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Role of 70-kDa Ribosomal Protein S6 Kinase, Nitric Oxide Synthase, Glycogen Synthase Kinase-3β, and Mitochondrial Permeability Transition Pore in Desflurane-induced Postconditioning in Isolated Human Right Atria

Sandrine Lemoine, Ph.D.,* Lan Zhu, M.D., Ph.D.,* Gallic Beauchef, Ph.D.,† Olivier Lepage, M.D.,‡ Gérard Babatasi, M.D., Ph.D.,‡ Caline Ivascau, M.D.,‡ Massimo Massetti, M.D., Ph.D.,§ Philippe Galera, Ph.D.,‖ Jean-Louis Gérard, M.D., Ph.D.,# Jean-Luc Hanouz, M.D., Ph.D.**

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ABSTRACT

Background: Desflurane during early reperfusion has been shown to postcondition human myocardium. Whether it involves "reperfusion injury salvage kinase" pathway remains incompletely studied. The authors tested the involvement of 70-kDa ribosomal protein S6 kinase, nitric oxide synthase, glycogen synthase kinase (GSK)-3β, and mitochondrial permeability transition pore in desflurane-induced postconditioning.

Methods: The authors recorded isometric contraction of human right atrial trabeculae suspended in an oxygenated Tyrode's solution (34°C, stimulation frequency 1 Hz). After a 30-min hypoxic period, desflurane 6% was administered during the first 5 min of reoxygenation. Desflurane was administered alone or with pretreatment of rapamycin, a 70-kDa ribosomal protein S6 kinase inhibitor, NG-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, and atractyloside, the mitochondrial permeability transition pore opener. GSK-3β inhibitor VII was administered during the first few minutes of reoxygenation alone or in the presence of desflurane 6%, rapamycin, NG-nitro-L-arginine methyl ester, and atractyloside. Developed force at the end of a 60-min reoxygenation period was compared (mean ± SD). Phosphorylation of GSK-3β was measured using blotting.

Results: Desflurane 6% (84 ± 4% of baseline) enhanced the recovery of force after 60 min of reoxygenation when compared with the control group (54 ± 4%, $P < 0.0001$). Rapamycin (68 ± 8% of baseline), NG-nitro-L-arginine methyl ester (57 ± 8%), atractyloside (52 ± 7%) abolished desflurane-induced postconditioning ($P < 0.001$). GSK-3β inhibitor-induced postconditioning (84 ± 5%, $P < 0.0001$ *vs.* control) was not modified by desflurane (78 ± 6%), rapamycin (81 ± 6%), and NG-nitro-L-arginine methyl ester (82 ± 10%), but it was abolished by atractyloside (49 ± 6%). Desflurane increased the phosphorylation of GSK-3β (3.30 ± 0.57-fold increase in desflurane *vs.* control; $P < 0.0001$).

Conclusions: *In vitro*, desflurane-induced postconditioning protects human myocardium through the activation of 70-kDa ribosomal protein S6 kinase, nitric oxide synthase, inhibition, and phosphorylation of GSK-3β, and preventing mitochondrial permeability transition pore opening.

What We Already Know about This Topic

- ❖ Volatile anesthetics, administered at the time of reperfusion, exert a postconditioning protection from myocardial ischemia, although whether this involves the reperfusion injury salvage kinase (RISK) cascade of enzymes is not known

What This Article Tells Us That Is New

- ❖ Using human atrial trabeculae *in vitro*, desflurane produced postconditioning protection of ischemic functional injury, and this was reversed by antagonists of several enzymes in the RISK cascade

* Research Fellow, Laboratory of Experimental Anesthesiology and Cellular Physiology EA3212, † Research Fellow, Laboratory of Extracellular Matrix and Pathology, Institut Fédératif de Recherche ICORE146 Université de Caen Basse Normandie, Caen, France. ‡ Cardiac Surgeon, § Professor of Cardiac Surgery, Department of Cardiac and Surgery, ‖ Professor of Molecular Biology, Laboratory of Extracullar Matrix and Pathology, Centre Hospitalier Universitaire de Caen, Caen, France. # Professor of Anesthesiology, Department of Anesthesiology and Intensive Care, Centre Hospitalier Universitaire de Caen, and Université de Caen Basse Normandie. ** Professor of Anesthesiology, Chairman, Department of Anesthesiology and Intensive Care, Director of Laboratory of Experimental Anesthesiology and Cellular Physiology EA3212, Centre Hospitalier Universitaire de Caen, Caen, France, and Université de Caen Basse Normandie.

