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Relation between energy metabolism, glycolysis, noradrenaline release and duration of ischemia

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Abstract

We studied the effect of 12–36 min of global ischemia followed by 36 min of reperfusion in Langendorff perfused rabbit hearts (n = 26). Metabolism was determined in terms of peak and total release of purines (adenosine, inosine, hypoxanthine), lactate and noradrenaline during reperfusion; and myocardial content of nucleotides (ATP, ADP, AMP), glycogen and noradrenaline at the end of reperfusion. An inverse relationship (r = –0.79) existed between duration of ischemia and developed pressure post-ischemia. Early during reperfusion, after 12 min of ischemia, the purine concentration (peak release) increased 100× ($p < 0.01$), that of lactate and noradrenaline 10× ($p < 0.05$) . Total purine release rose with progression of the ischemic period (30× after 36 min of ischemia; $p < 0.01$), concomitant with a reduction in nucleotide content. Lactate release was independent from the duration of ischemia, although glycogen had declined by 30% ($p < 0.01$) after 36 min of ischemia. The acid insoluble glycogen fraction, which presumably contains proglycogen, increased substantially during short-term ischemia. Peak noradrenaline increased 100× and 200× ($p < 0.05$) after 24 and 36 min of ischemia, respectively. Total noradrenaline release due to various periods of ischemia mirrored its peak release. Function recovery was inversely related to total purine and noradrenaline efflux (both r = –0.81); it correlated with tissue nucleotide content (r = 0.84). In conclusion, larger amounts of noradrenaline are released only after a substantial drop in myocardial ATP. During severe ischemia ATP consumption more than limited ATP production by anaerobic glycolysis, is a key factor affecting recovery on subsequent reperfusion. In contrast to lactate efflux, purine and noradrenaline release are useful markers of ischemic and reperfusion damage. (Mol Cell Biochem **160/161**: 187–194, 1996)

Key words: ATP breakdown. catecholamine, glycogen, ischemia

Introduction

Many critical factors are involved in the onset of severe myocardial ischemic damage. The most important are: (1) loss of tissue adenine nucleotide with consequent failure to restore energy metabolism with reperfusion [1–6]; (2) accumulation of toxic metabolites [7–9]; (3) calcium overload [10]; (4) release of noradrenaline [11–15]. Data about these metabolic alterations, however, derive from a combination of several individual studies employing a variety of different experimental preparations and ischemic protocols. In addition, the analytical techniques utilized for enzymatic determination of catecholamine and of adenine nucleotide often are of low sensitivity [4, 8, 12, 16, 17].

There is therefore the need of systematic data analyzing in details the temporal sequences of the metabolic alterations which occur as ischemia progresses in the light of finding out the more suitable index of ischemic and reperfusion damage.

Isolated perfused heart preparation was selected for the present study because it allows the control of many variables that *in vivo* influence the severity of ischemic damage such as: myocardial perfusion, work load, substrate availability, neurohumoral factors.

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Materials and methods

Heart perfusion

Male New Zealand white rabbits (2.0–2.3 kg), maintained on a standard diet, were used. They were treated in conformity with the guiding principles of the American Physiological Society. The animals were stunned by a blow on the head; the hearts were quickly removed and perfused as previously described [18]. The perfusion solution was heated to 37°C, bubbled with 95% O_2 and 5% CO_2 and transported at a rate of 22 ml/min to the aortic cannula with a Gilson Minipuls 2 rotary pump. The hearts were jacketed (40–44°C) to provide a constant myocardial temperature of 37°C, independent of coronary flow, checked by an Ellab thermometric probe (model CTD 85) in the pulmonary artery. They were paced using suprathreshold rectangular pulses at 0.1 ms duration at a rate of 180 beats/min.

After a 30-min period of aerobic equilibration (22 ml/min), the hearts were randomly divided into four groups: Control, these hearts (n = 7) were aerobically perfused for a further period of 60 min (no ischemia); the other hearts were made ischemic by stopping the coronary flow for: (A) 12 min (n = 7); (B) 24 min (n = 6); (C) 36 min (n = 6). At the end of ischemia, coronary flow was reestablished and all ischemic hearts were reperfused for 36 min. Left ventricular pressure was determined from a fluid-filled balloon inserted into the ventricular cavity as previously described [18].

