Late preconditioning against stunning is not mediated by increased antioxidant defenses in conscious pigs

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Tang, Xian-Liang, Yumin Qiu, Julio F. Turrens, Jian-Zhong Sun, and Roberto Bolli. Late preconditioning against stunning is not mediated by increased antioxidant defenses in conscious pigs. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H1651–H1657, 1997.—Previous studies in conscious pigs have demonstrated that a sequence of ten 2-min coronary occlusion/2-min reperfusion cycles renders the heart relatively resistant to myocardial stunning 24 h later [late preconditioning (PC) against stunning] by an unknown mechanism. Since oxygen radicals contribute importantly to myocardial stunning and since antioxidant enzymes have been reported to be upregulated 24 h after PC in dogs and rabbits, we tested the hypothesis that late PC against stunning is related to an increase in endogenous antioxidant defenses. Chronically instrumented conscious pigs underwent a sequence of ten 2-min coronary occlusion/2-min reperfusion cycles (preconditioned group, $n = 11$) or received no intervention (control group, $n = 5$). Twenty-four hours later, pigs were killed and the myocardial levels of Mn superoxide dismutase (SOD), Cu-Zn SOD, catalase, glutathione (GSH) peroxidase, GSH reductase, GSH, GSH disulfide, α-tocopherol, and ascorbate were measured. There were no differences in any of the enzymatic or nonenzymatic antioxidants between the ischemic and nonischemic regions in the preconditioned group or between the control and the preconditioned group. Thus, when a marked protection against stunning was present (24 h after PC), no alteration in antioxidant defenses was observed. These results indicate that, in conscious pigs, late PC against myocardial stunning is not mediated by increased endogenous antioxidant defenses, thereby refuting one of the major current hypotheses regarding this phenomenon.

superoxide dismutase; catalase; glutathione peroxidase; glutathione reductase; tocopherol; ascorbate

PREVIOUS STUDIES in conscious pigs (38) have demonstrated that a sequence of ten 2-min coronary occlusion/2-min reperfusion cycles induces severe myocardial stunning, but when the same sequence is repeated 24 h later, the severity of stunning is markedly reduced [late preconditioning (PC) against stunning]. More recent studies (3, 24) have demonstrated a similar phenomenon in conscious rabbits. In both conscious pigs and rabbits, a protective effect is consistently observed and is of substantial magnitude in every animal, resulting in a 50–60% decrease in the overall severity of myocardial stunning (3, 24, 38).

The mechanisms of late PC against stunning remain largely unknown. The fact that this phenomenon has a delayed onset (requiring >6 h to develop), lasts for at least 72 h, and disappears within 6 days after the PC ischemia (40) seems to be consistent with the time course of synthesis and degradation of cytoprotective protein(s). The two classes of proteins that have been proposed as the most likely candidates as mediators of protection are heat stress proteins (HSPs) and antioxidant enzymes. Although HSP 70 is upregulated 24 h after the PC ischemia in our conscious pig model (38), this association does not prove causality and may represent an epiphenomenon based on recent findings in rabbit models of late PC (15). A potential role of increased antioxidant defenses in late PC is supported by the notion that reactive oxygen species (ROS) contribute importantly to the pathogenesis of myocardial stunning (2, 4, 21, 41). Furthermore, late PC against stunning in this conscious pig model is completely abolished when animals are treated with antioxidants during the first sequence of coronary occlusion-reperfusion cycles, indicating that the development of the cardioprotective response is triggered by the oxidative stress associated with the first ischemia-reperfusion challenge (39). There is considerable evidence that exposure to an oxidative stress can induce antioxidant enzymes, such as catalase and Mn superoxide dismutase (SOD), in a variety of systems (6, 10, 14, 19, 31, 33, 34, 36, 37, 44, 46) and that increased expression of Mn SOD can protect against oxidant injury (43). In the setting of myocardial ischemia and reperfusion, previous studies have reported that a sequence of four 5-min coronary occlusions results (24 h later) in increased myocardial activity of Mn SOD in dogs (18) and of Mn SOD and Cu-Zn SOD in rabbits (15), concomitant with increased resistance against cell death (late PC against infarction) (20, 25). An increase in Mn SOD levels and in resistance to anoxia has also been reported in isolated rat myocytes preconditioned 24 h earlier with two 5-min periods of anoxia followed by reoxygenation (48). A possible role of antioxidant defenses in the pathogenesis of late PC is further supported by the observation that pretreatment of rats with endotoxin (7) or interleukin-1 (which are known to increase ROS generation) (8) results in increased resistance to ischemia-reperfusion injury 24–36 h later and that this tolerance is associated with increased myocardial activity of catalase or glucose 6-phosphate (G-6-*P*) dehydrogenase (8). Because of these facts, it seems plausible to speculate that late PC against stunning is mediated by an increase in endogenous antioxidant defenses triggered by the initial oxidative stress incurred on *day 1*, which would result in a lesser oxidative stress (and consequently lesser ventricular dysfunction) on *day 2*.

