EFFECT OF SARCOLEMMLAL RUPTURE ON MYOCARDIAL ELECTRICAL IMPEDANCE DURING OXYGEN DEPRIVATION.

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ABSTRACT
Plasma membrane disruption is a characteristic feature of cell death induced by hypoxia or ischemia. Here, we investigated whether analysis of tissue electrical impedance allows detection of on-going cell membrane rupture and necrotic cell death in hypoxic or ischemic myocardium. Twenty-eight isolated rat hearts were submitted to 5 h of ischemia (n=8) or hypoxia (n=20). Myocardial electrical impedance and LDH release were monitored. The time-course of hypoxia-induced cell death was modified by altering pH (pH 7.4 or 6.4, 5 h), or by adding glycine 3-10 mM. Ischemia and hypoxia induced an increase in electrical impedance, followed by a plateau, and later by a reduction. During hypoxia, LDH release started after a prolonged lapse of time (80.00±8.37 min at pH 7.4 and 122.50±11.82 at pH 6.4). The onset of LDH release was followed by the onset of the late reduction in electrical impedance, and both were delayed by acidic pH (p<0.05) and by glycine (p<0.05). The time of onset of LDH release and of late electrical changes were significantly correlated (r=0.752, p<0.001).
In separate experiments, induction of sarcolemmal rupture with Triton X-100 (n=6) mimicked the late effects of ischemia or hypoxia on tissue impedance. The protective effects of glycine and acidosis on membrane disruption were confirmed (propidium iodide) in energy-deprived HL-1 cardiomyocytes. These results describe for the first time a late fall in electrical impedance in myocardium submitted to prolonged oxygen deprivation, and demonstrate that this fall allows detection of on-going cell necrosis.

KEYWORDS: Tissue resistivity; Phase angle; Hypoxia; Ischemia; Cell death
INTRODUCTION

Disruption of plasma membrane is a characteristic feature of the final stage of hypoxic and ischemic necrotic cell death (15; 16; 19; 20). Breakdown of the plasma membrane permeability barrier is preceded by a short metastable state which includes bleb formation, changes in lipid fluidity and alterations in phospholipid composition (4; 11; 15; 19; 20; 35). During the last state of cell death, blebs increase in size, colloid osmotic swelling occurs, mitochondrial permeability transition begins, and lysosomes disintegrate (15; 19; 20; 39). This state culminates with physical rupture of a bleb which causes rapid equilibration of intra and extracellular contents (19; 20; 39).

Elucidation of the molecular mechanisms of necrotic cell death in ischemic myocardium and development of treatments able to delay it require methods to determine the time course of cell death in cell cultures or myocardial tissue. Two main approaches have been used for this purpose. The first one is based on the ability of some dyes (i.e., calcein, propidium iodide, Newport green, etc.) to enter or exit the cell only if sarcolemmal integrity is lost. Methods based on this approach allow monitoring of necrotic cell death in cell preparations but not in intact myocardial tissue (12; 20). The second approach uses detection in the extracellular space of large intracellular molecules (i.e., lactate dehydrogenase (LDH), troponine, etc.) that cannot leave the cell while plasma membrane is intact (12; 23; 24; 28). This approach has been used in cells and in isolated hearts submitted to hypoxia, but cannot be used during severe ischemia when the extracellular space is inaccessible.

Analysis of changes in electrical impedance shortly after death or removal of the organ from the animal have been used for more than 80 years to determine passive electrical properties of several tissues, including kidneys, brain, liver and spleen (18; 32;
Measurement of myocardial electrical impedance (Z) allows an overall estimation of the passive electrical properties of the tissue. In a very simplified way, tissue circuits contain three main elements corresponding to the intracellular compartment, the extracellular compartment, and the cell membranes (14). The intracellular and the extracellular resistances are arranged “in parallel”, and cell membranes act both as a resistance and as a capacitator connecting them. Membrane rupture and colloid osmotic edema should be, thus, expected to result in a reduction in total resistance of the tissue, more taking into account the fact that both membrane rupture and colloid osmotic swelling have been suggested to occur almost simultaneously (20).

