Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cardiomyocytes of rats

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Inserte, J., G. Taimor, B. Hofstaetter, D. Garcia-Dorado, and H. M. Piper. Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cardiomyocytes of rats. Am. J. Physiol. Heart Circ. Physiol. 278: H94–H99, 2000.—Oxidative stress may cause apoptosis of cardiomyocytes in ischemic-reperfused myocardium. We investigated whether ischemia-reperfusion modifies the susceptibility of cardiomyocyte induction of apoptosis by oxidative stress. Ischemia was simulated by incubating isolated cardiomyocytes from adult rats in an anoxic, glucose-free medium, pH 6.4, for 3 h. Annexin V-fluorescein isothiocyanate/propidium iodide staining and the detection of DNA laddering were used as apoptotic markers. H2O2 (7.5 μmol/l) induced apoptosis in 20.1 ± 1.8% of cells under normoxic conditions but only 14.4 ± 1.6% (n = 6, P < 0.05) after ischemia-reoxygenation. This partial protection of ischemic-reoxygenated cells was observed despite a reduction in their cellular glutathione content, from 11.4 ± 1.9 in normoxic controls to 2.9 ± 0.8 nmol/mg protein (n = 3, P < 0.05). Elevation of end-ischemic glutathione contents by pretreatment with 1 mmol/l N-acetylcysteine entirely protected ischemic-reoxygenated cells against induction of apoptosis by H2O2. In conclusion, ischemia-reperfusion can protect cardiomyocytes against induction of apoptosis by exogenous oxidative stress. This endogenous protective effect is most clearly demonstrated when control and postischemic cardiomyocytes are compared at similar glutathione levels.

simulated ischemia-reoxygenation; hydrogen peroxide; glutathione

Both necrosis and apoptosis have been shown to contribute to cell death induced by myocardial ischemia-reperfusion (9, 13). Apoptosis is a process of transcriptionally regulated, programmed cell death. The mechanisms by which ischemia-reperfusion may initiate programmed cell death are not well known. Recently, we have demonstrated that, in isolated adult cardiomyocytes from rats, conditions of simulated ischemia-reoxygenation cannot induce apoptosis (26). It seems therefore reasonable that exogenous factors in ischemic-reperfused myocardium might contribute to the induction of apoptosis. Reactive oxygen species (ROS) may play a role. ROS are indeed able to induce apoptosis in various cell types (16, 24, 25), and they are generated in excess in reperfused myocardium (3, 8, 18, 31). Cardiomyocytes represent only one of the potential sources of ROS. Sources exogenous to cardiomyocytes include neutrophils with a high capacity for producing ROS (2).

There are reasons to expect that ischemia can increase the susceptibility of cardiomyocytes to apoptosis induced by exogenous ROS. This is because ischemia reduces cellular levels of glutathione, superoxide dismutase, catalase, and other components of the cellular antioxidant defense (1, 7). It seems also possible, however, that ischemia disturbs the cellular metabolism in such a way that the transcriptionally regulated processes of programmed cell death are in fact impaired compared with normoxic control conditions.

The purpose of this study was to investigate experimentally whether ischemia influences the susceptibility of cardiomyocytes to apoptosis induced by exogenous ROS. Isolated rat cardiomyocytes were used as an experimental model because in this model the influence of nonmyocyte cells is excluded. The cells were exposed to H2O2, a generator of ROS, under normoxic control conditions and after simulated ischemia. The dependency of the apoptotic response on the cellular antioxidant defense was studied by variation of cellular glutathione contents. The results show that, in the absence of other external factors, the susceptibility of cardiomyocytes to apoptosis induced by exogenous oxidative stress is reduced after ischemic conditions.

**METHODS**

Cell isolation and short-term culture. Ventricular heart muscle cells were isolated from adult male Wistar rats (200–250 g) and plated in medium 199 (Sigma, St. Louis, MO) on 60-mm culture dishes preincubated overnight in medium 199 with 4% FCS (GIBCO), as described previously (20). Four hours after plating, the dishes were washed with a modified phosphate-free Tyrode medium (in mmol/l: 140 NaCl, 3.6 KCl, 1.2 MgSO4, 1 CaCl2, and 20 HEPES, pH 7.4). As a result of medium change, nonattached cells were removed, resulting in cultures of 85 ± 3% quiescent rod-shaped cells.