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The aim of the present study was to test this hypothesis using the same conscious pig model in which we have previously demonstrated late PC against stunning. We measured the myocardial levels of all major enzymatic and nonenzymatic antioxidants 24 h after ischemic PC. The conscious pig model was selected to avoid the confounding factors associated with open-chest preparations (21, 41) and also to correlate the levels of antioxidants with the presence of functional protection (38). To provide a comprehensive assessment of the effect of ischemic PC on antioxidant defenses, the analysis was extended to include Mn SOD, Cu-Zn SOD, catalase, glutathione (GSH) peroxidase, GSH reductase, GSH, GSH disulfide (GSSG), α-tocopherol, and ascorbate.

METHODS

A total of 18 pigs was used for this study. The experimental preparation and techniques have been previously described in detail (38–40). The study was performed in accordance with the guidelines of the Committee on Animals of Baylor College of Medicine and with the *Guide for the Care and Use of Laboratory Animals*.

Experimental preparation. Domestic pigs of either sex (weight at surgery, 27 ± 2 kg) were premedicated with ketamine hydrochloride (20 mg/kg im) and atropine (0.04 mg/kg im). Twenty to thirty minutes later, anesthesia was induced with methohexital sodium (7.5 mg/kg iv), after which the animals were intubated and anesthesia was maintained with 0.5–1.0% methoxyflurane. A left thoracotomy was performed under sterile conditions at the level of the fifth intercostal space. Tygon catheters were placed in the left atrium and femoral artery. A hydraulic occluder and a Doppler flow velocity probe were implanted around the middle left anterior descending coronary artery (LAD). A 10-MHz pulsed Doppler ultrasonic crystal was sutured to the epicardial surface in the center of the region to be rendered ischemic. Two insulated copper wires were sutured to the right ventricle to record the electrocardiogram. All wires and catheters were tunneled under the skin and exteriorized through small incisions on the back. The chest was closed in layers, and a small tube was left in the thorax to drain air and fluid postoperatively. Antibiotics were administered intravenously before surgery and daily for 7 days thereafter (cefazolin, 30 mg/kg twice a day, and gentamicin, 0.7 mg/kg twice/day). Arterial blood gases, hematocrit, rectal temperature, and heart rate were measured daily after instrumentation to ensure that the animals had fully recovered from the surgical procedure. The catheters were flushed daily until the end of the protocol. All pigs were allowed to recover for a minimum of 10 days after surgery.

Experimental protocol. Conscious pigs were studied while lying quietly in a cage in a quiet, dimly lit room. Aortic and left atrial pressures, LAD blood flow velocity, systolic wall thickening, and the electrocardiogram were monitored simultaneously on an eight-channel, direct-writing oscillograph (Gould Brush System 200).

Pigs were assigned to a control or a preconditioned group. Control pigs were not subjected to coronary artery occlusion-reperfusion. In the preconditioned group, pigs underwent a sequence of ten 2-min LAD occlusion/2-min reperfusion cycles, which were performed by inflation or deflation of the hydraulic balloon occluder. This protocol has been previously shown (38–40) to induce a consistent and powerful protection against myocardial stunning 24 h later in this animal preparation. Complete coronary occlusion was documented by the LAD blood flow velocity decreasing to zero and by the appearance of dyskinesia in the wall-thickening tracings. To measure regional myocardial blood flow, radioactive microspheres were injected, as previously described (5), at 30–60 s into the fifth LAD occlusion.

