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Original Research Communication

Adrenocorticotrope Hormone Fragment (4-10) Attenuates the Ischemia/Reperfusion–Induced Cardiac Injury in Isolated Rat Hearts

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ABSTRACT

The aim of our study was to investigate the contribution of the adrenocorticotropic hormone fragment, ACTH (4-10), on the recovery of postischemic cardiac function. Effects of ACTH (4-10) on caspase-3 activity, cardiomyocyte and endothelial apoptosis, and HO-1 protein expression were studied. Rats were treated with various doses of ACTH (4-10), and then 12 h later, anesthetized, hearts were isolated, perfused, and subjected to 30-min ischemia followed by 120-min reperfusion. Cardiac function including heart rate, coronary flow, aortic flow, and left ventricular developed pressure were recorded. After 120-min reperfusion, 200 μg/kg of ACTH (4-10) significantly improved the recovery of aortic flow, coronary flow, and left ventricular developed pressure from their untreated control values of 15.3 ± 0.9 ml/min, 6.5 ± 0.9 ml/min, and 10 ± 0.6 kPa to 20.7 ± 1.3 ml/min, 24.8 ± 1.8 ml/min and 13.7 ± 0.7 kPa, respectively. Heart rate did not show significant changes during reperfusion. ACTH (4-10) treatment resulted in a reduction in infarct size, caspase 3 activity, apoptosis, and an increase in HO-1 expression. When ACTH (4-10) was given at the moment of reperfusion, the drug failed to improve the postischemic recovery of the myocardium. Thus, ACTH (4-10) can be a useful tool for the prevention of the development of ischemia/reperfusion-induced injury. *Antioxid. Redox Signal.* 9, 1851–1861.

INTRODUCTION

In the past three decades, a number of studies have been devoted to understanding the mechanisms of ischemia/reperfusion–induced damage, and extensive research has been conducted to find effective treatment for ischemic failure. To our knowledge, relatively little attention has been paid on the effects of adrenocorticotropic hormone and its fragments, especially ACTH (4-10), on the recovery of postischemic cardiac function and infarct size. Melanocortins (including α-β-, and γ-melanocyte–stimulating hormones) are derived from a larger precursor molecule, the pro-opiomelanocortin peptide (POMC). The adrenocorticotropic hormone is a peptide that contains 39 amino acids (1-39). Its active fragment contains 13 amino acids: α-MSH (1–13), and within this fragment, ACTH (4–10). Melanocortins exert their effects by activating seven-transmembrane domain G protein–coupled receptors. These receptors have a wide and varied distribution and are found and function in several organs (38, 39, 44).

A few years ago, α-melanocyte–stimulating hormone

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(α-MSH) was described to have a protective effect on renal cells (12), gut (46), lung (15), and brain tissue (20) against ischemia/reperfusion–induced injury. Furthermore, melanocortins including α-MSH are able to exert protective effects in a model of permanent coronary artery occlusion (6, 7). Moreover, melanocortin peptides have peculiar, adrenal-independent antiinflammatory activity (8, 9, 17, 26, 27). In our previous study, we demonstrated that melanocortin peptides (*i.e.*, α-MSH) significantly attenuated the harmful consequences of myocardial ischemia, including arrhythmias, apoptotic and necrotic cell death, and impaired cardiac function (40). Yet, α-MSH, as an endogenous derivate, is a double-edged sword because of the unexpected hormonal side effects. Recently, α-MSH was reported to have protective effects on renal cells, gut, lung, and brain against ischemia/reperfusion–induced damage. The major problem is unexpected hormonal side effects and toxicity of ACTH. Besides hypersensitivity reactions, the toxicity of ACTH is attributable primarily to the increased secretion of corticosteroids. Moreover, ACTH isolated from animal pituitaries contains significant amounts of vasopressin, which can lead to life-threatening hyponatremia. Therefore, the goal of our experiment was to investigate whether ACTH (4–10) could afford cardiac protection against ischemia/reperfusion–induced cardiac damage without any adrenocorticotropic side effects. Our investigation focused on the structure of ACTH with the most protective and fewest reverse effects regarding ischemia/reperfusion–induced injury. Thus, the finding of a similar hormone, derivate, or fragment of derivative without any adrenocorticotropic effect would be an optimal tool for the treatment of an episode of myocardial ischemia/reperfusion. To achieve this goal, first we disclosed by selection of the fraction of ACTH molecule that has similar properties without any adrenocorticotropic effects. The shortest fragment, which displayed pressor and tachycardic responses, is the MSH "core," included His-Phe-Arg-Trp amino acids [γ-MSH-(5-8)], which is identical to ACTH (6–9) (38). Obviously, ACTH (4–10) also contains the His-Phe-Arg-Trp "core." Therefore, we analyzed whether α-MSH or ACTH (1–24) can attenuate ischemia/reperfusion–induced injury, and ACTH (4–10) (which also includes the His-Phe-Arg-Trp fragment) possesses a cardioprotective effect.

The main objectives of the present study were to examine the effects of ACTH (4–10) pretreatment on the postischemic recovery in isolated rat, including (a) the recovery of myocardial function, (b) the incidence of reperfusion-induced arrhythmias, (c) myocardial infarct size, (d) cardiomyocyte and endothelial apoptosis, (e) caspase-3 activity, and (f) HO-1 expression. In additional studies, ACTH (4–10) was given at the onset of reperfusion to determine its direct or indirect effect, if any exists, on the recovery of postischemic cardiac function.

METHODS

Isolated working heart preparation

All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised 1985). Sprague–Dawley rats weighing 275–300 g were subcutaneously injected with 0, 50, and 200 μg/kg of ACTH (4–10) (Sigma, Budapest, Hungary) in saline buffer, and after 12 h, rats were anesthetized with sodium pentobarbital (80 mg/kg b/w, i.p.) and anticoagulated with heparin sodium (500 IU/kg b.w, i.p.) injection. After thoracotomy, the heart was excised, the aorta was cannulated, and the heart was perfused (at 37°C) according to the Langendorff method for a 5-min washout period at a constant pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs–Henseleit bicarbonate buffer (millimolar concentration: sodium chloride, 118; potassium chloride, 4.7; calcium chloride, 1.7; sodium bicarbonate, 25; potassium biphosphate, 0.36; magnesium sulfate, 1.2; and glucose, 10). The Langendorff preparation was switched to the "working" mode after the washout period, as previously described by Yamamoto *et al*. (42) and modified by Tosaki and Braquet (36). Any isolated heart that showed cardiac disturbances (ventricular arrhythmia and fibrillation) before the induction of ischemia was excluded from this study.

