol treatment did not induce significant changes in the ventricular hemodynamic parameters evaluated, including

Table 2. *Effects of 7-day isoproterenol treatment on ventricular weight and arterial and LV hemodynamic parameters of rats*

	Control Rats	Isoproterenol-Treated Rats
LV weight/body weight, mg/g	1.88±0.08	2.45±0.08*
LV hypertrophy, %		30.3
Mean arterial pressure, mmHg	104±5	109±3
Heart rate, beats/min	264±9	274±4
LV systolic pressure, mmHg	139±5	140±7
LV end-diastolic pressure, mmHg	8.0±1.1	10.6±1.2
dP/dt_{max}, mmHg/s	5,907±745	5,051±751
dP/dt_{min}, mmHg/s	−4,212±696	−4,009±545

Values are means ± SE; $n = 6$ rats/group. LV, left ventricular; dP/dt_{max} and dP/dt_{min}, positive and negative first derivatives of LV pressure. *$P < 0.01$ vs. control rats (by *t*-test).

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LVSP, LVEDP, dP/dt_{max} and dP/dt_{min}, in anesthetized animals (Table 2).

Oral treatment with thalidomide did not modify the body weights of treated animals (isoproterenol: 282 ± 10 g vs. isoproterenol + thalidomide: 274 ± 3 g, $P > 0.05$ by *t*-test). In addition, thalidomide did not prevent the development of LV hypertrophy induced by isoproterenol treatment (isoproterenol: 2.81 ± 0.14 mg/g vs. isoproterenol + thalidomide: 2.88 ± 0.10 mg/g, $P > 0.05$ by *t*-test).

Effects of isoproterenol treatment on aortic gene and protein expression of proinflammatory cytokines. Isoproterenol treatment increased mRNA and protein expression of proinflammatory cytokines IL-1β (124% and 125%, respectively) and IL-6 (231% and 40%, respectively) in aortic homogenates compared with control animals (Fig. 1, *A–D*). However, there were no differences in aortic mRNA and protein expression of TNF-α between control and isoproterenol-treated rats (Fig. 1, *E* and *F*).

Effects of isoproterenol treatment on the protein expression of the p65 NF-κB subunit and IκB-α. β-Adrenergic stimulation with isoproterenol for 7 days increased the nuclear-to-cytosolic protein expression ratio of the NF-κB p65 subunit (+51%; Fig. 2*A*). In addition, the cytosolic protein expression of IκB-α was significantly smaller in aortic tissue of isoproterenol-treated rats (−52%) than in control animals (Fig. 2*B*).

Nuclear NF-κB activation in aortas from isoproterenol-treated rats. Isoproterenol treatment significantly increased NF-κB DNA binding activity in nuclear extracts of the rat aorta (31% over the control), as evaluated by EMSA (Fig. 3, *A* and *B*). Supershift analysis indicated that the p65/p50 heterodimer was the major DNA-protein complex present in nuclear extracts of the aorta (data not shown).

Vascular reactivity to phenylephrine in aortas of isoproterenol-treated rats: role of superoxide anion and NF-κB activity. The R_{max} induced by phenylephrine was increased in aortas from isoproterenol-treated rats compared with control (isoproterenol: 93 ± 1.1% vs. control: 55 ± 6.3% with contraction to KCl, $P < 0.05$ by *t*-test), as previously described (17). Possible roles of superoxide anion and NF-κB activity on this effect were evaluated by SOD and NaSal administration, respectively, in some aortic rings. Both SOD and NaSal incubation significantly reduced the phenylephrine-induced contraction only in isoproterenol-treated rats (Fig. 4, *A* and *B*). After SOD and NaSal incubation, there were no significant differences between R_{max} values to phenylephrine of control and isoproterenol-treated groups (with SOD, control: 50 ± 6.3% vs. isoproterenol: 65 ± 13.9% with contraction to KCl, $P > 0.05$ by *t*-test; with NaSal, control: 59 ± 13.8% vs. isoproterenol: 69 ± 10.1% with contraction to KCl, $P > 0.05$ by *t*-test). A comparison of dAUCs indicated that the effects of SOD and NaSal were higher in aortic rings from isoproterenol-treated rats compared with control rats (Fig. 4*C*). Moreover, dAUCs for SOD and NaSal incubations were almost similar in aortic rings from isoproterenol-treated rats (Fig. 4*C*).