Tissue preparation. Twenty-four hours later, the pigs were anesthetized with pentobarbital sodium (35 mg/kg iv), intubated, and ventilated with a positive-pressure respirator. The heart of each pig was exposed through a left thoracotomy and freed of adhesions. Pigs were then given heparin (5,000 U iv) and killed with a bolus of saturated KCl solution. The heart of each pig was excised immediately after death, and the aorta was perfused with ice-cold normal saline for 2 min at 100 mmHg to wash out all intravascular blood. Transmural samples (1–2 g) were rapidly removed from the center of the ischemic-reperfused region injury [anterior left ventricular (LV) wall] and from the nonischemic region (posterior LV wall), snap frozen, and stored in liquid nitrogen until use. The center of the ischemic-reperfused region was identified on the basis of the coronary anatomy.

To measure myocardial levels of SOD, catalase, GSH peroxidase, GSH reductase, GSH, and GSSG, the frozen tissue samples were homogenized on ice with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 4 vol 50 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. The homogenates were then ultracentrifuged at 150,000 *g*, and the supernatant was stored in aliquots at −70°C for measurement of antioxidant levels.

To measure myocardial content of α-tocopherol and ascorbate, the frozen tissue samples were ground to a fine powder with a mortar and pestle that was kept on dry ice. The ground tissue powder was homogenized in chelexed water using a hand-held tissue shredder at 4°C. To determine α-tocopherol, an internal standard tocopherol acetate was added before homogenization, and the homogenates were transferred to an extraction tube. The extraction procedure was an adaptation of the method of Burton et al. (9). To determine ascorbate, the homogenates were immediately treated with 0.65 ml of 6% trichloroacetic acid and centrifuged at 8,000 *g* for 5 min, and the supernatant was collected for ascorbate assays.

The sediment of the homogenate was saved for counting the radioactivity of the samples. Care was taken to ensure that the samples did not contain an admixture of ischemic and reperfused and nonischemic myocardium. Accordingly, the ratio of myocardial blood flow to the ischemic region during occlusion to simultaneous blood flow to the nonischemic region was estimated in each sample. Radioactive counts per minute per gram of tissue were measured in the samples from the two regions, and the counts per minute per gram for the nonischemic samples were averaged. Only samples of ischemic-reperfused tissue in which the counts per minute per gram were <10% of the average in the corresponding nonischemic samples were used for this study. This ensured that the samples of ischemic-reperfused myocardium assayed for antioxidants consisted of severely ischemic tissue (<10% of nonischemic flow).

Biochemical assays. Assays of SOD, catalase, GSH peroxidase, GSH reductase, GSH, and GSSG were carried out in a DU-65 Beckman spectrophotometer. Protein concentration was measured with the Folin phenol reagent, following the procedure described by Lowry et al. (22). Catalase activity was measured with the rate of H_2O_2 decomposition at 240 nm ($\epsilon = 40\ M^{-1} \cdot cm^{-1}$). Because this reaction follows a pseudo-first-order kinetic, an arbitrary unit of catalase was defined as the amount of enzyme that decomposes 1 μmol H_2O_2/min between 10.5 and 9.5 mM (1, 42). The assay of SOD activity

Table 1. *Enzymatic and nonenzymatic antioxidants in control and preconditioned groups*

Antioxidant	Control		Preconditioned	
	Anterior wall	Posterior wall	Ischemic region	Nonischemic region
Total protein, mg/g tissue	32.7 ± 4.0	33.1 ± 3.0	28.5 ± 2.1	30.2 ± 1.8
Total SOD, U/mg protein	9.4 ± 1.3	8.9 ± 0.7	9.1 ± 0.7	8.5 ± 0.7
Mn SOD, U/mg protein	5.6 ± 0.9	5.5 ± 0.8	5.4 ± 0.5	5.6 ± 0.5
Cu-Zn SOD, U/mg protein	3.8 ± 1.0	3.4 ± 0.5	3.3 ± 0.3	3.1 ± 0.3
Catalase, U/mg protein	30.1 ± 5.4	29.2 ± 4.3	29.9 ± 3.5	28.2 ± 2.6
GSH peroxidase, mU/mg protein	21.3 ± 3.9	19.3 ± 4.6	21.9 ± 3.9	19.0 ± 3.1
GSH reductase, mU/mg protein	16.3 ± 1.8*	16.2 ± 1.7*	12.4 ± 0.8	12.5 ± 0.6
GSH, nmol/mg protein	27.1 ± 1.8	28.2 ± 1.5	25.4 ± 1.1	25.5 ± 1.1
GSSG, nmol/mg protein	1.5 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
GSSG-to-GSH ratio	0.056 ± 0.002	0.052 ± 0.002	0.047 ± 0.002	0.054 ± 0.004
α-Tocopherol, μg/g tissue	9.9 ± 2.5	7.1 ± 1.2	9.4 ± 0.6	8.3 ± 0.4
Ascorbate, μg/g tissue	26.2 ± 12.5	21.0 ± 10.5	24.3 ± 5.3	18.5 ± 4.4