Biochemical assays

Analysis of coronary effluent

Coronary effluent fractions were collected in cooled vials (0°C) at regular intervals during the entire period of reperfusion for purine, catecholamine and lactate determinations. Two ml of perfusate were added to 30 µl 10 mM $Na_2S_2O_5$ for catecholamine assay. Another aliquot (0.5 ml) was added to 1.0 ml $HClO_4$ (6%) for the assay of lactate. Treated and untreated samples were stored at –80°C. Purines were determined with our reverse-phase high-pressure liquid chromatography (HPLC) method with detection at 254 nm [19, 20]. Noradrenaline was determined by reverse-phase HPLC with electrochemical detection on perfusate extracted with Al_2O_3 as previously described by us [21]. L-lactate was enzymatically determined by the method of Noll [22].

Tissue determinations

At the end of the experiments, the hearts were clamped with precooled Wollenberger tongs and stored in liquid nitrogen. The frozen tissue was homogenized with $HClO_4$. The mixture was thawed and centrifuged; an aliquot of the supernatant fraction was neutralized with KOH, and analyzed for nucleotide and catecholamine content. Nucleotides were determined by HPLC using the method of Harmsen *et al.* [23]. After Al_2O_3 extraction, noradrenaline was assayed as described above. Another aliquot was neutralized with NaOH, and analyzed enzymatically for glycogen content [24, 25], using rabbit-liver glycogen as the standard.

In the acid precipitate, protein and glycogen were assayed. Protein was measured according to Bradford [26] using bovine serum albumine as the standard. A factor of 70 mg protein/g wet weight was used to express release data, based on heart weight, per gram protein. Glycogen was determined as described before [24, 25].

Statistics

Data are reported as mean ± S.E.M. A one-way analysis of variance was first carried out to test for any differences between all groups. If a difference was established, each of the groups was compared with the control group (aerobic control), using the unpaired *t*-test with Bonferroni correction. $p < 0.05$ was considered significant.

Results

Mechanical changes

During 60 min of control aerobic perfusion, mechanical function was well maintained since developed pressure declined only by 15% (n.s.). The recovered pressures, measured at the end of reperfusion after different ischemic periods are depicted in Fig. 1. Compared with the control group, there was a complete recovery of developed pressure in group A, while, with prolongation of ischemia, it significantly decreased in groups B and C (to 66% and 38% of control, respectively; $p < 0.01$, see Fig. 1). There was an inverse relationship ($y = 61.69–1.04x$; $r = –0.79$, $p < 0.001$) between the duration of ischemic period and the recovered pressure during reperfusion.

Diastolic pressure in the control hearts remained 0 mmHg during the whole time of perfusion. The same was true for the hearts in group A. However, in groups B and C it rose to 24 ± 11 and 44 ± 14 mmHg, respectively ($p < 0.001$ vs control, at the end of reperfusion) (Fig.1).

Metabolite release

When the hearts were perfused under aerobic conditions, small amounts of purine (adenosine, inosine and hypoxanthine), noradrenaline and lactate were present in coronary

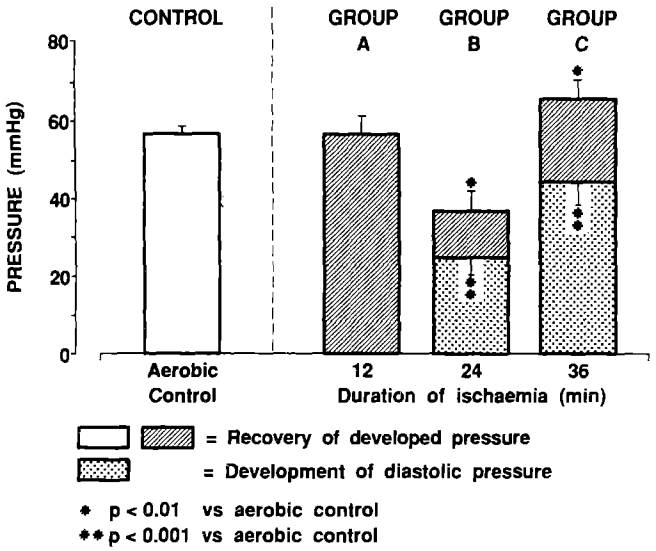

Fig. 1. Recovery of developed pressure and development of diastolic pressure after various periods of ischemia: decline in mechanical recovery and increment in diastolic tension are only evident after prolonged ischemia. For the non-ischemic controls (aerobic perfusion), the 60 min perfusion values were used. Mean values ± S.E.M. (n = 6–7) are depicted. Pre-intervention left ventricular systolic pressure was 63.1 ± 2.5 mmHg and diastolic pressure was 0 mmHg (n = 26).

