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Cyclic GMP in the perfused rat heart

Effect of ischaemia, anoxia and nitric oxide synthase inhibitor

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Abstract

Working rat hearts perfused with 5.5 mM glucose were submitted to a 10-min period of no-flow ischaemia or anoxia. Both conditions stimulated glycogenolysis, activated phosphorylase and increased cyclic GMP content, although the time course of these changes differed in anoxia and ischaemia. Changes in cyclic GMP content were not correlated with glycogenolysis or phosphorylase activation. Perfusion with 1 μM L-nitroarginine methylester, an inhibitor of nitric oxide synthase, decreased cGMP concentration under normoxic conditions and abolished the ischaemia-induced increase in cGMP. The inhibitor decreased the coronary flow without affecting the overall working performance of the hearts under normoxic conditions.

Key words: Cyclic AMP; Cyclic GMP; Ischaemia; Glycogen; Heart; Nitric oxide

1. Introduction

Under normal conditions, the energy consumed by the heart is provided by the oxidation of substrates that are taken up from the circulation, so that the endogenous stores of triglycerides and glycogen are not used for energy production [1–3]. In contrast, glycogen becomes a predominant source of energy during ischaemia or hypoxia when myocardium shifts to anaerobic glycolysis for its energy supply [2,3]. This shift, the well-known 'Pasteur effect', results from changes in the concentration of metabolic regulators such as adenine nucleotides and inorganic phosphate that act at the level of glycogen phosphorylase and 6-phosphofructo-1-kinase, a key enzyme of glycolysis [4,5]. Second messengers such as cyclic AMP (cAMP), inositol 1,4,5-trisphosphate (IP_3), and calcium may play a role in this short-term metabolic adaptation. For instance, the effects of cAMP or calcium on glycogen breakdown and glycogen phosphorylase activation have been studied in hypoxic myocardium [6–8].

To the best of our knowledge, nothing is known about changes in the content of cardiac cyclic GMP (cGMP) during ischaemia. If present, such modifications could have significant physiological and metabolic consequences, because cGMP is known to regulate cAMP phosphodiesterase [9,10]. cGMP could also control, via the cGMP dependent protein kinase, the intracellular calcium concentration and the phosphorylation state of several proteins [9,11,12].

In this work, we studied the variations in cAMP, cGMP, and IP_3 concentration in rat hearts submitted to ischaemia or anoxia, and we compared these variations to the time course of glycogen breakdown and the accumulation of glycolytic intermediates.

2. Experimental

2.1. Perfusion protocol

Hearts from male Wistar fed rats (180–250 g) were perfused under 'working' conditions [13,14] with a preload of 10 cm water and an afterload of 90 cm water. The hearts were perfused at 37°C with a Krebs–Henseleit buffer containing 5.5 mM glucose as the sole substrate, and in equilibrium with a 95% O_2/5% CO_2gas phase. After 15 min of perfusion, the hearts were submitted to either a global no-flow ischaemia, by clamping the perfusate supply to the hearts, or a period of anoxia, by perfusing the same medium in equilibrium with 95% N_2/5% CO_2gas phase. The hearts were freeze-clamped at the times indicated. Heart rate and aortic pressure were continuously monitored on a Gould recorder via a Statham transducer connected to a side-arm of the aortic cannula. Aortic and coronary outputs were measured with a graduated glass cylinder. The work produced by the heart was calculated from the product of the cardiac output (in kilograms) and the afterload (in meters) and was expressed by unit of time as the hydraulic power ($kg \cdot m \cdot min^{-1}$) [13].

2.2. Analytical procedures

ATP [15], hexose 6-phosphates (i.e. the sum of glucose 6-phosphate and fructose 6-phosphate) [16] and fructose 1,6-bisphosphate (Fru-1,6-P_2) [17] were measured enzymatically in neutralized perchloric acid extracts. Fructose 2,6-bisphosphate (Fru-2,6-P_2) was measured in alkaline extracts [18]. Glycogen content was measured enzymatically in an alkaline extract and expressed as glucose equivalents [19]. cAMP,

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Abbreviations: ATP, adenosine triphosphate; cAMP, 3′,5′-cyclic adenosine monophosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; IP_3, inositol trisphosphate.

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cGMP and IP_3 were measured by immunoassays with the correponding assay kits purchased from Amersham, UK. Glucose phosphorylation and glycolysis were evaluated by the rate of detritiation of [2-^{3}H]- and [3-^{3}H]glucose, respectively [20,21]. The active form of glycogen phosphorylase (phosphorylase *a*) was measured at 25°C as described [19]. One unit of enzyme activity corresponds to the formation of 1 μmol of product per min under the assay conditions.