In other studies, 50 and 200 μg/L of ACTH (4–10) were directly perfused into the myocardium at the onset of reperfusion ("late administration") for 10 min, and cardiac function was monitored.

Induction of global ischemia

For the measurement of cardiac function, including heart rate (HR), coronary flow (CF), aortic flow (AF), left ventricular developed pressure (LVDP), the incidence of reperfusion-induced ventricular fibrillation (VF), ventricular tachycardia (VT), infarct size, cardiac apoptosis, and protein expression, a model of myocardial ischemia/reperfusion was used. Thus, after 10-min aerobic perfusion of the heart, the atrial inflow and aortic outflow lines were totally clamped at a point close to the origin of the aortic cannula. After 30 min, the reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. An epicardial ECG was recorded throughout the experimental period with two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the presence or absence of reperfusion-induced VF. Hearts were considered to be in VF if an irregular undulating baseline was apparent on the ECG. If VT and VF developed and the sinus rhythm did not spontaneously return within the first 2 min of "nonworking" Langendorff reperfusion, hearts were electrically defibrillated with a defibrillator by using two silver electrodes and 15-V square-wave pulse of 1 ms duration and reperfused. Then, after the first 10 min of Langendorff reperfusion, hearts were further reperfused by switching to "working" mode for an additional 110 min. VT was defined if the numbers of consecutive premature ventricular beats were five or more. Aortic and coronary flow rates were measured by a timed collection of the aortic and coronary effluents that dripped from the heart. Before ischemia and during reperfusion, HR, CF, and AF rates were registered. LVDP was measured with a computer acquisition system (AD Instruments, Castlehill, Australia).

Determination of infarct size

Hearts for infarct-size measurement were perfused, at the end of each experiment, with 15 ml of 1% triphenyl tetrazolium (TTC) solution in phosphate buffer (88 m*M* Na_2HPO_4, 1.8 m*M* NaH_2PO_4) *via* the side arm of the aortic cannula and then stored at −70°C for later analysis. Frozen hearts were sliced transversely (34), in a plane perpendicular to the apico-basal axis, into 2- to 3-mm thick sections, weighed, blotted dry, placed between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single-pass flat-bed scanner (Hewlett-Packard, Palo Alto, CA). With the use of the NIH Image 1.61 image-processing software, each digitalized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels (14). The areas were measured by computerized planimetry software; these areas were multiplied by the weight of each slice, and then the results were summed to obtain the weight of the risk zone (total weight of the left ventricle, in milligrams) and the infarct zone (in milligrams). Infarct size was expressed as the ratio, in percentage, of the infarct zone to the risk zone.

Determination of cardiomyocyte and endothelial cell apoptosis

The formaldehyde-fixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick), and deparaffinized with a graded series of xylene and ethanol solutions. Immunohistochemical detection of apoptotic cells was carried out by using TUNEL, in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA (25). The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody followed by an FITC-conjugated rabbit anti-sheep IgG as a secondary antibody, as described by the manufacturer (Apop Tag Plus; Oncor Inc., Gaithersburg, MD). The sections ($n = 5$) were washed in PBS 3 times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing α-sarcomeric actin (Sigma-Aldrich Biotec, Inc.) followed by staining with TRITC-conjugated rabbit anti-mouse IgG (200:1 dilution) (30). For detection of apoptosis in endothelial cells, the sections were first stained with TUNEL (FITC staining). The sections were then incubated with rabbit polyclonal anti–von Willebrand factor (Sigma-Aldrich Biotec Inc., St. Louis, MO) as a primary antibody followed by incubation with tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG as a secondary antibody. The fluorescence staining was viewed with laser confocal microscopy (Fluoview; Olympus, Tokyo, Japan). For quantitative purposes, the number of TUNEL-positive cardiomyocytes and endothelial cells was counted in 100 high-power fields (HPF; magnification, ×600) from the endocardium through the epicardium of the mid portion of the left ventricular free wall in five sections from each heart (16, 35). Representative confocal images show von Willebrand factor–positive endothelial cells (strong red staining of the cytosol) that are negative for TUNEL staining (absence of green staining in the nucleus), as well as those positive for TUNEL staining.

Measurement of caspase III activity by immunohistochemistry

The free-floating sections of the heart were first incubated with biotinylated goat anti-caspase-3 antibody (Sigma; diluted 1:1,000) overnight at 4°C. The immunologic and immunocytochemical characteristics of the antibody were published earlier (24). Sections were then transferred into a solution of biotinylated rabbit antibody (Vector Laboratories, Burlingame, CA; diluted 1:200) for 50 min at room temperature, and then avidin-biotinylated peroxidase complex (ABC; Vector Laboratories, Burlingame, CA; diluted 1:100) for 4 h at room temperature, and completed with a diaminobenzidine chromogen reaction (23). Before the antibody treatments, sections were kept in 10% normal goat serum (Vector Laboratories) for 50 min. All incubations were performed under continuous gentle agitation, and all of antibodies were diluted in 10 m*M* phosphate-buffered saline (PBS, pH 7.4), to which 0.1% Triton X-100 and 1% normal rabbit serum (Vector Laboratories) were added. Sections

