Inhibition of myocardial glucose uptake by cGMP

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Inhibition of myocardial glucose uptake by cGMP. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1443–H1449. 1998.—Guanosine 3',5'-cyclic monophosphate (cGMP), a second messenger of nitric oxide (NO), regulates myocardial contractility. It is not known whether this effect is accompanied by a change in heart metabolism. We report here the effects of 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP), a cGMP analog, on regulatory steps of glucose metabolism in isolated working rat hearts perfused with glucose as the substrate. When glucose uptake was stimulated by increasing the workload, addition of the cGMP analog totally suppressed this stimulation and accelerated net glycogen breakdown. 8-BrcGMP did not affect pyruvate dehydrogenase activity but activated acetyl-CoA carboxylase, the enzyme that produces malonyl-CoA, an inhibitor of long-chain fatty acid oxidation. To test whether glucose metabolism could also be affected by altering the intracellular concentration of cGMP, we perfused hearts with Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, or with 5-nitroso-N-acetylpenicillamine (SNAP), a NO donor. Perfusion with L-NAME decreased cGMP and increased glucose uptake by 30% whereas perfusion with SNAP resulted in opposite effects. None of these conditions affected adenosine 3',5'-cyclic monophosphate concentration. Limitation of glucose uptake by SNAP or 8-BrcGMP decreased heart work, and this was reversed by adding alternative oxidizable substrates (pyruvate, β-hydroxybutyrate) together with glucose. Therefore, increased NO production decreases myocardial glucose utilization and limits heart work. This effect is mediated by cGMP, which is thus endowed with both physiological and metabolic properties.

MATERIALS AND METHODS

Perfusion protocol. Hearts from fed male Wistar rats (250–280 g; anesthetized with 60 mg/kg pentobarbital sodium i.p.) were perfused in the working mode for 20 min (33). The perfusion medium was a recirculating Krebs-Henseleit buffer (2 mM calcium) in equilibrium with a 95% O2-5% CO2 gas phase, containing 5 mM glucose as sole substrate. Hearts were perfused at two different workloads, namely with 10 cmH2O preload and 60 cmH2O afterload (referred to in the text as "low-load condition") or with 15 cmH2O preload and 120 cmH2O afterload (referred to as "high-load condition"). Previous experiments with the same model have indeed shown that increasing the workload stimulates glucose utilization (12).

The effects of the NO-cGMP pathway on glucose metabolism were investigated at both workloads by two different protocols. In the first protocol, hearts were perfused with 0.1 mM 8-BrcGMP (Sigma). In the second protocol, hearts were perfused with 100 µM Nω-nitro-L-arginine methyl ester (L-NAME, Sigma), an inhibitor of NO synthase, or 50 µM 5-nitroso-N-acetylpenicillamine (SNAP, ICN Laboratories), a NO donor. All substances were added at the beginning of the perfusion.
Physiological parameters (aortic pressure and aortic and coronary outputs) were measured at regular intervals. Heart work was calculated from the product of peak systolic pressure and cardiac output (expressed as hydraulic power in g·min⁻¹ per g of wet wt) (12, 33).

Analytic procedures. At the indicated times, the perfused hearts were freeze-clamped between aluminum blocks pre-cooled in liquid nitrogen. The concentration of glycogen, expressed as glucose equivalents (16) hexose 6-phosphates and fructose 1,6-bisphosphate (3), was measured enzymatically in deproteinized samples. Lactate was measured in perfusate samples (3). cGMP and cAMP were measured by radioimmunoassay (Amersham) (10).

Glucose uptake (i.e., glucose transport and phosphorylation) was estimated by the detritiation rate of [2-³H]glucose (6), whereas the flux through PFK-1 was estimated by the detritiation rate of [3-³H]glucose (17) (both tracers from Amersham). In either case, tracer amounts (2 µCi/100 ml) of tritiated glucose were added at the beginning of the perfusion period. After 5 min of equilibration, perfusate samples were taken every 5 min to separate tritiated water from tritiated glucose by column chromatography (6).

