New insights on myocardial pyridine nucleotides and thiol redox state in ischemia and reperfusion damage

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Abstract

Objective: to investigate the changes of pyridine nucleotides and thiol redox state in cardiac tissue following ischemia and reperfusion. NADH / NAD and NADPH / NADP redox couples were specifically studied and the influence of NADPH availability on cellular thiol redox was also investigated. Methods: isolated rabbit hearts were Langendorff perfused and subjected to a protocol of ischemia and reperfusion. An improved technique for extraction and selective quantitation of pyridine nucleotides was applied. Results: ischemia and reperfusion induced an increase in diastolic pressure, limited recovery in developed pressure and loss of creatine phosphokinase. Creatine phosphate and ATP were decreased by ischemia and only partially recovered during reperfusion. NADH was increased (from 0.36±0.04 to 1.96±0.15 μmol/g dry wt. in ischemia, P<0.001), whereas NADPH decreased during ischemia (from 0.78±0.04 to 0.50±0.06 μmol/g dry wt., P<0.01) and reperfusion (0.45±0.03 μmol/g dry wt.). Furthermore, we observed: (a) release of reduced (GSH) and oxidised glutathione (GSSG) during reperfusion; (b) decreased content of reduced sulphydryl groups during ischemia and reperfusion (GSH: from 10.02±0.76 to 7.11±0.81 nmol/mg protein, P<0.05, and to 5.48±0.57 nmol/mg protein; protein-SH: from 280.42±12.16 to 135.11±17.00 nmol/mg protein, P<0.001, and to 190.21±11.98 nmol/mg protein); (c) increased content in GSSG during reperfusion (from 0.17±0.02 to 0.36±0.02 nmol/mg protein, P<0.001); (d) increased content in mixed disulphides during ischemia (from 6.14±0.13 to 8.31±0.44 nmol/mg protein, P<0.01) and reperfusion (to 9.87±0.82 nmol/mg protein, P<0.01). Conclusions: under severe low-flow ischemia, myocardial NADPH levels can decrease despite the accumulation of NADH. The reduced myocardial capacity to maintain NADPH/NADP redox potential can result in thiol redox state changes. These abnormalities may have important consequences on cellular function and viability. © 2000 Published by Elsevier Science B.V.

Keywords: Energy metabolism; Free radicals; Ischemia; Reperfusion

1. Introduction

The lack of oxygen induced by myocardial ischemia impairs the oxidative metabolism of the myocytes with accumulation of metabolic intermediates in the form of reducing equivalents and protons. In particular, the reduced form of nicotinamide adenine dinucleotide (NADH), the essential coenzyme for all the oxidative enzymatic reactions, accumulates and is only utilised in reactions such as lactate formation from pyruvate, which, under ischemia, cannot be used in the Krebs cycle [1–3]. A general assumption is that nicotinamide adenine dinucleotide phosphate (NADP) — the cofactor of the anabolic enzymatic reactions — accumulates in the reduced form (NADPH), following the same pattern of the NADH/NAD redox couple. However, this assumption has never been spe-
cifically investigated and is based on old findings, when methods for determining these pyridinic nucleotides were not selective.

The primary aim of our study was to investigate NADPH/NADP changes under ischemia and reperfusion. To this end, we have applied a recently developed, improved technique for extraction and selective quantitation of purinic and pyridinic nucleotides [4].

Moreover, we have studied the influence of NADPH availability on the cellular thiol redox state. Cellular sulphydryl and glutathione constitute a defense against oxidative damage and they actually undergo marked changes during ischemia and reperfusion [5–9]. Reducing equivalents, in the form of NADPH or reduced sulphydryls, are in an equilibrium with reduced/oxidised glutathione (GSH/GSSG) which is controlled only by the reaction constants of the respective enzymes: no thermodynamic barrier prevents, within the system, the transfer of electrons, if a component is depleted or accumulated.