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Correspondence to Dr. Hanouz: Département d'Anesthésie-Réanimation Chirurgicale, Centre Hospitalier Universitaire de Caen, Avenue Côte de Nacre, 14033 Caen Cedex, France. hanouz-jl@chu-caen.fr. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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MYOCARDIAL postconditioning is elicited by the administration of halogenated volatile anesthetic at the onset of reperfusion, and it could be a promising therapeutic strategy in myocardial protection against ischemia–reperfusion damage.[1–4] The reperfusion injury salvage kinase pathway specifically activated at the time of myocardial reperfusion confers powerful cardioprotection.[5] Desflurane has been shown to exert cardioprotection *via* many pathways during ischemia-reperfusion.[6–8] However, whether myocardial anesthetic postconditioning involves "reperfusion injury signaling kinase" pathway remains incompletely studied. We recently showed that desflurane-induced postconditioning, in human myocardium *in vitro*, was mediated by the activation of the prosurvival phosphatidylinositol-3-kinase (PI3K)-Akt and extracellular receptor kinase 1/2 signaling pathway, original members of the reperfusion injury signaling kinase pathway.[4] Both PI3K-Akt and extracellular receptor kinase 1/2 could phosphorylate 70-kDa ribosomal protein S6 kinase (p70S6K), which is an important regulator of protein translation. Krolikowski *et al.*[2] reported that isoflurane could limit infarct size when applied at reperfusion in the hearts of rabbit through a mechanism involving nitric oxide synthase (NOS) and p70S6K. In the isolated perfused hearts of rat, glycogen synthase kinase (GSK)-3β, a direct Akt downstream target, has been demonstrated to mediate convergence of myocyte protection signaling through the inhibition of mitochondrial permeability transition pore (mPTP) opening in isoflurane-induced postconditioning.[9] Finally, mPTP seems to be a critical determinant of cell death in ischemia–reperfusion injury, and the prevention of mPTP opening has been reported in isoflurane-induced postconditioning in animals model.[9,10] The aim of this study was to determine whether desflurane-induced postconditioning might involve p70S6K, GSK-3β, NOS, and mPTP as intermediate steps in its protective signaling pathway in isolated human right atrial trabeculae.

Materials and Methods

After approval of the local medical ethics committee (Comité de Protection des Personnes Nord Ouest III, Caen, France) and written informed consent, right atrial appendages were obtained during cannulation for cardiopulmonary bypass from patients scheduled for routine coronary artery bypass surgery and aortic valve replacement. All patients received total intravenous anesthesia with propofol, remifentanil, and pancuronium. Patients with chronic atrial arrhythmia and with diabetes mellitus treated with insulin or oral hypoglycemic agents were excluded from the study.[4,8]

Human Atrial Trabeculae Model of Hypoxia–Reoxygenation Injury

Experimental Conditions. Right atrial trabeculae (one per appendage) were dissected and suspended vertically between an isometric force transducer (MLT0202; ADInstruments, Sydney, Australia) and a stationary stainless clip in a 200-ml jacketed reservoir filled with freshly prepared Tyrode's modified solution containing 120 mM NaCl, 3.5 mM KCl, 1.1 mM $MgCl_2$, 1.8 mM NaH_2PO_4, 25.7 mM $NaHCO_3$, 2.0 mM $CaCl_2$, and 5.5 mM glucose. The jacketed reservoir was maintained at 34°C by a thermostatic water circulator (Polystat micropros; Bioblock, Illkirch, France). The bathing solution was insufflated with carbogen (95% O_2–5% CO_2), resulting in a pH of 7.40 and a partial pressure of oxygen of 600 mmHg. Isolated muscles were field stimulated at 1 Hz by two platinum electrodes with rectangular wave pulses of 5-ms duration 20% above threshold (CMS 95107; Bionic Instrument, Paris, France).

Trabeculae were equilibrated for 60–90 min to allow stabilization of their optimal mechanical performance at the apex of the length active isometric tension curve (L_{max}). The force developed was measured continuously, digitized at a sampling frequency of 400 Hz, and stored on a writable compact disc for analysis (PowerLab; ADInstruments).

At the end of the experiment, the muscle cross-sectional area was calculated from its weight and length, assuming a cylindrical shape and a density of 1. To avoid core hypoxia, trabeculae included in the study should have a cross-sectional area less than 1.0 mm^2, a force of contraction normalized per cross-sectional area (FoC) more than 5.0 mN/mm^2, and a ratio of resting force/total force less than 0.45.

Experimental Protocol. At the end of the stabilization period, the trabeculae were randomly assigned (sealed envelopes) to one of the experimental groups. In all groups, hypoxia–reoxygenation was performed by replacing 95% O_2–5% CO_2 with 95% N_2–5% CO_2 in the buffer for 30 min, followed by a 60-min oxygenated recovery period. In the control group (n = 10), trabeculae were exposed to the hypoxia reoxygenation protocol alone (fig. 1).

In the desflurane treatment groups, desflurane was delivered to the organ bath by the gas flow passing through a specific calibrated vaporizer. Desflurane concentration in the carrier gas phase was measured with an infrared calibrated analyzer (Capnomac; Datex, Helsinki, Finland). Desflurane was administered at a concentration of 6% (n = 6) during the first 5 min of reoxygenation (fig. 1), and this concentration corresponds to 1.0 minimum alveolar concentration desflurane in adult humans at 37°C.

Mechanisms involved in desflurane-induced postconditioning were studied in the presence of desflurane 6%, 15 μM rapamycin, a p70S6K inhibitor (desflurane + rapamycin; n = 6), 200 μM NG-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor (desflurane + L-NAME; n = 6), and 50 μM atractyloside (Atract), the mPTP opener (desflurane + Atract; n = 6). Pharmacologic agents were administered 5 min before, throughout, and 10 min after desflurane exposure. Desflurane 6% was chosen because we have previously shown that 6% was the optimal concentration to induce postconditioning in human myocardium, *in vitro*.[4]

In additional groups, muscles were exposed to 15 μM rapamycin (n = 6), 200 μM L-NAME (n = 6), 50 μM Atract