2.3. Statistical analysis

All the values are means ± S.E.M. for the number of different preparations given in the legends, and are expressed per g of dry weight (g.d.w.). Unpaired two-tailed Student's *t*-test was used to determine statistically significant ($P < 0.05$) differences between groups.

3. Results

3.1. Comparison of anoxia and ischaemia

A comparison was first made of the effects of ischaemia and anoxia on glycogen breakdown (Fig. 1A), glycogen phosphorylase activation (Fig. 1B) and cyclic nucleotides concentration (Fig. 2). Both ischaemia and anoxia stimulated glycogen breakdown with an average rate of about 6 $\mu mol \cdot min^{-1} \cdot g^{-1}$d.w. The rate of glycogenolysis was faster during the first 5 min of anoxia than during the corresponding period of ischaemia. Accordingly, glycogen phosphorylase was activated more rapidly in anoxia than in ischaemia (Fig. 1B). However, this activation was transient in anoxia, whereas it was slower and persisted during the 10 min period of ischaemia.

cAMP increased within 1 min of ischaemia (Fig. 2A). This increase was transient and cAMP returned to control values within 2 min, suggesting that the maximal activation of phosphorylase reached at 4 min of ischaemia and onwards (Fig. 1B) cannot be attributed to a cAMP-dependent mechanism. In anoxia, no significant changes in the concentration of cAMP could be detected (Fig. 2B). Therefore, and in general agreement with previous report [22], phosphorylase activation does not seem to be dependent on cAMP in anoxia as in ischaemia. Messengers other than cAMP should thus be involved for the activation of phosphorylase, and, as already suggested [23], Ca^{2+} is a possible candidate. IP_3 is probably not involved in Ca^{2+} mobilization under these conditions, because its concentration was 4.2 ± 0.05 pmol/g under normoxic conditions and remained unaltered during ischaemia.

The changes in cGMP content were also measured and Fig. 2A shows that cGMP increased within 1 min of ischaemia and remained elevated during the 10 min period of ischaemia. In anoxia, no significant change in the concentration of cGMP could be detected at 2 and 4 min, and the concentration of cGMP became elevated only towards the end of the anoxic period (Fig. 2B).

The concentration of some glycolytic intermediates was also compared in ischaemic and anoxic hearts (Table 1). The concentration of hexose 6-phosphates increased almost 3-fold during ischaemia, whereas it slightly decreased during anoxia, with the lowest values found at 5 min. The concentration of Fru-1,6-P_2 increased during both ischaemia and anoxia; however, the changes were larger in anoxia than in ischaemia. In agreement with previous work [14], the concentration of Fru-2,6-P_2, a regulator of heart glycolysis under aerobic conditions, was not different in ischaemic and anoxic hearts (average value: 14 ± 2 nmol/g.d.w.) and did not differ from the values found in normoxic hearts, thus ruling out Fru-2,6-P_2 as a key element in the stimulation of heart glycolysis during ischaemia and anoxia. No difference between ischaemia and anoxia was found in the fall of ATP concentrations.