Data are means ± SE. SOD, superoxide dismutase; GSH, glutathione; GSSG, GSH disulfide. *The activity of GSH reductase tended to be higher in control pigs ($n = 5$) compared with preconditioned pigs ($n = 11$), but the difference was not statistically significant; this difference was caused by 1 control pig having high levels of GSH reductase (23.2 mU/mg protein in the anterior wall and 22.5 mU/mg protein in the posterior wall).

was carried out in 50 mM potassium phosphate and 0.1 mM EDTA (pH 7.8) at 550 nm. One unit of SOD was determined as the amount of enzyme that inhibited by 50% the rate of 20 μM cytochrome *c* reduction by 0.5 mM xanthine and xanthine oxidase (initial rate, 0.025 absorbance U/min) (29, 42). Sodium azide (0.2 mM) was added to the system to suppress cytochrome-*c* oxidase activity. To determine only Mn SOD activity, 1 mM sodium cyanide was used to inhibit Cu-Zn SOD activity. Cu-Zn SOD activity was determined by subtracting Mn SOD activity from the total activity (42). Glutathione peroxidase was measured after the rate of NADPH oxidation at 340 nm ($\epsilon = 6.22\ mM^{-1} \cdot cm^{-1}$) in a buffer containing 1 mM reduced GSH, 5 U/ml of glutathione reductase, 1 mM sodium azide, 0.2 mM NADPH, 1 mM EDTA, and 50 mM potassium phosphate (pH 7.0) incubated for 5 min at room temperature. The reaction was started by adding 0.1 mM *t*-butyl hydroperoxide (13). A unit was defined as the amount of sample that oxidized 1 μmol of NADPH/min (42). GSH reductase was determined by monitoring the oxidation of NADPH at 340 nm, 37°C, in a system containing (in mM) 50 tris(hydroxymethyl)aminomethane (Tris) and 0.25 EDTA (pH 8.0), 3.3 GSSG, and 0.1 NADPH. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH/min (42). For measuring myocardial concentration of GSH and GSSG, 1 ml of homogenate was denatured by dilution with 3 ml of perchloric acid to a final concentration of 1 M acid, 2 mM EDTA. The samples were centrifuged and neutralized with KOH. GSH and GSSG were measured by use of *o*-phthalaldehyde as a fluorescent reagent. The method takes advantage of the reaction of GSH with *o*-phthalaldehyde at pH 8.0 and of GSSG with *o*-phthalaldehyde at pH 12.0; GSH can be complexed to *N*-ethylmaleimide to prevent interference of GSH with the measurement of GSSG (17).

Myocardial levels of α-tocopherol were determined by first extracting tissue homogenates with sodium dodecyl sulfate, ethanol, and *n*-heptane (9). Before analysis, the heptane was removed under a stream of nitrogen and the samples were dissolved in methanol. The extracts were then analyzed by high-performance liquid chromatography using a Beckman pump equipped with a C_{18} reverse-phase column (250 × 4.6 mm) and a guard column. α-Tocopherol was eluted with 98% methanol and detected by a Gilson dual-wavelength spectrophotometer at 294 and 270 nm and 0.01 and 0.005 absorbant unit full scale, respectively. Data were collected and integrated using Hewlett-Packard ChemStation.

Myocardial levels of ascorbate were assayed by the Roe and Kuether (35) method, which involves forming the hydrozone derivatives of oxidized ascorbic acid. The sample was totally oxidized by incubation with Norit charcoal for 15 min at room temperature, which converted the ascorbic acid to the dehydroascorbic acid. The sample was then centrifuged at 11,500 *g* for 5 min. We added 10 μl of 10% thiourea and 80 μl of 2% 2,4-dinitrophenylhydrazine to the 0.25 ml of supernatant. The sample was incubated at 57°C for 45 min. Color was then produced by adding 85% sulfuric acid. Absorbance was determined using a spectrophotometer set at 520 nm.