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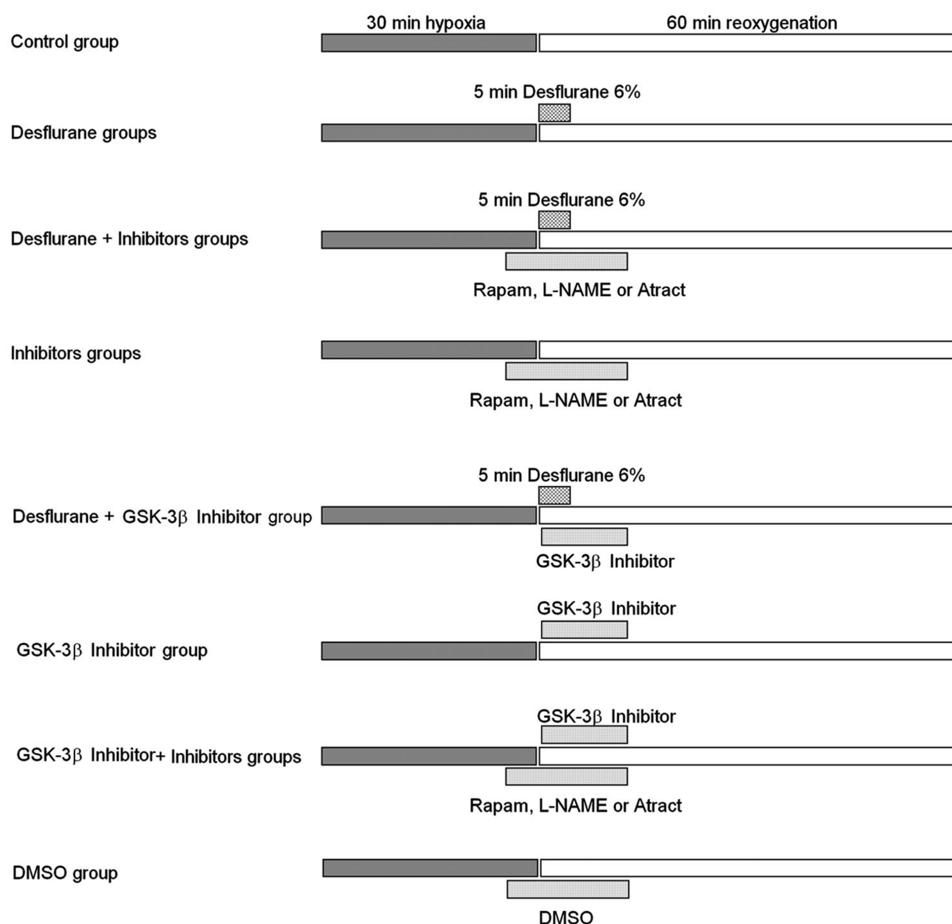

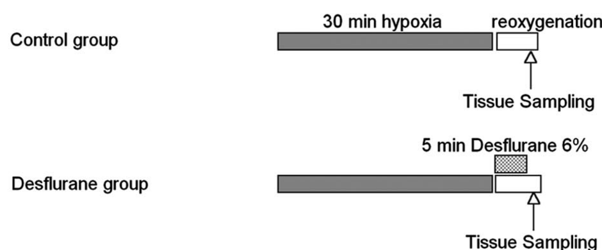

Fig. 1. Schematic diagram depicting the experimental protocol. (*A*) Contracting muscle experimental protocols. In the desflurane plus inhibitor, glycogen synthase kinase (GSK)-3 β inhibitor plus inhibitor, and inhibitor groups, rapamycin (Rapam) was administered at 15 μM, NG-nitro-L-arginine methyl ester (L-NAME) was administered at 200 μM, atractyloside (Atract) was administered at 50 μM, and GSK-3β inhibitor was administered at 20 μM. Dimethyl sulfoxide (DMSO) was administered at 0.1%. (*B*) Western blot experimental protocols.

(n = 6), and 0.1% dimethyl sulfoxide (DMSO; n = 6), 5 min before and in the first 15 min of reoxygenation (fig. 1).

Pharmacologic agents and DMSO were administered 5 min before, throughout, and 10 min after desflurane exposure. The concentrations of rapamycin, L-NAME, and Atract have been demonstrated to inhibit p70S6K, NOS, and open mPTP.[11–13] Rapamycin is dissolved in DMSO, and the volume of DMSO never exceeded 0.1% of the total bath volume.

In the GSK-3β inhibitor (GSK-3β Inh) treatment groups, atrial trabeculae underwent the hypoxia reoxygenation protocol and were exposed during the first 15 min of reoxygenation to 20 μM GSK-3β inhibitor VII alone (GSK-3β Inh; n = 6), in presence of desflurane (desflurane + GSK-3β Inh; n = 6), 15 μM rapamycin (GSK-3β Inh + rapamycin; n = 6), 200 μM L-NAME (GSK-3β Inh + L-NAME; n = 6), and 50 μM Atract (GSK-3β Inh + Atract; n = 6). Rapamycin, L-NAME, and Atract were ad-

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ministered 5 min before and throughout GSK-3β Inh administration (fig. 1).

Chemicals. Rapamycin and GSK-3β Inh VII were purchased from Calbiochem (VWR International, Fontenay sous Bois, France), and L-NAME, Atract, and DMSO were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Desflurane was purchased from GlaxoWellcome (Marly-le-Roi, France).

Western Blot Analysis

The right atrial appendage was pinned in a chamber (5 ml) containing Tyrode's modified solution, oxygenated with 95% O_2–5% CO_2, and maintained at 34 ± 0.5°C (Polystat micropros; Bioblock). The preparation was stimulated at a frequency of 1 Hz.

In all groups, after a 90-min equilibration period, hypoxia was performed by replacing 95% O_2–5% CO_2 with 95% N_2–5% CO_2 in the buffer for 30 min, followed by a 5-min oxygenated recovery period (control; n = 5) or by 5-min exposure to desflurane 6% (n = 5) (fig. 1).