Thalidomide treatment for 7 days partially prevented the changes in vascular reactivity induced by isoproterenol (Fig. 5*A*) and did not modify the endothelium-dependent relaxation to ACh (10 μM) (ACh, isoproterenol: 85 ± 2.1%, $n = 5$, vs. isoproterenol + thalidomide: 85 ± 1.5%, $n = 6$, $P > 0.05$ by *t*-test). In addition, as shown in Fig. 5*B*, thalidomide treatment was able to significantly reduce the increased activity of NF-κB in nuclear extracts of isoproterenol-treated aortas to control levels.

DISCUSSION

The present study, for the first time, demonstrated that overstimulation of β-adrenoceptors induced by 7-day isoproterenol treatment leads to increased gene and protein expression of vascular proinflammatory cytokines IL-1β and IL-6 without changes in aortic TNF-α levels, which is associated with altered vascular reactivity and oxidative stress. In addition, isoproterenol treatment induced the nuclear protein expression and activity of NF-κB as well as downregulated its inhibitor IκB-α in the vasculature. Both oxidative stress and increased NF-κB activity seem to be mechanisms involved in the altered vascular function induced by sustained β-adrenergic stimulation.

The development of a local inflammatory process is one potential trigger of endothelial dysfunction (15) and can play a pivotal role in the progression of vascular damage associated with cardiovascular diseases as hypertension (39), heart failure (1), and atherosclerosis (38). Because the inflammatory response is associated with cytokine release, cytokines may have an important role in the vascular injury induced by inflammation.

The present study demonstrated that 7-day isoproterenol treatment increases mRNA and protein expression of the proinflammatory cytokines IL-1β and IL-6 in the rat aorta. A previous study (8) has demonstrated that epinephrine, at physiological concentrations, is able to increases the secretion of IL-1β by monocytes in vitro. In addition, Murray et al. (33) found an increase of myocardial gene and protein expression of IL-1β, IL-6, and TNF-α in 7-day isoproterenol-treated rats. However, the present data suggested that aortic mRNA and protein levels of TNF-α were not altered by isoproterenol treatment. In agreement, a previous study (32) has indicated a lack of correlation between circulating TNF-α and norepinephrine levels in heart failure.

Mechanisms underlying the stimulation of vascular inflammatory cytokines during increased catecholamine levels are not yet well established. Prabhu et al. (35) demonstrated that β-adrenergic blockade after myocardial infarction is able to reduce the myocardial expression of TNF-α and IL-1β. In addition, epinephrine synergizes with IL-1β, inducing gene expression and the production of proinflammatory cytokines IL-6, IL-8, and IL-13 in cultured human mast cells, and this effect was downregulated by the β-adrenoceptor antagonist propranolol (12). These data reinforce a role for β-adrenocep-

Fig. 1. *A–F*: mRNA (*A*, *C*, and *E*) and protein (*B*, *D*, and *F*) expression of IL-1β (*A* and *B*), IL-6 (*C* and *D*), and TNF-α (*E* and *F*) in aortas from control (CT) and 7-day isoproterenol (ISO)-treated rats. mRNA expression is presented as a percentage of the mean value obtained in CT rats by analyzing the critical threshold numbers corrected by critical threshold readings of corresponding internal GAPDH controls. *B*, *D*, and *F* also show representative Western blot autoradiographies (*top*). α-Actin was used as the internal control for IL-1β, IL-6, and TNF-α protein expression. Bars are means ± SE. Numbers of animals are indicated in the bars. *$P < 0.05$, ISO vs. CT (by *t*-test).

