

## Catecholamine-induced cardiac mitochondrial dysfunction and mPTP opening: protective effect of curcumin

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<sup>1</sup>Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediene Bab Ezzouar, El Alia, Alger, Algérie; <sup>2</sup>Institut National de la Santé et de la Recherche Médicale, U-769, Châtenay-Malabry; and <sup>3</sup>University Paris-Sud, IFR141, UMR-S769, Châtenay-Malabry, France

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**Izem-Meziane M, Djerdjouri B, Rimbaud S, Caffin F, Fortin D, Garnier A, Veksler V, Joubert F, Ventura-Clapier R.** Catecholamine-induced cardiac mitochondrial dysfunction and mPTP opening: protective effect of curcumin. *Am J Physiol Heart Circ Physiol* 302: H665–H674, 2012. First published November 18, 2011; doi:10.1152/ajpheart.00467.2011.—The present study was designed to characterize the mitochondrial dysfunction induced by catecholamines and to investigate whether curcumin, a natural antioxidant, induces cardioprotective effects against catecholamine-induced cardiotoxicity by preserving mitochondrial function. Because mitochondria play a central role in ischemia and oxidative stress, we hypothesized that mitochondrial dysfunction is involved in catecholamine toxicity and in the potential protective effects of curcumin. Male Wistar rats received subcutaneous injection of 150 mg·kg<sup>-1</sup>·day<sup>-1</sup> isoprenaline (ISO) for two consecutive days with or without pretreatment with 60 mg·kg<sup>-1</sup>·day<sup>-1</sup> curcumin. Twenty four hours after, cardiac tissues were examined for apoptosis and oxidative stress. Expression of proteins involved in mitochondrial biogenesis and function were measured by real-time RT-PCR. Isolated mitochondria and permeabilized cardiac fibers were used for swelling and mitochondrial function experiments, respectively. Mitochondrial morphology and permeability transition pore (mPTP) opening were assessed by fluorescence in isolated cardiomyocytes. ISO treatment induced cell damage, oxidative stress, and apoptosis that were prevented by curcumin. Moreover, mitochondria seem to play an important role in these effects as respiration and mitochondrial swelling were increased following ISO treatment, these effects being again prevented by curcumin. Importantly, curcumin completely prevented the ISO-induced increase in mPTP calcium susceptibility in isolated cardiomyocytes without affecting mitochondrial biogenesis and mitochondrial network dynamic. The results unravel the importance of mitochondrial dysfunction in isoprenaline-induced cardiotoxicity as well as a new cardioprotective effect of curcumin through prevention of mitochondrial damage and mPTP opening.

curcumin; isoprenaline; cardiac injury; mitochondria function; mitochondrial morphology and permeability transition pore

CIRCULATING CATECHOLAMINES closely correlate with the severity and poor prognosis in heart failure and are thus considered to play a critical role in the development of cardiovascular diseases (11). Excessive release of catecholamines induces myocardial hypertrophy, myocyte damage, and contractile dysfunction, resulting in infarct-like necrosis of the heart muscle.

Oxidative stress due to increased generation of reactive oxygen species (ROS) plays a recognized role in the catecholamine-induced cardiotoxicity (24, 41). Indeed, cat-

echolamines can easily undergo oxidation with production of unstable catecholamines-O-quinones, giving rise to a subsequent production of superoxide radicals that was proposed to be responsible for catecholamine-induced cardiotoxicity. The superoxide radicals can be reduced by superoxide dismutase to hydrogen peroxide, which damages the membrane integrity. Imbalance between ROS production and cellular antioxidant defenses initiates subcellular alterations leading to cell death, cardiomyopathy, and heart failure (12, 37, 41).

Mitochondrial dysfunction is widely acknowledged as a seminal event in necrotic and apoptotic cell death and is emerging as a key mediator of ischemia-reperfusion (I/R) injury and catecholamine toxicity in the heart. Alterations in Ca<sup>2+</sup> handling and ROS clearly contribute to mitochondrial dysfunction and to opening of the mitochondrial permeability transition pore (mPTP), a high-conductance pore of the mitochondrial membranes. Opening of mPTP causes uncoupling of the mitochondria and swelling of the matrix, leading to rupture of the mitochondrial membranes, release of reduced equivalents and proapoptotic factors, and ultimately cell death (2). Increased vulnerability of the mPTP is an early event in pathological processes leading to heart failure (25). In recent years, mPTP has been considered a therapeutic target for cardioprotection during cardiac diseases, especially those associated with I/R (35).

Curcumin (CUR), a polyphenol responsible for the yellow color of the spice turmeric, at relatively low concentrations, is an effective anti-inflammatory agent and exhibits cardioprotective effects (49). The antioxidant properties of curcumin seem to be essential for its pleiotropic biological activities. Curcumin inhibits lipid peroxidation and effectively scavenges superoxide anion and hydroxyl radicals. It was also shown to interact with the mPTP (28). The cardioprotective effect of curcumin against catecholamine-induced cardiotoxicity has been established for a long time (32) and involves its antioxidant properties (24). Moreover, curcumin inhibits nuclear factor- $\kappa$ B activation, protects cardiac cells against I/R injury (14), and stabilizes the cytoskeleton through the increased expression of the heat shock protein Hsp27 (39). However, no study has been performed yet to demonstrate the implication of mitochondria as a possible target of the cardioprotective effect of curcumin.

Because mitochondria play a major role in cardiac dysfunction and cell death, the aim of the present study was 1) to characterize the mitochondrial dysfunction induced by catecholamines and 2) to investigate whether curcumin induces cardioprotective effects against catecholamine-induced cardiotoxicity by preserving mitochondrial function. To this aim, we

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investigated whether curcumin could protect the heart against isoprenaline-induced cell death, alteration of mitochondrial respiration, dynamics, and biogenesis, and prevent opening of mPTP and cell death.

## MATERIALS AND METHODS

**Animal treatments.** Male Wistar rats weighing 230–280 g were used. All experiments were approved by the Université Paris-Sud Institutional Care Committee and conformed to the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament.) Authorizations to conduct animal experiments were obtained from the French Ministère de l'Agriculture, de la Pêche et de l'Alimentation (no. 92–284, June 27, 2007). Rats were randomly divided into three groups of five animals each and treated as follows: 1) the control group was given normal saline subcutaneously one time a day for two consecutive days; 2) isoprenaline (ISO) group: 150 mg·kg<sup>-1</sup>·day<sup>-1</sup> ISO was administered subcutaneously for 2 days to induce cardiac damage; and 3) CUR + ISO group: curcumin (CUR; 60 mg·kg<sup>-1</sup>·day<sup>-1</sup>) was administered intraperitoneally for 2 days, 30 min before ISO injection. In additional studies, CUR (60 mg·kg<sup>-1</sup>·day<sup>-1</sup>) was administered alone intraperitoneally for 2 days (CUR group). Twenty four hours after the last treatment period, all rats were anesthetized by intraperitoneal injection of pentobarbital (150 mg/kg) before death.

A supraphysiological dose of catecholamine was used to induce rapid infarct-like damages to the myocardium. The relatively low dose of curcumin was chosen in the range of the cardioprotective effects described in the literature (27, 39).

After death, hearts were quickly harvested. After left ventricle isolation, part was immediately used for mitochondrial respiration measurements, and other parts were rapidly frozen and kept at –80°C for biochemical analysis or fixed in 10% phosphate-buffered formalin for light microscopic studies.