Then, atrial samples were frozen in liquid nitrogen and stored at −80°C before protein extraction and Western blot analysis. Frozen tissue samples were extracted into the extraction buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin-pepstatin A-aprotinin and homogenized with a Polytron. The homogenates were centrifuged at 10,000*g* for 30 min, the supernatant was decanted, and the protein concentration was determined using the BCA protein assay (Bradford colorimetric method; Bio-Rad, Marnes-la-Coquette, France). Extracted protein samples were reduced with 100 mM threo-1,4-dimercapto-2,3-butanediol and denatured at 95°C for 5 min. Denatured proteins (30 μg/lane) from human atrial tissues were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred on nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline with Tween 20 (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 10% nonfat dry milk at room temperature.

The membranes were incubated with a rabbit monoclonal antibody recognizing phospho GSK-3β (Ser 9) and a rabbit monoclonal antibody recognizing GSK-3fl total (1/1000 dilution; Cell Signaling Technology, Ozyme, Saint Quentin Yvelines, France), one night at 4°C. After washing in Tris-buffered saline with Tween 20, the blots were incubated with a secondary antibody (goat anti-rabbit, 1/1,000 dilution) coupled to peroxidase (Santa Cruz Technology, Tebu-Bio, Le Perray en Yvelines, France) for 1 h at room temperature. The blots were washed again in Tris-buffered saline with Tween 20, and the bands were detected using chemiluminescence reagent (Pierce Perbio Science, Brebieres, France) before exposure to photography film. The Western blots of each group were stripped and probed again with an antibody against β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equivalent loading.

The developed films were scanned, and the band densities were quantified using NIH ImageJ software (Research Service Branch, National Institutes of Mental Health, Bethesda, MD). Phospho-GSK-3β (Ser9) was normalized to GSK-3β total level.

Statistical Analysis

The endpoint of the study was the recovery of FoC at 60 min of reoxygenation (FoC_{60}, expressed as percent of baseline). Power analysis calculated a group size of n = 5 to detect a difference of 40% in FoC (control and inhibitors group: FoC_{60} = 50 ± 9% of baseline; desflurane 6% group: FoC_{60} = 90 ± 9% of baseline) with a power of 0.8 at an α-level of 0.05. The number of experiments per group was calculated based on a one-way ANOVA with four control and inhibitors groups and one desflurane 6% group. Data are expressed as mean ± SD. Baseline values of main mechanical parameters, age, preoperative left ventricular ejection fraction, and FoC_{60} were compared by univariate ANOVA with group factor as the independent variable. If the *P* value was less than 0.05, a Bonferroni *post hoc* analysis was performed. Within-group data were analyzed over time using two-way ANOVA for repeated measures and Bonferroni *post hoc* analysis with group factor and time (baseline; hypoxia 5, 10, 20, and 30 min; and reoxygenation 5, 10, 20, 30, 40, 50, and 60 min) as independent variables.

In Western blotting, the band densities for protein of interest were then normalized to that of the band for β-tubulin in the same sample and then normalized to the mean of control tissues, defined as 1 arbitrary unit ± SD.

Statistical comparisons were made by the use of ANOVA for repeated measures and Bonferroni *post hoc* analysis. All *P* values were two-tailed, and a *P* value of less than 0.05 was required to reject the null hypothesis. Statistical analysis was performed using Statview 5 software (Deltasoft, Meylan, France).

Results

There was no statistical difference between groups for patients' demographic data, preoperative treatments, and left ventricular ejection fraction (table 1). Eighty-eight human right atrial trabeculae and 10 right atrial appendages were studied. There were no differences in baseline values for L_{max}, cross-sectional area, ratio of resting force to total force, and force of contraction normalized per cross-sectional area between groups (table 2).

Effects of Desflurane on Hypoxia-Reoxygenation

In the control group, reoxygenation resulted in a partial recovery of FoC_{60} (54 ± 4% of baseline; fig. 2). When compared with the control group, desflurane 6% (84 ± 4% of baseline; $P < 0.0001$) significantly increased FoC_{60} (fig. 2).

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Table 1. Control Values of Main Mechanical Parameters of Human Right Atrial Trabeculae

Experimental Groups	L_{max} (mm)	CSA (mm²)	FoC (mN/mm²)	RF/TF
Control (n = 10)	5.7 ± 1.3	0.65 ± 0.31	21 ± 14	0.38 ± 0.15
Desflurane (n = 6)	7.5 ± 0.6	0.60 ± 0.26	28 ± 18	0.30 ± 0.10
Desflurane + rapamycin (n = 6)	6.5 ± 1.2	0.55 ± 0.21	30 ± 17	0.24 ± 0.08
Desflurane + L-NAME (n = 6)	6.3 ± 1.8	0.47 ± 0.15	22 ± 5	0.36 ± 0.12
Desflurane + Atract (n = 6)	7.8 ± 2.2	0.47 ± 0.12	28 ± 10	0.31 ± 0.11
Desflurane + GSK-3β Inh (n = 6)	5.9 ± 1.1	0.52 ± 0.19	29 ± 7	0.23 ± 0.04
GSK-3β Inh (n = 6)	6.6 ± 1.5	0.54 ± 0.23	30 ± 13	0.23 ± 0.07
GSK-3β Inh + rapamycin (n = 6)	5.5 ± 0.8	0.50 ± 0.20	19 ± 5	0.34 ± 0.09
GSK-3β Inh + L-NAME (n = 6)	5.2 ± 1.0	0.31 ± 0.17	19 ± 3	0.41 ± 0.20
GSK-3β Inh + Atract (n = 6)	5.7 ± 1.2	0.53 ± 0.16	23 ± 5	0.31 ± 0.10
Rapamycin (n = 6)	7.2 ± 1.7	0.47 ± 0.17	27 ± 7	0.28 ± 0.08
L-NAME (n = 6)	6.0 ± 1.7	0.37 ± 0.17	25 ± 11	0.38 ± 0.08
Atract (n = 6)	7.0 ± 1.7	0.40 ± 0.20	31 ± 13	0.41 ± 0.05
DMSO (n = 6)	5.7 ± 0.8	0.54 ± 0.21	18 ± 3	0.30 ± 0.10

Data are mean ± SD.