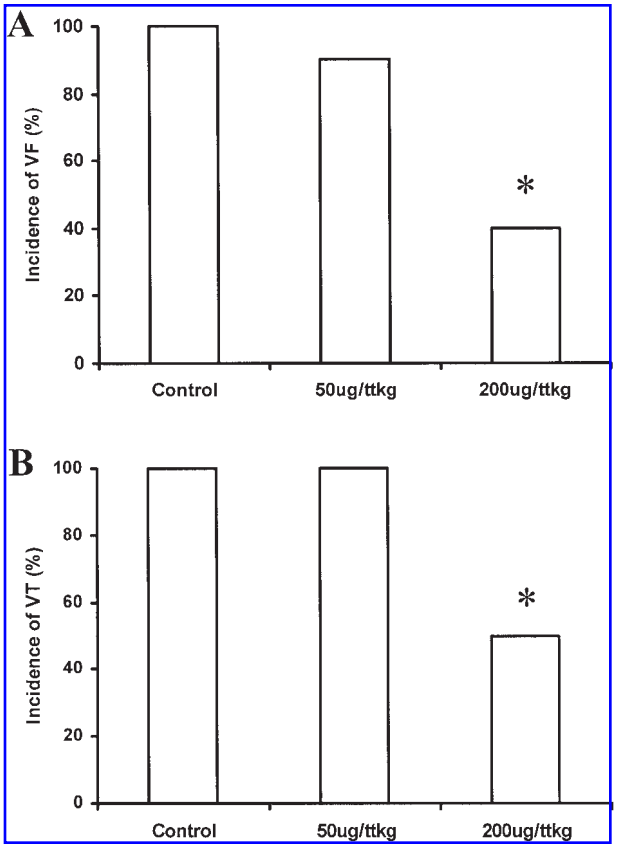

FIG. 1. Effects of various doses of ACTH (4–10) on the incidence (percentage) of VF (A) and VT (B) in isolated rat hearts. Isolated hearts ($n = 10$ in each group) were obtained from rats treated subcutaneously with 0 (drug-free control), 50, or 200 μg/kg ACTH (4–10). Then hearts were isolated and subjected to 30-min ischemia followed by 120-min reperfusion. *$p <$ 0.05 compared with the untreated drug-free control values.

were mounted on gelatin-coated slides and covered with Permount neutral medium (Fluka, Buchs, Switzerland).

Western blot method

Total protein (50 μg) in the Clontech Extraction buffer was added to an equal volume of sodium dodecylsulfate (SDS) buffer and boiled for 10 min before being separated on 12% SDS polyacrylamide gels in running buffer (25 m*M* Tris, 192 m*M* glycine, 0.1% (wt/vol) SDS, pH 8.3) at 200 V. The Precision plus Protein Kaleidoscope standards (10 μl) (Bio-Rad Laboratories, CA) were used as molecular-weight standards. The gel was transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 100 V for 1 h in transfer buffer (25 m*M* Tris base, 192 m*M* glycine, 20% (vol/vol) methanol, pH 8.3). After blocking the membranes for 1 h in Tris-buffered saline (TBS-T) (50 m*M* Tris, pH 7.5, 150 m*M* NaCl) containing 0.1% (vol/vol) Tween-20 and 5% (wt/vol) nonfat dry milk, blots were incubated overnight at 4°C with the primary antibody. Membranes were washed 3 times in TBS-T before incubation for 1 h with horseradish peroxide (HRP)-conjugated secondary antibody diluted 1:2,000 in TBS-T and 5% (wt/vol) nonfat dry milk. Western blots were developed with the ECL Detection Reagents 1 and 2 (Amersham Biosciences, NJ) and exposed to Kodak X-OMAT (Amersham Biosciences, NJ) film.

Statistical analysis

HR, CF, AF, LVDP, infarct size, caspase-3, and HO-1 activities were expressed as mean value ± SEM. A two-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free group by Bonferroni correction. A different procedure, because of the nonparametric distribution, was used for the distribution of discrete variables, such as the incidence of VF and VT. Thus, the χ^2 test was used to compare individual groups.

RESULTS

Effect of ACTH (4–10) on cardiac arrhythmias

Figure 1 shows the incidence of reperfusion-induced VF (Fig. 1A) in isolated hearts obtained from rats subcutaneously treated with 0 (untreated control), 50, and 200 μg/kg of ACTH (4–10), 12 h before the isolation of hearts and induction of ischemia and reperfusion. Thus, our results show that the incidence of reperfusion-induced ventricular fibrillation was significantly reduced from its untreated control value of 100% to 90%, and 40% ($p < 0.05$), respectively. The reduction of the incidence of reperfusion-induced VT (Fig. 1B) followed the same pattern. The incidence of reperfusion-induced ventricular tachycardia was significantly reduced from its untreated control value of 100% to 100% and 50% ($p < 0.05$) with the doses of 50 and 200 μg/kg of ACTH (4–10). These data and our previous results represent (40) that the higher dose of ACTH (4–10) was