Measurement of enzyme activities. To measure PDH activity, frozen samples (−100 mg) of ventricles were homogenized in 9 vol of a buffer (0.1 M potassium phosphate, 2 mM EDTA, and 1 mM dithiothreitol, pH 7.3) containing 0.1% (wt/vol) Triton X-100 and 50 µl/ml fresh rat serum, and then frozen at −20°C. The homogenates were thawed and centrifuged (3,000 g for 30 s), and the active form was immediately assayed in the supernatant. Total and active PDH were assayed by a coupling reaction with arylamine N-acetyltransferase as described (23). The proportion of PDH in the active form was expressed as a percentage of the total activity, which was determined following incubation of the supernatant with purified pig heart PDH phosphatase and in the presence of 1 mM Ca²⁺ and 25 mM Mg²⁺ (22). Total PDH activity was not affected by the various perfusion protocols and was equal to 4.3 ± 0.2 µmol·min⁻¹·g⁻¹.

For the measurement of ACC activity, frozen samples (−100 mg) of ventricles were homogenized in 3 vol of a buffer containing 50 mM Tris·HCl (pH 7.4), 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM phenyl methanesulfonyl fluoride, 0.1 mM benzamidine, 0.1 mM leupeptin, 1 µg/ml aprotinin, 0.1 mM 1,4-tosylamino-2-phenylethyl-chloro-methyl ketone, and 0.1 mM chloro-3-tosylamido-amino-2-heptanehydrochloride. The homogenate was centrifuged (20,000 g for 20 min) at 4°C, and the proteins in the supernatant were fractionated with polyethylene glycol (PEG) 8000. The 2.5–6% (wt/vol) PEG fraction was washed with the homogenization buffer in which mannitol had been replaced by 10% (wt/vol) glycerol and supplemented with 10% (wt/vol) PEG 8000. The resulting pellet was resuspended in the same buffer. ACC activity was assayed by measuring the incorporation of radioactive acetyl units into lipids in the presence of an excess of purified fatty acid synthase and 10 mM magnesium citrate to measure maximal activity (4). The activity was measured at 37°C in a final volume of 0.2 ml containing 60 mM HEPES at pH 7.5, 2.5 mM EGTA, 0.25 mM dithioerythritol, 2 mM Mg-ATP, 10 mM KHCO₃, 0.5 mM NADPH, 4.25 mg/ml of bovine serum albumin, 60 µM butyryl-CoA, 100 µM [¹⁴C]acetyl-CoA (400,000 cpm/ml), and 10 mM magnesium citrate plus the sample. After 6 min of incubation at 37°C, the reaction was stopped by adding 100 µl of 10 M NaOH. Saponification and extraction of fatty acids were performed as described (1).

Statistical analysis and expression of results. The data are expressed per gram of wet weight, except where otherwise stated, and are means ± SE. The number of hearts used in each group is indicated (as n) in the appropriate legends. An analysis of variance with Bonferroni correction for variance diversity was used to evaluate the statistical significance of differences. A value of P < 0.05 was considered as statistically significant.

RESULTS

Effect of 8-BrcGMP on glucose metabolism. The functional parameters of the preparation were similar to those found previously (12) and were stable during the whole experimental periods. Submitting hearts to increased workload enhanced hydraulic power and coronary flow. This was accompanied by a significant increase in glucose uptake, flux through PFK-1, and lactate output (Table 1). No significant change in glycogen content (Table 1) and hexose 6-phosphate concentration (Fig. 1) was observed.

The effects of 8-BrcGMP were investigated under these two workload conditions. In low-load conditions, addition of 8-BrcGMP significantly increased hydraulic power and coronary output (Table 1). The only significant effects of 8-BrcGMP on glucose metabolism in low-load conditions were a 65% increase in hexose 6-phosphate concentration (Fig. 1) and a 20% increase in glycogen content (Table 1). Under high-load conditions, addition of 8-BrcGMP blunted the stimulation of hydraulic power observed in the corresponding controls. Such an effect on external work was associated

Table 1. Effect of 8-BrcGMP on hydraulic power, coronary output, glucose uptake, glycolytic flux, lactate output, and glycogen content in hearts perfused at two workload conditions with or without 8-BrcGMP