effluent. Peak release of these metabolites was observed during the first minute of reperfusion (groups A, B and C, Fig. 2). Purine release, which was very low in control hearts (0.044 ± 0.004 μmol/min/g protein), became 100-fold higher after 12 min of ischemia (group A; $p < 0.01$). If the ischemic period was prolonged from 12 to 24 min (group B) it increased and it doubled after 36 min of ischemia (group C, Fig. 2A). The peak releases of individual purines are shown in Table 1. Inosine represented the major component of total purine released in the aerobic period (42%). After various ischemic periods (groups A, B and C), release of all purines increased. In particular, during reperfusion, inosine rose to 63% in group C, at the expense of adenosine.

Peak noradrenaline release (Fig. 2B) rose 10 fold in group A ($p < 0.05$ vs Control) and by an extra two orders of magnitude in groups B and C. Peak lactate release reached already a plateau in group A ($p < 0.01$ vs Control, see Fig. 2C).

Noradrenaline and lactate release declined to baseline in a few minutes (4 min for group B) whereas purine release persisted substantially longer (7–8 min for group B; Fig. 3).

Figure 4 shows the total release of the compounds determined in perfusate collected during 36 min of reperfusion. The behaviour observed for purines was quite similar to that described for their peak release (compare Figs. 2A and 4A). Total noradrenaline release in group A was not different from that in control (Fig. 4B), whereas peak release in group A exceeded that in control 10-fold (Fig. 2B). No increment in total lactate release was observed after ischemia in groups A, B and C; all values were comparable to those obtained for

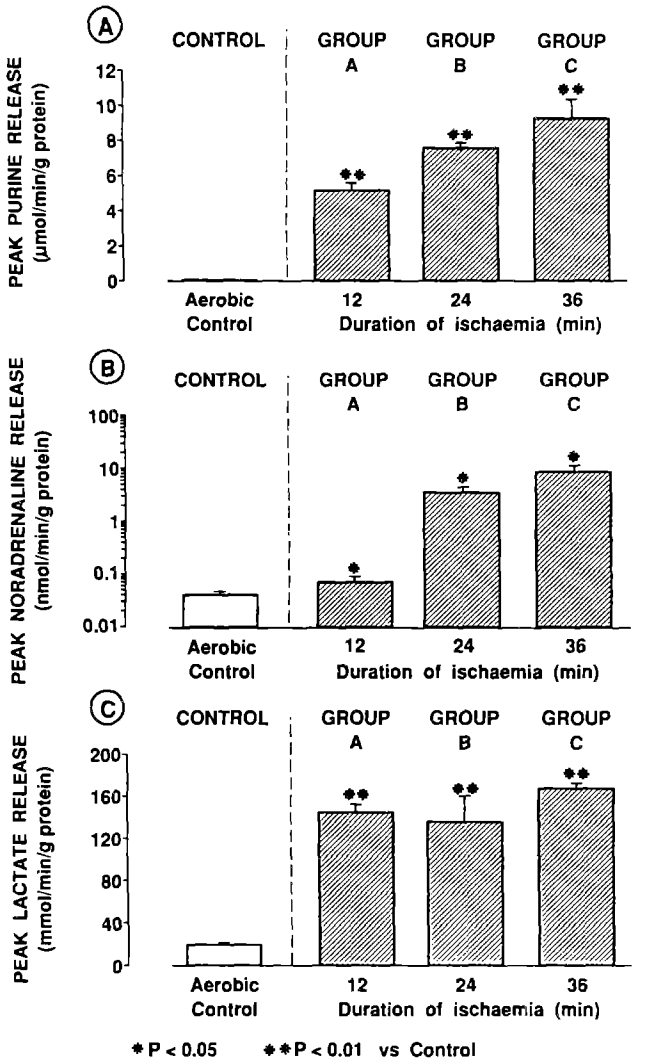

Fig. 2. Differences in peak release pattern of purines, noradrenaline and lactate after various periods of ischemia. Purines comprise adenosine, inosine and hypoxanthine. Please, note that the catecholamine data are given on a logarithmic scale. For other details, see legend to Fig. 1.

aerobic control perfusion (Fig. 4C), contrasting the behaviour of peak lactate release (Fig. 2C).