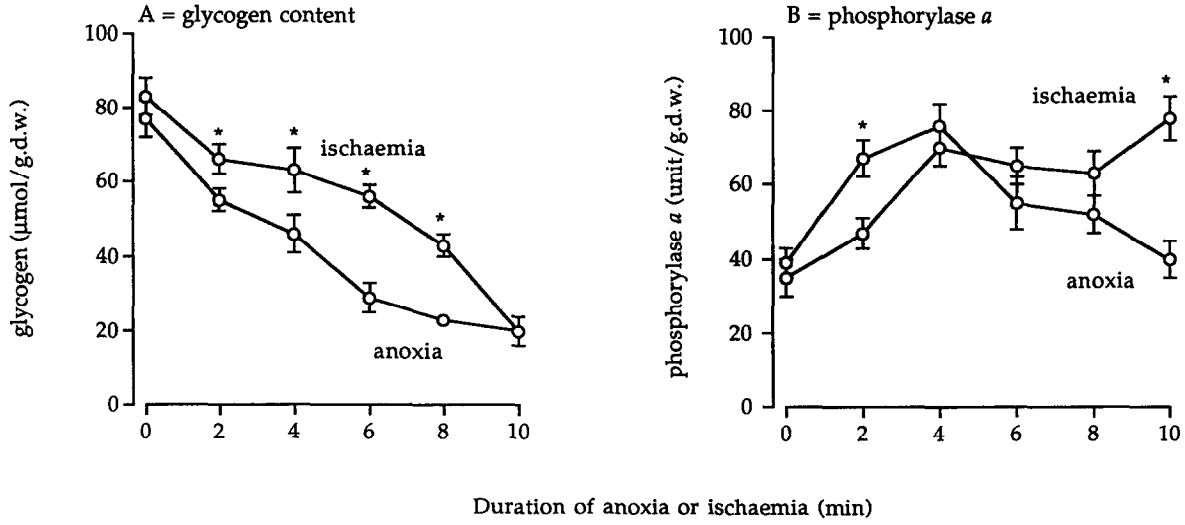

Fig. 1. Time course of glycogen breakdown (A) and phosphorylase activation (B) in rat hearts submitted to ischaemia or anoxia. The hearts were perfused for 15 min under normoxic conditions before ischaemia or anoxia. They were freeze-clamped after the indicated periods of ischaemia or anoxia. The values are means ± S.E.M. for at least five different preparations. *Statistically significant difference between ischaemia and anoxia at the same time point.

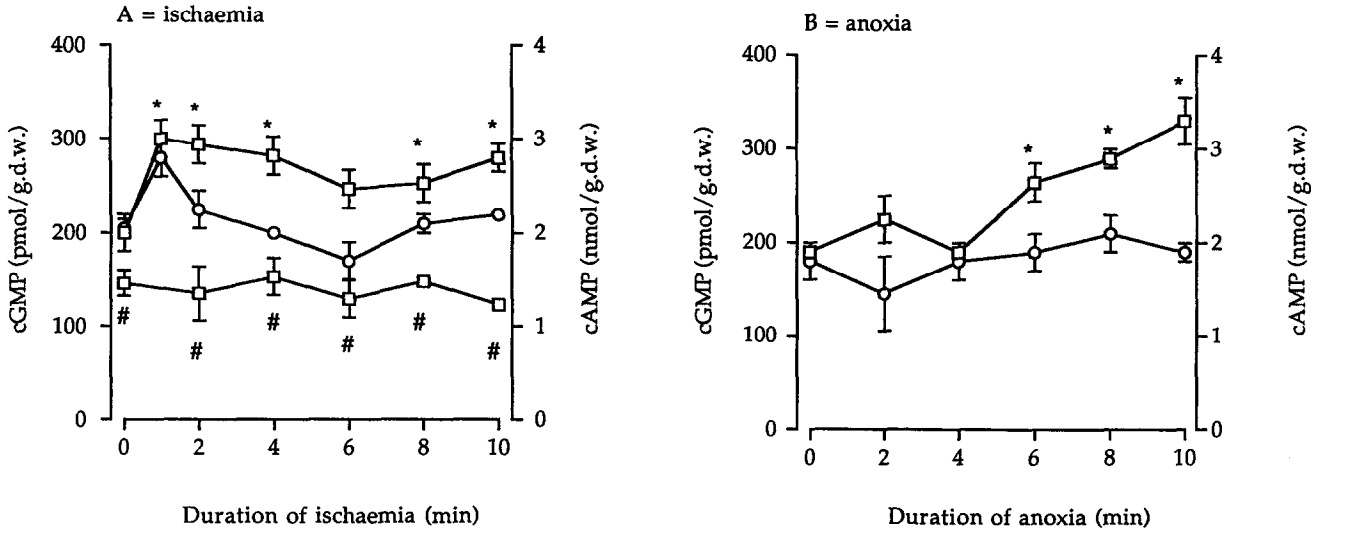

Fig. 2. Time course of the changes in cAMP and cGMP concentration in rat hearts submitted to ischaemia (A) or anoxia (B). The hearts were perfused for 15 min under normoxic conditions before ischaemia or anoxia. The hearts were freeze-clamped after the indicated periods of ischaemia or anoxia. cAMP (open circles) and cGMP (open squares, control hearts; filled squares, hearts with L-NAME) contents were measured. Some hearts were perfused for 5 min before ischaemia with 1 μM L-NAME. The values are means ± S.E.M. for at least five different preparations. *Statistically significant different from the corresponding value at 0 min. #Statistically significant different from the corresponding values without L-NAME.