The aim of this work was to assess whether analysis of tissue electrical impedance can allow assessing membrane rupture in vivo. For this purpose, we analyzed the time course of changes in myocardial electrical impedance in isolated rat hearts submitted to hypoxia or ischemia. The time course of cell death in hypoxic hearts was determined by monitoring LDH release in the coronary effluent. The studies were also performed under conditions that are known to modify the progression of hypoxic injury as acidification of the hypoxic buffer (21; 29; 38) or addition of glycine (12; 20). The effects of these interventions on sarcolemmal rupture during energy depletion were confirmed in a cell line derived from murine cardiac myocytes (HL-1).

MATERIAL AND METHODS

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication Nº. 85-23, revised 1996). All procedures were approved by the Ethics Committee of our institution.
1. Studies in isolated rat hearts

1.1. Experimental preparation

Thirty-six adult male Sprague-Dawley rats (250-350 g) were killed by an intraperitoneal overdose of pentobarbital. The hearts were quickly removed and retrogradely perfused through the aorta with an oxygenated (95% O2 – 5% CO2) Krebs solution at 37°C (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, CaCl2 2.5, NaHCO3 25, KH2PO4 1.2, and glucose 11, pH 7.4). After removing both atria, complete atrioventricular conduction block was induced by crushing the His bundle with a thin forceps. Right ventricle and interventricular septum were opened by a longitudinal incision from the cardiac base to the apex, and opened hearts were pinned to a silicon membrane placed at the bottom of an organ bath, exposing the endocardial surface of the left ventricle (23; 28). A 2.0 silk-snare was placed in the septum of the hearts and connected to an isometric force transducer (FSG-01, SG-M DC bridge amplifier module, Experimentia, London, UK). Resting tension was 1 g. Preparations were paced from the base of the hearts using rectangular pulses of 2.5 ms duration and 4 V of amplitude, at 400 ms basic cycle length.

1.2. Myocardial electrical impedance

A four-electrode probe placed in the septum was used to measure myocardial electrical impedance and its two components, tissue resistivity (\(\rho\)) and phase angle (\(\theta\)), at 7 KHz, as previously described (23; 28). The impedance probe consisted of a linear array of four platinum electrodes (length: 5 mm, diameter: 0.4 mm), placed at an interelectrode distance of 2.5 mm. An alternating current (10 \(\mu\)A) was applied through the outer pair of electrodes, and the in phase components of voltage were recorded.
continuously through the inner pair of electrodes (High-input impedance lock-in amplifier, Princeton Applied Research, model 5110, USA). The frequency of the applied current was 7 kHz because this frequency maximizes differences in phase angle measurements between normal and ischemic myocardium without impairing the discriminating value of tissue electrical resistivity (2). Tissue resistivity ($\rho$) was calculated from the relation $R = k\rho$, where $R$ is the in phase component of $V$ with respect to $I$, and $k$ is the cell constant of the electrode, by measuring the electrical resistance of a 0.9% NaCl solution at 25ºC, which has a known resistivity of 70 $\Omega$·cm. The onset of the late changes in myocardial electrical impedance was determined as the time of intersection of the two tangent lines superimposed on the original trace recordings. Since the weight of the extracellular component in total tissue resistivity is larger at low frequencies, to rule out this influence, additional impedance measurements were performed at higher frequencies (100 kHz) and compared with those measurements obtained at 7 kHz (25).