Our hypothesis was that the cellular capacity to maintain the NADPH/NADP redox potential could be involved in the changes of GSH and total sulphydryl groups (total-SH) known to occur during myocardial ischemia and reperfusion: its pattern may be differently affected than that of NADH, by oxygen deprivation, thus becoming a determinant of reduced/oxidised glutathione (GSH/GSSG) and thiols cellular balance. To this end, we have employed a rabbit Langendorff model of ischemia and reperfusion, which has already been proved to characterise peculiar aspects of myocardial defences and oxidative stress [5,7,10–12].

2. Methods

2.1. Ischemia and reperfusion model

Thirty-eight male New Zealand rabbits (2.0–2.3 kg), maintained on a standard diet, were used. The animals received humane care in compliance with the European Guidelines 86/609/CEE. They were sacrificed — after a previous light pentobarbital anaesthesia — by cervical dislocation with a blow to the neck and the hearts were immediately removed and placed in cold perfusion buffer (4°C). Hearts (5.4 ± 0.4 g wet wt.) were perfused, using the constant-flow Langendorff technique, with a Krebs–Henseleit buffer containing in mM: NaCl 115.0; NaHCO₃ 25.0; KCl 4.0; KH₂PO₄ 0.9; MgSO₄ 0.65; CaCl₂ 1.7 and D-glucose 11.0, as previously described [7]. The perfusion solution was warmed at 37°C, bubbled with 95% O₂ and 5% CO₂, and transported to the aortic cannula with a Gilson Minipuls rotary pump. The hearts were maintained at a constant myocardial temperature of 37°C independently from coronary flow. The hearts were paced using suprathreshold rectangular pulses at 0.1-ms duration at a rate of 180 bpm during all experimental phases.

After 30 min of aerobic perfusion at a constant coronary flow of 22 ml/min (equilibration period), the isolated rabbit hearts were subjected to 60 min of global low-flow ischemia (0.5 ml/min), followed by 30 min reperfusion (22 ml/min).

To better investigate the kinetics of NADPH/NADP changes and the thiol redox state under ischemia and reperfusion conditions, perfusion was discontinued at different periods: at the end of ischemia (n = 7), at 3 min (n = 7), at 10 min (n = 8) and at the end (n = 12) of reperfusion. Another group of hearts (n = 4) — representing the aerobic control hearts — was aerobically perfused for 120 min, corresponding to the longest experimental protocol, including the stabilisation period. During each experiment, mechanical function was continuously monitored and aliquots of coronary effluent were timely collected for creatine phosphokinase (CPK), lactate and GSH and GSSG determinations. At the end of each perfusion, hearts were frozen with pre-cooled Wollenberger tongs and stored in liquid nitrogen until tissue determinations were carried out.

2.2. Mechanical function

Mechanical function was measured as systolic and diastolic pressures.

To obtain an isovolumetrically beating preparation, a fluid-filled balloon was inserted into the left ventricular cavity via the atrium and connected by a fluid-filled polyethylene catheter to a Hewlett Packard transducer (Mod. 1290 A OPT 002). At the beginning of the experiments, the amount of fluid in the balloon was adjusted to obtain a diastolic pressure of <1.0 mmHg.

Coronary perfusion pressure was also measured.

2.3. Biochemical measurements

2.3.1. Creatine phosphokinase release

Creatine phosphokinase release was measured in coronary effluent fractions after collection in pre-cooled vials (4°C), at regular intervals during the whole protocol. CPK activity was assayed spectrophotometrically by the method of Oliver [13].