**Preparation of tissue homogenate.** Hearts were homogenized in 10 volumes (wt/vol) of ice-cold phosphate buffer (50 mM, pH 7.4) using a Polytron homogenizer (Ultra-turrax T25). The homogenates were then centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was used for biochemical assays. Protein content was determined using BSA as standard (4).

**Histological examinations.** Formalin-fixed left ventricular tissues were dehydrated in ascending grades of alcohol and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin and examined under light microscopy (Carl Zeiss). Other serial 5- $\mu$ m sections were stained with 1 g/ml Hoechst 33258 for 30 min at room temperature and visualized under fluorescence microscopy (Carl Zeiss, Thornwood, NY). Apoptotic morphological cells were counted in 10 visual fields of 5 different areas.

**Markers of cardiac stress and metabolic enzymes.** Creatine kinase activity was assayed using a Teco commercial kit according to the manufacturer's instructions (CK-NAC reagents; Teco Diagnostics). Lactate dehydrogenase (LDH) activity was assayed by the method of Wahlefeld (45). The malondialdehyde (MDA) level was assessed according to Ohkawa et al. (34). Myeloperoxidase (MPO), a marker of neutrophil infiltration, was measured in the heart according to Krawisz et al. (22). Catalase activity was determined by measuring the rate of decomposition of H<sub>2</sub>O<sub>2</sub> according to Aebi (1). Glutathione (GSH) content in supernatant of heart homogenate was evaluated by the method of Boyne and Ellman (3). Frozen tissue samples were weighed and homogenized in ice-cold buffer, and enzyme activities were determined as described previously (10). Complex I activity was measured in heart homogenized in ice-cold buffer containing 10 mmol/l Tris base (pH 7.2), 75 mmol/l sucrose, 225 mmol/l mannitol, 100  $\mu$ mol/l EDTA, and 0.1% Triton X-100. To measure NADH-coenzyme Q reductase activity with decylubiquinone as electron acceptor, samples were incubated in 25 mmol/l phosphate buffer (pH 7.5), 2.5 mg/ml BSA, and 100  $\mu$ mol/l decylubiquinone at 30°C.

Activity was reported as a rotenone-insensitive decrease in NADH absorbance at 340 nm. Citrate synthase activity was measured in the presence of acetyl-CoA, oxaloacetic acid, and DTNB according to Ref. 38. Cytochrome c oxidase (COX) activity was determined by measuring the disappearance of reduced cytochrome c at 550 nm according to Ref. 48.

**Real-time quantitative RT-PCR analysis.** Total RNA was isolated from rat left ventricle (50–100 mg) using the Trizol reagent technique (Invitrogen, Cergy Pontoise, France). Oligo(dT) first-strand cDNA was synthesized from 5  $\mu$ g total RNA using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using the SYBR Green technology on a Light Cycler rapid thermal cycler (Roche Diagnostics) as previously described (15). Forward and reverse primers were each designed in a different exon of the target sequence, eliminating the possibility of amplifying genomic DNA as described previously (15, 16). Amplification was allowed to proceed from 30 to 40 cycles, each consisting of denaturation at 95°C for 10 s, annealing at 60°C nuclear respiratory factor 2, mitochondrial transcription factor A, COX subunits I and IV, and mitofusin 1 (Mfn1) and 2 (Mfn2), or 58°C peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), optic atrophy protein 1 (OPA1), and dynamin-related protein 1 (Drp1) from 5 to 9 s and extension at 72°C from 5 to 19 s depending on the target gene. Cyclophilin A and acidic ribosomal protein were used for normalization with Genorm software.

**Western blot analysis.** Hearts of control, ISO, and CUR + ISO were homogenized in a lysate buffer containing (in mM): 50 HEPES, 20 KCl, 1 EDTA, 1 EGTA, 5  $\alpha$ -glycerolphosphate, 1 orthovanadate, 1 dithiothreitol (DTT), 5 NaPPi, 2 phenylmethylsulfonyl fluoride (Sigma-Aldrich Chimie), cocktail of protease inhibitors (Calbiochem Set V EDTA free), and 0.1% Triton X-100. Protein extracts (50  $\mu$ g) were loaded on a 10% SDS-polyacrylamide gel. Blots were first incubated with a primary antibody for rat total or phospho-Drp1 (dilution 1:1,000; Chemicon International) and then with an anti-rabbit IgG, horseradish peroxidase-linked antibody (dilution 1:2,500; Cell Signaling). Drp1 protein was revealed with an enhanced chemiluminescent substrate (SuperSignal West Dura; Pierce Biotechnology).

**In situ study of mitochondrial respiration.** Mitochondrial respiration was studied in situ in freshly saponin-skinned fibers as previously described (23, 42). Briefly, fibers were separated from the endocardial surface of the left ventricle under a binocular microscope in skinning solution (see below) at 4°C and permeabilized in the same solution with 50  $\mu$ g/ml of saponin for 30 min. After being placed in respiration solution (see below) for 10 min to wash out adenine nucleotides and creatine phosphate, permeabilized separated fibers were transferred to a 3-ml water-jacketed oxygraphic cell (Strathkelvin Instruments, Glasgow, UK) equipped with a Clark electrode under continuous stirring. The respiration and skinning solutions contained the following (in mM): 2.77 CaK<sub>2</sub>EGTA, 7.23 K<sub>2</sub>EGTA (100 nM free Ca<sup>2+</sup>), 1 free Mg<sup>2+</sup>, 20 taurine, 0.5 DTT, and 20 imidazole (pH 7.1). The skinning solution also contained (in mM) 50 potassium methanesulfonate (160 mM ionic strength), 5.7 Na<sub>2</sub>ATP, and 15 phosphocreatine while the respiration solution contained 10 glutamate, 4 malate, 3 phosphate, 90 potassium methanesulfonate, 10 sodium methanesulfonate (ionic strength 160 mM), and 2 mg/ml fatty acid-free BSA. Experiments were performed at 22°C. ADP-stimulated respiration ( $V_{ADP}$ ) above basal oxygen consumption ( $V_0$ ) was measured by stepwise addition of ADP. Maximal respiration rate ( $V_{max}$ ) was  $V_{ADP} + V_0$ . Oxidation-phosphorylation coupling was determined by calculating the acceptor control ratio (ACR) as  $V_{max}/V_0$ . Respiratory chain complexes were successively investigated with specific substrates and inhibitors [10 mM succinate and 2 mM amytal (for complex II), 0.5 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine + 0.5 mM ascorbate (for complex IV), and 4 mM azide (to inhibit complex IV)]. Respiration rates are given in  $\mu$ mol O<sub>2</sub>·min<sup>-1</sup>·g dry wt<sup>-1</sup>. Two or three determinations were performed for each heart and each protocol.

Table 1. Anatomic data from control, ISO and CUR + ISO groups

Group	Control	ISO	CUR + ISO	CUR
<i>n</i>	20	19	15	5
Body wt before treatment, g	241 ± 6	242 ± 8	251 ± 9	232 ± 14
Body wt after treatment, g	251 ± 6	229 ± 8*	243 ± 7	243 ± 14
Control, %	100	91	97	97
Heart wt, g	0.83 ± 0.03	1.05 ± 0.05**	0.99 ± 0.05*	0.99 ± 0.05
Control, %	100	127	119	119
Heart wt/body wt, mg/g	3.3 ± 0.12	4.58 ± 0.14***	4.07 ± 0.21***	3.62 ± 0.13##
Control, %	100	139	123	110
Protein/wet wt, %	18.1 ± 0.46	17.44 ± 3.43	16.44 ± 2.05	ND

Values are means ± SE; *n*, no. of rats. ISO, isoprenaline; CUR, curcumin. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 vs. the control group. #*P* < 0.05 and ##*P* < 0.01 vs. the ISO group. ND, not determined.