1.3. Lactate dehydrogenase (LDH) release and myocardial water content

LDH is a cytoplasmatic enzyme that, due to its high molecular weight (3), can not leave the cytoplasm unless plasmatic membrane is broken. Thus, enzyme release was determined in samples taken from the effluent by a spectrophotometric method at 340 nm, as previously described (23; 28). Water content was measured in hearts submitted to ischemia/reperfusion at the end of the experiments. Tissue samples were weighted before and after desiccation at 100 ºC for 24 h, and myocardial water content was determined as the difference between wet and dry weight, and was expressed as ml of water per 100 g of dry weight.
1.4. Experimental protocol

Following 40 min of equilibration, in 8 isolated rat hearts, the effects of ischemia on myocardial electrical impedance (7 kHz and 100 kHz) and developed tension were analyzed. Ischemia was performed by stopping the normoxic perfusion through the aortic cannula during 5 h. During the ischemic period, temperature was kept at 36.5-36.8 ºC by immersing the hearts in a hypoxic solution at pH 7.4 (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, CaCl2 2.5, NaHCO3 25, KH2PO4 1.2, and sucrose 11; pH 7.4, bubbled with 95% N2 -5% CO2). In 20 hearts the effects of prolonged (5 h) hypoxia was analyzed. In 10 of the hearts, hypoxic perfusion was performed at pH 6.4 (in mM: NaCl 139.5, KCl 4.7, MgSO4 1.2, CaCl2 2.5, NaHCO3 3.5, KH2PO4 1.2, and sucrose 11; pH 6.4, bubbled with 95% N2 -5% CO2). In the remaining 10 hearts, hypoxic perfusion through the aorta was performed at pH 7.4 (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, CaCl2 2.5, NaHCO3 25, KH2PO4 1.2, and sucrose 11; pH 7.4, bubbled with 95% N2 -5% CO2). In both types of hypoxia, 5 hearts were used as controls, whereas in the remaining 5 hearts glycine 3 mM (for pH 6.4) or 10 mM (for pH 7.4) was added to the hypoxic solution. Changes in developed tension and myocardial electrical resistivity and phase angle were continuously recorded during the whole experiments, and cell injury was evaluated by measurement of LDH release during the entire hypoxic period. In two additional hearts, membrane rupture was also analyzed by propidium iodide (PI) staining of nuclei and with confocal microscopy. PI is a membrane-impermeant dye that stains the nuclei by intercalating into DNA molecules only when plasmatic membrane is broken (20). In these two hearts, a hypoxic Krebs solution (pH 6.4) containing PI (5 µg/ml) was infused through the aorta, after 120 and 260 min of hypoxia at pH 6.4, respectively. Hearts were fixed by perfusion with 4% paraformaldehyde, cut in 5 µm sections and analyzed by confocal microscopy.
To further analyze the relationship between the late changes in electrical impedance observed during prolonged ischemia or hypoxia and membrane rupture, 6 additional hearts were perfused with Triton X-100, a manoeuvre known to cause sarcolemmal disruption. Hearts were first perfused for 10 minutes with normoxic Krebs containing the inhibitor of contractility 2,3-butanedione monoxime (BDM) 30 mM to avoid that hypercontracture occurring upon sarcolemmal disruption could affect impedance measurements, and then for 30 minutes with normoxic Krebs containing in addition Triton X-100 0.2% (n=3) or 0.05% (n=3). Finally, PI (5 µg/ml) was added to the Krebs and hearts were fixed in 4% paraformaldehyde, sliced and analyzed by confocal microscopy as described in the previous section.

2. Studies in HL-1 cardiomyocytes

2.1. Cell culture

HL-1 cardiac myocytes, an atrial-derived mouse cardiomyocyte cell line obtained from Claycomb WC, were culture under a 5 % CO₂ atmosphere in Claycomb medium supplemented with 10 % fetal bovine serum (JRH Biosciences), 4 mM L-Glutamine (INVITROGEN), 100 units/ml penicillin (INVITROGEN), 100 µg/ml streptomycin (INVITROGEN), and 100 µM norepinephrine (Sigma). Cells were seeded at a 20,000 cells/cm² density in a culture flasks precoated with 25 µg/ml fibronectin / 0.02 % gelatin solution, until a 70-80 % confluence was achieved, as previously described (7).