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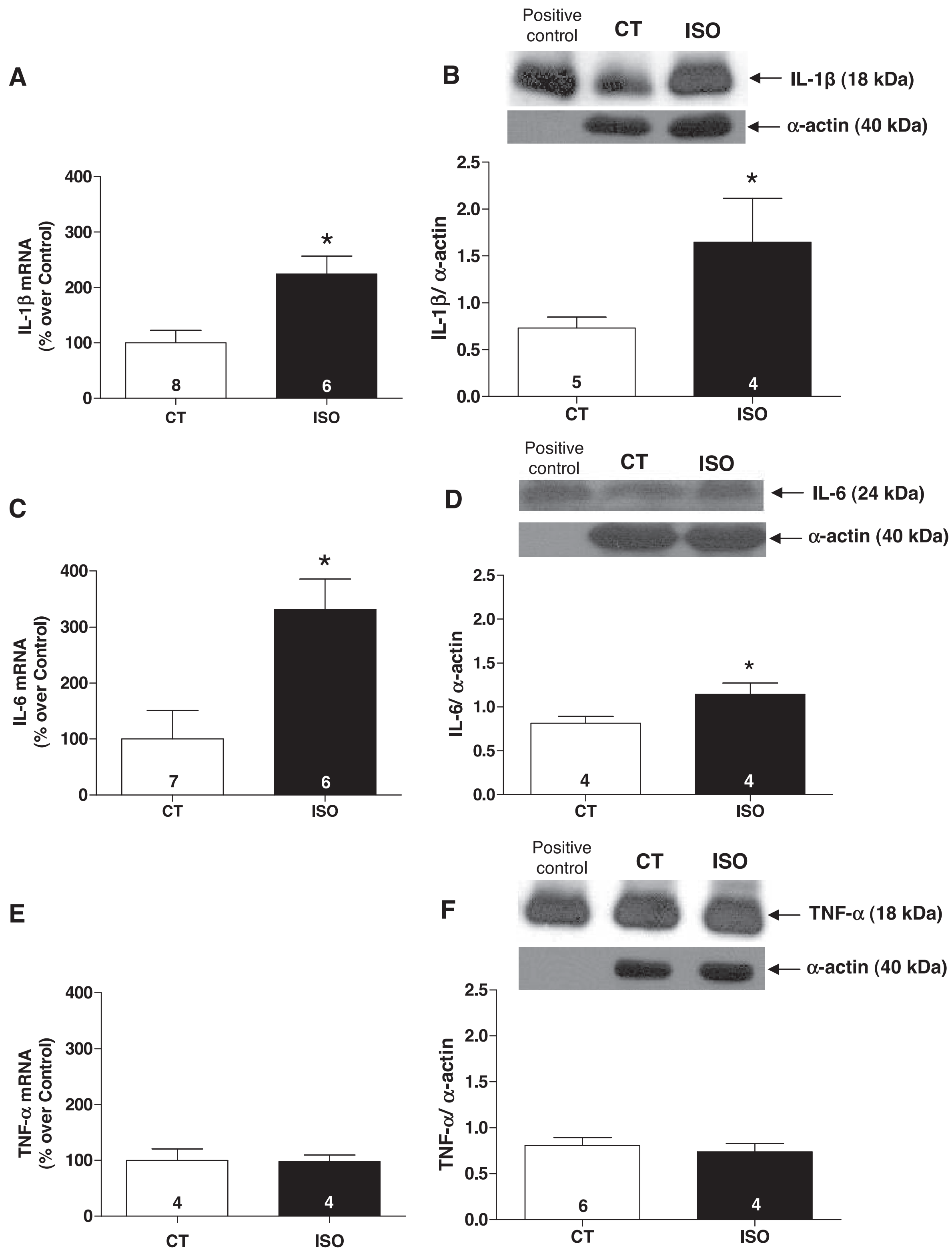
tors in inducing cytokine production during increased levels of circulating catecholamines. Nevertheless, it was described that in some cases, catecholamines can induce an immunoregulatory effect, suppressing the inflammatory response (25, 40).