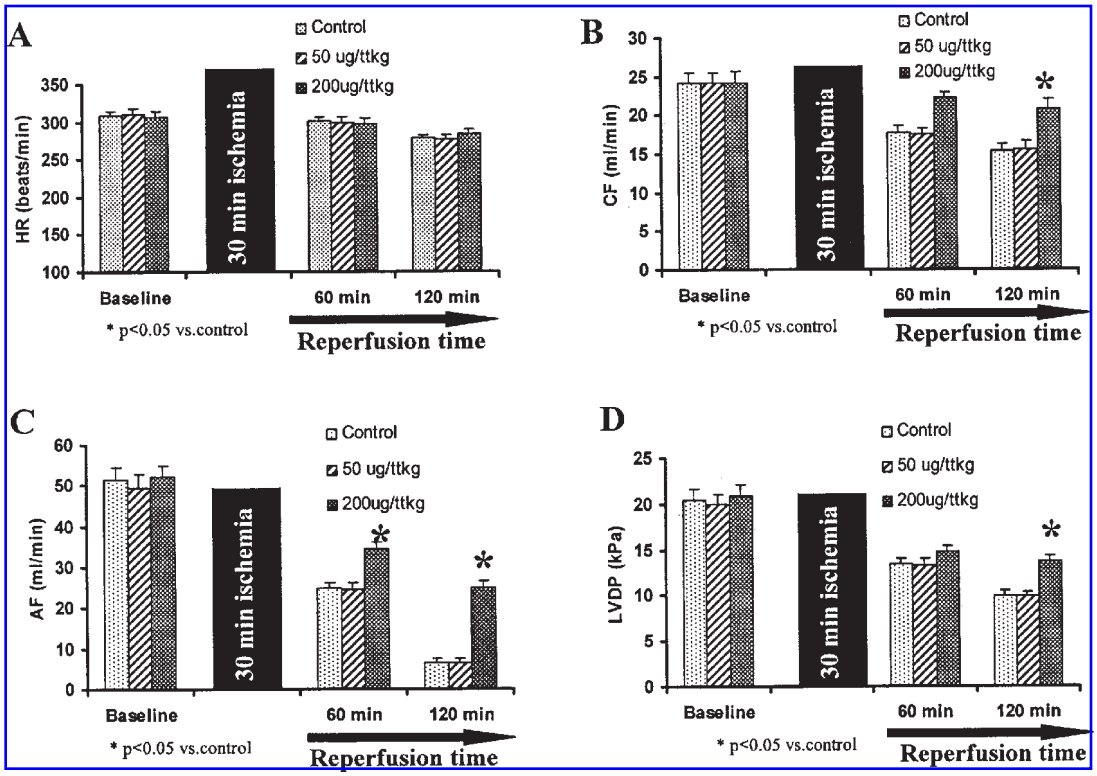

FIG. 2. Effects of different doses of ACTH (4–10) on heart rate (HR), coronary flow (CF), aortic flow (AF), and left ventricular developed pressure (LVDP). Rats were treated subcutaneously with two different doses (50 and 200 μg/kg) of ACTH (4–10) 12 h before the induction of ischemia and reperfusion. HR, CF, AF, and LVDP were measured before the induction of ischemia and during postischemic reperfusion. Results are expressed as mean ± SEM of six hearts in each group.

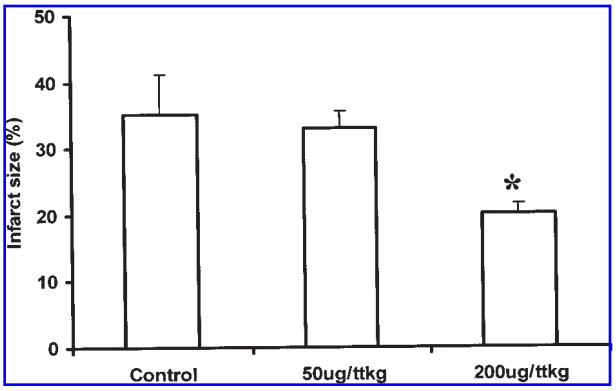

FIG. 3. Effects of different doses of ACTH (4–10) on infarct size in isolated rat hearts subjected to 30 min of ischemia followed by 120 min of reperfusion. Isolated hearts were obtained from rats treated *in vivo* with 0 (drug-free control), 50, and 200 μg/kg of ACTH (4–10). Then hearts were isolated and subjected to 30 min of ischemia followed by 120 min of reperfusion; $n = 6$ in each group, mean ± SEM. $^*p < 0.05$ compared with the untreated drug-free control value. Representative slices of infarct size are shown above each bar. *White areas*, Infarcted tissues with TTC staining.

able to reduce the incidence of VF and VT; however, it was not so effective as 200 μg/kg of α-MSH (ACTH 1–13).

Effects of ACTH (4–10) on cardiac function

Rats treated with various doses of s.c. ACTH (4–10) had significantly improved postischemic contractile function compared with the drug-free controls. It can be easily explained by the reduction of the incidence of reperfusion-induced VF and VT. Interestingly, ACTH (4–10) at the doses used had no effect on heart rate (Fig. 2). However, AF was increased from its drug-free ischemic/reperfused control value of 6.5 ± 0.9 to 6.5 ± 1 ml/min (NS) and 24.8 ± 1.8 ml/min ($p < 0.05$) with the concentrations of 50 and 200 μg/kg of ACTH (4–10), respectively (Fig. 2C). Postischemic CF (Fig. 2B) and LVDP (Fig. 2D) also showed significant improvement in cardiac function in hearts obtained from rats treated with 50 and 200 μg/kg of ACTH (4–10). The lower dose of ACTH (4–10) failed to improve the postischemic recovery in HR, CF, AF, and LVDP (Fig. 2).

Extent of infarct size

Figure 3 shows infarct size in the control hearts (35.3 ± 6%). In hearts treated with 200 μg/kg of ACTH, a marked reduction in infarct size [20.16 ± 1.5% ($p < 0.05$)] at the end of reperfusion was observed, suggesting that ACTH (4–10) can prevent ischemia/reperfusion–induced injury. The lower concentration (50 μg/kg) of ACTH (4–10) failed to reduce infarct size (33.0 ± 2.6%).

Measurement of caspase-3 activity

Visualization of the caspase-3 protein after the immunoperoxidase reaction revealed decreased activity of these proteins in treated hearts subjected to 30 min of ischemia followed by 2 h of reperfusion. Immunoreactive caspase-3 was localized mainly in the cytoplasm of cardiomyocytes and vascular smooth muscle cells. Figure 4 shows caspase-3 activity in hearts obtained from rats treated with 0 (untreated control), 50, and 200 μg/kg of ACTH (4–10), and subjected to ischemia/reperfusion. Caspase activity, using immunohistochem-