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hydraulic Power, g·m·min⁻¹ per g wet wt</th>
<th>Coronary Output, ml·min⁻¹·g⁻¹</th>
<th>Glucose Uptake, µmol·min⁻¹·g⁻¹</th>
<th>Glycolytic Flux, µmol·min⁻¹·g⁻¹</th>
<th>Lactate Output, µmol·min⁻¹·g⁻¹</th>
<th>Glycogen Content, µmol/g</th>
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<tbody>
<tr>
<td>Low load</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Without 8-BrcGMP</td>
<td>25 ± 3</td>
<td>8.5 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>11.0 ± 0.5</td>
</tr>
<tr>
<td>With 8-BrcGMP</td>
<td>35 ± 3*</td>
<td>12.0 ± 0.5*</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>13.5 ± 1.0*</td>
</tr>
<tr>
<td>High load</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without 8-BrcGMP</td>
<td>60 ± 4†</td>
<td>15.0 ± 3.0†</td>
<td>1.4 ± 0.1†</td>
<td>1.3 ± 0.2†</td>
<td>0.5 ± 0.1†</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>With 8-BrcGMP</td>
<td>42 ± 7*</td>
<td>16.0 ± 2.0</td>
<td>0.9 ± 0.1*</td>
<td>0.9 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
<td>7.5 ± 0.5†</td>
</tr>
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Values are means ± SE; n = number of hearts. Hearts were perfused for 20 min at two different workloads with or without 0.1 mM 8-bromoguanosine-3'5'-cyclic monophosphate (8-BrcGMP). Glucose uptake and glycolytic flux were measured by the detritiation rate of [2-³H]glucose and [3-³H]glucose, respectively. At the end of perfusion, hearts were freeze-clamped for glycogen measurement. *P < 0.05 vs. corresponding value without 8-BrcGMP; †P < 0.05 vs. corresponding value in low-load conditions.
with a significant decrease in glucose uptake and flux through PFK-1 and a concomitant decrease in glycogen content (Table 1). The stimulation of net glycogen breakdown in such condition illustrates the need to provide glucosyl units for glycolysis from endogenous stores when the uptake of extracellular glucose is limited. As shown on Table 1, the effects of 8-BrcGMP on glucose uptake and flux through PFK-1 were of similar extent, indicating that all the glucose taken up was glycolyzed and that glucose uptake indeed controlled glycolysis in the presence of the cGMP analog.

Because PDH is a major regulatory step for glucose oxidation, whereas ACC regulates fatty acids oxidation, both enzymatic activities were measured. Our aim was to determine whether the inhibition of glucose metabolism by cGMP corresponded to a metabolic shift from glucose to fatty acid consumption or was related to a diminution of the overall myocardial metabolism. As shown in Fig. 2, the percentage of PDH in the active form was ~45% at low load and increased to 95% at high load. Addition of 8-BrcGMP did not significantly affect PDH activity in both groups. In the low-load conditions, ACC activity was ~30 mU/mg of protein, and this value was not affected by increasing the workload (Fig. 3). However, ACC activity was approximately doubled at both loads after addition of 8-BrcGMP (Fig. 3). Thus PDH activity was affected by workload but not by 8-BrcGMP, whereas the opposite was observed with ACC.

Finally, to test whether 8-BrcGMP could interact with other mechanisms of increased glucose uptake, hearts were perfused at low load with glucose together with 0.1 µM insulin. Addition of insulin increased glucose uptake from 0.8 ± 0.1 to 1.2 ± 0.1 µmol·min⁻¹·g⁻¹ (P < 0.01), and addition of 0.1 mM 8-BrcGMP together with insulin reduced glucose uptake to the control value (0.8 ± 0.1 µmol·min⁻¹·g⁻¹): P < 0.01 vs. insulin-treated hearts without 8-BrcGMP). Thus 8-BrcGMP may interfere with the stimulation of glucose uptake induced by different physiological mechanisms, namely increased workload and addition of insulin.

Effect of NO modulators on glucose uptake. Because both NO synthase inhibitors and NO donors regulate cGMP concentration (2, 7, 10), we investigated whether these substances could also affect the rate of glucose metabolism in our model. We measured the effects of L-NAME, an inhibitor of NO synthase, and SNAP, a NO donor, on heart work, glucose uptake, glycogen content, and cGMP concentration in hearts perfused at both loads. Results are summarized in Table 2.
At low load, addition of L-NAME or SNAP significantly altered cGMP concentration, but they did not affect glucose uptake (Table 2). SNAP increased coronary flow and hydraulic power, as was observed with 8-Br- cGMP in the same conditions (Table 2). At high load, however, addition of L-NAME increased hydraulic power and glucose uptake by 30% and decreased cGMP concentration by 25% (Table 2). Opposite effects were observed with SNAP, which decreased hydraulic power and glucose uptake by 30% and increased the concentration of cGMP by 40% (Table 2). Glycogen content was not affected by L-NAME but was significantly decreased by SNAP (Table 2).