2.2. Experimental interventions

The experiments were performed in HL-1 cells plated at a 20,000 cells/cm² density in culture flasks. Once cells reached confluence they were harvested in a 0.05 %
trypsin-EDTA solution, dispersed by agitation, separated into 1 ml aliquots at a density of 0.5 – 1 x 10^6 cells/ml and collected by centrifugation at 500 g. Cell pellets were submitted to 4-4.5 h of simulated ischemia by suspending them in a buffer containing (in mM): NaCl 140, KCl 3.6, CaCl_2 2, MgSO_4 1.2, HEPES 20, NaCN 2, 2-deoxyglucose 20, at 37°C, both at pH 7.4 and 6.4.

2.3. Cell viability

Cell death was assessed using PI staining and flow cytometry. PI fluorescence was measured using a FACScalibur flow cytometer (Becton Dickinson Inc., San Jose, CA. USA) calibrated weekly for fluorescence and light scatter using fluorescence beads. At the end of the experiment, 1 μg/ml PI were added at each sample, and HL-1 cells were identified on the basis of forward and sideward scatter parameters in the logarithmic mode. For each sample 20,000 cells were collected. Data were analyzed with CELLQuest™ software (Becton Dickinson) and results were expressed as a percentage of PI positive HL-1 cells.

3. Statistical analysis

Statistical analysis was performed using commercial available software (SPSS for Windows 8.0). Data are expressed as mean±SEM. Repeated measures analysis of variance (ANOVA) was used to assess differences in the time course of LDH release and electrical impedance during hypoxia between groups. ANOVA followed by Tukey test was used to compare the onset of the changes in tissue electrical impedance and LDH release. Student’s t test was used to analyze differences in cell death between control and glycine-treated groups in HL-1 cells. Differences were considered to be significant when p<0.05.
RESULTS

1. Studies in isolated rat hearts

1.1. Effects of ischemia

Rigor onset in hearts submitted to ischemia occurred at 18.45±0.86 min. In these hearts, ischemia also caused a marked increase in myocardial resistivity determined at 7 kHz (from 119.91±7.10 to 506.03±72.03 Ω·cm at 80 min, p<0.001) and a decrease in phase angle (from −1.55±0.34 to -18.43±5.09° at 80 min, p<0.001), which continued with a plateau or a phase with low slope (Fig. 1). This was interrupted by an abrupt decrease in tissue resistivity and an increase in phase angle, whose onset occurred at 215.00±11.90 and 213.75±13.13 min, respectively (Fig. 1). Similar results were obtained when myocardial electrical impedance was analyzed at 100 kHz, with no differences in the onset of the late changes respect to measures performed at 7 kHz (205.00±14.43 min for resistivity and 206.25±15.73 for phase angle) (Fig. 1).

1.2. Effects of hypoxia

The effects of hypoxia were qualitatively equivalent to those of ischemia. Developed tension decreased during hypoxia at pH 7.4, to reach a minimal value several minutes later. Rigor contracture, detected as an abrupt increase in diastolic tension occurred between 4 and 6 min after hypoxic perfusion at pH 7.4.

Hypoxia at pH 7.4 induced a marked increase in tissue resistivity (from 119.39±6.79 to 159.97±11.48 Ω·cm at 20 min, p<0.001) and a decrease in phase angle (from -2.78±0.55 to -4.82±0.44° at 50 min, p<0.001). A plateau phase was reached at about 20-
25 min, which lasted only for about 30 min. Thereafter, myocardial resistivity begins to continuously decrease and phase angle increases.

During the first 60 min of hypoxia there was no significant release of LDH. However, thereafter, there was a progressive increase in LDH release. Myocardial water content at the end of 5 h of hypoxia at pH 7.4 was 604.85±31.11 ml/100 g dry tissue.