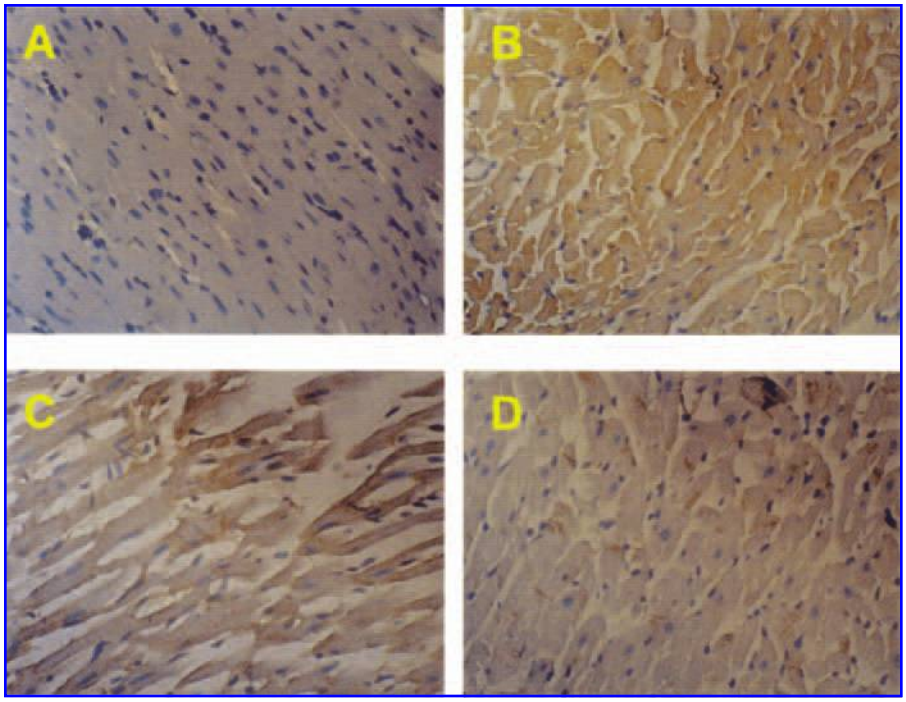

FIG. 4. Caspase-3 activity detected by immunohistochemistry. (**A**) Nonischemic aerobically perfused heart. (**B**) Drug-free heart subjected to 30-min ischemia followed by 120 min of reperfusion (drug-free control). (**C**, **D**) Rats were subcutaneously treated with 50 or 200 μg/kg ACTH (4–10) 12 h before ischemia, and then hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion. The immunohistochemistry and sampling were done at the end of the reperfusion period.

istry, was reduced in treated subjects indicating by a reduction in brown staining intensity (see Fig. 4) in the myocardium. The results of caspase-3 activity were confirmed by Western blot analysis (see later).

Cardiomyocyte and endothelial apoptosis

The results show that TUNEL-positive nuclei were condensed; representing apoptotic cells (Fig. 5). Similar to myocardial infarct size, the numbers of apoptotic cardiomyocytes and endothelial cells were reduced significantly when hearts were pretreated by the higher dose of ACTH (4–10). Total numbers of cardiomyocytes at 100 high-power fields, which cover almost all the mid portion of left ventricular free wall, were examined for detecting apoptotic cells. The data were expressed in counts/100 high-power field and not in percentage of apoptotic cells. After treatment with 200 μg/kg of ACTH (4–10), the numbers of endothelial and cardiomyocyte apoptotic cells were also significantly reduced. However, the lower dose of ACTH (4–10) did not cause a significant change in apoptotic cell death.

Effects of ACTH (4–10) on the expression of caspase-3 and HO-1

The ischemia/reperfusion–induced caspase-3 expression was decreased by the higher dose of ACTH (4–10) (Fig. 6), and Western blot analysis confirmed the semiquantitative immunohistochemistry results. In addition, we examined whether HO-1 signaling is involved in ACTH (4–10) treatment. Figure 6B shows that pretreatment with the higher dose (200 μg/kg) of ACTH (4–10) increased the expression of HO-1, whereas the housekeeping gene expression (GAPDH) was unchanged (Fig. 6C). Thus, the expression of HO-1 could be related to the protective effect of ACTH (4–10).