We then investigated whether the decrease in glucose utilization observed in SNAP-perfused hearts was the cause or the consequence of the decrease in hydraulic power. If it was the cause, then the addition of alternative oxidizable substrates, such as pyruvate and β-hydroxybutyrate, should prevent the functional decline. Therefore, SNAP-treated hearts perfused with glucose together with pyruvate and β-hydroxybutyrate (5 mM each) were compared with hearts perfused with glucose alone. As shown in Fig. 4, external work was similar and remained stable during the whole protocol in both groups perfused without the NO donor. However, addition of SNAP rapidly induced a progressive decline of hydraulic power in hearts perfused with glucose alone, whereas the NO donor had no significant effects in hearts perfused with pyruvate and β-hydroxybutyrate (Fig. 4).

Finally, because cGMP may alter cAMP concentration by regulating cAMP-PDE (34), we measured cAMP in control hearts as well as in hearts perfused with L-NAME or SNAP. No statistically significant difference in cAMP concentration was observed among the three groups (2.72 ± 0.13, 2.98 ± 0.18, and 2.84 ± 0.12 nmol/g in controls, SNAP-perfused hearts, and L-NAME-perfused hearts, respectively). The effects observed when modulating cGMP concentration were thus not mediated by changes in cAMP.
PKG. This is supported by the observation that 8-BrcGMP is a potent activator of PKG but has no effect on cAMP-PDE (8, 20) and that cAMP concentration was not affected by 8-BrcGMP, L-NAME, and SNAP in our experiments. Both physiological and metabolic effects of 8-BrcGMP will be discussed.

Effects of 8-BrcGMP and NO modulators on physiological parameters. In this study, we compared two workloads: a low-load condition, which corresponds to a situation of minimal energetic demand (12, 33), and a high-load condition, which is closer to the load conditions found in the intact animal. This comparison actually allows one to assess the role of external work on heart metabolism. Moreover, addition of the cGMP analog or of NO modulators at both load conditions allows one to compare their effects on the organ in its basal state and in a condition of increased energetic demand.

From our data, it appears that 8-BrcGMP and SNAP exert two main physiological properties: vasodilatation and decrease of myocardial contractility. However, one effect can mask the other, and the experimental evidence mainly depends on the workload. Indeed these two parameters are often related in our preparation, because oxygen delivery (which is directly proportional to coronary flow in saline-perfused hearts) is one of the determinants of external work (5, 26). Therefore, both 8-BrcGMP and SNAP increased heart work at low load as a consequence of their vasodilatory effect and despite their reported negative inotropic effect (2, 7). At high load, however, coronary flow was already fully stimulated so that the main effect of 8-BrcGMP and SNAP was a decrease of heart work. By comparing these two conditions, we can thus determine the effects of the cGMP analog and the NO donor on both the vasculature (at low load) and the myocardium (at high load).

The increase in hydraulic power that concerned the effects of L-NAME observed at high load despite the decrease in coronary flow illustrates the prevalence of the effect on myocardial contractility under these conditions. This effect was related to a decreased concentration of cGMP and to an increased glucose uptake.

Finally, 8-BrcGMP had no effect on apparent glucose uptake in low-load condition. However, the fact that coronary output was increased by the analog (in the same conditions indicates that glucose extraction was indeed decreased in hearts perfused with 8-BrcGMP.

Effects of 8-BrcGMP and NO modulators on metabolic parameters. Comparison of the rates of detritiation of [2-3H] and [3-3H]glucose indicates that, in conditions of stimulated glucose metabolism, the flux through PFK-1 (detritiation of [3-3H]glucose) was inhibited to the same extent as glucose uptake (detritiation of [2-3H]glucose). This suggests that the flux through PFK-1 was not limiting but rather a consequence of an upstream inhibition. It is therefore conceivable that glucose uptake represents a major site of inhibition by cGMP. On the other hand, in a low-load condition, addition of 8-BrcGMP significantly increased the concentration of hexose 6-phosphates (Fig. 1) without changing the concentration of fructose 1,6-bisphosphate (not shown). This suggests that, in condition of low energetic demand and “basal” glucose uptake, inhibition may also occur at the level of PFK-1. PFK-1 inhibition by 8-BrcGMP seems, however, minimal. Indeed, in the same experimental model, a 50% inhibition of the flux through PFK-1, resulted in a fourfold increase in hexose 6-phosphate concentration (14), i.e., 5–10 times the increase observed in the present work.