1.3. Effects of acidosis and glycine during hypoxia.

Acidosis during hypoxia (pH 6.4) delayed the onset of rigor contracture (p<0.001). No significant differences in the changes induced by both types of hypoxia in developed or diastolic tension were observed in the groups treated with glycine (rigor onset for hypoxia at pH 6.4 was 16.60±1.76 in controls and 15.38±3.79 min in hearts treated with glycine 3 mM; for hypoxia at pH 7.4 rigor onset was 4.54±0.63 in controls and 4.95±0.62 min in hearts treated with glycine 10 mM).

As occurred with hypoxia at pH 7.4, acid hypoxia caused a marked increase in tissue resistivity (from 120.89±4.34 to 145.09±11.82 Ω·cm at 50 min of hypoxia, p<0.001), and a decrease in phase angle (from –1.78±0.27 to -3.5±0.84º at 50 min, p<0.001) (Figs. 2 and 3). As seen for rigor onset, the onset of the plateau in myocardial electrical impedance was delayed compared with hearts submitted to hypoxia at pH 7.4, and it was reached at about 40-50 min (Fig. 2). The pH during hypoxia determined the duration of this plateau, which lasted for about 100 min in the group of hearts submitted to hypoxia at pH 6.4 compared with the duration of 30 min observed in the group treated at pH 7.4. Thereafter, myocardial resistivity begins to continuously decrease and phase angle increases (Fig. 2). The onset of this last decrease in resistivity and increase in phase angle was significantly delayed by acidosis (tissue resistivity: from 87.00±25.62 min in hypoxia at pH 7.4 to 192.00±14.71 min during acidic hypoxia;
p=0.0075; phase angle: from 82.60±21.89 to 207.00±11.47 min, p<0.001) (Figs. 2, 3 and 4). Treatment with glycine did not modify the initial changes in tissue electrical impedance. However, the onset of the last changes in tissue impedance was significantly delayed by glycine in the group submitted to hypoxia at pH 6.4 (Figs. 3 and 4), with the same trend observed in the group submitted to hypoxia at pH 7.4 (Figs. 4 and 5).

During the first 60 min of acid hypoxia there was no significant release of LDH. However, thereafter, and as happened at pH 7.4, there was a progressive increase in LDH release, that was less marked in the hypoxic group treated at pH 6.4. Treatment with glycine during hypoxia attenuated LDH release in both types of hypoxia (Fig. 3), and significantly delayed the onset of LDH release in the group submitted to hypoxia at pH 6.4 (from 122.50±11.82 min in control hearts to 184.00±11.66 min of hypoxia in hearts treated with glycine 3 mM, Student’s t test, p=0.0081), showing the same trend during hypoxia at pH 7.4 (from 80.00±8.37 min in control hearts to 92.00±15.62 min in hearts treated with glycine 10 mM, p-NS).

The onset of LDH release in both groups of hypoxia correlated well with the onset of the delayed changes in electrical tissue impedance (resistivity: r=0.752, p=0.0003, y=0.503x+42.79; phase angle: r=0.766, p=0.0002, y=0.491x+41.59) (Fig. 5).

Propidium iodide staining disclosed minimal necrotic cell death in one heart in which hypoxic perfusion (pH 6.4) was stopped at 120 min (approximately 60 min before the late fall in electrical impedance) and extensive necrosis in another heart in which hypoxia lasted for 260 min (approximately 60 after the late fall in electrical impedance) (Fig. 6).

Myocardial water content at the end of 5 hours of acid hypoxia was significantly lower than that observed in hearts submitted to hypoxia at pH 7.4 (p<0.05). Treatment with glycine did not modify myocardial water content at the end of 5 hours of hypoxia.
at any pH (pH 6.4: 513.70±22.44 ml/100 g dry tissue in control hearts and 493.51±27.10 in hearts treated with glycine 3 mM; hypoxia at pH 7.4: 604.85±31.11 ml/100 g dry tissue in control hearts; 619.87±26.66 in glycine-treated hearts; p-NS).

