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# Cyclic GMP, cyclic AMP, glucose at birth, and maturation of rat liver mitochondria

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**The improvement in mitochondrial functions which normally occurs in newborn rat liver in vivo during the few hours following delivery is inhibited by a glucose injection at birth (Meister, R., Comte, J., Baggetto, L., G., Godinot, C. and Gautheron, D.C. (1983) Biochim. Biophys. Acta 722, 36–42). To test whether this improvement could be correlated to changes in cyclic nucleotides, the levels of cAMP and cGMP have been measured during the 2 h following birth. At birth, a short rise followed by a decrease of cAMP occurs, then a significant increase of cAMP level is observed between 45 min and 2 h. The cAMP level for animals injected at birth with glucose is lower than for control animals at each time studied. The cGMP level is not significantly affected in control animals, while in glucose-treated animals a significant decrease of cGMP is observed in the postnatal 2 h. The present work shows also that the glucose-induced inhibition of mitochondrial maturation is mimicked by injection at birth of either 8-Br-cGMP or nitroprusside. The latter transiently increases intracellular cGMP. In contrast, the glucose-induced inhibition is prevented by the injection at birth of either dbcAMP or alkylxanthines together with glucose (Comte, J., Meister, R., Baggetto, L.G., Godinot, C. and Gautheron, D.C. (1986) Biochem. Pharmacol. 35, 2411–2416). It is concluded that the postnatal improvement of mitochondrial functions is stimulated by cAMP and inhibited by cGMP, and that glucose-induced inhibition of the maturation is at least partly supported by a decrease in cAMP but not correlated to an increase in cGMP.**

## Introduction

ATP production in the fetal rat liver is mainly glycolytic, glucose being supplied by the mother's blood [1]. The capacity of liver mitochondria for ATP synthesis coupled to substrate oxidation is low at birth [2,3]. It increases rapidly to reach an adult value a few hours after delivery [4]. The respiratory control ratio with succinate as substrate is about 1.9 at birth and 3.2 two hours after birth [5]. ATP production progressively shifts to an aerobic type, while many enzyme inductions occurring during the first 12 h of aerian life permit the onset of liver glycogenolysis and gluconeogenesis [6].

These metabolic changes appear correlated with an abrupt fall of insulinemia and a rise of glucagonemia at birth. The insulin/glucagon ratio decreases from 10 before birth to 1, one hour after

Abbreviations used: cAMP, cGMP, respectively adenosine and guanosine 3',5'-cyclic monophosphate; 8-Br-cGMP, 8-bromo-cGMP; dbcAMP, dibutyryl cAMP; cAMP-PDE, cAMP phosphodiesterase; RCR, respiratory control ratio.

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birth [7]. A concomitant increase of the cAMP level in the liver occurs in the few days following delivery [8].

We have shown that a glucose injection given to the newborn immediately at birth delays the postnatal improvement of mitochondrial functions [5], and that the simultaneous injection at birth of glucose and either cAMP or alkylxanthines –drugs known to increase the cellular cAMP level – prevents this inhibitory effect of glucose [9]. Glucose injection at birth is known to increase insulinemia [10,11] and thus to antagonize both the observed fall of the insulin/glucagon ratio and probably also the increase of the liver cAMP level. However, in our results, the cAMP level was only slightly lowered in the liver of glucose-treated neonates at 45 min and 2 h after birth. The decrease did not seem sufficient to explain either the inhibitory effect of glucose or the positive effect of alkylxanthines on the maturation of liver mitochondria [9].

We have therefore investigated the role of cGMP at birth since glucose, or rather glucose metabolites, have been shown to increase the cGMP level in hepatocytes [12], and since insulin itself, at least in the adult liver, increases cGMP [13]. The present work demonstrates the inhibitory effect of cGMP on mitochondrial maturation. It also shows that the inhibitory effect of glucose is partly supported by a decrease of cAMP, but is not correlated to an increase in cGMP that could be expected from the known effects of a glucose injection in the adult.