**Isolation of mitochondria and measurement of mitochondrial swelling.** Mitochondria were isolated from all experimental groups by differential centrifugation as previously described (19). Isolated cardiac mitochondria were resuspended in swelling buffer containing (in mM): 120 KCl, 5 KH<sub>2</sub>PO<sub>4</sub>, 20 MOPS, and 10 Tris-HCl, pH 7.4 to a final protein concentration of 0.25 mg/ml. Mitochondrial swelling was followed by changes in absorbance at 520 nm, after addition of 20 μM Ca<sup>2+</sup> (46). The protein yield for isolated mitochondria was (mg protein/g heart tissue) 6.94 ± 0.32 for control, 6.55 ± 0.48 for ISO, and 6.12 ± 0.41 for CUR + ISO (not significant).

**Preparation of adult rat ventricular myocytes.** Adult rat ventricular myocytes were dissociated by retrograde perfusion of healthy isolated heart with collagenase as previously described (44) with slight modifications. Freshly isolated cells were plated on laminin-coated culture dishes at a density of 5 × 10<sup>5</sup> cells/dish and switched to serum-free medium for 2 h. Cells were then incubated for 48 h with ISO (5 × 10<sup>-7</sup> M) and with or without curcumin (5 × 10<sup>-6</sup> M) or their vehicle (control). Five independent cultures were used for each condition.

**Mitochondrial network analysis and mPTP experiments.** Fluorescence experiments were performed with a Carl Zeiss LSM-510 confocal microscope using a ×63 1.4 plan Apochromat objective lens. For mitochondrial network analysis, fresh cardiomyocytes were infected 48 h with an adenovirus containing green fluorescent protein and a sequence targeted to COX IV subunit (36) (gift from Dr. G. Szabadkai). Fluorescence was excited with the 488-nm line, and images were collected through a 505- to 550-nm band pass filter. To reconstruct three-dimensional images of the cells, serial images of individual cardiomyocytes were collected in the *z*-axis. Next, data were imported, and images were deconvoluted using Autoquant X software (Media Cybernetics, Bethesda, MD). Volumes of cardiomyocytes and individual mitochondrion were obtained with IMARIS software (Bitplane, Zurich, Switzerland) using the Imaris MeasurementPro module. Fluorescence emitted from mitochondria was analyzed by the software, which allows, by surface segmentation methods, to identify individual objects corresponding to individual mitochondria. Characteristics of individ-

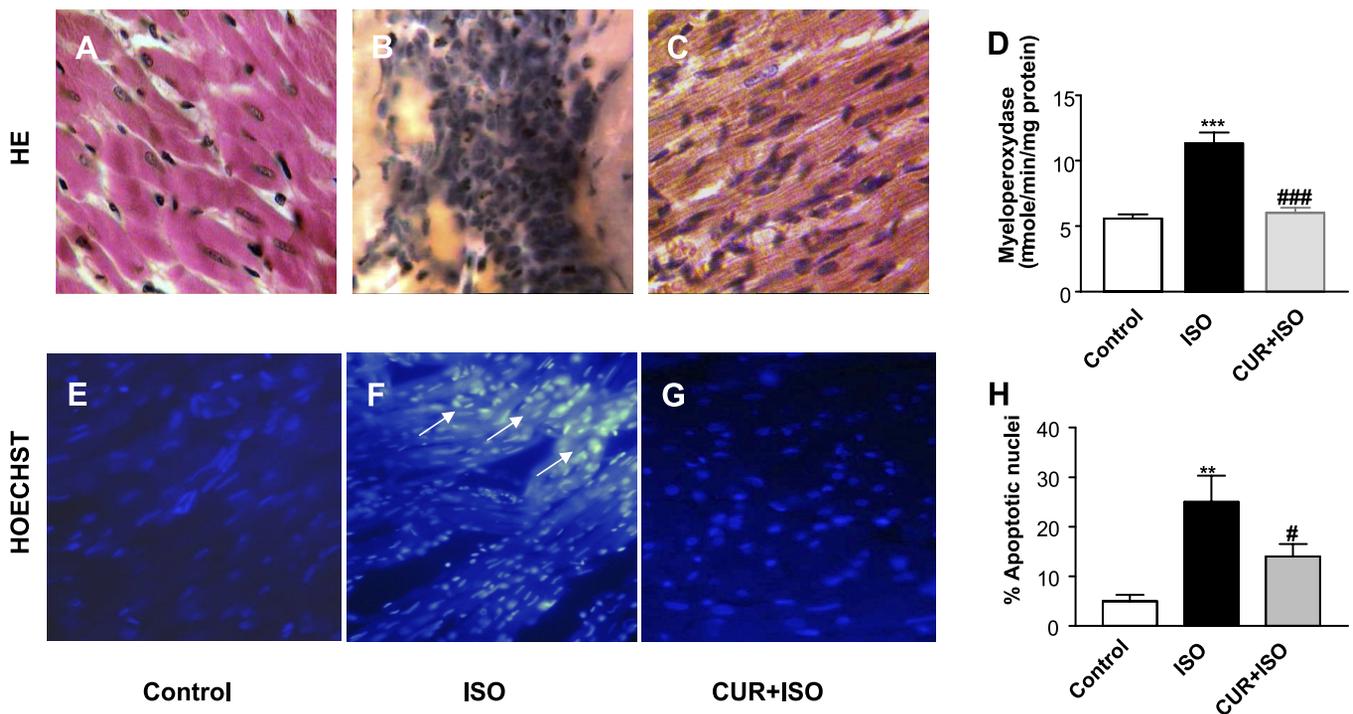


Fig. 1. Curcumin (CUR) protects the heart against apoptosis and oxidative stress. Heart tissues were processed for hematoxylin and eosin (HE) staining. *A*: normal aspect of myocardial tissue in control rat. *B*: acute extensive myofibrillar degeneration, with infiltration of neutrophil granulocytes and interstitial edema in isoprenaline (ISO)-treated hearts. *C*: mild degenerative changes of myocardial tissue in rats pretreated with CUR + ISO. *D*: effect of curcumin on myeloperoxidase activity in ISO-treated hearts (*n* = 5). Cell apoptosis was assessed by Hoechst staining for control (*E*), ISO (*F*)-, and CUR + ISO-treated (*G*) rats. *H*: summary data showing Hoechst-positive cells (%total counted cells) from 10 visual fields of 5 different areas. Values are means ± SE. \*\**P* < 0.01 and \*\*\**P* < 0.001 vs. the control group. #*P* < 0.05 and ###*P* < 0.001 vs. the ISO group (*n* = 3). Original magnification ×1,000 for A–C and ×400 for E–G.