Tissue changes

The myocardial content of nucleotides (sum of ATP, ADP, and AMP), noradrenaline and glycogen is shown in Fig. 5. In groups A, B and C, total nucleotide content decreased ($p < 0.01$) compared with aerobic control (to 76%, 43% and 21%, respectively, see Fig. 5A). The ATP, ADP and AMP levels are specified in Table 1. There was a significant decline in ATP and ADP levels, proportional with the duration of the ischemic period. In contrast, AMP content decreased in group A ($p < 0.05$), and increased in groups B ($p < 0.01$) and C (n.s.).

Table 1. Purine release as well as nucleotide content after various periods of ischemia

Variable	Aerobic control	Ischemic period (min)		
		12 (Group A)	24 (Group B)	36 (Group C)
Peak release (μmol/min/g protein)				
Adenosine	0.015 ± 0.002	0.89 ± 0.12	0.92 ± 0.28	1.30 ± 0.70
Inosine	0.020 ± 0.002	3.09 ± 0.26**	4.82 ± 0.53**	5.84 ± 0.84**
Hypoxanthine	0.009 ± 0.001	0.84 ± 0.06**	1.87 ± 0.15**	2.14 ± 0.11 **
Total release (μmol/36'/g protein)				
Adenosine	0.49 ± 0.07	1.29 ± 0.15*	3.14 ± 0.71**	3.26 ± 0.63**
Inosine	0.76 ± 0.09	6.19 ± 0.34**	15.11 ± 1.76**	19.08 ± 2.55*
Hypoxanthine	0.37 ± 0.03	1.55 ± 0.08**	4.75 ± 0.41**	5.87 ± 0.52**
Tissue content (μmol/g protein)				
ATP	35.2 ± 1.5	27.2 ± 1.3	13.3 ± 2.2**	5.12 ± 1.04**
ADP	5.94 ± 0.31	4.35 ± 0.12**	3.07 ± 0.13**	2.22 ± 0.21 **
AMP	1.01 ± 0.11	0.52 ± 0.06*	1.81 ± 0.12**	1.16 ± 0.23
Energy charge	0.905 ± 0.005	0.915 ± 0.004	0.804 ± 0.021**	0.675 ± 0.044**

The values pertain to the 36-min reperfusion period, either early (peak release), over the whole period (total release), or to the end (myocardial content). The data show major ATP breakdown after the longer periods of ischemia. Energy charge is [(ATP) + 0.5 (ADP)]/[(ATP)+(ADP)+(AMP)]. Mean ± S.E.M.; n = 5–7. *$p < 0.05$, **$p < 0.01$ vs control values

Besides myocardial nucleotide content, the phosphocreatine (PCr) tissue level was determined. In the control group, it amounted to 56.3 ± 3.4 μmol/g protein at the end of experiment. No significant differences were observed due to ischemia/reperfusion.

Energy-charge data are useful to complete the view on myocardial energetic status in the reperfused hearts after various ischemic periods. The aerobic control value was 0.905 ± 0.005. Only in groups B and C energy charge decreased, to 88% and 75% with respect to control ($p < 0.01$, Table 1).

Panels B and C of Fig. 5 depict the noradrenaline and glycogen content. They show a decrease with the prolongation of the ischemic period from 12 min to 24 and 36 min. The decrements in noradrenaline content were not statistically significant. Total glycogen decreased to 49% and 30% in groups B and C, respectively ($p < 0.001$ vs Control, Fig . 5C). Acid-insoluble glycogen increased from 7.3 ± 0.4 mg/g protein in the control group to 17.1 ± 1.6 (group A, $p < 0.001$), 12.3 ± 1.9 (group B, $p < 0.05$) and 7.7 ± 1.3 mg/g protein (group C, n.s.)