2.3.2. Creatine phosphate (CP) and ATP

Creatine phosphate (CP) and ATP were extracted from frozen tissue, both ground (in 0.4 N HClO₄, at liquid nitrogen temperature) and homogenised using an Ultra-Turrax. Supernatants obtained from centrifugation were used for high-performance liquid chromatography (HPLC, Waters 600E multisolvant and Mod. 990 photodiode array detector) analysis. Detection was performed at 205 nm for CP and at 260 nm for ATP, as previously described [4].
2.3.3. Pyridinic nucleotides (NADH, NAD, NADPH, NADP)

Pyridinic nucleotides (NADH, NAD, NADPH, NADP) were extracted from frozen tissue. Oxidised and reduced nucleotides were extracted differently: for oxidised pyridinic nucleotides (NAD and NADP), the existing, previously described procedure was applied [4]. Samples were ground (mixed with 0.4 N HClO₄, at liquid nitrogen temperature) and homogenised using an UltraTurrax. Homogenates were then centrifuged at 4000 g for 10 min at 4°C, and the supernatant, adjusted to pH 6.0–6.5 with 6 N KOH, was used for high-performance liquid chromatography analysis (HPLC, Waters 600E multisolvant and Mod. 990 photodiode array detector). Separations were performed on a Supelchem C₁₈, 3-μm RP column (0.46×15 cm). The mobile phase consisted of a gradient of buffer A (0.1 M KH₂PO₄, 5 mM tetrabutylammonium hydrogen sulphate, 2.5% (v/v) acetonitrile, pH 6.0) and buffer B (0.1 M KH₂PO₄, 5 mM tetrabutylammonium hydrogen sulphate, 25% (v/v) acetonitrile, pH 5.5). Detection was performed at 260 nm. Identification of peaks was confirmed by co-elution with standards used at known concentrations, enzymatic shifts and spectral analysis.

An alkaline extraction of samples was used for NADH and NADPH. Pre-weighed portions of frozen tissue were ground in phenol buffer (approximately 500 mg per 3 ml of 0.64 M phenol, 0.07 M phosphate buffer solution, pH 7.8), added to 3 ml of chloroform and allowed to thaw in the dark at 4°C. Homogenates were then centrifuged at 4000 g for 10 min at 4°C and the aqueous phase was washed five times with 5 ml diethyl ether to remove residual traces of phenol and organic material. Extracts were then used for chromatography, as described above.

2.3.4. Reduced and oxidised glutathione

Reduced and oxidised glutathione was measured in fresh coronary effluent or in portions of frozen left ventricular tissue, after deproteinisation (3 M HClO₄) and neutralisation of supernatant with 2 M K₂CO₃. A sample of the neutralised extract was analysed for total GSH by the method of Tietze [14]. GSSG was measured after the preliminary reaction of GSH with 20 mM N-ethylmaleimide followed by complete removal of unreacted sulphydryl reagent with diethyl ether.

2.3.5. Myocardial content of sulfhydryl groups

Myocardial content of sulfhydryl groups was measured using the method of Sedlack and Lindsay [15]. Absorbance was read at 412 nm and a molar extinction coefficient of 13600 M⁻¹ cm⁻¹ was used. The acid-soluble thiol group content, as an expression of non-protein sulfhydryl groups (non protein-SH) was similarly determined, but in the supernatants obtained after denaturation of homogenates with ice-cold 50% trichloroacetic acid, as samples.

Protein sulphydryl groups (protein-SH) were determined by subtracting the acid-soluble thiol group content from the total-SH content.

2.3.6. Myocardial content of glutathione-bound mixed disulphides

Myocardial content of glutathione-bound mixed disulphides was determined in portions of frozen left ventricular tissue by the method described by Thayer [16].

2.4. Protein determination

Protein determination was carried out according to the method of Bradford [17], using bovine serum albumin as standard.

2.5. Reagents

High-grade reagents were obtained from Sigma Chemical (USA).

2.6. Statistical analysis

Data are reported as means±S.E.M. The Student t-test (with Bonferroni’s correction) was used and results were considered to be significant if P<0.05.

3. Results

3.1. Time-course of myocardial damage during ischemia and reperfusion

During aerobic perfusion, the mechanical function of isolated rabbit hearts did not change: developed pressure was maintained after 120 min and diastolic pressure remained constantly close to 0 mmHg. In aerobic control conditions, CPK, as well CP, ATP (data not shown), GSH, GSSG, protein-SH, and pyridinic nucleotides contents (Figs. 2–4) remained constant during the whole duration of the experimental protocol.