Atract = atractyloside; CSA = cross-sectionnal area; DMSO = dimethyl sulfoxide; FoC = force of contraction normalized per cross-sectional area; GSK-3β Inh = glycogen synthase kinase-3β inhibitor; L_{max} = maximal length at the apex of the length active isometric tension curve; L-NAME = NG-nitro-L-arginine methyl ester; RF/TF = ratio of resting force on total force.

Effects of Rapamycin, L-NAME, and Atractyloside

As shown in figure 2, the desflurane-induced enhanced recovery of FoC_{60} (desflurane: 84 ± 4% of baseline) was abolished in the presence of rapamycin (desflurane + rapamycin: 68 ± 8% of baseline; $P < 0.001$ *vs.* desflurane group), L-NAME (desflurane + L-NAME: 57 ± 8% of baseline; $P < 0.0001$ *vs.* desflurane group), and Atract (desflurane + Atract: 52 ± 7% of baseline; $P < 0.0001$ *vs.* desflurane group).

When compared with the control group (control: 54 ± 4% of baseline), rapamycin alone (54 ± 6% of baseline; $P = 0.90$), L-NAME alone (51 ± 3% of baseline; $P = 0.36$), Atract alone (53 ± 7% of baseline; $P = 0.91$), and DMSO (54 ± 7% of baseline; $P = 0.98$) did not significantly modify FoC_{60} (fig. 2).

Role of Glycogen Synthase Kinase-3β in Desflurane-induced Postconditioning

Fifteen minutes exposure at the beginning of reoxygenation to GSK-3β Inh alone resulted in a significant increase in FoC_{60} (84 ± 5% of baseline) when compared with the control group ($P < 0.0001$), and it was not different from that measured in the desflurane group (*vs.* 84 ± 4% of baseline with desflurane, $P = 0.92$). Desflurane-induced enhanced recovery of FoC_{60} was not modified by coinfusion of GSK-3β Inh (78 ± 6% of baseline; $P = 0.06$ *vs.* desflurane; fig. 2).

Effect of Rapamycin, L-NAME, and Atractyloside on GSK-3β Inhibitor Treatment

The enhanced recovery of FoC_{60} after the administration of GSK-3β Inh (84 ± 5% of baseline) was not modified in the presence of rapamycin (81 ± 6% of baseline, $P = 0.36$ *vs.* GSK-3β Inh) and L-NAME (82 ± 10% of baseline, $P = 0.43$ *vs.* GSK-3β inh). In contrast, the enhanced recovery of FoC_{60} after the administration of GSK-3β inhibitor was abolished in the presence of Atract (49 ± 6% of baseline, $P < 0.0001$ *vs.* GSK-3β Inh; fig. 2).

Phosphorylation of Glycogen Synthase Kinase-3β

In Western blot analysis, desflurane 6% did not modify protein expression of GSK-3β total (1.02 ± 0.04 relative to control in desflurane *vs.* control; $P > 0.05$). In contrast, tissue samples obtained at 5 min of reperfusion and exposed to desflurane 6% showed a significant increase in the ratio of phospho GSK-3β (Ser 9) to GSK-3β total when compared with tissue samples that were not exposed to desflurane (3.30 ± 0.57-fold increase in desflurane *vs.* control; $P < 0.0001$; fig. 3).

Discussion

The current study shows that desflurane-induced postconditioning of human myocardium was mediated at least in part by: (1) the activation of p70S6K and NOS, (2) phosphorylation and inhibition of GSK-3β, and (3) inhibition of the opening of mPTP.

Our results showed that the administration of rapamycin, a p70S6K inhibitor, abolished the enhanced recovery of FoC_{60}, resulting from desflurane administration during early reoxygenation, suggesting that activation of p70S6K mediated desflurane-induced postconditioning of human myocardium, *in vitro*. It has been shown that ischemic postconditioning was mediated by p70S6K phosphorylation in isolated, perfused hearts of rat and mouse.[5,14] However, controversial data are available on its involvement in anesthetic-induced postconditioning. Thus, rapamycin abolished postconditioning induced by isoflurane at 1 minimum alveolar concentration,[2] but it did not modify postconditioning induced by isoflurane at 0.5 minimum alveolar concentration,[15] in rabbit *in vivo*. In addition, isoflurane-induced postconditioning resulted in phosphorylation and, thus, activation of p70S6K.[2] Furthermore, it has been shown that

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Table 2. Patients' Demographic Data, Preoperative Drug Treatments, and Preoperative Left Ventricular Ejection Fraction