Table 2. Effect of curcumin on metabolic enzymes and oxidative stress

Group	Control	ISO	CUR + ISO	CUR
LDH, IU/mg protein	2.99 ± 0.20	1.26 ± 0.10***	2.31 ± 0.19*###	ND
CK, IU/mg protein	4.69 ± 17	2.91 ± 0.14***	4.17 ± 0.11*###	ND
MDA, nmol/mg protein	0.95 ± 0.06	2.2 ± 0.2***	0.98 ± 0.08*###	0.87 ± 0.08##
GSH, nmol/mg protein	24.7 ± 1.1	15.5 ± 0.3***	22.6 ± 1.4##	20.8 ± 1.3##
Catalase, IU/mg protein	22.0 ± 0.9	10.3 ± 0.7***	15.5 ± 1.6*###	19.7 ± 2.1##
CxI, IU/g protein	255 ± 48	215 ± 25	258 ± 35	ND
COX, IU/g protein	2,765 ± 260	2,462 ± 133	2,284 ± 150	ND
CS, IU/g protein	587 ± 12	480 ± 43	556 ± 29	ND

Values are means ± SE; *n* = 5 rats in each group. Cardiac data: lactate dehydrogenase (LDH), creatine kinase (CK), complex I (CxI), citrate synthase (CS), and cytochrome oxidase (COX). Levels of lipid peroxidation [malondialdehyde (MDA)], antioxidant defenses measured as reduced glutathione (GSH) or catalase activity. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 vs. the control group. ##*P* < 0.01 and ###*P* < 0.001 vs. the ISO group. ND, not determined.

ual objects are obtained (surface, volume, etc.). By this approach, it is possible to calculate the distribution and average volume of individual mitochondria, as well as global mitochondrial network and cell volume.

For mPTP experiments, fresh cardiomyocytes were coloaded in culture medium with 5 μM rhodamine (Rhod-2) and 1 μM calcein (Invitrogen) for 45 min at room temperature and then washed with culture medium. Cardiomyocytes were then permeabilized with sapo-

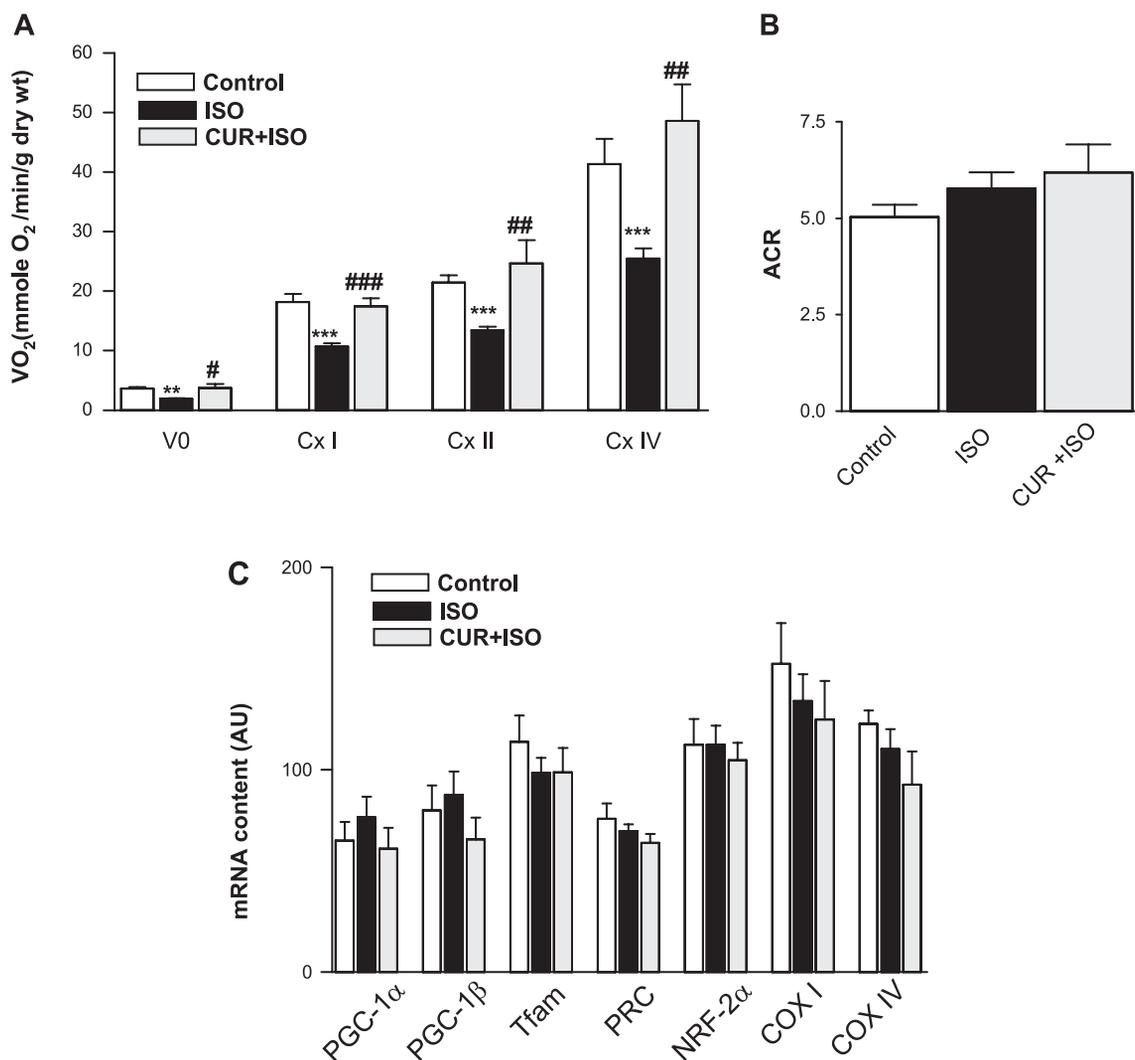


Fig. 2. Effect of curcumin on mitochondrial function and biogenesis in the heart of ISO-treated rats. *A*: basal respiration rate without ADP ( $\dot{V}O_2$ ) and respiration rates with glutamate + malate (complex I), succinate + amylal (complex II), and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine + ascorbate (complex IV); values are expressed in μmol of oxygen consumed per mg dry weight per minute. *B*: acceptor control ratio (ACR). *C*: effect of curcumin on mitochondrial biogenesis in ISO-treated rats. Real-time quantitative RT-PCR analysis of mRNA expression of peroxisome proliferator-activated receptor-γ coactivator 1α and 1β (PGC-1α and -1β, respectively), mitochondrial transcription factor A (Tfam), PGC-1-related coactivator (PRC), nuclear respiratory factor 2 DNA binding subunit α (NRF-2α), and cytochrome oxidase I and IV (COXI and COXIV, respectively). AU, arbitrary units. \*\**P* < 0.01 and \*\*\**P* < 0.001 vs. the control group. ##*P* < 0.01 and ###*P* < 0.001 vs. the ISO group. Values are means ± SE of 5 rats in each group.

nin (50  $\mu\text{g/ml}$ ) for 3 min and then washed in respiration solution (see above) for 5 min to eliminate cytosolic constituents and dyes before experiments. Rhod-2 was excited with the 568-nm line of an argon/krypton laser, and images were collected through a 560-nm long pass emission filter. Calcein fluorescence was excited simultaneously with the 488-nm line of the same laser, and images were collected through a 505- to 550-nm band pass filter. Rhod-2 and calcein signals were monitored after addition of 2  $\mu\text{M}$   $\text{Ca}^{2+}$  in the different conditions (control, ISO, and CUR + ISO) until mPTP opening was observed. In additional plates, cells were superfused with 1  $\mu\text{M}$  cyclosporin A (CsA) to check for specificity of the mPTP opening.

All of the data were analyzed by fitting both Rhod-2 and calcein curves with the following equation:  $y = y_{\infty} + (y_0 - y_{\infty})/[1 + (t/t_{\max})^{p_{\max}}]$ , where  $y_{\infty}$  is the value of the fluorescence for time ( $t$ ) =  $\infty$  and  $y_0$  the value at  $t = 0$  (after  $\text{Ca}^{2+}$  addition), and  $t_{\max}$  and  $p_{\max}$  are the time and the slope for fluorescence decrease, respectively, when maximal mPTP opening occurs (where 50% of the signal has decreased). The initial slope of signal decrease was defined as  $p_{\text{init}}$ . Thus  $t_{\max}$  and  $p_{\text{init}}$  give an index of the  $\text{Ca}^{2+}$  sensitivity of mPTP.