Statistical analysis. All data are reported as means ± SE. Antioxidant levels were analyzed by a two-way analysis of variance (ANOVA; group and region) followed by unpaired or paired Student's *t*-tests, as appropriate. All statistical analyses were performed with Statistical Analysis System software. Two-way ANOVA was performed using the procedure General Linear Models (GLM). Statistical significance was defined as $P < 0.05$.

RESULTS

Of the 18 pigs entered into the study, 5 were assigned to the control group and 13 to the preconditioned group. Two of the thirteen pigs in the preconditioned group were excluded because of ventricular fibrillation during reperfusion. Therefore, totals of 5 and 11 pigs were used for the assays in the control and preconditioned groups, respectively.

In the preconditioned group, the performance of complete LAD occlusion during the 10 coronary occlusion-reperfusion cycles was documented by the Doppler flow velocity probe and wall-thickening crystal recordings. The measurements of radioactivity in the sediment of the homogenates were 18 ± 8 counts/min (cpm)/g in the samples from the ischemic-reperfused region and 1,009 ± 358 cpm/g in the samples from the nonischemic region. The blood flow ratio between the two regions (1.8 ± 0.9%) confirms that blood flow in the samples of the ischemic-reperfused region was virtually zero during coronary occlusion.

The results are summarized in Table 1. Total proteins in the supernatant were 32.7 ± 4.0 and 33.1 ± 3.0 mg/g

of tissue in the anterior and posterior walls, respectively, in the control group (*n* = 5) and 28.5 ± 2.1 and 30.2 ± 1.8 mg/g of tissue in the ischemic and nonischemic regions, respectively, in the preconditioned group (*n* = 11). There were no statistical differences between the two regions of each group or between the two groups.

As shown in Table 1, there were no appreciable differences in total SOD activity, Mn SOD activity, or Cu-Zn SOD activity between the anterior and posterior walls of the LV or between the ischemic and nonischemic regions in preconditioned pigs. The activity of catalase and GSH peroxidase were also similar between the anterior and posterior walls in control pigs and between the ischemic and nonischemic regions in preconditioned pigs. The activity of GSH reductase tended to be lower in preconditioned pigs compared with that in controls, but the difference was not statistically significant; this difference was due to one pig having high levels of GSH reductase in the control group (Table 1 *legend*). The activity of GSH reductase was virtually identical between the two ventricular regions within each group (Table 1).

There were no appreciable differences between the two groups or between the two LV regions within the same group with respect to the myocardial concentration of GSH and GSSG, the GSSG-to-GSH ratio, the myocardial concentration of α-tocopherol, or the myocardial concentration of ascorbate (Table 1).

DISCUSSION

The goal of the present study was to determine whether late PC against myocardial stunning in conscious pigs is associated with increased antioxidant defenses. We measured the myocardial levels of all major enzymatic (Cu-Zn SOD, Mn SOD, catalase, GSH peroxidase, and GSH reductase) and nonenzymatic (GSH, α-tocopherol, and ascorbate) antioxidants at 24 h after ischemia, when the protective effects of PC against stunning are maximal (40). The results show that none of the antioxidants examined were increased in the ischemic-reperfused myocardium. This indicates that late PC against stunning in conscious pigs is not mediated by an increase in antioxidant defenses, thereby refuting one of the major current hypotheses regarding the pathogenesis of this phenomenon. Although previous studies have examined changes in antioxidant enzymes late after ischemia or hypoxia in isolated cardiomyocytes (47, 48) or in anesthetized open-chest animals (15, 18), to our knowledge this is the first study to assess the effect of ischemia on antioxidant levels in a conscious animal preparation.