3.2. *Effects of L-nitroarginine methylester in ischaemia and normoxia*

Nitric oxide (NO) is known to increase the concentration of cGMP in certain tissues [24]. Therefore, we investigated whether the ischaemia-induced increase in cGMP content could be abolished by inhibition of NO synthase. Hearts were perfused with 1 μM L-nitroarginine methylester (L-NAME), a specific inhibitor of NO synthase [25], which was added to the perfusion medium 5 min before the onset of ischaemia. Fig. 2A shows that the inhibitor not only decreased the normoxic values of cGMP content but also that it completely prevented the ischaemia-induced increase in cGMP. L-NAME was without effect on the concentration of cyclic AMP (average value: 2.1 ± 0.2 nmol/g.d.w.)

Since NO is known to affect vascular resistance [24], it was important to check whether the function and metabolism of normoxic hearts, as well as the metabolic response to ischaemia were affected by L-NAME. Table 2 shows that, under normoxic condition, L-NAME was without effect on all the functional parameters studied (peak aortic pressure, heart rate, aortic flow, hydraulic power), except for the coronary flow, which was significantly decreased. This is consistent with the known vasoconstrictive effect of inhibitors of the endothelial NO synthase [26]. This also shows that the hearts perfused

Table 1
Concentration of hexose 6-phosphates, fructose 1,6-bisphosphate, ATP and glycogen in hearts submitted to ischemia or anoxia

Conditions	Duration	Hexose-6-P	Fru-1,6-P_2	ATP	Glycogen
	(min)	(μmol/g)	(μmol/g)	(μmol/g)	(μmol/g)
Ischaemia	0	1.05 ± 0.07	0.19 ± 0.02	18 ± 2	83 ± 5
	5	2.73 ± 0.20*	0.25 ± 0.02	14 ± 1*	50 ± 5*
	10	2.75 ± 0.15*	0.23 ± 0.03	8 ± 0.5*	20 ± 4*
Ischaemia with L-NAME	0	1.40 ± 0.20	0.06 ± 0.01#	18 ± 2	78 ± 4
	5	2.65 ± 0.25*	0.11 ± 0.02#	13 ± 2*	58 ± 3*
	10	2.80 ± 0.20*	0.18 ± 0.01*	10 ± 1*	30 ± 4*
Anoxia	0	1.13 ± 0.10	0.21 ± 0.03	20 ± 2	77 ± 7
	5	0.60 ± 0.07*	0.33 ± 0.04*,#	12 ± 1*	43 ± 2*
	10	0.93 ± 0.10*	0.40 ± 0.33*,#	10 ± 1	20 ± 1*

The hearts were perfused for 15 min in normoxic conditions before ischaemia or anoxia. They were freeze-clamped after the indicated periods of ischaemia or anoxia. Some hearts were perfused for 5 min before ischaemia with 1 μM L-NAME. The values are means ± S.E.M. for at least 6 different preparations. *$P < 0.05$, versus 0 min, same treatment; #$P < 0.05$, versus ischaemia alone at the same time.

Table 2
Effects of L-NAME on the function and the metabolism of glucose in the normoxic working rat heart

	Controls	L-NAME
Peak systolic pressure (cm H_2O)	100 ± 10	105 ± 10
Heart rate (beat/min)	280 ± 25	300 ± 15
Aortic flow (ml/min per g)	145 ± 7	160 ± 13
Coronary flow (ml/min per g)	80 ± 7	60 ± 5*
Hydraulic power (kg.m/min per g)	0.202 ± 0.015	0.198 ± 0.020
[2-^{3}H]Glucose detritiated (μmol/min per g)	7.8 ± 0.8	8.0 ± 0.6
[3-^{3}H]Glucose detritiated (μmol/min per g)	7.2 ± 0.8	7.9 ± 0.5
Lactate release (μmol/min per g)	8.0 ± 0.8	8.0 ± 0.9

The hearts were perfused for 15 min in normoxic conditions with or without 1 μM L-NAME. The values are means ± S.E.M. for at least 6 different preparations. *$P < 0.05$ versus control.

with L-NAME displayed a better 'working' efficiency, because the same hydraulic power was performed despite a decreased coronary flow.