TNF-α, IL-1β, and IL-6 gene expression is often generated by a mechanism that is regulated by NF-κB, which, in turn, also produces mediators with action at the vascular level that contribute to the inflammatory response, constituting an am-



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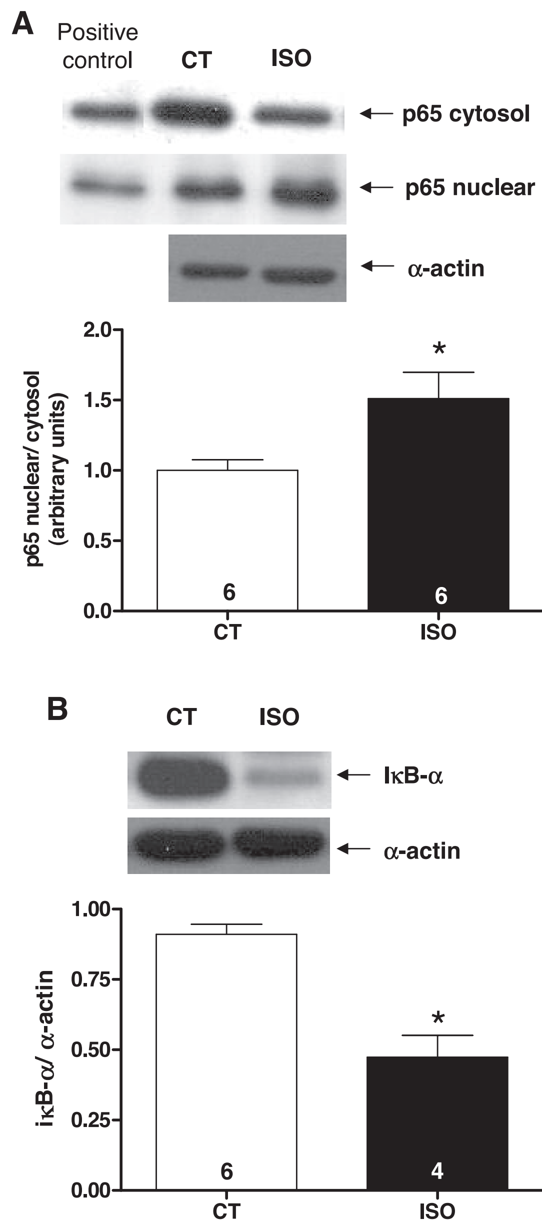

Fig. 2. *A* and *B*: representative Western blot autoradiographies (*top*) and densitometric analysis [*bottom*; in arbitrary units (AU)] of the p65 nuclear-to-cytosolic protein expression ratio (*A*) and IκB-α cytoplasmatic protein expression (*B*) in aortas from CT and 7-day ISO-treated rats. α-Actin was used as the internal control for IκB-α and p65 protein expression. Bars are means ± SE. Numbers of animals are indicated in the bars. *$P < 0.05$, ISO vs. CT (by *t*-test).

plification cascade (43). In a previous study (17), our laboratory demonstrated that 7-day isoproterenol treatment is able to increase anion superoxide production associated with uncoupled endothelial NO synthase in the rat aorta. In endothelial cells, NF-κB is a prime target for reactive oxygen species, and its activation has been linked with endothelial dysfunction (16). Upon activation, NF-κB stimulates inflammatory responses by increasing the expression of specific genes including, e.g., cytokines, adhesion molecules, chemokines, and enzymes as iNOS and COX-2 (14, 34, 43). Thus, NF-κB activation associated with increased superoxide anion levels could be involved in the vascular effects induced by 7-day isoproterenol treatment.