Correlation between function and metabolism

The linear correlations between the recovery of developed pressure upon reperfusion and metabolite release (peak and total) as well as tissue content are reported in Table 2. A linear inverse and significant relationship existed between recovery of function and peak release of purines, noradrenaline and lactate, although in the last case, the correlation coefficient is low. The myocardial nucleotide and glycogen contents correlated strongly with developed pressure. Total lactate release and noradrenaline tissue content exhibited no linear relation with recovered mechanical function.

Discussion

Energy metabolism

We observed, in line with literature [6, 27–29], that reperfusion was unable to restore basal levels of cardiac adenine nucleotide content (Fig. 5A). This is especially true for ATP stores (see Table 1). In contrast to ATP, phosphocreatine was resynthetized during reperfusion. The finding of a declined AMP content in the hearts reperfused after 12 min of ischemia could be explained with the increased AMP dephosphorylation which occurs in anoxic and ischemic conditions. On the other hand, more severe ischemia causes large ATP catabolism with a probable congestion of the breakdown pattern and consequent AMP accumulation. More specifically, this accumulation could be due to increased cytosolic Pi which inhibits the activity of 5'-nucleotidase, the enzyme responsible for cardiac adenosine production [30].

Our data reveal that the lacking recovery in tissue adenine nucleotides, compared with aerobic levels, was balanced with purines lost during the reperfusion period (Table 1). This indicates that a portion of ATP, consumed during ischemia,

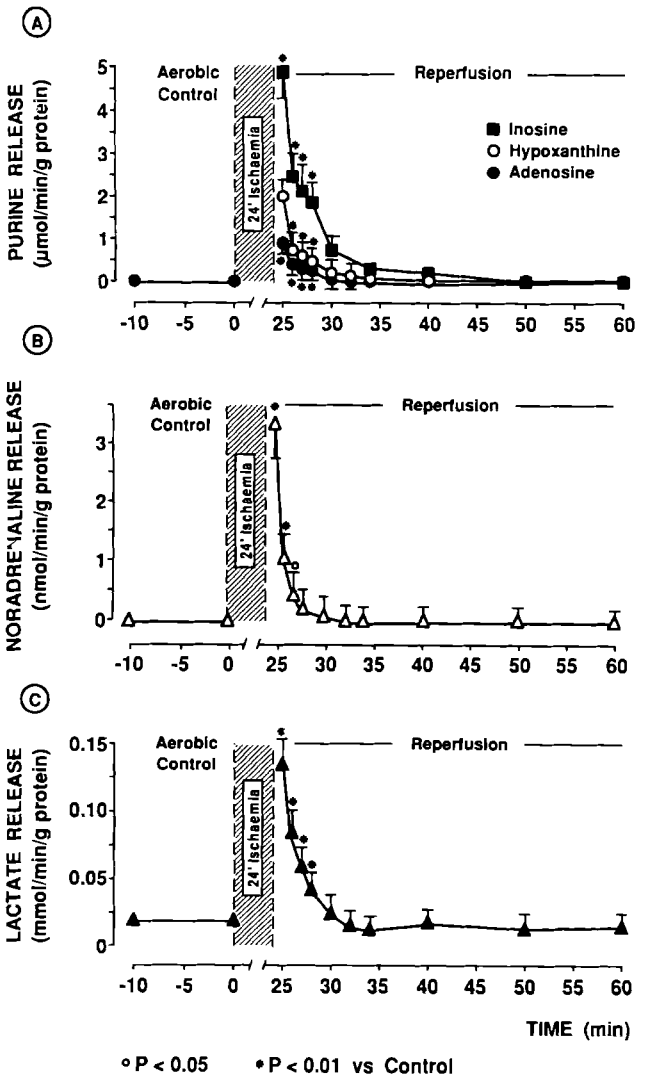

Fig. 3. Time course of metabolite release after 24 min of ischemia. The efflux of noradrenaline and lactate is already back to baseline in 4 min, when purine release is still elevated. For other details, see legend to Fig. 1.