**Statistical analysis.** All data were expressed as means  $\pm$  SE and were subjected to one-way ANOVA followed by Student-Newman-Keuls's test. All results with  $P < 0.05$  were considered statistically significant.

## RESULTS

**Curcumin attenuated ISO-induced cardiac hypertrophy, apoptosis, and oxidative stress.** ISO-treated rats showed a significant increase in heart weight by 27% and heart-to-body weight ratio by 39% compared with the control group that was partially prevented by curcumin (Table 1). No significant difference was observed for protein content, showing that edema was not responsible for increased heart weight. As expected, histological evaluation of ISO-treated hearts showed extensive granulomas with necrotic fibers and infiltrated inflammatory cells compared with control (Fig. 1, A and B). Curcumin pretreatment resulted in marked structural improvement of myonecrosis and edema compared with ISO-treated rats (Fig. 1C) and a decrease of neutrophil infiltration as reflected by myocardial MPO activity (Fig. 1D). The nuclear morphology was also analyzed using Hoechst 33258 staining assay to check for apoptosis. As shown in Fig. 1, E and F, ISO increased the number of apoptotic nuclei by 20% ( $P < 0.001$ ) compared with control. Curcumin reduced nucleus abnormalities associated with cell death (Fig. 1, G and H). No differences were detected between the control group and the curcumin-pretreated group. Isoprenaline significantly decreased cardiac LDH and CK levels ( $P < 0.001$ , Table 2). ISO induced an increase in tissue MDA content ( $P < 0.001$ ) and a decrease

in GSH and catalase activities ( $P < 0.001$ ) compared with control (Table 2). Pretreatment with curcumin prevented all of the deleterious effects of ISO. Curcumin alone had no effect on anatomical parameters and oxidative stress (Tables 1 and 2). All of these data confirm the antioxidant and protective effects of curcumin on ISO-induced cardiac injury.

**Curcumin preserved cardiac mitochondrial respiration under ISO treatment and had no effect on mitochondrial biogenesis.** To investigate the involvement of mitochondria in ISO-induced cardiotoxicity and in curcumin-induced cardioprotection, the oxygen consumption of permeabilized cardiac fibers was measured (Fig. 2). In ISO treated-rats, basal ( $P < 0.01$ ) and maximal ( $P < 0.001$ ) respiration rates on complex I were significantly reduced by 47 and 41%, respectively, compared with control fibers. Similarly, a significant decrease ( $P < 0.05$ ) in the respiration on complex II (37%) or IV (38%) was observed (Fig. 2A), whereas the ACR was not altered (Fig. 2B). Addition of cytochrome *c* did not rescue maximal respiration rates, showing the intactness of the outer mitochondrial membrane in functioning mitochondria [ $V_{\text{cyt}}/V_{\text{max}}$ :  $1.10 \pm 0.03$  ( $n = 11$ ) in control vs.  $1.10 \pm 0.02$  ( $n = 14$ ) in ISO]. Pretreatment with curcumin preserved basal as well as complex I, complex II, and complex IV respiration rates.

mRNA levels of proteins involved in mitochondrial biogenesis (PGC-1 $\alpha$  and its downstream targets) did not differ between groups, showing that the beneficial effects of curcumin were not related to changes in mitochondrial biogenesis (Fig. 2C). These results prompted us to investigate other mitochondrial targets such as mitochondrial remodeling, fusion and fission, and mPTP opening.

**Curcumin reduced mPTP opening induced by ISO.** Mitochondrial swelling following calcium addition, measured as changes in absorbance in isolated mitochondria, reflects the opening of the mPTP (Fig. 3A). Isoprenaline induced a large increase in the swelling rate of mitochondria by 268% compared with the control group ( $P < 0.001$ ), and pretreatment with curcumin markedly reduced the rate of mitochondrial swelling (Fig. 3B).

To decipher whether the beneficial effects of curcumin on mPTP opening were due to systemic or local effects, adult rat ventricular myocytes were incubated for 48 h with ISO and with or without curcumin. Opening of the mPTP was determined in saponin-permeabilized cardiomyocytes, using calcein probe to follow mPTP opening and Rhod-2 fluorophore to monitor mitochondrial calcium. Increased Rhod-2 fluorescence indicated uptake of calcium in mitochondria after addition of 2

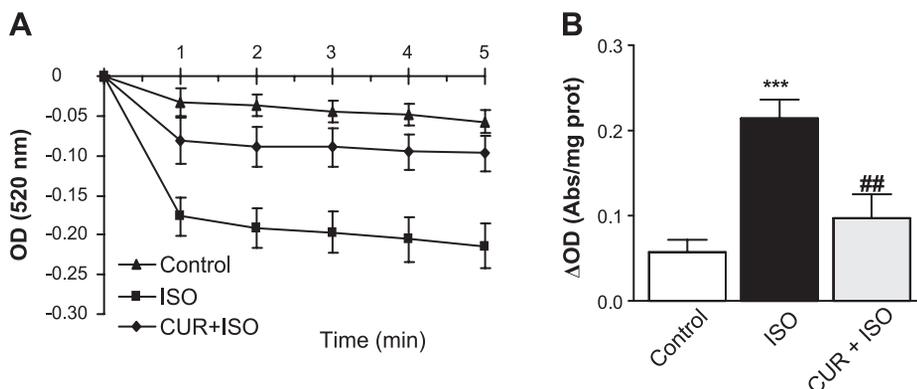


Fig. 3. Effect of curcumin on swelling of heart mitochondria. A: mean changes in absorbance at 520 nm following calcium addition for mitochondria from control, ISO-, and CUR + ISO-treated rats. OD, optical density. B: mean values at 5 min. \*\*\* $P < 0.001$ , versus control group; \*\* $P < 0.01$ , versus ISO group.

$\mu\text{M}$  calcium and progressive mitochondrial calcium loss correlated with calcein leakage and indicated mPTP opening (Fig. 4, A–C). Involvement of the mPTP was confirmed by the addition of CsA, the known inhibitor of the mPTP, which delayed these decreases. After treatment with ISO, the maximal time of mPTP opening ( $t_{\text{max}}$ ) appeared significantly earlier for Rhod-2 ( $628 \pm 29$  s,  $n = 37$  vs.  $870 \pm 62$  s,  $n = 26$  in control,  $P < 0.01$ ) and for calcein ( $574 \pm 32$  s,  $n = 37$  vs.  $818 \pm 67$  s,  $n = 26$ ,  $P < 0.01$ ) (Fig. 4, D and E), indicative of an increase in mPTP  $\text{Ca}^{2+}$  sensitivity. Isoprenaline also induced a significant increase of the slope at opening ( $p_{\text{init}}$ ) by 75% ( $P < 0.001$ ) and 30% ( $P < 0.05$ ) for Rhod-2 and calcein, respectively, compared with control cardiomyocytes (Fig. 4, F and

G). Pretreatment with curcumin significantly delayed mPTP opening and fully restored the slope to control values ( $P < 0.05$ ). Acute addition of curcumin alone ( $5 \mu\text{M}$ ) had no direct effect on mPTP opening (results not shown). Taken together, these results show that curcumin exerts a protective effect on mPTP opening in the heart by a cellular rather than systemic effect.