Methodological considerations. We utilized a conscious porcine model in which PC is induced with a sequence of ten 2-min coronary occlusions, because this is the preparation in which the phenomenon of late PC against myocardial stunning has been previously described (38) and characterized (39, 40). The cardioprotective effects observed in this model are powerful and very consistent, with virtually every pig exhibiting decreased severity of myocardial stunning 24 h after PC (38–40). Because previous studies (40) have documented that cardioprotection is fully developed at this time, we elected to measure antioxidant levels at 24 h after PC. To avoid any possible contamination of myocardial antioxidant enzymes with circulating red blood cell enzymes, the heart was thoroughly perfused with ice-cold saline for 2 min before we obtained the tissue samples. Even if sporadic red blood cells were still present in the tissue, their impact on our measurements should have been similar in ischemic and nonischemic samples. Particular care was taken to avoid contamination of ischemic samples with nonischemic tissue. Only samples with a blood flow <10% of simultaneous nonischemic flow during coronary occlusion were utilized for this study (see RESULTS).

No previous study has assessed the effect of ischemic PC on the myocardial content of antioxidants in a conscious animal preparation. We reasoned that the abnormal conditions associated with anesthetized open-chest models [e.g., surgical trauma, exaggerated ROS generation after ischemia and reperfusion (21), elevated catecholamine levels, fluctuations in body temperature (41), inflammatory reaction to thoracotomy with possible release of cytokines] could affect the response of antioxidant enzymes to ischemia and reperfusion. For example, Hoshida et al. (18) found that in dogs subjected to thoracotomy, the myocardial content of Mn SOD increased spontaneously in the nonischemic myocardium during the first 24 h of recovery after surgery, presumably as a result of surgery-induced release of cytokines. The use of a conscious animal model obviates any potentially confounding influence of the factors associated with open-chest preparations. Furthermore, the use of a conscious model enabled us to directly correlate the functional protection observed 24 h after ischemic PC (38–40) with the changes (or lack thereof) in antioxidant defenses.

Previous studies of the effect of early PC on antioxidants. The data regarding the effect of the early phase of PC on antioxidant enzymes are conflicting. Using open-chest rabbits preconditioned with a 5-min coronary occlusion followed by 10 min of reperfusion and then subjected to a 30-min occlusion, Turrens et al. (42) found no change in the activity of Cu-Zn SOD, Mn SOD, catalase, GSH peroxidase, GSH reductase, or total glutathione at 5 min after the 30-min occlusion. On the other hand, using open-chest dogs preconditioned with four 5-min coronary occlusion/10-min reperfusion cycles, Hoshida et al. (18) reported that the activity of Mn SOD was significantly increased 5 min after PC (26% in the subendocardium and 36% in the subepicardium), although the content of Mn SOD protein declined (−17% in the subendocardium and −5% in the subepicardium); this increase in Mn SOD activity was accompanied by a small (4–7%) but statistically significant increase in GSH peroxidase activity and a decrease in GSH reductase activity (10–14%). These changes disappeared by 3 h after PC. The reason for the discrepancy between these results and those of Turrens et al. (42) is unknown. Das et al. (12) found an increase in the number of antioxidant enzymes, including Mn SOD, catalase, GSH peroxidase, and GSH reductase at 6 h of

reperfusion after a 1-h coronary occlusion in porcine hearts that were preconditioned with four 5-min coronary occlusions interspersed with 10 min of reperfusion compared with hearts that were not preconditioned. Because of the intervening 60 min of coronary occlusion after PC, these results (12) cannot be compared with those of Turrens et al. (42). In a subsequent study in isolated, buffer-perfused rat hearts, Das et al. (11) observed that a sequence of 4 cycles of 5-min ischemia/10-min reperfusion was associated with induction of catalase and Mn SOD mRNAs and resulted in an increase in the activity of Mn SOD, peroxisomal catalase, and GSH peroxidase 60 min after the PC protocol. It is not known whether the difference between these results and those of Turrens et al. (42) reflects differences in species, models, or the timing of antioxidant measurement.