The NF-κB subunit family in mammals is composed of p50, p65 (RelA), cRel, p52, and RelB, which can form homo- and heterodimers, with the most prominent being the p65/p50 heterodimer (18). In the present study, isoproterenol treatment increased the translocation of the p65 subunit from the cytoplasm to the nucleus in the rat aorta. These data suggested that chronic β-adrenergic stimulation may increase the nuclear activation of the p50/p65 heterodimer in the aorta.

The NF-κB dimer is retained in the cytoplasm in an inactive state through an interaction with the inhibitory molecule IκB. Upon stimulation, IκB is rapidly phosphorylated and degraded. The released NF-κB can then translocate to the nucleus and activate target genes (18). In the present study, aortas from isoproterenol-treated rats showed diminished protein content of IκB-α compared with the control group, a mechanism that can be involved in the increased translocation of NF-κB to the nucleus. To evaluate if β-adrenergic overstimulation through isoproterenol treatment is able to stimulate the nuclear activity of NF-κB, we performed an EMSA for NF-κB in aortas from control and isoproterenol-treated rats. An increase of NF-κB

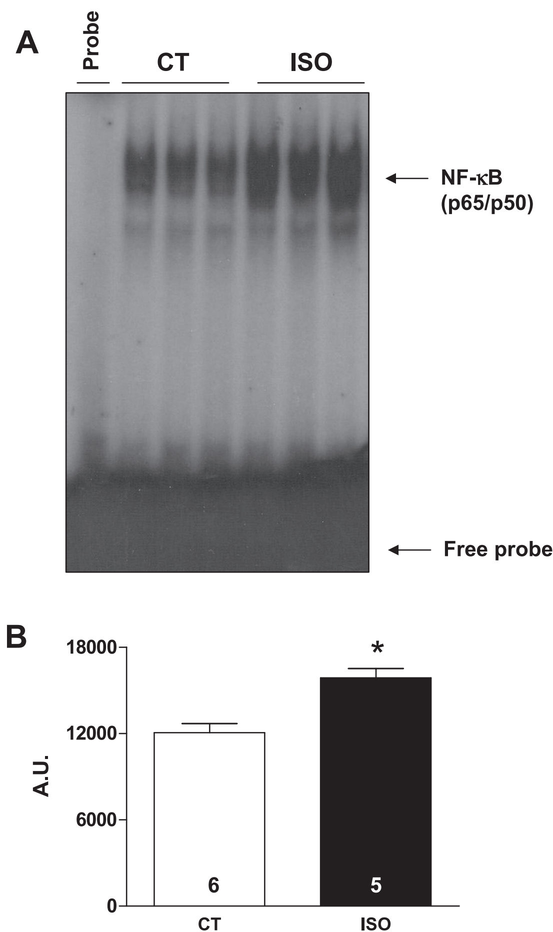

Fig. 3. EMSA for NF-κB in nuclear extracts of the aorta. *A*: nuclear protein (10 μg) was extracted from aortas of CT and 7-day ISO-treated rats. The position of the specific NF-κB-DNA binding complex (p65/p50 bands) is indicated. *B*: densitometric analysis of p50/p65 bands in nuclear extracts of the aorta from CT and 7-day ISO-treated rats. Bars are means ± SE. Numbers of animals are indicated in the bars. *$P < 0.05$, ISO vs. CT (by *t*-test).