Curcumin did not modify proteins involved in mitochondrial dynamic and mitochondrial network. mPTP opening properties can also be influenced by mitochondrial morphology (33), and ISO can modify mitochondrial morphology through Drp1 phosphorylation (9). To assess whether changes in the mPTP properties could be due to changes in mitochondrial dynamics,

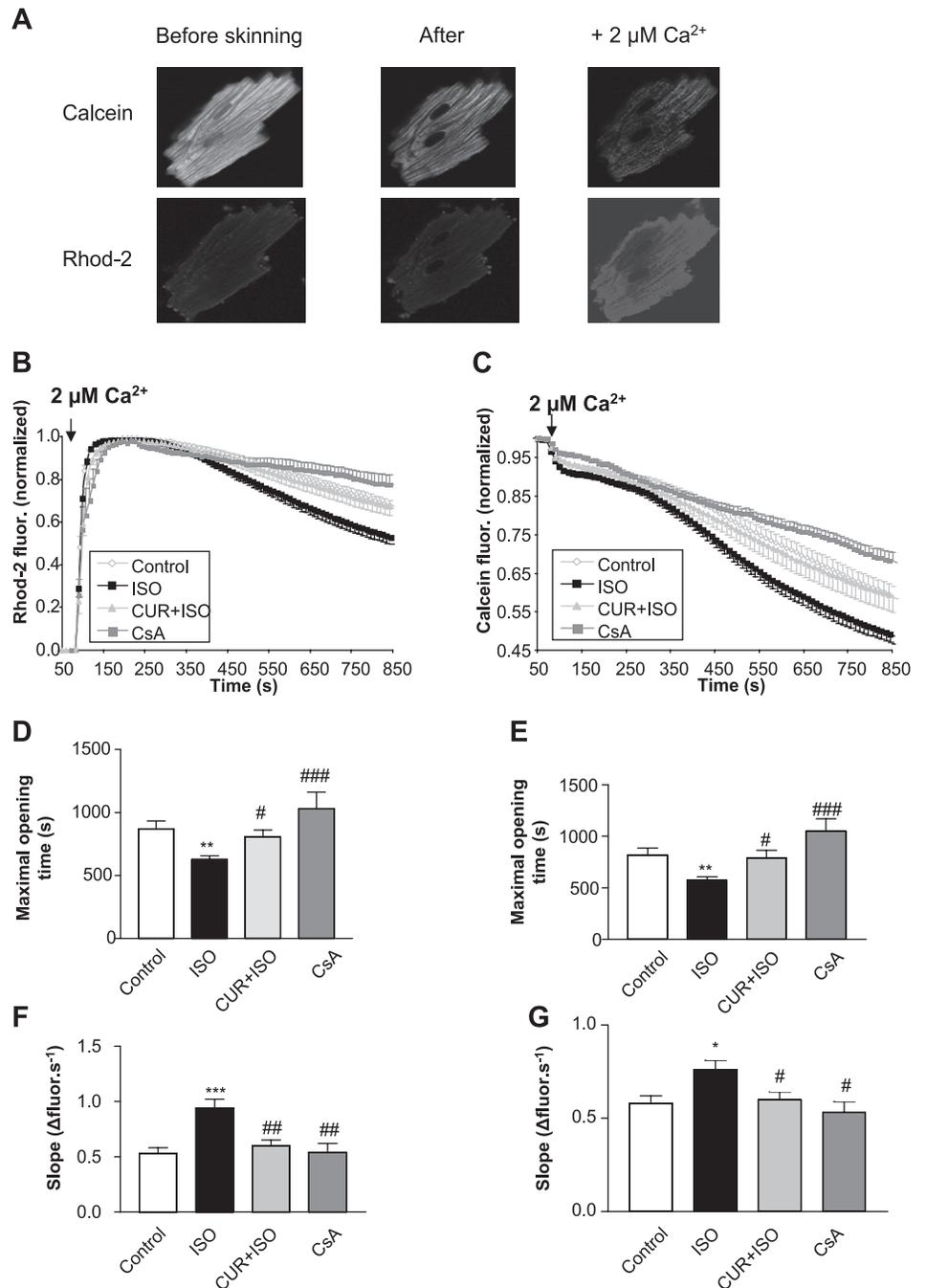


Fig. 4. Effect of curcumin on mitochondrial permeability transition pore (mPTP) of ISO-treated cardiomyocytes. A: representative images of calcein and rhodamine (Rhod-2) fluorescence before and after skinning and after  $2 \mu\text{M}$  calcium. B: mitochondrial Rhod-2 fluorescence intensities reflecting mitochondrial  $\text{Ca}^{2+}$  uptake in saponin-permeabilized cardiomyocytes. C: mitochondrial calcein fluorescence in permeabilized cardiomyocytes reflecting the opening of the mPTP. Maximal opening time for Rhod-2 (D) and calcein (E) probes and slopes at initial mPTP opening for Rhod-2 (F) and calcein (G). Values are means  $\pm$  SE of 5 rats in each group. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. control. # $P < 0.05$ , ## $P < 0.01$ , and ### $P < 0.001$  vs. ISO-treated cardiomyocytes.

we next assessed the expression of the mitochondrial fusion/fission regulatory proteins Opa1, Drp1, and Mfns-1 and -2. Their expression was not significantly changed in the heart of ISO-treated rats (Fig. 5A). Protein kinase A (PKA)-mediated phosphorylation of Drp1 induces sequestration of DRP1 in the cytosol, decreases the fission rate of mitochondria, and may play a protective role upon apoptotic stress (6, 9). As expected, ISO treatment significantly ( $P < 0.05$ ) increased the phosphorylation of Drp1, which was, however, maintained in curcumin-pretreated rats (Fig. 5B).

Using fluorescence imaging and three-dimensional reconstruction, we examined whether ISO treatment could alter the mitochondrial network morphology (Fig. 6A). ISO induced an increase in the total mitochondrial volume ( $P < 0.05$ , Fig. 6B) and in the volume of individual mitochondria ( $P < 0.05$ , Fig. 6C). The population of small mitochondria decreased compared with control, whereas larger mitochondria were increased ( $P < 0.05$ , Fig. 6D). Pretreatment of cardiomyocytes with curcumin was without effect, in line with Drp1 phosphorylation results. ISO tended to increase individual cell volume ( $t$ -test,  $P < 0.05$ ), whereas addition of curcumin had no significant effect (Fig. 6E).

## DISCUSSION

The present results show that, in addition to inflammation, apoptosis, cell damage, and oxidative stress, ISO treatment also induces mitochondrial dysfunction, mitochondrial swelling, and opening of the mPTP. These deleterious effects were prevented by pretreatment with curcumin. Beneficial effects of curcumin on mitochondrial function occurred without changes in mitochondrial dynamic proteins, network morphology, and mitochondrial biogenesis. Altogether, these results evidence that mitochondria and mPTP are involved in catecholamine

cardiotoxicity and are an additional target for the cardioprotective effects of curcumin.

The present study shows that curcumin prevented ISO-induced cardiac hypertrophy, oxidative stress, inflammatory events, necrosis, and neutrophil infiltration and protected cardiomyocytes from cellular injury. Several studies have shown cardioprotective effects of curcumin in different models of catecholamine-induced oxidative stress (14, 31, 39) at doses close to the one used in this study, whereas higher doses ( $400 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) were less effective (39). Most of these protective effects are usually attributed to the ability of curcumin to enhance antioxidant defenses and to scavenge ROS, which can protect cells from necrosis and apoptosis (32).