From the onset of low-flow ischemia, developed pressure progressively decreased leading to contractile quiescence within 5 min After 30 min, diastolic pressure began to rise up to 45.7±3.1 mmHg by the end of ischemia. Reperfusion resulted in a further increase in diastolic pressure (65.2±4.6 mmHg at the end of reperfusion) and in a poor recovery of developed pressure (38.7% of baseline) (Fig. 1).

A progressive and massive CPK release was observed during reperfusion, indicating a marked cellular damage (Fig. 1).

In ischemic conditions, CP markedly decreased from 38.9±1.5 (aerobic conditions) to 6.1±1.0 μmol/g dry wt. (P<0.001) and similarly ATP from 16.95±1.44 to 3.98±0.67 μmol/g dry wt. (P<0.001). At reperfusion, CP partially recovered (18.4±2.0 μmol/g dry wt., after 3
Fig. 1. Left ventricular pressure expressed as mean values of systolic (filled circles) and diastolic (empty circles) pressures, creatine phosphokinase (CPK) release, reduced (GSH) and oxidised glutathione (GSSG) release in the coronary effluent in isolated hearts subjected to ischemia and reperfusion.

Fig. 2. Myocardial content of reduced and oxidised nicotinamide-adenine-dinucleotide (NADH and NAD), and their ratio (NADH/NAD) in hearts subjected to ischemia and reperfusion. Hearts were aerobically perfused for 120 min (n=4), or, after the stabilisation period, subjected to a global low-flow ischemia for 60 min (ischemic hearts, n=7), or reperfused for 3 (n=7), 10 (n=8) or 30 (n=12) min.

3.2. Myocardial content of pyridinic nucleotides

3.2.1. Reduced and oxidised nicotinamide-adenine-dinucleotide

Ischemia resulted in an accumulation of NADH (from 0.36±0.04 μmol/g dry wt. to 1.96±0.15 μmol/g dry wt., \( P<0.001 \)), which did not completely return to baseline during reperfusion (0.83±0.09 μmol/g dry wt., \( P<0.001 \), vs. ischemia; Fig. 2). Conversely, 60 min of ischemia induced a decrease in myocardial levels of NAD from 2.73±0.05 μmol/g dry wt. to 1.22±0.09 μmol/g dry wt. (\( P<0.001 \)) (Fig. 2). Re-oxygenation of the hearts did not completely recover NAD levels (1.85±0.09 μmol/g dry wt., after 3 min, 1.85±0.14, after 10 min, and 2.02±0.15, at the end of reperfusion). An increase of NADH/NAD ratio after ischemia (from 0.140±0.012 to 1.70±0.22, \( P<0.001 \)) indicated an impaired oxidative utilisation of reducing equivalents at the level of citrate synthetase in the Krebs cycle. This increase was only partially recovered after 30 min of reperfusion (0.42±0.04, \( P<0.001 \), vs. ischemia) (Fig. 2).

3.2.2. Reduced and oxidised nicotinamide-adenine-dinucleotide phosphate

Ischemia resulted in a decrease of NADPH tissue contents (from 0.78±0.004 μmol/g dry wt. to 0.50±0.06 μmol/g dry wt., \( P<0.01 \)), which were not counteracted by reperfusion (0.45±0.03 μmol/g dry wt.; Fig. 3). NADP was reduced after 60 min of ischemia (from 0.13±0.01 μmol/g dry wt. to 0.09±0.01 μmol/g dry wt., \( P<0.05 \)) and, differently from NADPH, reperfusion completely recovered NADP levels from the first minutes (0.12±0.01,
3.3. Thiol redox state