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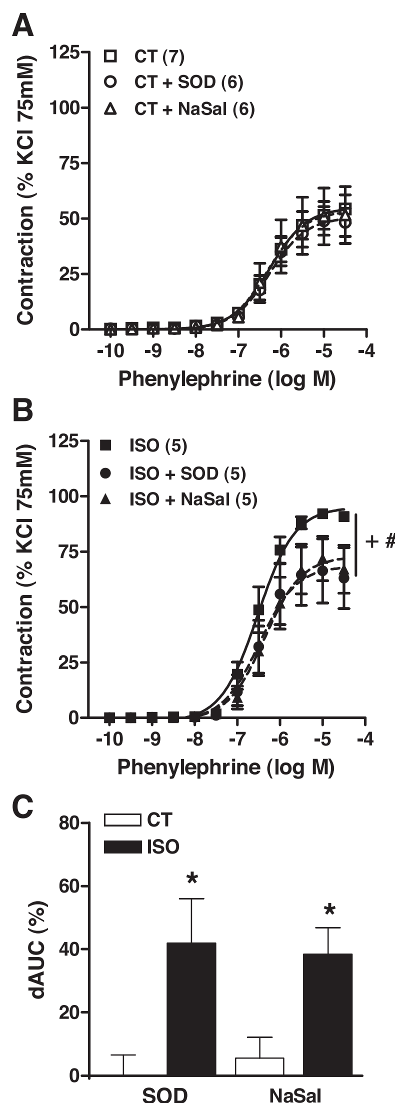

Fig. 4. *A* and *B*: effects of SOD (150 U/mL) and sodium salicylate (NaSal; 5 mM) on concentration-response curves to phenylephrine in aortic rings from CT (*A*) and ISO-treated (*B*) rats. Results are means ± SE and are expressed as percentages of responses to 75 mM KCl. Numbers of animals are indicated in parentheses. $+P < 0.05$, ISO + SOD vs. ISO; $\#P < 0.05$, ISO + NaSal vs. ISO (by two-way ANOVA). *C*: bar graph showing the differences in areas under the concentration-response curves (dAUCs) to phenylephrine in aortic rings in the absence or presence of SOD or NaSal. dAUC values are means ± SE and are expressed as percentages of the difference of corresponding AUCs for nonincubated aortas. $*P < 0.05$, ISO vs. CT (by *t*-test).

DNA binding activity was observed in aortas from isoproterenol-treated rats compared with control rats. Together, these data suggest that a major activation of NF-κB might be involved in the downstream cascade activated during prolonged β-adrenergic stimulation in the aorta.

Vascular function was assessed through the vasoconstrictor response to the α_1-adrenergic agonist phenylephrine in isolated aortas. Chronic β-adrenergic stimulation with isoproterenol increased the contraction to phenylephrine. The increased vasoconstrictor response to phenylephrine was normalized by SOD administration. This result reinforce our previous results showing that the changes in the reactivity to vasoconstrictors agents induced by isoproterenol treatment for 7 days seems to be due to an alteration of endothelial modulation of the contractile response to phenylephrine associated with NO and superoxide anion imbalance (17). In a similar magnitude, the NF-κB inhibition through the incubation of aortic rings with NaSal (5 mM) also significantly reduced the hyperreactivity to phenylephrine of isoproterenol-treated rats. It is well known that the nonsteroidal anti-inflammatory drug NaSal inhibits NF-κB activation in a variety of cell types, and this effect is independent on any interference with COX activity (21, 24, 31). This anti-inflammatory effect of NaSal has been observed in a range of concentrations that correlates with amounts in plasma for optimal clinical treatment of inflammation (1–5 mM). More recently, Marra et al. (31) demonstrated that treatment with NaSal (5 mM) inhibited NF-κB activation in PDGF-treated smooth muscle cells without affecting their viability. In our study, we used the same dose and period of incubation as Marra et al. (31), which strongly suggest that the