## Material and Methods

### *Reagents*

The 'cAMP and cGMP assay kits' were from Amersham, 8-Br-cGMP from Boehringer-Mannheim, and sodium nitroprusside from Fluka. All other reagents were of the highest grade available. Pentoxifylline was a gift from the Laboratoire Hoechst, France.

### *Animals, treatments and mitochondrial respiration measurements*

Timed pregnant Wistar rats were obtained from the Institut Universitaire de Technologie, as previously described [5]. They were killed by decapitation on the morning of the 22nd day. Fetuses were delivered and the umbilical cord tied. Each litter (12–15 animals) was divided into three or four equal groups, allowing the study of two or three treatments and their control. Each neonate received at birth either 25 mg glucose or an equivalent volume (50 $\mu$l) of 0.9% NaCl intraperitoneally per 5 g animal. In other treatments, neonates received either 10 $\mu$g of nitroprusside per animal or 8-Br-cGMP (0.5, 2.5 or 5 $\mu$mol/kg). Pentoxifylline was injected at the therapeutic dose of 10 mg/kg at birth and 1 h after birth. Neonates were killed 2 h after birth.

Mitochondrial purification and respiratory control ratio (RCR) estimation were as described [9]. The RCR represents the ratio between the rate of substrate oxidation in the presence and the absence of added ADP. This ratio is a good criterion to estimate the functional capacity of mitochondria for ATP synthesis [14].

### *Estimation of cAMP and cGMP levels in neonate livers*

Livers were rapidly removed either from newborn rats or from 10-min-, 20-min-, 45-min- or 2-h-old neonates of the same litter, and frozen in liquid nitrogen within 10 s of animal death. Frozen livers were homogenized at 4°C in 1.5 ml of 6% trichloracetic acid in a Potter-Elvehjem homogenizer fitted with a glass pestle. The suspension was centrifuged at $2000 \times g$ for 15 min. Supernatants were extracted five times with water-saturated ethyl ether to remove trichloracetic acid, and lyophylized overnight. The dry residues were dissolved in 400 $\mu$l of 50 mM Tris/4 mM EDTA (pH 7.5) and 10 $\mu$l of 1 M Tris (pH 7.6) were added if necessary. Levels of cAMP were determined in 10-$\mu$l extracts, and levels of cGMP in 100 $\mu$l, using Amersham cAMP and cGMP radioimmunoassay kits, which essentially follow the procedure of Steiner et al. [15]. Protein contents were measured by the method of Lowry et al. [16] in the solution obtained after dispersion of the pellet in M NaOH vigorously shaken with sand. Bovine serum albumin was used as a standard.

### *Statistical analysis*

One- or two-way analysis of variance was used. In Figs. 1 and 2 the statistical significance of the *F*