3.3.1. Reduced and oxidised glutathione release

Fig. 1 shows glutathione release during ischemia and reperfusion. GSH was markedly released and it was maintained at high rate up to the end of reperfusion (4.997 ± 0.399 nmol/min/g wet wt. vs. 0.516 ± 0.089 nmol/min/g wet wt.; \(P<0.001\)). GSSG baseline release was 0.098 ± 0.027 nmol/min/g wet wt.; it peaked during early reperfusion (1.15 ± 0.3 nmol/min/g wet wt. at 3

\[ P<0.05 \text{ and } \] 
\[ P<0.01 \text{ ischemia vs. reperfusion} \]
\[ P<0.05, ** P<0.01 \text{ and } *** P<0.001 \text{ reperfusion vs. ischemia} \]

Fig. 4. Myocardial content of reduced glutathione (GSH), protein-SH, oxidised glutathione (GSSG), and glutathione-mixed disulphides, in hearts subjected to ischemia and reperfusion. See Fig. 2 for further details.
nmol/mg protein, respectively; \( P<0.001 \) from a baseline value of 0.17±0.02 nmol/mg protein in aerobic hearts (Fig. 4).

3.3.3. Myocardial content of glutathione-bound mixed disulphides

Mixed disulphide levels significantly increased from 6.14±0.13 nmol/mg protein, under aerobic conditions, to 8.31±0.44 at the end of ischemia \( (P<0.01) \). With re-oxygenation, they slightly decreased from 11.08±0.93 nmol/mg protein, at the first minute, to 9.87±0.82 nmol/mg protein, at the end of reperfusion (Fig. 4).

4. Discussion

Our data demonstrate for the first time that ischemia induces metabolic alterations which can lead to a reduced capacity to maintain NADPH/NADP redox potential. We have observed that during severe, low-flow ischemia, myocardial NADPH is decreased and its pattern is inverse to that of NADH.

Up to date, NADPH levels were believed to parallel those of NADH [18,19]. This assumption was mainly due to the lack of methods able to adequately discriminate the specific nucleotides. Indirect measurements, such as the indicator-metabolite methods [20], based on the determination of specific metabolites (i.e. pyruvate/malate for the NADPH/NADP redox couple), rely on the assumptions that these reactions are at the equilibrium and the relevant equilibrium constants are extrapolated from empirical observations. Chemiluminescence and enzyme cycling methods have poor sensitivity to discriminate between NADH/NAD and NADPH/NADP redox couples, due to the limited specificity of the enzymes employed in signal generation and amplification [21,22]. Surface spectroscopy is considered to measure mitochondrial NADH, but a component of the signal is due to NADPH, whose spectrum is not distinguishable at the wavelengths usually employed (e.g. 337 nm for excitation and 425 nm for emission) [18–23].

Our method of extraction and HPLC detection overcomes the above-mentioned limitations, being precise, repetitive and sensitive in distinguishing NADPH from NADH [4].

4.1. Why does ischemia result in decrease of NADPH content?

Several hypotheses can explain an impaired metabolic capacity to generate NADPH or its usage during ischemia and reperfusion:

(i) during the early phases of ischemia, glucose is preferentially used to produce energy through the anaerobic glycolysis, which is maximally stimulated by a reversal of the inhibition of glycolysis by ATP on 6-phosphofructo-1-kinase [2,3], i.e. the Pasteur’s effect. On the contrary, the hexose monophosphate shunt is likely to play a role during the early phases of reperfusion; in fact, the activity of glucose-6-phosphate dehydrogenase, the first enzyme in the hexose monophosphate shunt, is regulated by cytosolic GSSG [24], which only accumulates after re-flow;

(ii) glycogen is the major source of glucose for glycolysis during ischemia [2,3]. Consequently, a depletion of glycogen, proportional to the degree of the ischemic injury, occurs during the late phase of ischemia [2,3]. This has been demonstrated by us and other authors using in vitro models of ischemia and reperfusion similar or identical to the one used in the current study [25,26]. In turn, the depletion of glycogen implies the lack of availability of glucose for the re-synthesis of NADPH;