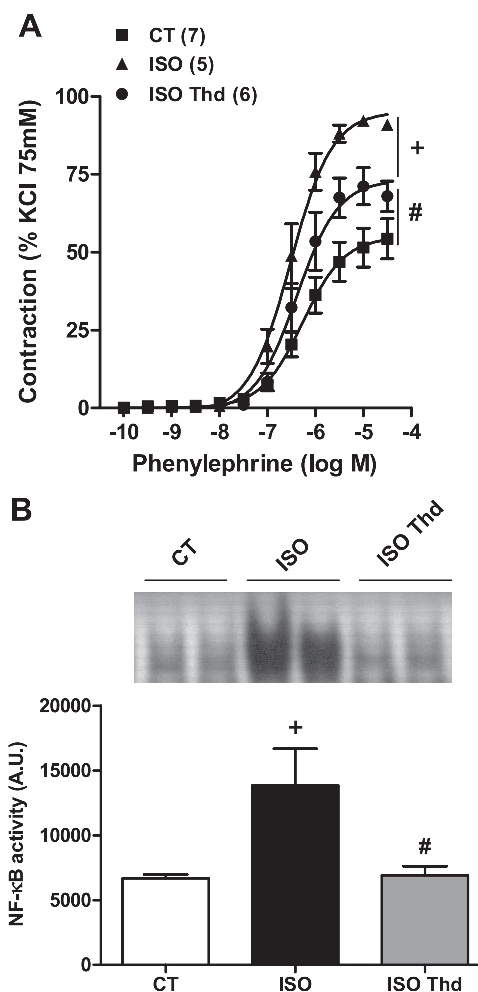

Fig. 5. *A*: concentration-response curves to phenylephrine in aortic rings from rats treated for 7 days with vehicle (CT), ISO, or ISO plus thalidomide (Thd). Results are means ± SE and are expressed as percentages of responses to 75 mM KCl. Numbers of animals are indicated in parentheses. *B*: representative (*top*) and densitometric analysis (*bottom*) of an EMSA for NF-κB in nuclear extracts of the aorta. Nuclear protein (10 μg) was extracted from the rat aorta of vehicle (CT), ISO-treated, and ISO + Thd-treated rats. Bars are means ± SE. Three animals were used per group. $+P < 0.05$, ISO vs. CT; $\#P < 0.05$, ISO + Thd vs. ISO (by two-way ANOVA in *A* or one-way ANOVA in *B*).

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results showing the effects of NaSal (5 mM, 1 h) on the vascular reactivity of the rat aorta is via NF-κB inhibition. In addition, we chronically inhibited the NF-κB pathway by cotreatment of isoproterenol-treated rats with thalidomide for 7 days. Thalidomide is an immunomodulatory drug, although its exact mechanism of action is still not clear. It probably acts through the suppression TNF-α, the modulation of other inflammatory mediators, and inhibition of NF-κB (22). According, there are previous works demonstrating that both acute incubation and oral thalidomide treatment are able to inhibit NF-κB activity (27, 30). Oral administration of thalidomide was able to improve the increase vascular reactivity to α-adrenergic agonist in the aorta, concomitant with a significant reduction of the increased NF-κB activity induced by isoproterenol treatment. Taken together, the present data suggest that the higher NF-κB activity found in the aorta after isoproterenol treatment seems to be involved in the altered vascular function in this experimental model.

In the heart, Chandrasekar et al. (9) demonstrated that isoproterenol treatment can induce the activation of NF-κB in a β_2-adrenoceptor-dependent manner, suggesting a role for the activation of β-adrenoceptors contributing to a proinflammatory state of the cardiovascular system. However, in the vasculature, this mechanism is not clear. The NF-κB activation pathway is induced by a variety of immunity mediators. In regard to vascular function, important stimuli are proinflammatory cytokines such as TNF-α and IL-1β and oxidative stress (18). In the present study, it was observed that SOD and NaSal improved the vascular reactivity to phenylephrine in aortic rings from isoproterenol-treated rats in a similar magnitude. It is known that oxidative stress can lead to IKK activation and subsequent IκB-α phosphorylation and NF-κB activation (26). Thus, this could be the mechanism associated with the upregulation of NF-κB in aortas from isoproterenol-treated rats. In addition, it is important to note that cytokines such as IL-1β can activate NF-κB by causing oxidative stress (4, 26). Upon stimulation, NF-κB activates the gene expression of cytokines that, in turn, activate NF-κB activity. As the gene expression of IL-1β and IL-6 was increased by isoproterenol treatment, these factors can induce an amplification cascade to activate NF-κB and could mediate the vascular effects of NF-κB activation in the vascular function.