Previous studies of the effect of late PC on antioxidants. To date, only one investigation (18) has examined the effects of ischemia on myocardial antioxidants 24 h later. In the aforementioned study by Hoshida et al. (18), in which open-chest dogs were preconditioned with a sequence of four 5-min coronary occlusion/10-min reperfusion cycles, at 24 h after PC there was an increase in Mn SOD activity (29 and 24% in the subendocardium and subepicardium, respectively) and protein (~40 and 10% in the subendocardium and subepicardium, respectively), with no significant changes in Cu-Zn SOD, GSH peroxidase, or GSH reductase activities. The reason for the apparent discrepancy between these findings and our present results is unknown. The divergent results may be secondary to differences in species (dogs vs. pigs), experimental preparations (open-chest vs. conscious animals), or PC protocols (four 5-min occlusions vs. ten 2-min occlusions). It should be noted that in the Hoshida et al. (18) study, both the activity and the protein content of Mn SOD increased gradually for 24 h after PC even in the nonischemic myocardium (~17% in the subendocardium and 24% in the subepicardium), a finding that was not observed in our present study. This spontaneous increase in Mn SOD, which may reflect the effects of surgical trauma, consequent inflammation, and concomitant cytokine release, complicates the assessment of the specific effects that ischemia per se may exert on antioxidant enzymes. In recent preliminary communications by Heads et al. (15, 16), an increase in the activities of both Cu-Zn SOD and Mn SOD, without changes in protein content, was reported in open-chest rabbits preconditioned 24 h earlier with four 5-min occlusions. The difference between these findings and our present results may be due to the different PC protocols, to the aforementioned effects of the open-chest preparation, or to the method used by Heads et al. (15, 16) to measure SOD activity (in-gel zymography).

Besides in vivo ischemia, a number of manipulations have been reported to induce delayed modulation of antioxidant enzymes. Using a model of late PC in neonatal rat myocytes subjected to hypoxia and reoxygenation, Yamashita et al. (47) found evidence that the increased resistance to hypoxic injury 24 h after PC was mediated by induction of Mn SOD. In a model of PC in isolated adult rat myocytes, Zhou et al. (48) reported that the activity of Mn SOD was augmented both early (2 h) and late (24 h) after exposure to two brief (5 min) bouts of anoxia; this increase was associated with augmented resistance to a 60-min anoxic insult. An increase in the activity of antioxidant enzymes (Mn SOD, Cu-Zn SOD, catalase, GSH peroxidase, G-6-*P* dehydrogenase) has also been reported at 24–72 h after pharmacological PC with interleukin-1 (27) and endotoxin (28) in rats and with amphetamine in pigs (26). It is not possible to directly compare the effects of ischemic PC and of pharmacological agents on antioxidant enzymes. Furthermore, the differences between the experimental models used in these studies [cultured cardiomyocytes (47, 48), isolated rat hearts (27, 28), or isolated pig hearts subjected to 60 min of LAD occlusion and cardioplegic arrest (26)] and the model used in the present study (conscious pigs) preclude comparisons of the results. The effects of oxidative stress in isolated cell models have been conflicting. For example, Lu et al. (23) found that exposure to H_2O_2 induced an increase in the activities of SOD, catalase, and GSH peroxidase 18 h later in bovine vascular endothelial cells. In contrast, Wiese et al. (45) recently reported that exposure of various mammalian cell lines to relatively low doses of H_2O_2 results in markedly increased resistance to an H_2O_2 challenge several hours later and that this adaptation to oxidative stress is not associated with any significant increase in the activity of classic antioxidant enzymes, such as Cu-Zn SOD, Mn SOD, catalase, or GSH peroxidase.

Antioxidant hypothesis. The hypothesis that late PC against myocardial stunning is mediated by increased antioxidant defenses has gained widespread support, because it is predicated on a number of pertinent considerations. First, there is convincing evidence that myocardial stunning is caused in part by the generation of ROS during reperfusion (2). Thus it would seem reasonable to hypothesize that protection against stunning could be due to increased antioxidant capacity. Second, as discussed above, previous studies in open-chest dogs (18) and rabbits (15, 16) have concluded that ischemic PC induces Mn SOD 24 h later, which is associated with increased resistance to myocardial infarction (20, 25). Third, oxidative stress is known to upregulate antioxidant enzymes in a variety of biological systems (6, 14, 33, 34, 36, 37, 44, 46), including the heart (10, 31). Because reperfusion after brief ischemia is associated with a burst of ROS generation (2, 4, 21), it seems plausible to speculate that this increased oxidative stress could lead to an increase in antioxidant defenses in the myocardium. Fourth, administration of endotoxin or interleukin-1 (which are thought to generate ROS) has been shown to result in upregulation of myocardial antioxidant enzymes (7, 8, 27, 28) concomitant with increased myocardial resistance to ischemia-reperfusion injury. Finally, the fact that late PC against stunning requires >6 h to develop and disappears between 3 and 6 days thereafter (40) strongly suggests that it is mediated by the synthesis of cardioprotective

proteins (such as antioxidant enzymes), which are then degraded over the next few days.