Groups and Heart Disease	Age (yr)	Preoperative Drug Treatments	LVEF (%)
Control AVR (n = 4); CABG (n = 6)	68 ± 10	ACE (6), ßAB (5), BZD (2), CA (0), COR (2), FUR (0), STA (5), NT (1)	52 ± 12
Desflurane AVR (n = 3); CABG (n = 3)	67 ± 6	ACE (2), ßAB (2), BZD (1), CA (1), COR (2), FUR (1), STA (2), NT (0)	70 ± 11
Desflurane + rapamycin AVR (n = 4); CABG (n = 2)	63 ± 11	ACE (4), ßAB (4), BZD (2), CA (0), COR (0), FUR (0), STA (4), TNT (0)	53 ± 3
Desflurane + L-NAME AVR (n = 2); CABG (n = 4)	66 ± 14	ACE (2), ßAB (4), BZD (0), CA (2), COR (0), FUR (0), , STA (4), NT (0)	60 ± 10
Desflurane + Atract AVR (n = 6); CABG (n = 0)	75 ± 6	ACE (2), ßAB (3), BZD (1), CA (0), COR (3), FUR (1), STA (2), NT (1)	56 ± 23
Desflurane + GSK-3β Inh AVR (n = 3); CABG (n = 3)	67 ± 9	ACE (2), ßAB (2), BZD (1), CA (0), COR (0), FUR (0), STA (1), TNT (0)	65 ± 5
GSK-3β Inh AVR (n = 2); CABG (n = 4)	60 ± 15	ACE (6), ßAB (5), BZD (1), CA (1), COR (0), FUR (0), STA (4), NT (0)	68 ± 6
GSK-3β Inh + rapamycin AVR (n = 4); CABG (n = 2)	76 ± 10	ACE (2), ßAB (2), BZD (0), CA (0), COR (2), FUR (0), STA (5), NT (1)	72 ± 3
GSK-3β Inh + L-NAME AVR (n = 3); CABG (n = 3)	75 ± 7	ACE (3), ßAB (3), BZD (1), CA (0), COR (2), FUR (2), STA (6), NT (2)	74 ± 4
GSK-3β Inh + Atract AVR (n = 3); CABG (n = 3)	74 ± 5	ACE (3), ßAB (1), BZD (1), CA (2), COR (1), FUR (2), STA (6), NT (1)	65 ± 1
Rapamycin AVR (n = 5); CABG (n = 1)	69 ± 6	ACE (2), ßAB (3), BZD (0), CA (1), COR (2), FUR (0), STA (6), NT (0)	54 ± 6
L-NAME AVR (n = 3); CABG (n = 3)	69 ± 12	ACE (6), ßAB (6), BZD (0), CA (0), COR (0), FUR (0), STA (4), NT (0)	51 ± 3
Atract AVR (n = 2); CABG (n = 4)	61 ± 12	ACE (5), ßAB (3), BZD (0), CA (0), COR (0), FUR (0), STA (3), NT (0)	53 ± 8
DMSO AVR (n = 2); CABG (n = 4)	56 ± 3	ACE (1), ßAB (2), BZD (0), CA (0), COR (0), FUR (0), STA (1), NT (0)	71 ± 0
Western blot: control AVR (n = 4); CABG (n = 1)	57 ± 18	ACE (2), ßAB (5), BZD (1), CA (0), COR (1), FUR (1), STA (2), NT (1)	65 ± 11
Western blot: desflurane AVR (n = 3); CABG (n = 2)	62 ± 9	ACE (2), ßAB (1), BZD (0), CA (1), COR (1) FUR (1), STA (5), NT (0)	57 ± 18

The number in parentheses after heart disease and drug abbreviation indicate the number of patients. Age and preoperative left ventricular ejection fraction (LVEF) are expressed as mean ± SD.

ACE = angiotensin-converting enzyme inhibitors; Atract = atractyloside; AVR = aortic valve replacement; ßAB = ß-adrenergic blocking drugs; BZD = benzodiazepine; CA = calcium channel antagonists; CABG = coronary artery bypass graft; COR = amiodarone; DMSO = dimethyl sulfoxide; FUR = furosemide; GSK-3β Inh = glycogen synthase kinase-3β inhibitor; L-NAME = NG-nitro-L-arginine methyl ester; NT = nitroglycerin; STA = statins.

p70S6K was downstream of PI3K/Akt activation.[9] Because we have previously shown that Akt activation mediated desflurane-induced postconditioning,[4,16] it could be hypothesized that Akt may activate p70S6K in response to desflurane-induced postconditioning. Nevertheless, in the current study, rapamycin only partially inhibited the desflurane-induced postconditioning. It could be hypothesized that p70S6K is not a limiting pathway in postconditioning. Further studies are required to examine precisely the role of p70S6K in desflurane-induced postconditioning.

A 30-min administration of desflurane before or after prolonged ischemia induced cardioprotection through nitric oxide production, in the heart of rabbit *in vivo*.[17,18] However, it remained uncertain that brief administration of desflurane could result in nitric oxide production. The current study shows that the cardioprotective effect resulting from a 5-min administration of desflurane during early reoxygenation was abolished by L-NAME. This strongly suggests that desflurane-induced postconditioning activates NOS and, thus, nitric oxide production. Moreover, postconditioning by isoflurane could involve specifically endothelial NOS because Krolikowski *et al.* demonstrate that L-NAME abolished postconditioning by isoflurane, but pretreatment with either the selective inducible NOS antagonist or the selective neuronal NOS inhibitor did not inhibit postconditioning by isoflurane, leading them to suppose that endothelial NOS mediated cardioprotection by isoflurane during early reperfusion.[2] Tsang *et al.*[5] showed that PI3K/Akt activation was upstream of endothelial NOS activation in ischemic postconditioning. Because we have previously shown that Akt activation mediated desflurane-induced postconditioning, it can be hypothesized that it also resulted in endothelial NOS activation and nitric oxide generation. However, the current study could not determine whether nitric oxide generation