In the present work, 7-day isoproterenol treatment produced LV hypertrophy. However, this treatment did not change mean arterial pressure or heart rate or significantly alter LVSP, LV diastolic pressure, dP/dt_{min}, and dP/dt_{max} in anesthetized rats. Thus, the present results confirm previous studies (7, 42) showing that in rats receiving the same time and dose of isoproterenol as used in the present study there are no developments of overt heart failure, despite cardiac hypertrophy and overstimulation of β-adrenoceptors.

Murray et al. (33) demonstrated that 7-day isoproterenol treatment induces increases of proinflammatory cytokines in the heart. In vitro studies have shown that cytokines can induce hypertrophy (44) and extracellular matrix remodeling (32). Therefore, cardiac morphological alterations induced by sustained β-adrenergic stimulation can be associated with actions of cytokines. In the present study, the cotreatment of the isoproterenol group with thalidomide did not significantly alter LV weight, suggesting that at the dose and time used in the present study, the immunomodulatory effect of thalidomide was not able to reverse the increase in ventricular mass induced by chronic β-adrenergic stimulation. However, we did not evaluate histopathological or ultrastructural parameters of cardiac myocytes. Loh et al. (29) studied the effects of thalidomide on the acute myocardial injury induced by isoproterenol (85 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 days). Thalidomide, in same dose as used in the present study, not only significantly reduced myocardial injury but also markedly improved cardiac function, which was significantly worsen by isoproterenol treatment. By reducing LVEDP, thalidomide might have improved the perfusion to the subendocardium, thereby reducing the myocardial injury. As the low dose of isoproterenol used here did not affect ventricular hemodynamics, it is possible that this beneficial effect of thalidomide could not be observed in the heart.

As the isoproterenol treatment did not significantly change arterial and ventricular hemodynamic parameters, it is suggested that the vascular alterations observed seems to be dependent on a mechanism induced by isoproterenol treatment directly in the vasculature and not to hemodynamic effects. Thus, this seems to be an important experimental model to study interactions between an altered vascular response and a proinflammatory process without hemodynamic changes. Therefore, as endothelial dysfunction is a key feature in the progression of compensated cardiac hypertrophy to chronic heart failure, contributing to vasoconstriction, this is an important model in which to study the vascular mechanisms associated with the early stages of cardiac hypertrophy.

In summary, the present data suggest that chronic β-adrenergic stimulation is associated with increased vasoconstrictor reactivity and elevated gene and protein expression of proinflammatory cytokines and NF-κB activity in the rat aorta. Oxidative stress induced by isoproterenol treatment probably induces the upregulation of NF-κB, which, in turn, stimulates vascular proinflammatory cytokine gene expression (Fig. 6). Both oxidative stress and the vascular proinflammatory state may play key roles in the progression of vascular damage induced by chronic β-adrenergic stimulation, which is associated with the progression to heart failure.

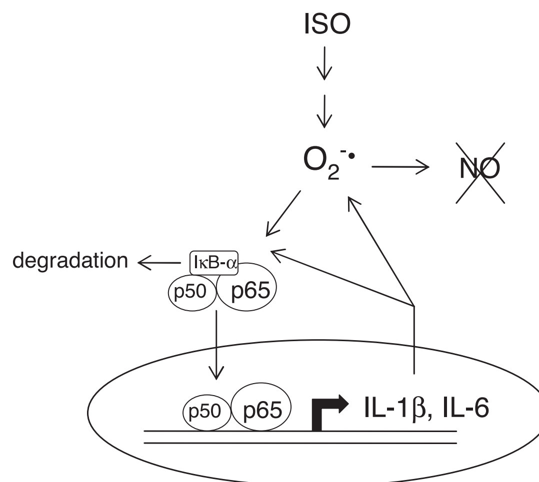

Fig. 6. Scheme showing the proposed effects for altered vascular function induced by prolonged ISO treatment in the rat aorta. NO, nitric oxide.

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GRANTS

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