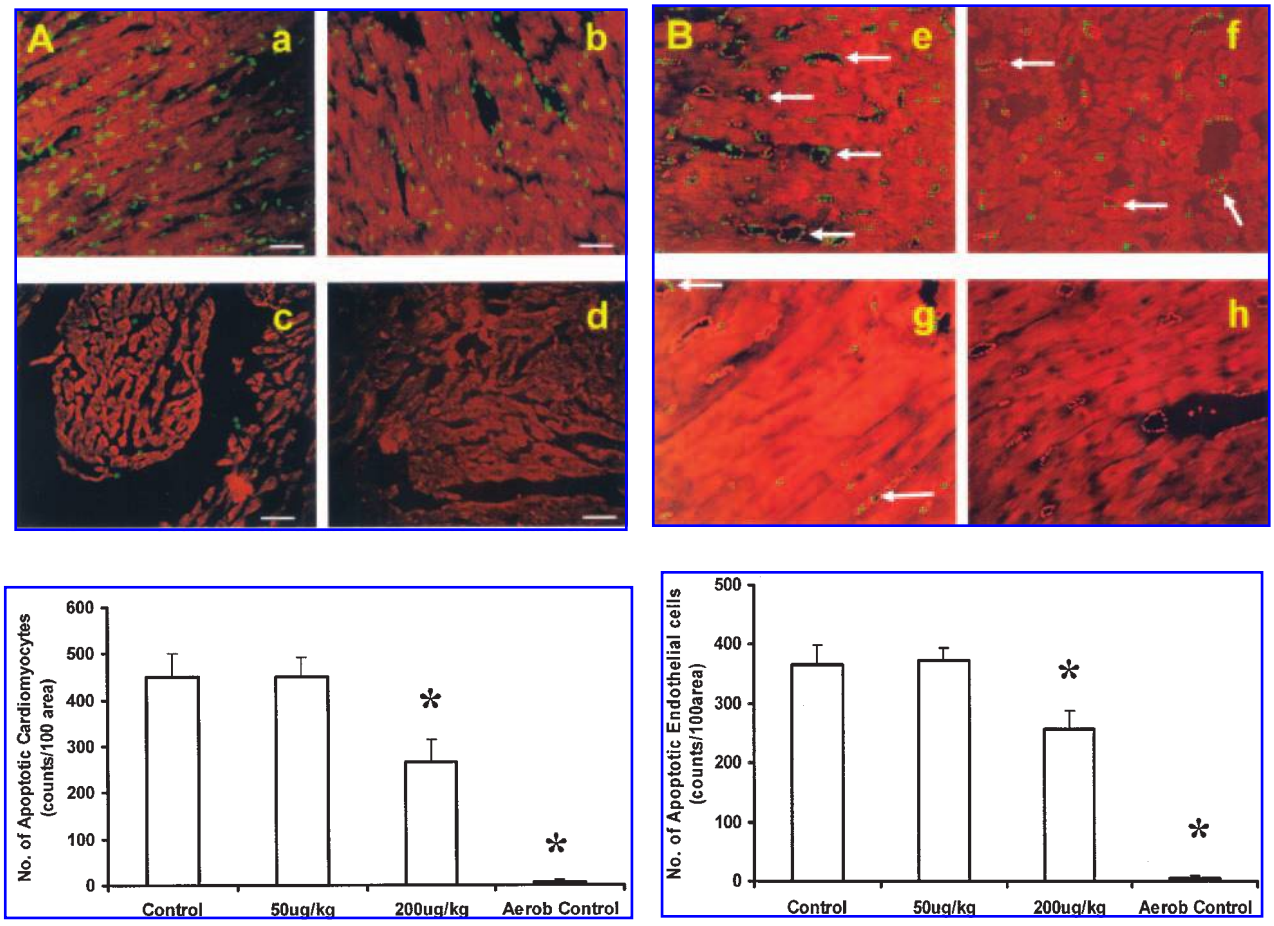

FIG. 5. TUNEL assay for apoptotic cardiomyocytes (A) and endothelial cells (B). (**a–d**) Double immunofluorescence staining for α-sarcomeric actin (specific for cardiomyocyte; red fluorescence) and TUNEL-positive (green fluorescence). (e–h) Double immunofluorescence staining for von Willebrand factor (specific for endothelial cells; red fluorescence) and TUNEL-positive (green fluorescence). (**a, e**) Untreated ischemia/reperfused (drug-free control). (**b, f**) Treated with 50 μg/kg ACTH (4–10). (**c, g**) Treated with 200 μg/kg ACTH (4–10). (**d, h**) Nonischemic aerobically perfused heart. Data are expressed at counts/100 high-power fields. Bar graphs show the extent of cell death by apoptosis; $n = 4$ in each group, mean ± SEM. *$p < 0.05$ compared with the untreated drug-free control value. Immunohistochemistry and sampling were carried out at the end of reperfusion period. *Arrows* (white), Endothelial apoptotic cells around coronary vessels.

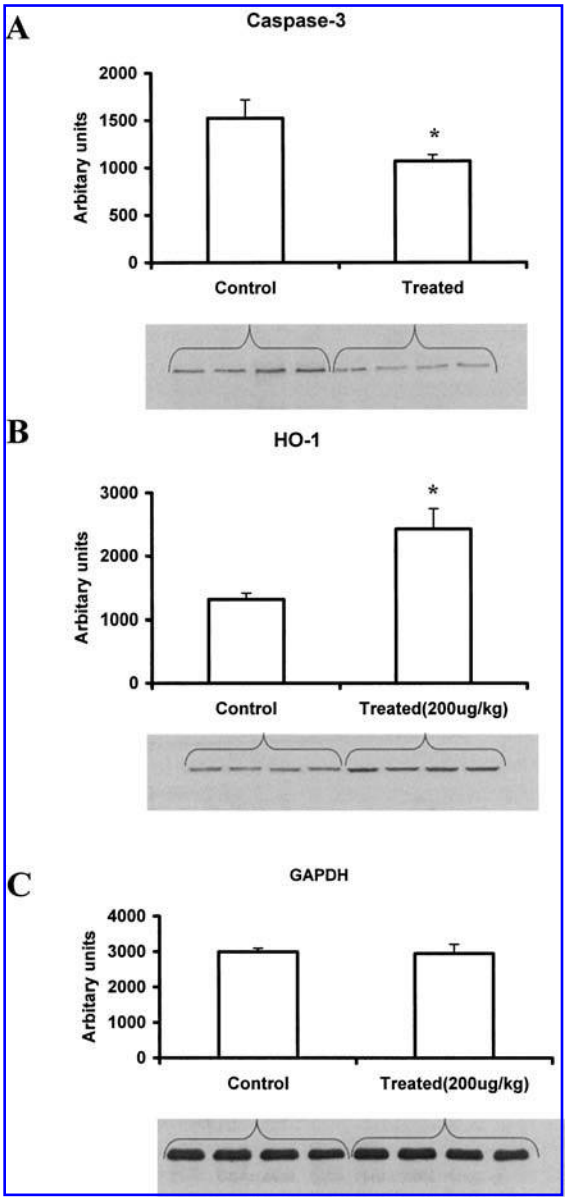

FIG. 6. Effects of the 200-μg/kg ACTH (4–10) on caspase-3 and HO-1 protein expression. Blots were scanned, normalized, and the averages (mean ± SEM) of four samples are shown on the upper panels; the representative blots are shown on the lower panels. In each lane, protein concentration was 60 μg. GAPDH(C) was used to demonstrate equal protein loading. Samplings were done at the end of reperfusion period (n = 4 in each group, mean ± SEM; *$p < 0.05$ compared with the untreated age-matched, drug-free control values.

Effects of ACTH (4–10) on cardiac function ("late administration")

In additional studies, the drug perfused at the onset of reperfusion failed to improve the postischemic recovery in HR, CF, AF, and LVDP (Fig. 7). The incidence of reperfusion-induced VF and VT was also not significantly reduced (data not shown) in comparison with the control values.

DISCUSSION

Underlying the electrophysiologic dysfunction of acute myocardial ischemia is a dysfunction of biochemical homeostasis, including various endogenous substances that alter cell–cell coupling and signaling. After the ischemic period, without any doubt, reperfusion, although a prerequisite for the survival of ischemic cells, is not without risk. Thus, components of the reperfusion process might temporary increase the presumption of ventricular arrhythmias and heart failure, decreasing the chance of the myocardial recovery to steady-state contractile function. In view of this, the utmost importance lies in protecting the tissue from ischemia/reperfusion–induced damage. Many factors play a critical role in ischemia and reperfusion, but the relative importance of these factors is uncertain and controversial. If we provoke a change in only one pathologic factor, then the modification of the signal-transduction pathway can beneficially modulate the recovery of postischemic cardiac function.

The goal of our experiments was to study whether ACTH (4–10) pretreatment could afford cardiac protection against ischemia/reperfusion–induced cardiac damage. Thus, we determined that ACTH (4–10) is able (a) to improve postischemic cardiac function, (b) to reduce the incidence of reperfusion-induced ventricular fibrillation and tachycardia, (c) to decrease the myocardial infarct size, (d) to attenuate the apoptotic endothelial and myocardial cell death, and (e) to influence caspase-3 activity and HO-1 signaling. In addition, (f) we studied whether the effect of ACTH (4–10) originates from its direct action on the myocardium or its indirect effect.