Glucose phosphorylation and glycolysis, as measured by the rate of detritiation of [2-^{3}H]- and [3-^{3}H]glucose, respectively, were not significantly affected by perfusion with L-NAME. The addition of L-NAME 5 min before ischaemia seemed to slower the ischaemia-induced glycogenolysis (Table 1) although the difference was not statistically significant. Similarly the activation of phosphorylase was not significantly affected by L-NAME (not shown). However, in the treated group, the concentration of Fru-1,6-$P_2$was lower than in the control group under both normoxic and ischaemic conditions (Table 1). Moreover, the concentration of hexose 6-phosphates under normoxic conditions tended to be slightly larger in the presence of L-NAME than in the controls (Table 1).

L-NAME is not only an inhibitor of NO synthase but has also recently been reported to antagonize muscarinic receptors [27]. Therefore, we tested *N*-nitro-L-arginine, a more specific inhibitor of NO synthase without anticholinergic effects and we compared its effects with those of L-NAME. Table 3 shows that, under normoxic conditions, L-NAME and *N*-nitro-L-arginine decreased cGMP content and the coronary flow to the same extent. However, the addition of 10^{-3} M L-arginine on top of 10^{-6} M L-NAME could prevent the fall in both cGMP concentration and coronary flow that were caused by L-NAME alone. These results indicate that, in our model, L-NAME is indeed acting as an inhibitor of NO synthase.

4. Discussion

4.1. Increase in cGMP during ischaemia and anoxia

This work is the first report of an ischaemia- or anoxia-induced increase in cGMP content in hearts. As to the mechanism responsible for this increase in cGMP content, the inhibitory effect of L-NAME, a known specific inhibitor of NO synthase, suggests that heart cGMP concentration is controlled, at least in part, by NO. Indeed, the ischaemic-induced increase in cGMP was completely prevented by the inhibitor. However, the basal cGMP content was only partly decreased by the inhibitor, and other substances, such as the auricular natriuretic peptide (ANP), are known to influence the concentration of cGMP in heart. The mechanism by which NO synthase is activated in ischaemia remains to be elucidated. One possibility is the alteration of the intracellular Ca^{2+} content during ischaemia. Indeed, it has been demonstrated that the constitutive NO synthase can be activated by Ca^{2+}-dependent mechanism in certain tissues [28]. Although the presence of inducible and constitutive NO synthase has been documented for the heart, little is known about the control of the activity of the constitutive enzyme in this tissue.

4.2. Control of glycolysis by ischaemia, anoxia and L-NAME

Both ischaemia and anoxia are known to stimulate glycolysis [29]. The regulation is exerted mainly at the level of 6-phosphofructo-1-kinase and glucose transport [29]. However, as previously described [29,30], the changes in the concentrations of glycolytic intermediates observed during ischaemia and anoxia differed and might indicate that glycolysis was more stimulated during anoxia, than in ischaemia, where a relative inhibition of glycolysis gradually builts up. This progressive inhibition of glycolysis during severe ischaemia has been proposed to result from lactate accumulation, which supposedly inhibits glyceraldehyde-3-phosphate dehydrogenase, although the molecular mechanism of this inhibition remains unknown [31].

The data presented indicate that the overall metabolism of glucose and glycogen was little affected by L-NAME both under normoxic and ischaemic conditions. However, in the presence of L-NAME, the concen-

Table 3
Effects of L-NAME, N-nitro-L-arginine and L-arginine on cyclic GMP content and coronary flow in the normoxic working rat heart

	cGMP	Coronary flow
	(nmol/g d.w.)	(ml/min per g.d.w.)
Controls	0.21 ± 0.01	86 ± 9
10^{-6} M L-NAME	0.17 ± 0.01*	60 ± 5*
10^{-6} M L-NoArg	0.16 ± 0.01*	65 ± 5*
1 mM L-Arg + L-NAME	0.22 ± 0.01	85 ± 5
1 mM L-Arg	0.24 ± 0.02	87 ± 10

The hearts were perfused for 15 min in normoxic conditions with the indicated additions. The values are means ± S.E.M. for at least 4 different preparations. L-NoArg, *N*-nitro-L-arginine; L-Arg, L-arginine. *$P < 0.05$ versus control.

tration of Fru-1,6-P_2 was decreased compared with the controls. This could result from a regulation of the production, or the disposal, of Fru-1,6-P_2 by either NO or cGMP. It is worth mentioning in this context that NO has been reported to inhibit in vitro glyceraldehyde-3-phosphate dehydrogenase by auto-ADP-ribosylation of the enzyme [32,33]. One may speculate that this inhibition could also occur under certain conditions in vivo. A moderate inhibition of PFK-1 is not excluded because the concentration of hexose 6-phosphates increased slightly in the presence of L-NAME.

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