1.4. Effects of Triton X-100.

BDM caused an almost immediate inhibition of contractility. Perfussion with Triton X-100 induced, in the presence of BDM, only minor changes in diastolic tension, but caused a reduction in myocardial resistivity and an increase in phase angle, similar to the late changes in tissue electrical impedance observed during ischemia or hypoxia (Fig. 7). The effects of Triton X-100 on myocardial impedance were more marked at 0.2 % (resistivity: from 114.44±2.62 to 73.63±1.83 Ω·cm at 30 min, p=0.0002; phase angle: from –1.43±0.37 to 0.4±0.4º, p=0.0283) than at 0.05% (resistivity: from 121.87±3.55 to 105.25±9.86 Ω·cm at 30 min, p-NS; phase angle: from –1.63±0.29 to –1.53±0.54º, p-NS), and this correlated with a much more extensive cell necrosis, as indicated by PI staining, in hearts treated with 0.2% Triton X-100 as compared to hearts treated with Triton X-100 at 0.05% (Fig. 7).

2. Studies in HL-1 cardiomyocytes

Prolonged simulated ischemia at pH 7.4 for 4 h was associated to marked sarcolemmal disruption in HL-1 cardiomyocytes, as assessed by propidium iodide staining. Treatment with glycine 3 mM reduced membrane rupture (propidium iodide positive cells: 45.61±1.39 % in control simulated ischemia vs. 33.55±5.00 % in glycine-treated cells, p=0.0046). In contrast to simulated ischemia at pH 7.4, simulated ischemia at pH 6.4 (4.5 h) induced only minor sarcolemmal disruption as assessed by the percentage of propidium iodide cells, with no differences between groups (propidium
iodide positive cells: 13.84±0.42 % in control simulated ischemia vs. 15.89±1.94 % in glycine-treated cells, p=NS).

DISCUSSION

The present study describes for the first time the late fall in tissue electrical impedance in myocardium submitted to prolonged ischemia or hypoxia, and demonstrate that analysis of electrical tissue impedance allows detection and monitoring of myocardial necrosis during sustained oxygen deprivation.

Tissue electrical impedance during prolonged oxygen deprivation.

Tissue electrical impedance measurement (electrical resistivity and phase angle) is an overall estimation of the passive electrical properties of the tissue, that is composed by the resistance of the extracellular and the intracellular compartments, and the resistance of plasma membrane and its capacitance (14). Both membrane rupture and intracellular colloid osmotic edema should be expected to result in a reduction in total resistivity of the tissue, due to a reduction in membrane and intracellular resistances, respectively.

Changes in tissue electrical resistance in myocardial tissue have been previously described after short periods of ischemia or hypoxia not resulting in significant cell death until oxygen availability is restored upon subsequent reperfusion or reoxygenation. During myocardial ischemia, tissue resistivity is characterized by an small initial increase due to changes in extracellular resistance, probably due to a decrease in extracellular volume, followed, several minutes later, by a continuous and marked increase due to modifications in intracellular resistance (8; 17; 23; 28; 31). The same pattern of changes is observed in phase angle (5; 23; 28). During hypoxia, there is
a short initial decrease in tissue resistivity, most likely due to changes in interstitial edema, followed by a marked increase related to changes in intracellular compartment (27). After 30-45 minutes of ischemia or hypoxia, a stable plateau is reached in which values of myocardial resistivity are increased and phase angle is decreased respect to baseline (8; 17; 23; 27; 28; 31). However, the duration of this plateau, or what happens thereafter had not been previously described. In pigs submitted to occlusion of the left anterior descending coronary artery for one month, the infarct scar showed a reduced tissue resistivity compared with normal or acute ischemic myocardium (6; 10), but no data on the transition between the increased values of tissue resistivity during acute myocardial ischemia and the reduction observed in chronic infarction were available until now. Our present data demonstrate that after a prolonged ischemia or hypoxia, isolated rat hearts show an abrupt decrease in tissue resistivity and a marked increase in phase angle.