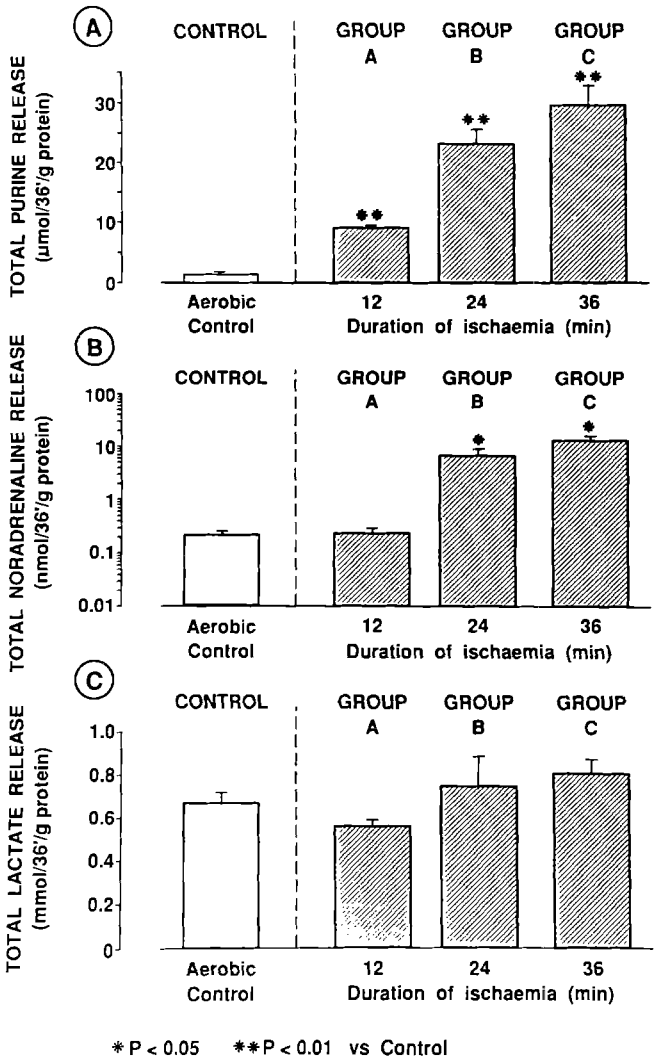

Fig. 4. Differences in total release pattern of purines, noradrenaline and lactate after various periods of ischemia. Depicted are the amounts released during 36 min of reperfusion. Purines are released in proportion with duration of ischemia. Major noradrenaline release takes place only after the longer periods of ischemia, but no-flow does not affect total lactate release. For other details, see legends to Figs. 1 and 2.

was not only degradated to ADP and AMP, but catabolized to adenosine, inosine and hypoxanthine which were immediately released from myocytes. Further degradation of hypoxanthine does not take place in rabbit heart [31].

After exhaustive ATP depletion during total ischemia [32], ATP levels can be partly restored during reperfusion from ADP by oxidative phosphorylation. But ATP resynthesis by the purine salvage pathway is too slow to play an important role in a relatively brief reperfusion period [6, 28, 29]. These data suggest that ATP degradation to purines during ischemia could slow high-energy phosphate restoration during reperfusion with delayed mechanical and metabolic cardiac recovery (stunned myocardium; ref. [27]). Our findings also emphasize the specificity of purine release to characterize the occurrence of myocardial ischemic conditions [33], where lactate release seems to be inadequate as a marker (Figs. 2 and 4).

Noradrenaline release and tissue content

The study of noradrenaline release rate is of relevance in oxygen deprivation conditions as already evidenced by the pionieristic work of Wollenberger A. *et al.* [34]. We found a large noradrenaline release at the first minute of reperfusion (peak release) after 12, 24 or 36 min of ischemia (Figs. 2B and 3B). This noradrenaline efflux represents washout of the neurotransmitter accumulated within the extracellular space during the ischemic period [14, 35, 36]. We observed also an increment of noradrenaline release during the whole reperfusion period (total release), after 24 and 36 min of