(iii) an impairment of energy-linked trans-hydrogenase reactions. Heart cells contain a high activity of nicotinamide nucleotide trans-hydrogenase which is involved in maintaining the NAD(P)H/NAD(P) redox potential [27]. In energised mitochondria, the kinetic properties of the enzyme sustain the so-called ‘forward reaction’, i.e. the reduction of NADP by NADH. This reaction is driven by the proton electrochemical gradient, thus, the protection against NADPH oxidation occurs with energy expenditure. Trans-hydrogenation is reversible: in fact, the kinetic properties of the enzyme and the stoichiometry of the proton extrusion are in keeping with an activation of the reverse transhydrogenase reaction by a decrease in the mitochondrial proton electrochemical gradient. Under mild anoxic conditions or in the early phases of ischemia, energy-linked transhydrogenase can produce energy at the expenses of the redox potential of NADPH. In prolonged ischemia — in which mitochondria are de-energised and ATP is no longer available — trans-dehydrogenation is inhibited also in the presence of an excessive depletion of NADPH [27]. Trans-hydrogenase can also be reversibly inactivated by GSSG [28];

(iv) an inadequate supply through NADP-linked substrates such as isocitrate, glutamate and malate. The block of the Krebs cycle for the anaerobic conditions is likely to affect many of these systems during severe ischemia [29]. Conversely, it is well known that a net production of alanine occurs in oxygen-deprived myocardium and a NADP-linked malic enzyme can be involved in the reactions leading to amino acid synthesis [30]. Even in the case of alanine, the net production is in the range of nmol/mg protein/h: therefore, it is unlikely that it can have an impact on the NADPH or thiols systems which are in the millimolar range;

(v) activation of NADPH-consuming pathways might also be of relevance. NADPH utilisation by energy-requiring biosynthetic processes is indeed blocked during ischemia and there is limited knowledge on other NADPH-linked reactions during progression of myocardial ischemic damage. One of such possibilities is the polyol pathway
whose inhibition during ischemia was hypothesised to be protective [31].

4.2. What can be the possible consequences of NADPH synthesis impairment?

Under conditions of impaired NADPH production, the possible utilisation of the cofactor can remain unbalanced. There is clear evidence that a highly-reduced state of NADP and of glutathione redox couples exerts a major protective function [32,33]. In particular, glutathione is involved in the protection against free-radical-induced damage, through the action of glutathione peroxidase, which maintains protein-bound sulfhydryl functions under reduced state or it regulates the function of specific enzymes by the formation of mixed disulphides.

In previous work [5–7,9], we have addressed the issue of cellular glutathione and thiol status during myocardial ischemia and reperfusion damage. We have demonstrated that ischemia induces a reduction of tissue GSH and protein sulphhydryl groups without accumulation of GSSG, and a specific decrease of the enzymatic activity of Mn-superoxide dismutase. Therefore, the defence mechanisms against oxidative stress, i.e. direct detoxification and GSH system, are both impaired. The readmission of oxygen during reperfusion can exert a toxic effect, inducing oxidative damage which is also demonstrated by tissue accumulation and release of GSSG. This pattern of changes has been consistently confirmed in vitro and in vivo, in different pathophysiological conditions [34,35], even in humans [36].

The results of the present study further extend our knowledge on the changes of the thiol redox state occurring during ischemia and reperfusion for two different aspects, those related to pyridine nucleotide abnormalities, and the ones related to the formation of mixed disulphides.

In view of these observations, the decrease in NADPH may contribute to explain the depletion of GSH and total-SH observed in ischemia that was difficult to justify considering the accumulation of protons and of reducing equivalents during the ischemic period.