Our present results, however, clearly (and unexpectedly) demonstrate that late PC against stunning is not associated with increased antioxidant defenses. These results do not rule out the possibility that different PC protocols [such as the four 5-min occlusions used by Hoshida et al. (18)] may result in increased antioxidant defenses. Our data, however, clearly demonstrate that a PC protocol that induces powerful protection against stunning 24 h later is not associated with increased antioxidant defenses. This indicates that the mechanism for the protection against stunning does not involve changes in antioxidant levels. Accordingly, other mechanisms (e.g., the induction of other cardioprotective proteins) must be responsible for this phenomenon.

Our finding that antioxidant defenses are not upregulated during late PC does not contradict the notion that ROS mediates stunning for the following reasons: *1*) ROS are not the sole culprit in the genesis of myocardial stunning (2); *2*) because the generation of ROS after reperfusion is closely related to the severity of the antecedent ischemia (4), any cellular adaptation that alleviates the severity of ischemia would be expected to diminish the intensity of ROS generation after reperfusion and thereby alleviate ROS-induced damage (2, 4); and *3*) it seems plausible that ROS generation initiates a cascade of reactions that culminates in the damage of critical cellular targets (2). In theory, the cellular adaptations associated with late PC may protect these targets without interfering with the upstream ROS-initiated reactions.

Potential limitations. We cannot rule out the possibility that in this study a transient upregulation of antioxidant enzymes may have occurred soon after the PC stimulus and disappeared by 24 h. If this were the case, however, the time course of the increase in antioxidant defenses would be unrelated to the time course of the protection, since, in this model, late PC against stunning is only partially manifest at 12 h after the initial ischemic challenge and achieves full expression at 24 and 72 h (40). Thus an upregulation of antioxidant defenses that resolves by 24 h after the ischemic stimulus could not be construed as the mechanism of late PC against stunning.

The transmural distribution of antioxidant enzymes was not assessed in this study. In principle, it is possible that changes limited to the subendocardium (the most ischemic layer) may have been obfuscated by the dilutional effect produced by the transmural sampling. In the aforementioned study by Hoshida et al. (18) in dogs, however, the activity of Mn SOD increased both in the subendocardium (+29%) and in the subepicardium (+24%). Because the transmural gradient of collateral perfusion is less in the porcine heart compared with that in the canine heart, it seems unlikely that a selective upregulation of antioxidant enzymes would occur in the former but not in the latter. Furthermore, previous studies using transmural sampling in pigs (12, 26) were able to detect an increase in antioxidant enzymes.

It could be argued that an increase in antioxidant defenses is unlikely to play a role in late PC against myocardial stunning in the pig, because the porcine heart lacks xanthine oxidase (30, 32). This argument, however, is refuted by the fact that we did observe a marked attenuation of myocardial stunning with antioxidant therapy in conscious pigs subjected to the same coronary occlusion protocol employed in this study (ten 2-min coronary occlusions) (39), indicating that ROS do play an important role in the pathogenesis of myocardial stunning in the porcine heart and implying that sources other than xanthine oxidase are responsible for the formation of these species.

In conclusion, the present study demonstrates that, in the conscious pig, late PC against myocardial stunning is not associated with any appreciable increase in the major enzymatic and nonenzymatic antioxidant defenses. This finding, which is somewhat surprising and contrary to our original expectations, indicates that ischemic PC does not upregulate antioxidant enzymes and that the increased resistance to ischemia-reperfusion injury 24 h after PC in this model is mediated by other, as yet undefined, mechanisms unrelated to an increase in endogenous antioxidant defenses.

We thank Gemma Wallis for excellent technical assistance.

This study was supported in part by National Heart, Lung, and Blood Institute Grants R01 HL-43151 and R01 HL-55757 (to R. Bolli) and American Heart Association Kentucky Affiliate Grants KY 96-GB-31 (to X.-L. Tang) and KY 96-GB-32 (to Y. Qiu).

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Received 10 January 1997; accepted in final form 20 May 1997.

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