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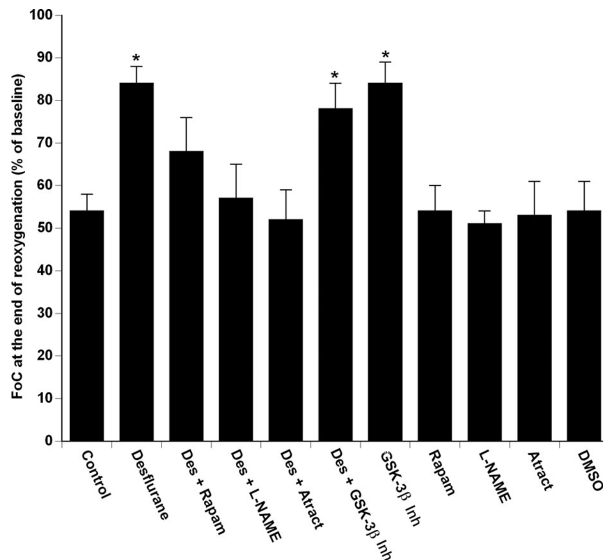

Fig. 2. Recovery of force of contraction (FoC) of isolated human right atrial trabeculae at the end of the 60-min reoxygenation period after 30-min hypoxia. Data are mean ± SD. * $P < 0.001$ *versus* control, Des + Rapam, Des + L-NAME, Des + Atract, GSK-3β Inh + Atract, Rapam, L-NAME, Atract, and DMSO groups. Atract = atractyloside; Des = desflurane; DMSO = dimethyl sulfoxide; GSK-3β Inh = glycogen synthase kinase-3β inhibitor; L-NAME = NG-nitro-L-arginine methyl ester; Rapam = rapamycin.

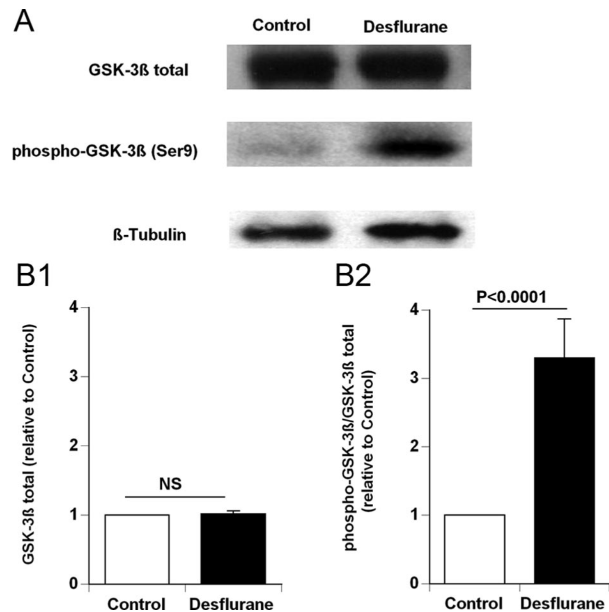

Fig. 3. (*A*) Western blot analysis showing levels of glycogen synthase kinase (GSK)-3β total, and glycogen synthase kinase 3β phosphorylated (Ser9) (phospho-GSK-3β) after 5 min of reoxygenation alone (control) or in the presence of 5 min of desflurane, 6% (desflurane). Western blot analysis was performed using specific antibodies against GSK-3β total, phospho-GSK-3β (Ser 9), and β-tubulin. (*B*) Histogram depicting the variation of GSK-3β total expression in control and 6% desflurane groups (relative to control) (*B.1*), and the phosphor-GSK-3β (Ser9)/GSK-3β total ratio (*B.2*) in the control group and desflurane group (relative to control). The relative GSK-3β total and phospho-GSK-3β protein levels were calculated by averaging the results obtained from five independent experiments and were normalized to the β-tubulin in each group. Densitometry analysis is expressed in arbitrary units. The relative phospho-GSK-3β protein levels were calculated by averaging the results obtained from five independent experiments and were normalized to the β-tubulin control in each group. Densitometry analysis is expressed in arbitrary units. Data are mean ± SD.

was required during the triggering or mediating phase of desflurane-induced postconditioning. Although the exact mechanism by which NOS mediates cardioprotection is unclear, the most probable mechanism seems to be *via* the enhancement of the production of free radicals by forming peroxynitrite, attenuating the calcium overload of mitochondria,[19] and importantly, inhibition of mPTP opening observed in response to nitric oxide generation.[20]

We also showed that selective inhibition of GSK-3β in the first few minutes of reoxygenation enhanced the recovery of FoC_{60} when compared with the control group, suggesting that GSK-3β inhibition at the onset of reoxygenation is cardioprotective in human myocardium, *in vitro*. Our results extend previous ones, which demonstrated that GSK-3β Inh administered 5 min before reperfusion induced cardioprotection in rat[21] and produced myocardial protection when administered shortly before reperfusion in rabbits.[15] Moreover, our results show that GSK-3β inhibition in addition to desflurane did not confer additive cardioprotection when compared with desflurane alone, suggesting that desflurane administered at the beginning of reoxygenation may have already inactivated GSK-3β. To our knowledge, no pharmacologic activator of GSK-3β has been synthesized to validate this hypothesis. In contrast, in rabbit *in vivo*, GSK-3β inhibition enhanced isoflurane-induced protection against infarction during early reperfusion.[15] These discrepancies may result from differences in species, in experimental models, in study endpoints (*i.e.*, myocardial function, infarct size, cell survival, protein expression, and apoptosis), and in the timing of administration of inhibitors. Nevertheless, in the current study, the Western blot analysis clearly showed that the phosphorylation activity of GSK-3β was increased by a brief administration of desflurane in early reoxygenation. These results strongly suggest that desflurane-induced postconditioning may be mediated by the inhibition (by phosphorylation)[22] of GSK-3β. Furthermore, in the isolated heart of rat, administration of isoflurane at the beginning of reperfusion leads to inhibition of GSK-3β, and this inhibition was dependent of Akt activation.[9]