Simulated ischemia and reoxygenation. For simulated ischemia, dishes were filled with 1 ml of the modified Tyrode medium at pH 6.4, gassed with 100% N2, and incubated at 37°C in gas-tight chambers in an atmosphere of 100%N2 for 3 h. Reoxygenation was performed by addition of 1 ml of CCT medium (modified medium 199 including Earle’s salts, 5 mmol/l creatine, 2 mmol/l L-carnitine, 5 mmol/l taurine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mmol/l cytosine-β-D-arabinofuranoside, pH 7.4). Time-matched normoxic controls were obtained with the use of air-equilibrated, instead of N2-saturated, modified Tyrode medium at pH 7.4.

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Experimental protocol. Three sets of experiments were performed. In the first set of experiments, the induction of apoptosis by different concentrations of H$_2$O$_2$ under normoxic conditions versus simulated ischemia-reoxygenation was compared. In these experiments, the cardiomyocytes were exposed to 3 h of simulated ischemia or normoxia and then reoxygenated by adding 1 ml of CCT medium supplemented with 2% H$_2$O$_2$ at final concentrations of 0, 1, 5, 7.5, 10, or 15 µmol/l.

The second set of experiments was performed to test the effect of N-acetylcysteine (NAC) pretreatment on glutathione content. NAC is a sulfhydryl group donor that is easily transported into the cells where it is deacetylated and increases the thiol pool, primarily by reduction of glutathione, in a dose-dependent manner (5). NAC, at final concentration of 0.01, 0.1, or 1 mmol/l, was added during the 4-h incubation period in medium 199. Next, cardiomyocytes were washed, subjected to 3 h of either simulated ischemia or normoxia, and reoxygenated by adding 1 ml of CCT medium.

Finally, in the third set of experiments, the effect of NAC pretreatment on the induction of apoptosis by H$_2$O$_2$ was analyzed under normoxic conditions and after simulated ischemia-reoxygenation. In these experiments, dishes were preincubated for 4 h with medium 199 with or without NAC at final concentrations of 0.01, 0.1, or 1 mmol/l, exposed to either normoxia or simulated ischemia for 3 h, and reoxygenated by adding 1 ml of CCT medium with or without 10 µmol/l of H$_2$O$_2$.

Determination of creatine phosphate contents. Experiments were terminated by addition of 1 ml of HClO$_4$ (1.2 mol/l) to the contents of the culture dishes. After neutralization, perchloric acid extracts of cultures were analyzed for creatine phosphate (CrP; see Ref. 15).

Analysis of genomic DNA. Six hours after intervention, i.e., either 3 h of simulated ischemia or addition of H$_2$O$_2$, DNA was extracted as described by Tanaka et al. (27). In a previous study (26), this time interval has been shown to be sufficient for detection of DNA laddering in isolated cardiomyocytes. Briefly, cardiomyocytes were harvested by centrifugation at 2,800 g for 5 min. After resuspension in lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris·HCl, 25 mmol/l EDTA, 0.5% SDS, and 100 mmol proteinase K, pH 8.0), myocytes were incubated for 3 h at 37°C. After phenol/chloroform extraction and ethanol precipitation, DNA was dissolved in TE buffer (10 µg). DNA samples were electrophoretically separated on 1.5% agarose gels for detection of DNA laddering in isolated cardiomyocytes. For fluorescent staining, 10 µl of annexin V-FITC (Boehringer Ingelheim) and 1 ng propidium iodide were added to the culture medium (2 ml) 2 h after intervention, i.e., either 3 h of simulated ischemia or addition of H$_2$O$_2$. It was shown before that this time interval is sufficient for apoptosis detection in cardiomyocytes (26). Cultures were then incubated for 10 min at 37°C in the dark and then analyzed by fluorescence microscopy. For quantification of apoptosis and necrosis, ~300 randomly distributed cells per dish were counted. The small number of necrotic cells at time 0 was subtracted from all following counts.

Analysis of glutathione content. After 3 h of simulated ischemia, modified Tyrode medium was discarded. The culture dishes were washed two times with ice-cold potassium phosphate buffer (0.2 mol/l, pH 7.1) containing 2 mmol/l EDTA and were then taken up in 340 µl of this buffer. After homogenization in a glass potter tube with a glass pestle at 4°C, and sonication for 15 s at 30 W (4°C), the cellular homogenate was deproteinized by adding 60 µl of sulfosalicylic acid (30%). The homogenate was centrifuged (5 min, 3,000 g) at 4°C, the supernatant was used for assays of GSH and GSSG, and the pellet was used for protein determination (4).