In our previous study, we found that melanocortin peptides (*i.e.*, α-MSH) significantly attenuated the life-threatening consequences of myocardial ischemia. In the present study, we endeavored to obtain more direct or indirect circumstantial evidence for an involvement of the ACTH fragment on the postischemic recovery of the heart. Thus, we investigated whether the smaller fragment of α-MSH could mimic the effect of melanocortin peptides in ischemic/reperfused myocardium. The action mechanisms of melanocortins remain speculative, and a possible explanation is based on previous investigations (38, 39). One of the protective effects of adrenocorticotropic hormones in myocardial infarct seems to be due to the capacity of these peptides to inhibit the overproduction of free radicals and to their antiinflammatory activity. Although it was not the aim of our study, it is of interest to note that clinical concepts recently have proven that the synthesis or release of endogenous inflammatory mediators could contribute to the protection. The antiinflammatory action of melanocortins is possibly related to the melanocortin-1 and melanocortin-3 receptors (MC1-R, MC3-R) (18, 19). Thus, the antiinflammatory effects of melanocortin peptides are also associated with a reduced production of proinflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, tumor necrosis factor (TNF) (27, 28), and with an enhanced genesis of the antiinflammatory IL-10 and of the angiogenic factor IL-8 (28). The role of melanocortin peptides on the immune system appears to be complex and depends on several concerted actions. Thus, exogenously applied α-MSH was found to inhibit the lipopolysaccharide (LPS)-induced release of proinflammatory TNF-α in whole blood under *in vitro* conditions (10). Furthermore, the

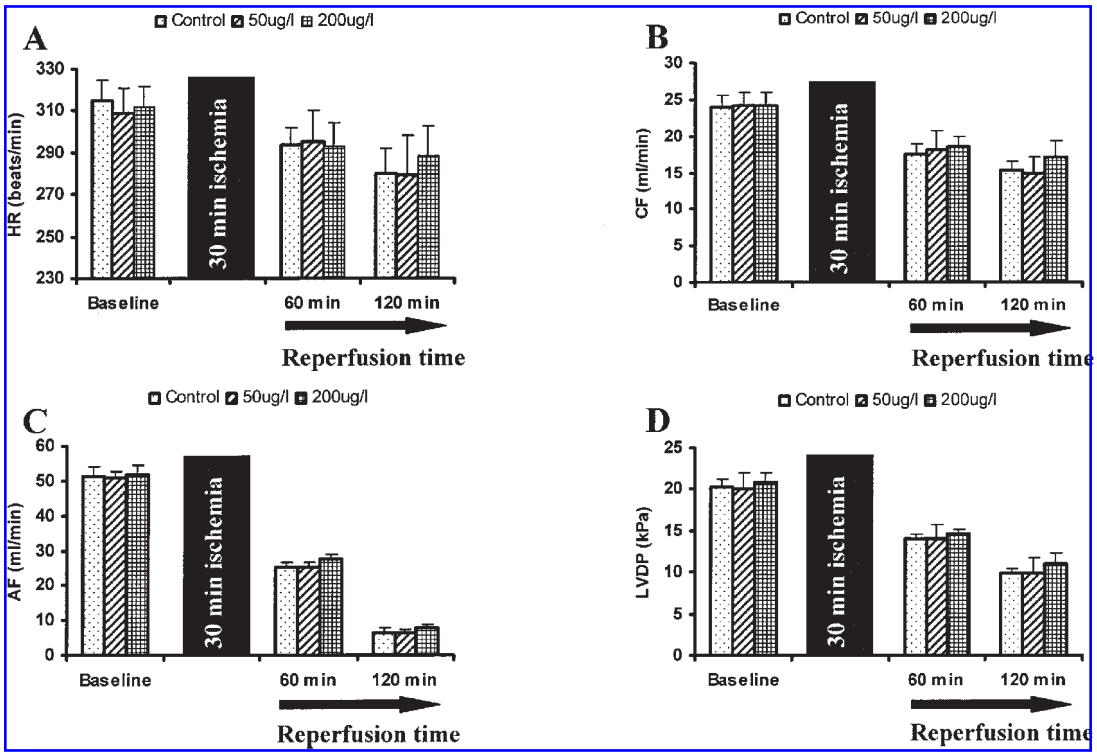

FIG. 7. Effects of ACTH (4–10) on HR, CF, AF, and LVDP, when the drug was given at the onset of reperfusion ("late administration"). Rat hearts were treated with 50 and 200 μg/L ACTH (4–10) immediately after ischemia and during the first 10 min of reperfusion. HR, CF, AF, and LVDP were measured before the induction of ischemia and during the postischemic reperfusion. Results are expressed as mean ± SEM of six hearts in each group.

POMC peptides are widely expressed in the body. Thus, POMC and POMC-derived ACTH peptides are produced by monocytes cells (32), melanocytes (11), and endothelial cells (33). Therefore, it is reasonable to believe that endogenous mediators such as α-MSH or ACTH fragments could contribute to the reduced release of inflammatory mediators in the heart and other organs (1, 2).

In many of the studies, interventions or drugs have been used before the induction of ischemia, often during the period of ischemia, and sometimes at the moment of reperfusion. Thus, many drugs (*e.g.*, beta blockers and calcium channel antagonists) and other interventions exert beneficial effects, thereby protecting the heart against the ischemia-induced cell injury, so that at the onset of reperfusion, the myocardium in treated groups is less severely injured. Because vulnerability of the myocardium to reperfusion-induced damage is proportional to the severity of the preceding period of ischemia, it is almost impossible to ascertain whether the obtained cardiac protection is a direct consequence of a reduction of reperfusion-induced damage or whether it is indirect and originates from other beneficial signal transduction mechanism(s). In our present study, ACTH (4–10) demonstrated cardiac protection when the drug was administered before the induction of ischemia, and this protection was lost if the drug was given at the moment of reperfusion. Therefore, we stress that the protection afforded by ACTH (4–10) may originate from other signal-transduction mechanism(s) that operate before the initiation of reperfusion.

Programmed cell death in the myocardium has been also linked to ischemia/reperfusion injury, as well as to extreme mechanical forces associated with an increase in ventricular loading. Moreover, hypoxia and ischemia activate the suicide program of cardiomyocytes and endothelial cells under *in vitro* and *in vivo* conditions. Apoptosis also frequently occurs as a harmful mechanism in both acute and chronic tissue injury, yet the signal transduction responsible for this action is not precisely known. The end points of our previous study showed that higher doses of α-MSH reduced apoptotic cell death (40). In the present study, we investigated whether ACTH (4–10), which also contains the His-Phe-Arg-Trp core, could influence the apoptotic capability of cardiomyocyte and endothelial cells.