The key role of mPTP in cardioprotection was established by Argaud *et al.*,[23] in rabbits *in vivo*, showing hat administration of mPTP inhibitors at the time of reperfusion limits infarct size to the same extent that ischemic postconditioning does. Although it has been shown that isoflurane- and sevoflurane-induced postconditioning were mediated by the inhibition of mPTP opening, in rabbit *in vivo*, and the isolated, perfused hearts of rat,[10,24] its role in desflurane-

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induced postconditioning has not been studied. In the current study, desflurane-induced postconditioning was abolished by mPTP opening, at the onset of reoxygenation, suggesting that desflurane administration in the first few minutes of reoxygenation prevents mPTP opening. This may be related to desflurane-induced improved resistance of mPTP to calcium-induced opening.[25] The inhibition of mPTP opening has been suggested to be downstream of the reperfusion injury signaling kinase pathway and more precisely of the PI3K/Akt pathway in ischemic postconditioning.[26] However, the exact interaction between mPTP and reperfusion injury signaling kinase pathway remains unclear.

In additional experiments, we investigated the relationship between GSK-3β and p70S6K, NOS, and mPTP. The current results showed that the enhanced recovery of FoC_{60} after GSK-3β Inh administration during early reoxygenation was abolished in the presence of Atract. This suggests that the effect of desflurane on the mPTP (*i.e.*, inhibition of mPTP opening) should be mediated through the inactivation of GSK-3β at reoxygenation. Moreover, Western blot analysis showed that desflurane administration resulted in increased phosphorylation and, thus, inhibition of GSK-3β (fig. 3). It has been shown that isoflurane-induced postconditioning protected against reperfusion damage by preventing opening of mPTP through inhibition of GSK-3β.[9] Furthermore, Akt activation has been shown to be upstream of mPTP in ischemic postconditioning in rat cardiomyocytes,[27] and GSK-3β inhibition has been shown to depend on Akt activation, in rabbit *in vivo*.[15] In addition, using transgenic mice, it has been shown that the inhibition of GSK-3β by postconditioning was required to prevent opening of the mPTP during reperfusion.[28]

In contrast, we showed that the cardioprotective effects induced by GSK-3β inhibition was unaffected by rapamycin and L-NAME, whereas these inhibitors abrogated desflurane-induced postconditioning. This strongly suggests that p70S6 kinase and NOS should be upstream of GSK-3β. However, the mTOR/p70S6K signal pathway has been suggested to regulate GSK-3β inhibition.[22,29] Opioid-induced GSK-3β inactivation at reperfusion was abrogated by blockade of the mTOR/p70S6K pathway with rapamycin, suggesting that this pathway plays a role in the action of opioid on GSK-3β inactivation at reperfusion.[21] To our knowledge, the interrelation between nitric oxide production and GSK-3β have never been investigated in cardioprotection. Rakhit *et al.*[19] also showed that increased bioavailability of nitric oxide may decrease mPTP opening and could induce cardioprotection. Nitric oxide has been shown to activate protein kinase C and open mitochondrial adenosine triphosphate-sensitive potassium ($mitoK_{ATP}$) channels, suggesting strong links between nitric oxide-induced cardioprotection, protein kinase C, and $mitoK_{ATP}$ channels.[30–32] Moreover, opening of $mitoK_{ATP}$ channel mediated GSK-3β inhibition–induced cardioprotection.[33] Finally, protein kinase C-ϵ could interact with the cardiac mPTP and inhibit its opening.[34] We have also previously shown that activation protein kinase C and $mitoK_{ATP}$ channels were involved in desflurane-induced postconditioning in human myocardium.[4]

Several limitations must be considered in the interpretation of the current results. First, the effects of anesthetics drugs,[35,36] diseases, or medical treatments received by the patients before obtaining the atrial appendages cannot be ruled out. Furthermore, although the experimental groups showed comparable demographic data (table 1), age has been shown to attenuate volatile anesthetic preconditioning.[37] However, the patients included in this study are representative of those patients in whom desflurane may be used during anesthesia. Importantly, our investigation included a control group that was equally affected by any one of these potentially modifying factors. Second, our experiments are performed under moderate hypothermia (34°C) to ensure stability of trabeculae over time. It has been shown that hypothermia may decrease the sensitivity of the $mitoK_{ATP}$ channels.[38] However, during surgical procedures, moderate hypothermia may occur. Third, we studied isolated contracting human atrial trabeculae but not myocardial ventricular infarct size (for ethical reasons). In addition, as described in myocardial preconditioning, the beneficial effects of postconditioning have also been described on reperfusion-induced arrhythmias[39] and myocardial conctractility.[40] Fourth, based on previous results from our laboratory,[4,8] the current study was powered to detect large differences between experimental groups. Consequently, small differences could not have been detected.

In conclusion, this study provides the first evidence of, *in vitro*, desflurane-induced postconditioning of the human myocardium exerting its effect *via* the activation of p70S6K and NOS, phosphorylation of GSK-3ß, and inhibition of opening of mPTP, at the onset of reoxygenation.

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