**Protective effects of acidosis and glycine.**

The present study discloses a narrow temporal association between the onset of the fall in electrical impedance and the onset of LDH release during sustained hypoxia. The fact that the temporal association between the fall in electrical impedance and LDH release is maintained when the latter is delayed by acidosis or glycine strongly suggests a cause-effect relationship between both events. Moreover, the effect of these interventions on sarcolemmal rupture induced by oxygen deprivation was confirmed in cell cultures submitted to prolonged metabolic inhibition. The observations that induction of membrane disruption with Triton X-100 mimicked the late effects of ischemia and hypoxia on electrical impedance, and that membrane disruption assessed by PI was intense after the late fall in electrical impedance but not before its onset, add
further support to the relationship between cell necrosis and the late changes in impedance. Although, the low frequency of the electrical current applied for analysis of tissue impedance rises the question of the possible contribution of changes in extracellular compartments to the measurements, a major contribution of these changes seems unlikely since the findings were the same when measurements were performed at 100 kHz.

LDH release into the coronary effluent is a global index of myocyte injury across the whole heart, while measures of tissue electrical impedance are more localized. However, due to the probe size (1 cm long, consisting in a linear array of four electrodes spaced 2.5 mm, each with a length of 5 mm and a diameter of 0.4 mm), impedance measures reflect changes from a relatively large area of myocardium, that in the rat ventricle may include the complete thickness of the ventricular wall.

In the present study, acidosis and addition of glycine were used to delay the progression of ischemic injury. The effectiveness of both interventions has been solidly established. Several studies have shown that extracellular acidosis delays ATP depletion (21; 29; 38). However, since acidosis markedly decreases conductivity of both gap junctions and hemichannels (membrane pores formed by connexins) (1; 34), the question can be raised of whether the changes in sarcolemmal permeability and tissue electrical resistance observed late during prolonged ischemia reflect a fast opening of hemichannels, and the protective effect of acidosis is due to interference with this phenomenon. However, the large molecular weight of LDH (3)(more than 20 times larger than the maximum size that can permeate connexin43 hemichannels (13)) makes this explanation extremely unlikely.
Glycine, a nonessential amino acid has been shown to be protective against cell death induced by oxygen deprivation in different cell types and tissues other than myocardium (9; 12; 20; 22; 26; 37; 40). We have recently shown that glycine protects isolated cardiomyocytes and rat hearts from reoxygenation-induced cell death by interfering with mitochondrial permeability transition (30). The present study extends those results by demonstrating a protective effect of glycine against myocardial necrosis also during the period of oxygen deprivation. Although the mechanism of action of glycine during this period can not be deduced from this study, the fact that, as occurred during reoxygenation, the effects of glycine are comparable to those of acidosis, a manoeuvre with a potent inhibitory effect on permeability transition, suggests that the permeability transition pore could be a target of glycine also during prolonged oxygen deprivation.

Implications

The present results demonstrate that analysis of electrical tissue impedance allows detection of ongoing myocardial necrosis. Monitoring of cell death during ischemia or hypoxia may prove very useful as a research tool in the elucidation of the mechanism of cell death during oxygen deprivation, as during heart preservation and transplantation, or in patients undergoing cardiac surgery. The potential application of this method must take into account certain inherent limitations. The fact that the onset of LDH release precedes the onset of the late changes in myocardial electrical impedance indicates that measurement of passive electrical properties of the tissue is a less sensitive parameter than enzyme release. However, in situations in which enzyme release can not be monitorized, electrical impedance would be a valuable tool. The limited spatial resolution of impedance measurements is another pitfall of the technique that, on the other hand, should be expected to increase its sensitivity. Finally, the invasive nature of
this technique limits its applicability, although the development of new intracavitary catheters able to detect electrical impedance by using the contact technique (36) could overcome this limitation.
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References


FIGURE LEGENDS

Figure 1. Mean changes (± SEM) induced by ischemia (5 h) on tissue electrical resistivity (upper panel) and phase angle (middle panel) determined at 7 (n=4) and 100 (n=4) kHz in isolated rat hearts. The onset of the late changes in tissue electrical impedance is indicated by the arrows.