Intracellular sulfhydryls, GSH cycle and NADPH are closely linked as shown in the scheme of Fig. 5. The hexose monophosphate shunt produces, through glucose-6-phosphate oxidation, the reducing equivalents (NADPH) for the action of glutathione reductase. This reaction is at equilibrium under physiologic conditions and does not require energy; therefore, a decrease of NADPH supply will directly affect glutathione status. GSH is then utilised by glutathione peroxidase to form GSSG in the detoxification processes. It follows that the changes of NADPH may affect GSH status, which is also in dynamic equilibrium with all cellular SH groups, known to modulate the activity of a number of proteins and enzymes [37,38].

At this stage, other sources of SH groups — such as sulphhydryl groups in the proteins — may become important for cell defence against oxidative damage, and they are crucial to reconstitute the reduced glutathione via glutathione-S-transferase, leading to mixed disulphide formation [37] (Fig. 4). The role of mixed disulphide formation has not been extensively investigated in the ischemic myocardium: so far, only data obtained during anoxia are available [39]. In the current study, we have demonstrated: (a) an increase of glutathione-bound mixed disulphides, together with a reduction of sulphhydryl cellular pool, already during ischemia; and (b) the occurrence of a true

![Fig. 5. Scheme representing the interdependence of intracellular sulphydrylic pools and pyridinic nucleotide redox couples.](https://academic.oup.com/cardiovascres/article-abstract/47/3/586/345862)
oxidative stress, in the form of increased GSSG accumulation and release, during reperfusion.

These abnormalities can have marked consequences on myocardial function. The involvement of thiol oxidation and/or alteration of redox state in proteins, such as ATPases and channels, is likely to alter cellular ionic homeostasis [40–42], leading to functional alterations, such as the increase of diastolic pressure, as we have observed in our study.

Thiol oxidation is also linked to myocardial dysfunction and impaired viability via the formation of disulphides and mixed disulphides. Thiol oxidation induces alterations of enzymatic activities, such as adenylate cyclase, pyruvate kinase and malate dehydrogenase, as well as alterations of contractile proteins [37,38]. A role of thiol oxidation in the initiation of the apoptotic process has also been hypothesised [43].

5. Possible limitations of the study

We used a model in which the external influences, such as circulating blood cells and the neuroendocrine system, are minimised to study the intracellular events occurring during ischemia and reperfusion. The in vivo conditions may actually be worsened by the influence of these circulating factors, representing sources of reactive oxygen species under pathological conditions. Moreover, our data are limited to conditions where the substrate is solely glucose: actually, this is not the case in vivo where the preferred substrates are fatty acids, at least under normoxic conditions. Nevertheless, much of our knowledge on pyridine nucleotides and on oxidative stress under ischemic and reperfusion conditions has been obtained with the same model.

Another possible limitation is the fact that we have measured total changes of myocardial pyridine nucleotides while the changes we have described might occur in subcellular compartments; this is unavoidable since subcellular fractionation is not possible under conditions which preserve the extremely biologically labile NADPH.

However, this limitation does not represent a major hindrance for the interpretation of the data. Measurement of NADH/NAD redox couple has always been considered to represent changes occurring in the mitochondrial compartment as it is about 80% of the total pool of this form of the coenzyme. On the contrary, the NADPH/NADP couple is considered more represented in the cytosol. Moreover, mitochondrial and cytosolic pyridine nucleotide pools are in an equilibrium controlled by a network of nicotinamide-nucleotide-dependent dehydrogenases interlinked by shared substrates and by selective permeability of the mitochondrial membrane for the metabolites involved. Thus, the carefully controlled mechanisms — which equilibrate the pools according to the nucleotide concentrations — render the total measurement a reliable index of the changes of pyridine nucleotide redox couples.

6. Conclusions

The results of this study show that, under severe low-flow ischemia, myocardial NADPH levels can decrease despite the accumulation of reducing equivalents (NADH). These changes are likely to affect the cellular thiol redox state. These abnormalities may have important consequences on cellular function and viability and are in keeping with the pathophysiologic role of oxidative stress in the progression of ischemia and reperfusion heart damage.

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