Mitochondria are the primary source of ROS production in cardiomyocytes as well as their primary target. Previous studies have shown that isoprenaline impairs cardiomyocyte structure through oxidative stress and induction of cell apoptosis (17), and mitochondrial respiratory dysfunction is one of the important features of oxidative stress-mediated cell death (47). We thus investigated mitochondrial function in situ with the advantage over isolated mitochondria to explore the whole mitochondrial population within its cellular environment. Previous study provides evidence that impairment of respiratory capacity of isolated mitochondria by isoprenaline-mediated oxidative stress selectively affects NADH oxidation (47). We show here using permeabilized fibers that basal respiration and respiration rates not only under complex I, but also under complexes II and IV, are affected in ISO-treated rat. Decreased respiration rates were not due to decreased oxidative phosphorylation or disruption of the outer mitochondrial membrane in functioning mitochondria because the ACR was not altered and cytochrome *c* addition did not rescue  $\text{O}_2$  consumption. Moreover, tissue activities of CxI, CxIV, and citrate synthase were

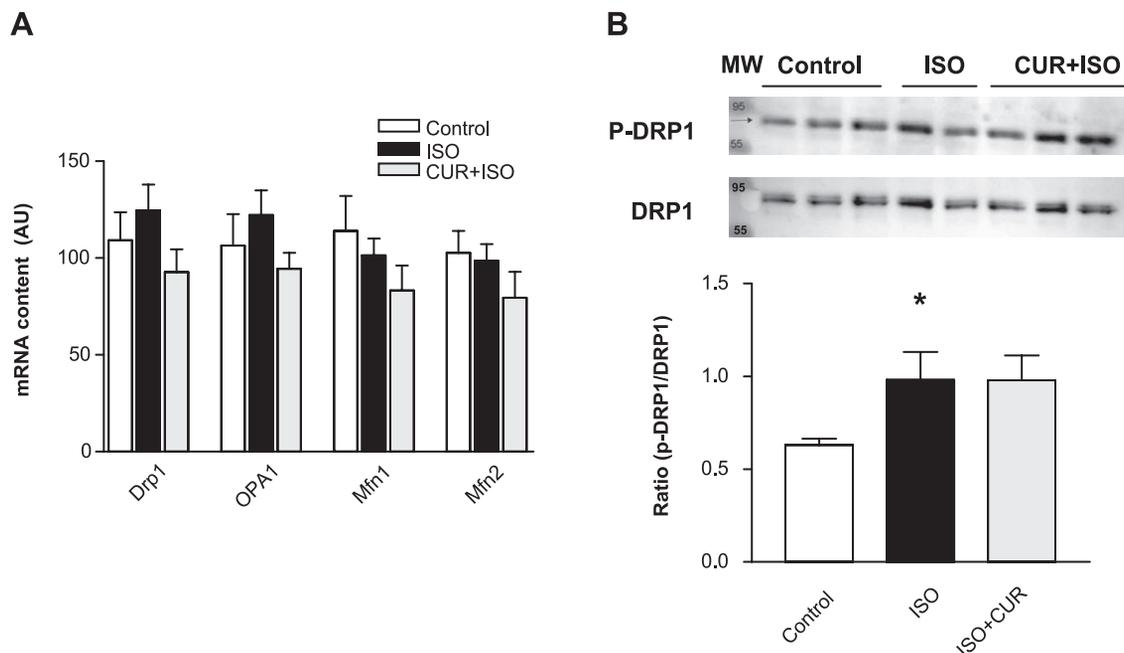


Fig. 5. Effect of curcumin on mitochondrial dynamics in the heart of control ISO- or CUR + ISO-treated rats. *A*: real-time quantitative RT-PCR analysis of mRNA expression of dynamin-related protein 1 (Drp1), optic atrophy protein 1 (OPA1), and mitofusins (Mfn1 and -2). *B*: phosphorylation (P) of DRP1 determined by Western blot analysis and densitometry. \* $P < 0.05$  vs. the control group. Values are means  $\pm$  SE of 5 rats in each group.

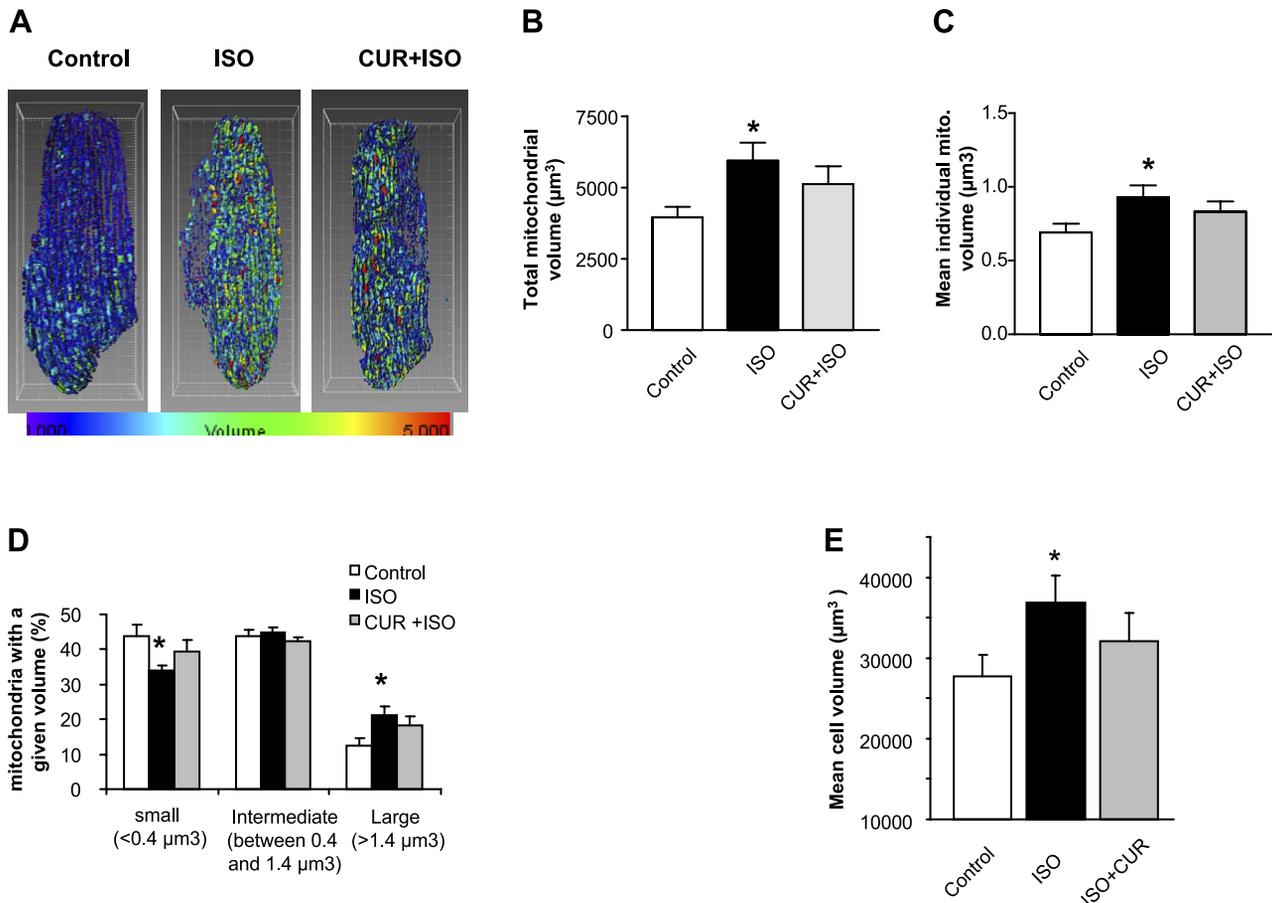
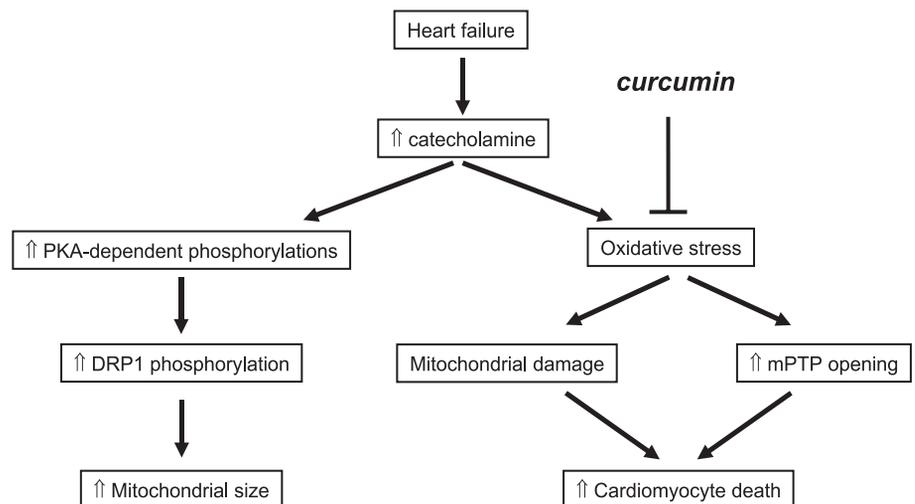