The effects of 8-BrcGMP on glycogen content are also of interest. At any time, glycogen content results from the balance between glycogen synthesis and breakdown. Heart work by itself did not significantly affect glycogen concentration, but addition of 8-BrcGMP resulted in opposite effects when we compared the two workloads. At low load, 8-BrcGMP increased glycogen content. This could result from an increase in hexose 6-phosphate, which is known to stimulate glycogen synthesis (14). By contrast, net glycogen breakdown was stimulated at high load in the presence of 8-BrcGMP. This might be related to the limitation of extracellular glucose uptake in these conditions. Again, this discrepancy illustrates the fact that the effects of cGMP depend on the workload.

Because glucose uptake results from both glucose transport and hexokinase activity, it remains to be demonstrated which step was inhibited by cGMP. If hexokinase had been inhibited by cGMP, a decrease in hexose 6-phosphate concentration would have been expected. The opposite was actually observed (Fig. 1). Thus this strongly suggests that cGMP affects glucose uptake by controlling glucose transport rather than hexokinase. This result is in agreement with the general belief that glucose transport limits glucose uptake and metabolism in the isolated working rat heart perfused with glucose alone (18). It is also consistent with the fact that stimulation of glucose uptake by increased workload or insulin results from recruitment of the glucose transporter GLUT4 from its intracellular stores to the plasma membrane (27, 31, 35, 37).

Besides the measurement of glucose fluxes, we also assessed the effects of 8-BrcGMP on major regulatory enzymes, namely PDH and ACC. The oxidative decarboxylation of pyruvate to acetyl-CoA by PDH is a point of no return in glucose metabolism, whereas malonyl-CoA, the product of ACC, inhibits the uptake and, hence, the oxidation of long-chain fatty acids in the mitochondria (1, 28). The activity of PDH was stimulated by increased workload, as expected (19), but was not affected by 8-BrcGMP, confirming that the main effect of 8-BrcGMP on glucose metabolism was exerted on glucose uptake. On the other hand, 8-BrcGMP activated ACC and we assume that this activation should inhibit β-oxidation. Therefore, the analog limits the consumption of both glucose and fatty acids rather than switching the metabolism from glucose to fatty acid consumption. The inhibition is exerted at early steps in both pathways, namely the uptake of glucose and the entry of fatty acids into mitochondria, and corresponds to a limitation of heart work. Thus the negative inotropic effect of cGMP observed at high load is matched with a concerted downregulation of the
overall metabolism, leading the heart toward a new equilibrium: without suppressing heart work, cGMP decreases the workload toward "basal" values. However, when pyruvate or ketone bodies were added to the perfusate, they prevented the functional decline, because the metabolism of these oxidizable substrates bypasses the molecular targets of cGMP.

In ischemic conditions, energy supply mainly depends on glucose utilization, and our previous data support a role for cGMP in ischemia. Indeed, cGMP concentration increases in ischemic hearts as a consequence of NO synthase activation (9, 10). We showed that the progressive decline of glucose uptake observed during low-flow ischemia could be largely prevented by addition of NO synthase inhibitors, which improved the postischemic functional recovery (13). From the present data, we conclude that activation of NO synthase in the ischemic heart increases cGMP concentration which, in turn, inhibits glucose uptake. Addition of inhibitors of NO synthase to the ischemic heart prevents such effects. It allows for a better uptake of extracellular glucose, a sparing of endogenous glycogen stores, and an improved functional recovery on reperfusion (13). Such inhibitors may also protect the hearts by limiting the production of free radicals from NO during ischemia-reperfusion (25, 30, 36).

In conclusion, our data show that cGMP controls metabolic fluxes in the heart, on top of its effects on contractility. cGMP indeed inhibits heart glucose metabolism by decreasing glucose uptake, and it may also inhibit fatty acid oxidation by activating ACC. cGMP thus exerts both inotropic and metabolic effects that are opposed to those observed with cAMP (11, 15). This opens a new field of investigation, namely the control of heart metabolism by the cGMP-dependent signaling pathway. Whether the metabolic effects of cGMP are restricted to the heart or apply to other tissues is certainly worthy of consideration.

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