Total glutathione content was quantified by using the glutathione reductase technique (11), which monitors the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) at 412 nm. Pure GSH dissolved in 4.5% sulfosalicylic acid solution was assayed for the purpose of calibration. Concentrations of 1, 2, 3, and 4 nmol GSH/cuvette were used. GSSG was assayed enzymatically after derivatization of GSH with 2-vinylpyridine. Pure GSSG dissolved in 4.5% of sulfosalicylic acid solution was assayed under identical conditions to ensure correct calibration. Concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 nmol GSSG/cuvette were used. GSH was calculated as total glutathione minus GSSG.

Protein analysis. Protein content was assayed in the acid precipitates with Coomassie brilliant blue according to the method of Bradford (4), using BSA as standard.

Statistics. Data are given as means ± SE from n different culture preparations. Comparisons involving multiple groups were performed by means of ANOVA. Statistical significance of difference between groups was assessed by Student-Newman-Keuls test. A critical P value of 0.05 was used for all tests; values above P = 0.05 were regarded as not significant (NS).

RESULTS

High-energy phosphates during simulated ischemia and reoxygenation. Under normoxic conditions, cardiomyocytes contained 20.1 ± 2.6 nmol CrP/mg protein. After 3 h of simulated ischemia, the content of CrP was reduced to 9.7 ± 3.4 nmol/mg protein (n = 3, P < 0.05). After 1 h of reoxygenation, cells had completely recovered their CrP (19.4 ± 5.1 nmol/mg protein). Pretreatment of cardiomyocytes with 1 mmol/l NAC did not alter cellular contents of CrP either under normoxic conditions or after 3 h of simulated ischemia (21.1 ± 7.6 and 9.7 ± 3.3 nmol/mg protein, respectively, P = NS with respect to corresponding values in nontreated cells).

Induction of apoptosis by oxidative stress under normoxia and simulated ischemia-reoxygenation. H$_2$O$_2$ induced apoptosis in a dose-dependent manner in cardiomyocytes under normoxic control conditions and after simulated ischemia-reoxygenation (Fig. 1A). The basal number of apoptotic cells in the absence of H$_2$O$_2$ was the same in both experimental groups (7.2 ± 1.4%...
results obtained with annexin V-FITC/propidium iodide staining (Fig. 1B). The intensity of the ladders increased progressively with increasing concentrations of H$_2$O$_2$. In the normoxic group, DNA laddering could be seen after exposure to 7.5 µmol/l H$_2$O$_2$, whereas in the group with simulated ischemia-reoxygenation DNA laddering was visible only at higher concentrations of H$_2$O$_2$ (10 µmol/l).

**Effect of NAC pretreatment on glutathione levels.** Three hours of simulated ischemia produced a 3.9-fold decrease of total glutathione contents compared with normoxic controls (11.4 ± 1.1 and 2.9 ± 0.4 nmol/mg protein, respectively, n = 3, P < 0.05; Fig. 2A). The ratio of GSH to GSSG, which is indicative of the redox state of the cells, also decreased during simulated ischemia from 17.6 ± 3.5 to 4.3 ± 0.9 (n = 3, P < 0.05; Fig. 2B). Pretreatment with NAC of cardiomyocytes subjected to 3 h of simulated ischemia produced a dose-dependent increase of the end-ischemic glutathione levels and of the ratio of GSH to GSSG. Pretreatment at a concentration of 1 mmol/l attenuated the drop below the normoxic control level of cellular glutathione contents after 3 h of simulated ischemia (8.4 ± 0.8, n = 3, NS). Normoxic cells pretreated with 1 mmol/l NAC did not significantly increase their glutathione content (Fig. 2A).

**Effect of NAC pretreatment on apoptosis induced by oxidative stress.** When normoxic cells pretreated with 1 mmol/l NAC were subjected to 10 µmol/l H$_2$O$_2$, the number of cells becoming apoptotic was not different compared with the absence of NAC pretreatment (18.8 ± 1.5 and 18.7 ± 1.4%, respectively, n = 6, NS). In contrast to this behavior of normoxic cells, cardiomyocytes exposed first to simulated ischemia and then to H$_2$O$_2$ were less prone to develop apoptosis when pretreated with NAC (Fig. 3A). The effect of NAC pretreatment was dose dependent. The attenuation of H$_2$O$_2$-induced apoptosis became significant in cells pretreated with 0.01 mmol/l NAC (18.2 ± 0.5 versus 20.95 ± 1.9% apoptotic cells, n = 6, P < 0.05). After pretreatment with 1 mmol/l NAC, ischemic-reoxygenated cardiomyocytes were entirely unresponsive to apoptosis induction with 10 µmol/l H$_2$O$_2$ (9.0 ± 0.3% apoptotic cells compared with 9.1 ± 0.7% among pretreated ischemic-reoxygenated cells without exposure to H$_2$O$_2$, n = 6, NS).