Figure 2. Representative recordings showing the changes induced by hypoxia at pH 6.4 (5 h) on tissue electrical resistivity (upper panel), phase angle (middle panel), and LDH release (lower panel) in a control rat heart. The onset of the late changes in tissue electrical impedance and of LDH release is indicated by the arrows.

Figure 3. Mean changes (± SEM) induced by hypoxia at pH 6.4 (5 h) on tissue electrical resistivity (upper panel), phase angle (middle panel), and LDH release (lower panel) in control rat hearts and in hearts treated with glycine. The onset of the late changes in tissue electrical impedance and of LDH release is indicated by the arrows.

Figure 4. Onset of LDH release and of the late changes in tissue electrical impedance (resistivity and phase angle) occurring during hypoxia at pH 7.4 or pH 6.4 in the presence or absence of glycine. * (p<0.01) shows significant effects of acidosis respect the corresponding group at pH 7.4. τ (p<0.05) indicates significant effects of glycine respect the corresponding group without glycine. # (p<0.01) shows significant differences between the onset of LDH release and the onset of the late changes in electrical impedance in each condition.

Figure 5. Relationship between the onset of LDH release and the onset of the late changes in tissue electrical resistivity (upper panel) and phase angle (lower panel) in isolated rat hearts submitted to hypoxia (both pH) both in the presence and absence of glycine.
Figure 6. Sections of rat hearts perfused with propidium iodide (confocal microscopy). (A) No labelling of cell nuclei was observed after 120 min of hypoxia (pH 6.4). (B) Intense labelling of cell nuclei after 260 min of hypoxia.

Figure 7. (A). Representative recordings showing the effects of Triton X-100 (0.05% and 0.2%) on myocardial electrical resistivity (upper panel) and phase angle (lower panel). (B) Sections of rat hearts perfused with propidium iodide (confocal microscopy). Note the concordance between the occurrence of a marked fall in myocardial electrical impedance and extensive sarcolemmal rupture.
Figure 1
Figure 2
Figure 3

**Resistivity (%)**
- **Control**
- **Glycine 3 mM**

**Phase angle (%)**
- **Control**
- **Glycine 3 mM**

**LDH (U/g dry tissue)**
- **Control**
- **Glycine 3 mM**

**MANOVA**
- $p < 0.001$
- $p = 0.011$
- $p = 0.030$
Onset of changes during prolonged hypoxia

Figure 4
Resistivity vs. LDH release

![Resistivity vs. LDH release graph](image)

- Control hypoxia (pH 6.4)
- Hypoxia + glycine 3 mM (pH 6.4)
- Control hypoxia (pH 7.4)
- Hypoxia + glycine 10 mM (pH 7.4)

$r = 0.752$
$p = 0.0003$

Onset resistivity decrease (min)

Phase angle vs. LDH release

![Phase angle vs. LDH release graph](image)

- Control hypoxia (pH 6.4)
- Hypoxia + glycine 3 mM (pH 6.4)
- Control hypoxia (pH 7.4)
- Hypoxia + glycine 10 mM (pH 7.4)

$r = 0.766$
$p = 0.0002$

Onset phase angle increase (min)

**Figure 5**
Figure 7

A

- Resistivity ($\Omega \cdot \text{cm}$)
- Triton X-100
- Triton X-100 0.2%
- Triton X-100 0.05%

B

- Phase angle ($^\circ$)
- Triton X-100 0.2%
- Triton X-100 0.05%