We stressed earlier that molecular mechanisms of apoptosis are complicated and diversified, and many factors have been suggested to play an important role in the apoptotic or necrotic process. A change in only one pathologic factor or downstream pathway could attenuate the development of cell death. Caspase-3 is an important apoptotic marker in the cellular and suicide cascade, which is well known and documented. Caspase-3 is a downstream effector of caspase-9, which is activated by cytochrome *c* by mitochondria or by caspase-8 (3). Caspase-3 protein also is related to the Bcl-2 family, Bax-like (22), Fas/FasL, TNF-α, TNF-α receptor (3), right ventricular dysplasia, and end-stage heart failure (13, 29, 31). Therefore, it is important to consider whether cardiac function and infarct size can be modified by caspase-3 down- or upregulation. However,

blockade of caspase activation with a specific inhibitor was reported to decrease infarct size after coronary artery ligation in rats (43), but the cause-and-effect relation between caspase-3 and cardiac function has not been established (13). It is important to note that drugs or natural derivatives, which could attenuate caspase activity, may contribute to avoid the harmful and destructive consequences of apoptosis. Treatment with a caspase-3 inhibitor improved survival and prevented ventricular dilation and dysfunction after permanent coronary artery occlusion. The results of our study showed, for the first time, that the beneficial effect of ACTH (4–10) is due to its ability to reduce caspase-3 activation, which is linked, at least in part, to the reduction of apoptotic cell death. Caspase-3 activity, shown by immunohistochemistry and Western blot analysis, was reduced in the ACTH (4–10)–treated group. Because caspase-3 activity was diminished, accordingly, the number of death cells of cardiomyocytes and endothelial cells were also reduced in the ACTH (4–10)–treated group. Moreover, one of the reasons for decreased apoptosis could be the overexpression of the HO-1 protein. HO-1 induction appears to be an important factor in ischemia/reperfusion protection of the myocardium. The role of heme oxygenase signaling in various disorders (ischemia/reperfusion, hypertension, cardiomyopathy, organ transplantation, endotoxemia, lung disease, and immunsuppression) has already been well documented (37). Zou *et al*. (46) showed that treatment with α-MSH at 1 h of reperfusion led to an increased HO-1 protein expression in a gut ischemia/reperfusion model. The exact antiapoptotic mechanism(s) of HO is (are) not clearly known but is speculative. One possible mechanism could be *via* the bilirubin system, because bilirubin has been shown to protect cardiomyocytes against oxidative damage (41, 45). Therefore, we investigated whether ACTH (4–10) is also able to affect the HO-1 protein level in our model. Without doubt, it is reasonable to believe that a reduction in programmed cell death limits infarct size in ACTH-treated rats. The importance of HO-1 expression could be a crucial factor in the postischemic cardiac protection of ACTH (4–10), because ACTH (4–10)–induced cardiac protection related to the HO-1 protein expression when the drug was administered before the isolation of hearts and the induction of ischemia and reperfusion. The mechanism(s) of reperfusion-induced damage and development of arrhythmias could be related to the endogenous carbon monoxide production *via* the HO-1 system, as was stressed by Bak *et al*. (5). The authors (5) demonstrated that HO-1 expression and its relation to endogenous carbon monoxide production significantly determined the development of reperfusion-induced VF in HO-1 knockout mice. Thus, interventions that are able to increase HO-1 expression (4) and parallel reduce apoptosis and/or necrosis could directly or indirectly result in a reduction in the incidence of reperfusion-induced arrhythmias. However, if the drug were perfused at the moment of reperfusion, the ACTH (4–10)–induced protection was lost, indicating that HO-1 expression and protection is time dependent and operated before the induction of ischemia. Thus, ACTH (4–10) could be a preventive tool against ischemia/reperfusion–induced damage.

In conclusion, the findings of the present study demonstrate that ACTH (4–10) could attenuate the destructive consequences of myocardial ischemia. The application of ACTH (4–10) before the induction of ischemia suggests that the observed protective effect could originate from the mechanism(s) operating before the induction of ischemia because the protection was lost when the drug was perfused at the moment and the first 10 min of reperfusion. The interpretation of our results must, of course, be limited by our observations in the rat heart and by the fact that we used an isolated preparation. The use of the isolated perfused myocardium, although offering the advantage of the ability to study direct cardiovascular responses, independent of various peripheral factors, is subject to the criticisms that it is a denervated and perfused ansanguineous solution. In addition to its denervated condition, the isolated perfused heart system can be criticized because of the absence of blood and its cellular components, such as leukocytes and platelets, which can influence cardiac function, apoptosis, and cell signaling, determining the recovery of the myocardium. However, considerably more experimentation must be done in the field of ischemia/reperfusion–induced injury under *in vivo* conditions to establish the most useful cardioprotective ACTH fragment without any adrenocorticotropic side effects. Thus, future studies should determine (beside realization of the exact mechanism) the "core" of adrenocorticotropic hormones with most beneficial effects and fewest side effects regarding ischemia/reperfusion injury in different organs, because these drugs may open a new and modern therapeutic approach to ischemia/reperfusion-induced diseases.

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ABBREVIATIONS

ACTH, adrenocorticotropic hormone; AF, aortic flow; CF, coronary flow; ECG, electrocardiogram; HO, heme-oxygenase; HR, heart rate; HRP, horseradish peroxide; I/R, ischemia/reperfusion; IL, interleukin; LPS, lipopolysaccharide; LVDP, left ventricular developed pressure; MC1-R, melanocortin-1 receptor; MC3-R, melanocortin-3 receptor; NIH, National Institutes of Health; POMC, proopiomelanocortin; SDS, sodium dodecylsulfate; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α; TRITC, tetrarhodamine isothiocyanate; TTC, triphenyl tetrazolium; ttkg, total tissue kilogram; VF, ventricular fibrillation; VT, ventricular tachycardia; α-MSH, α-melanocyte–stimulating hormone.

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