The percentage of necrotic cardiomyocytes in normoxic preparations was 7.3 ± 2.1%, without differences between groups. After simulated ischemia-reoxygenation, the percentage of necrotic cells increased significantly to 36.4 ± 3.6% (P < 0.05), without significant influence of NAC or H$_2$O$_2$ treatment (Fig. 3A).

Comparable results were obtained by DNA agarose electrophoresis (Fig. 3B). DNA ladders were clearly visible in the normoxic groups subjected to 10 µmol/l H$_2$O$_2$, with or without 1 mmol/l NAC pretreatment. No DNA laddering was observed when cardiomyocytes were treated with 1 mmol/l or 100 µmol/l NAC before being subjected to simulated ischemia and reoxygenation in the presence of 10 µmol/l H$_2$O$_2$.
The central question of the present study was whether the susceptibility of cardiomyocytes to apoptosis induced by exogenous oxidative stress would be changed after exposure to simulated ischemia-reoxygenation. The main finding is that cardiomyocytes are less susceptible to the induction of apoptosis by H$_2$O$_2$ after simulated ischemia-reoxygenation. Full protection against H$_2$O$_2$-induced apoptosis is achieved if the ischemic loss of glutathione is prevented.

It has been demonstrated by studies from our laboratory and other laboratories that fundamental aspects of ischemia-reperfusion injury can be simulated in an isolated cardiomyocyte model and that this model allows causal analysis in greater depth than possible in intact tissue (12, 14, 22, 23, 28). We showed previously that, for isolated cardiomyocytes devoid of the influences by the surrounding tissue, conditions of simulated ischemia (up to 18 h) and reoxygenation (up to 12 h) were not sufficient to induce apoptosis (26). Only the progressive development of necrosis was observed. The lack of development of apoptosis in cardiomyocytes in response to simulated ischemia and reoxygenation is in contrast to the ability of exogenous factors to induce apoptosis in these cells.

In the present study, the prooxidant H$_2$O$_2$ was used at low concentrations, which caused apoptosis but not necrosis of cardiomyocytes. To determine apoptosis in the cell population, the cells were stained with annexin V-FITC/propidium iodide staining 2 h after H$_2$O$_2$ addition or after reoxygenation, given in percentages of all cells. Data are means ± SE of 6 independent preparations. *Differences from nonpretreated normoxic control with $P < 0.05$.

**DISCUSSION**

**Fig. 2.** Glutathione contents after simulated ischemia. A: total glutathione contents; B: ratio of GSH to GSSG. Cardiomyocytes were preincubated with 0, 0.01, 0.1, and 1 mmol/l N-acetylcysteine (NAC) for 4 h. Preincubation was followed either by normoxia or by 3 h of simulated ischemia (Anoxia). Thereafter, glutathione contents were determined. Data are means ± SE of 3 independent preparations. *Differences from nonpretreated normoxic cells with $P < 0.05$.

**Fig. 3.** Apoptosis and necrosis in normoxic and anoxic/reoxygenated cardiomyocytes after adjustment of cellular glutathione contents. Cardiomyocytes were preincubated with 0, 0.01, 0.1, and 1 mmol/l NAC for 4 h. Preincubation was followed by normoxia or 3 h of simulated ischemia. Thereafter, 10 µmol/l H$_2$O$_2$ were added to the culture medium of normoxic cells (Normoxia) and of cells reoxygenated after 3 h of simulated ischemia (Anoxia-Reoxygenation). A: quantification of apoptotic and necrotic cells by annexin V-FITC/propidium iodide staining 2 h after H$_2$O$_2$ addition or after reoxygenation, given in percentages of all cells. Data are means ± SE of 6 independent preparations. *Differences from nonpretreated normoxic control with $P < 0.05$. B: DNA laddering. DNA was extracted 6 h after H$_2$O$_2$ addition or after 6 h of reoxygenation. Five micrograms per lane were separated on 1.5% agarose gels. Marker (M) was λ-DNA, Hind III digested. DNA was stained by ethidium bromide.
V-FITC/propidium iodide. As an additional parameter for apoptosis, internucleosomal DNA cleavage was assayed by the DNA-laddering phenomenon after gel electrophoretic separation of DNA. In agreement with our previous experience, both methods gave basically the same results.