Fig. 6. Effect of curcumin on morphology of the mitochondrial network in rat cardiomyocytes revealed by confocal fluorescent microscopy. *A*: 3-dimensional reconstruction of adult rat cardiomyocytes infected with an adenovirus encoding green fluorescent protein (GFP) targeted to mitochondria. *B*: total mitochondrial volume. *C*: mean individual mitochondrial volume. *D*: mitochondrial volume distribution. *E*: mean cell volumes. \**P* < 0.05 vs. the control group.

not significantly decreased. Taken together, this suggests that decreased respiration rates per milligram of tissue are due to a proportion of defective mitochondria that have lost their inner and outer membrane integrity, leading, for example, to release of reducing equivalents that prevents rescue by cytochrome *c* addition (13). These defective mitochondria are most probably predominantly localized in severely injured or dead cardiomyo-

cytes. The lack of effect of cytochrome *c* addition on respiration does not preclude that cytochrome *c* was released from nonrespiring mitochondria. Although we did not measure, it is an early event in the process of apoptosis. Pretreatment with curcumin completely restored mitochondrial respiration by preserving cell and mitochondrial integrity, in line with their preserved ultrastructure previously described in a similar model (24).

Fig. 7. Proposed mechanism for the beneficial effect of curcumin on catecholamine-induced cardiac injury. PKA, protein kinase A.



Several mechanisms have been implicated in the regulation of PGC-1 $\alpha$  expression and mitochondrial biogenesis, including increased production of reactive nitrogen species and catecholamines (43). We thus explored whether catecholamine cardiotoxicity was associated with alteration of mitochondrial biogenesis by measuring the mRNA expression of PGC-1 $\alpha$ , the major transcriptional regulator of the mitochondrial biogenesis, and of its downstream targets. Neither ISO nor CUR + ISO significantly affected PGC-1 $\alpha$  or - $\beta$  or their downstream targets, suggesting that alterations in mitochondrial biogenesis are not involved.

It has been shown that a change in mitochondrial shape can contribute to mitochondrial defects and mitochondrial mPTP opening (33). Mitochondrial network morphology is the result of fusion and fission events that allow the cell to maintain homogeneous mitochondrial population (7) by exchanging mitochondrial components and eliminating nonfunctional mitochondria (40). Mitofusins and OPA1 are involved in fusion of outer and inner mitochondrial membranes, respectively, whereas Drp1 is involved in mitochondrial fission, an early apoptotic event (20, 26). Catecholamine activates the PKA, and PKA-induced phosphorylation of Drp1 induces its sequestration in the cytosol thus inhibiting mitochondrial fission, a mechanism thought to be protective against apoptosis (6, 9). Whereas in ISO-treated hearts we found no change in the expression of proteins of the mitochondrial dynamics, we observed an increase in the number of large mitochondria and in the mean mitochondrial volume in relation with the increase in Drp1 phosphorylation. Changes in mitochondrial morphology were not prevented by curcumin, suggesting that the beneficial effects of curcumin do not involve mitochondrial network remodeling. Moreover, maintained Drp1 phosphorylation suggests that curcumin does not interfere with the phosphorylation process induced by PKA.

Alternatively, loss of functioning mitochondria can be due to mitochondrial swelling, opening of the permeability transition pore, and depletion of reducing equivalents. Increased mPTP sensitivity to calcium can even precede changes in respiration and can play an important role in cell death (5). mPTP opening causes release of reducing equivalent, cytochrome *c*, and other proapoptotic proteins and loss of membrane potential that may lead to apoptotic cell death (13, 18). ISO treatment accelerated mPTP opening assessed either in mitochondria from treated animals or in ISO-treated isolated cardiomyocytes. Pretreatment with curcumin largely delayed and prevented ISO-induced opening of the mPTP and mitochondrial swelling. Thus, either in vivo or in vitro, curcumin was able to blunt the deleterious effects of ISO on mPTP opening, suggesting a direct beneficial effect on cardiomyocytes. ISO-induced cardiotoxicity has also been associated with alterations in Ca<sup>2+</sup> handling, itself being tightly related to the generation of ROS (8, 21). Oxidative stress and Ca<sup>2+</sup> mishandling induced by ISO can favor mPTP opening. It was shown recently that heart failure can be precipitated by Ca<sup>2+</sup> overload and mitochondria-dependent increase in myocyte necrosis and could be prevented by loss of cyclophilin D, the mitochondrial target of CsA that prevents mPTP opening (30). The increased opening susceptibility of the mPTP observed in the present study may be responsible for impairment in respiration and increased apoptosis involved in the cardiotoxicity of catecholamine. The cardioprotective effect of curcumin on mitochondrial respira-

tion and apoptosis could thus be mediated at least in part by its beneficial effect on inflammation, oxidative stress, and mPTP opening. There are limitations to the present investigation that need to be further addressed. For example, whether mPTP plays a major role in the cardioprotective effect of curcumin could be studied by evaluating the effects of an mPTP inhibitor or using, for example, cyclophilin D knockout mice (29). Moreover, whether curcumin can reverse the catecholamine-induced injury remains to be established.

In summary, ISO-induced cardiotoxicity involves inflammation, oxidative stress, mitochondrial dysfunction, alteration of mitochondria integrity, increase in mPTP opening, and cell damage. The cardioprotective effect of curcumin involves decreased oxidative stress and inflammation, and improved resistance to mPTP opening, leading to preservation of mitochondrial function and prevention of apoptosis (Fig. 7). Interestingly, increased circulating catecholamines, deregulation of intracellular Ca<sup>2+</sup> homeostasis, unbalance between pro-oxidant reactions and antioxidant defenses, and modification of the redox state are important events involved in hypertrophied and failing myocardium (8). Curcumin was shown to prevent heart failure in rats by inhibiting p300 histone acetyltransferase activity (27). Alternatively, it was shown that heart failure can be prevented by decreasing the open probability of mPTP (30). The present results show that curcumin may also protect the heart by preserving mitochondrial function and mPTP opening. Inhibition of the mPTP may thus be a new mechanism by which curcumin exerts its cardioprotective effects.

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#### DISCLOSURES

No conflicts of interest are declared by the authors.

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