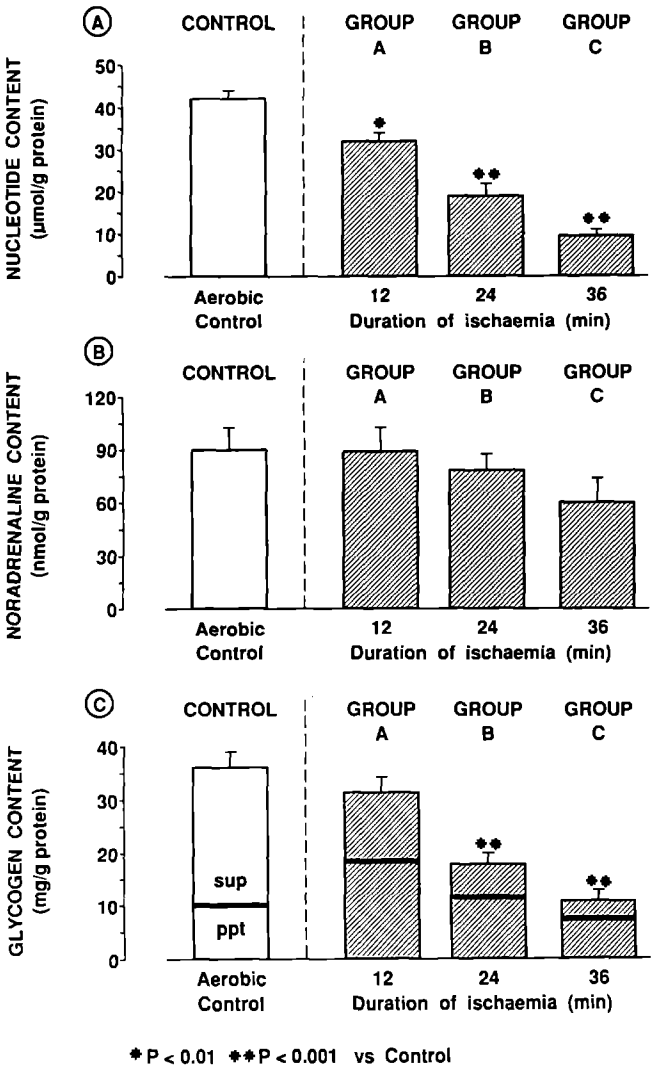

Fig. 5. Decline in tissue nucleotide, noradrenaline, and glycogen content after various periods of ischemia. Adenine nucleotides comprise ATP, ADP and AMP. Glycogen found in the acid-soluble (sup) and acid-insoluble (ppt) fractions is given in the upper and lower portions of the bars, respectively. The ratio sup/ppt in the post-ischemic groups differed from Control ($p < 0.001$). The data show substantial nucleotide and glycogen (acid-soluble) breakdown with increased duration of ischemia, with minor changes in noradrenaline content. The shorter period of ischemia induces an increase in the acid-insoluble glycogen fraction, which presumably comprises proglycogen. For other details, see legend to Fig. 1.

ischemia (Fig. 4B), and a balanced, progressive, but not significant, decline in myocardial noradrenaline content (Fig. 5B). On the other hand, it should be recalled that noradrenaline released during reperfusion represents only a small portion, from 0.1–14%, of the basal tissue content.

Based on the positive relationship between total noradrenaline release and total purine release ($r = 0.80$, $p < 0.001$) and on the inverse correlation with ATP myocardial levels ($r = -0.75$, $p < 0.001$) and glycogen content ($r = -0.71$, $p < 0.001$), we propose the following influence of endogenous noradrenaline on energetic and carbohydrate metabolism during myocardial ischemia. Released noradrenaline stimulates glycogenolysis by cyclic-AMP formation and activation of phosphorylases, as earlier suggested [13, 37]. Metz and Bernauer [16] hypothesized that noradrenaline release is not essential for the activation of anaerobic glycogenolysis, but is important for stimulating anaerobic glycolysis [14]. However, these authors found no evidence for an alternative regulating mechanism during ischemia [16].

Our data also suggest that cardiac noradrenaline, e.g. by activation of a phosphatase such as mitochondrial ATPase, could affect breakdown of ATP stores, exacerbating the ischemic consumption of high-energy phosphates. However, little is known about the effect of noradrenaline on metabolism during myocardial ischemia, in contrast to the great number of studies on its arrhythmogenic and inotropic effects upon reperfusion [11, 13–15). In the light of these novel data and observation, the metabolic role of noradrenaline released during ischemia deserves more attention. As a marker of ischemia, it is perhaps only useful after longer periods of ischemia (cf. Figs. 2B and 4B).