During simulated ischemia-reoxygenation, cardiomyocytes lost more than one-half of their glutathione contents, and in the remaining pool the ratio of GSH versus GSSG was markedly diminished. This finding is consistent with results from many other studies demonstrating the loss of GSH in energy-depleted myocardial cells (1, 6). Because GSH is a central element in the cellular antioxidant defense, its loss renders cells more prone to oxidative stress. In part of the experiments, cardiomyocytes were pretreated with the glutathione precursor NAC before ischemia. This has been shown to be an efficient procedure to increase myocardial glutathione content (5). In NAC-pretreated cells, the glutathione content at the end of simulated ischemia was much higher than in nontreated cells and came close to the glutathione content of normoxic control cells. Concomitantly, the ratio of GSH to GSSG at the end of simulated ischemia was increased in NAC-pretreated cells.

The degree of the postischemic apoptotic response to H2O2 was related in a dose-dependent manner to the magnitude of the end-ischemic cellular glutathione pool. This indicates that the induction of apoptosis is related to the extent of antioxidant defense (Fig. 4). This finding is consistent with the observation that apoptosis is elicited in a dose-dependent manner by the prooxidant H2O2. The importance of these results is only revealed if the ability of H2O2 to induce apoptosis is compared between ischemic-reoxygenated and normoxic cardiomyocytes, each with high glutathione levels. This comparison uncovers that, at a given antioxidant defense, ischemic-reoxygenated cardiomyocytes are much less susceptible to H2O2-induced apoptosis. In fact, reoxygenated cells are completely protected against H2O2-induced apoptosis if their end-ischemic glutathione reserves are brought to a close-to-normal level.

The antiapoptotic protective effect of an ischemic exposure resembles the protection provided by ischemic preconditioning. Furthermore, both phenomena could share the same common mechanisms. For example, ROS generated upon posts ischemic reperfusion could precondition the heart via activation of the mitogen-activated protein kinase cascade (17). However, the phenomenon described here presents conceptual differences with ischemic preconditioning. Ischemic preconditioning is defined as the protection provided by a brief ischemic episode against a subsequent period of ischemia (30). In the phenomenon described here, ischemia protects myocytes against exogenous noxious stimuli during reperfusion. The findings in the present study should therefore be distinguished from recent observations showing that repetitive brief episodes of ischemia (5 min) protect rat hearts against apoptosis induced by a subsequent 30-min ischemic period (19). They should also be distinguished from the protection afforded by brief metabolic inhibition against apoptosis induced by prolonged metabolic inhibition in isolated cardiomyocytes (10). In these cases, reduced apoptosis clearly appears as another manifestation of classic ischemic preconditioning.

Various studies performed during the last few years in different models have convincingly demonstrated the occurrence of apoptotic cell death in ischemic-reperfused myocardium (10, 13, 21), but the relative importance of apoptotic cell death compared with necrosis has remained controversial. These observations have raised questions about the nature of inducers of apoptosis and mechanisms leading to programmed cell death in ischemic or ischemic-reperfused myocardium. Apoptosis might be directly initiated by the sublethal injury of cardiomyocytes in an energy-depleted state or, alternatively, by exogenous apoptosis-promoting factors originating from blood or nonmyocytes in ischemic-reperfused tissue. We have shown in a previous (26) and in the present study that neither the metabolic depression of ischemia nor the process of metabolic alterations upon reoxygenation are sufficient causes for induction
of apoptosis in adult cardiomyocytes once these are separated from exogenous influences. The inducers of apoptosis in ischemic-reoxygenated myocardium must therefore be sought among factors exogenous to cardiomyocytes. The results of the present study indicate that the reoxygenated cardiomyocyte responds less than control, normoxic myocytes to such exogenous inducers of apoptosis. The phenomenon described in this study, i.e., that prolonged ischemia renders cardiomyocytes less susceptible to prooxidant-induced apoptosis, may correspond to a new form of endogenous protection induced by ischemia. It must be left to future studies to identify the mechanisms of protection against ischemia-reperfusion involved in classic ischemic preconditioning.

This study was partially supported by the Commission of the European Union, BIOMED 1 Program (PL95–1254).

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Received 4 March 1999; accepted in final form 3 August 1999.

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