Glycolytic metabolism

The glycolytic pathway is profoundly changed by ischemia. In the early phase of ischemia, glycogenolysis is stimulated by local catecholamine release [13]; phosphofructokinase is activated by an increase in ADP, AMP and Pi levels and a contemporary reduction in ATP content [37]. As a result, anaerobic glycolysis is accelerated, with ATP production and lactate accumulation [5]. We have studied alterations in gly-

Table 2. Correlation between function recovery and metabolic variables after various periods of ischemia

	Peak Concentration (per g protein)			Release (per 36′/g protein)			Tissue Content (per g protein)		
	Purine (μmol/)	NorA (nmol/)	Lactate (mmol/)	Purine (μmol/)	NorA (nmol/)	Lactate (mmol/)	Nucl (μmol/)	NorA (nmol/)	Glycogen (mg/)
vs Developed pressure	r –0.66	–0.81	–0.41	–0.81	–0.81	–0.32	0.84	0.21	0.84
	p < 0.001	< 0.001	< 0.05	< 0.001	< 0.001	n.s.	< 0.001	n.s.	< 0.001

Calculated are the correlation coefficients and their p-values for developed pressure vs peak and total release of metabolites, and vs tissue compounds. Function data, obtained at the end of normoxic control perfusion and after 36 min of reperfusion were used. Mean ± S.E.M. (n = 24–26). NorA = noradrenaline, Nucl = adenine nucleotides

colytic metabolism, determining myocardial glycogen content and lactate release upon reperfusion. In agreement with the literature [17], our results show that, after reperfusion, the glycogen content was reduced proportionally with the duration of ischemia. Furthermore, myocardial glycogen levels were closely correlated with the recovery of developed pressure after different ischemic periods (Table 2). We expected lactate release during reperfusion to be correlated with glycogen consumption. During 36 min of reperfusion, we observed that all reperfused hearts maintained a total lactate release similar to control hearts (Fig. 4C). However, at the first minute of reperfusion, all treated groups showed a high lactate release, compared with aerobic release. These peak releases were independent of the period of ischemia (12–36 min, Fig. 2C). Increased peak release is in apparent contrast with correspondent unchanged total release (cf. Fig. 2C and 4C), but is explained by lower-than-baseline lactate release after the early minutes of reperfusion (Fig. 3C).

We want to underline that lactate washout is independent from the duration of ischemia. Possible mechanisms include either a saturable lactate export from the myocardial cells or an inhibition of anaerobic glycolysis, without a stop in glycogenolysis. The first hypothesis is unproved because lactate accumulating in tissue was released approximately in proportion to the tissue concentration [38]. The latter hypothesis is supported by several studies: when ischemia is prolonged, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase are inhibited by excess NADH, acidosis and lactate accumulation [39–42]. Therefore, lactate production stops while other glycolytic products accumulate in myocytes, such as glucose-1-phosphate and glucose-6-phosphate [5, 41, 43, 44]. For this reason lactate as a quantitative marker for myocardial ischemia is unacceptable (see also Figs. 2C and 4C). Apstein *et al.* [45] already pointed at the limitations of lactate production as an index of myocardial ischemia.

A novel observation is the substantial increase in acid-insoluble glycogen due to 12 or 24 min of ischemia, followed by reperfusion. This fraction presumably comprises of proglycogen, a low-molecular-weight form of glycogen with the protein glycogenin attached to it [46]. Glycogenin primes glycogen biogenesis [47]. Further investigations have to substantiate that proglycogenin, described for skeletal muscle [46], does indeed occur in myocardial tissue, where it may be affected by ischemia/reperfusion.

Conclusion

Our data demonstrate that: (1) impaired restoration of myocardial function during reperfusion is correlated with a large loss of purines and consequent delay to reestablish adequate myocardial energy metabolism; (2) ATP production by anaerobic glycolysis is independent from the duration of ischemia; (3) cardiac noradrenaline release is possibly involved in aggravating energy waste during ischemia. These findings suggest that manipulations which increase energy saving during ischemia [5] or that inhibit purine release, with accelerated repletion of ATP stores on reperfusion [19, 29], are useful to improve mechanical function during post-ischemic reperfusion. In contrast to lactate release, purine and possibly noradrenaline release are useful markers of ischemic damage.

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