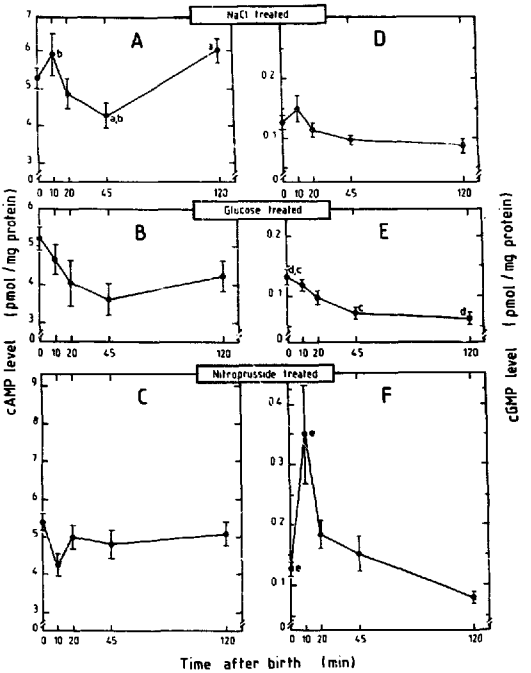


Fig. 1. Effects of glucose or nitroprusside treatment on the time-course of cAMP (left) and cGMP (right) levels in individual livers of rats treated at birth as indicated, and killed 0–120 min later. Values are in pmol cAMP and cGMP/mg protein, mean ± S.E., 26–34 animals/curve. One way analysis of variance for each curve shows significant differences for A ($P < 0.025$), E ($P < 0.001$) and F ($P < 0.01$). Pairwise comparisons are significant with couples: a, b ($P < 0.01$); c, d ($P < 0.001$) and e ($P < 0.03$). Two way analysis of variance shows a significant effect of the treatment between A and B: $F(1,46)$, $P < 0.01$.

ratio for each cyclic nucleotide and each treatment is indicated only if it is better than 5%. Then the significance of subsequent pairwise comparisons with the multiple *t*-test is shown.

## Results

### *Effect of nitroprusside and 8-Br-cGMP on mitochondrial maturation*

The role of cGMP in the maturation of newborn liver mitochondria was first investigated using nitroprusside, which is known to stimulate the soluble guanylate cyclase [17] and to increase intracellular cGMP. Table I shows the respiratory control ratios of mitochondria isolated from 2-h-old rats after various treatments. In experiments No. 1, rats were treated at birth with either glucose, or nitroprusside, or nitroprusside plus an alkylxanthine, pentoxifylline. Glucose inhibits the mitochondrial maturation process (the RCR being 66.6% of the control value). Nitroprusside also inhibits it, but even more strongly (RCR 45.4% of

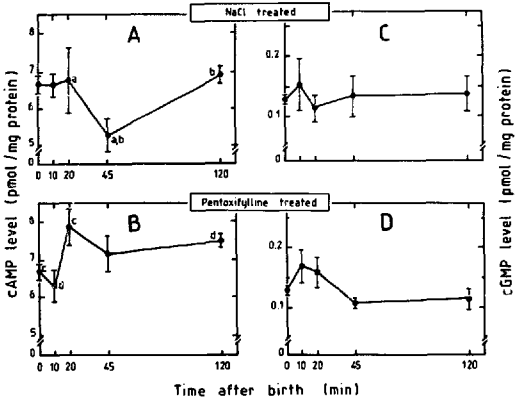


Fig. 2. Effects of pentoxifylline treatment on the time-course of cAMP and cGMP levels in individual livers of rats (see legend to Fig. 1). In this second series of experiments, the number of animals was 28–29/curve. One way analysis of variance shows significant differences for A and B ($P < 0.05$). Pairwise comparisons: a, b $P < 0.02$; c, d $P < 0.05$. Two way analysis of variance: between A and B, $F(1,50)$, $P < 0.01$.

TABLE I

RESPIRATORY CONTROL RATIO OF LIVER MITOCHONDRIA FROM 2-h-OLD NEONATES TREATED AT BIRTH AS INDICATED

Respiratory control ratios (RCR) are measured with succinate as substrate and are mean ± S.E. of $N$ experiments. Analysis of variance shows the following differences: for experiments 1, $F(3,12)$: $P < 0.001$; for experiments 2, $F(5,19)$: $P < 0.01$. Significance of pairwise comparisons: a, e $P < 0.01$; b, c, f, g $P < 0.001$; h, k not significant. The data of experiments No. 3 are taken from Ref. 9 for comparison with the present data.

| Expt. No. | Treatment at birth | $N$ | RCR | % of NaCl control values |
|---|---|---|---|---|
| 1 | NaCl controls | 4 | $2.82 \pm 0.16^{a,b}$ | 100 |
| | Glucose | 4 | $1.88 \pm 0.20^{a}$ | 66.6 |
| | Nitroprusside | 4 | $1.28 \pm 0.04^{b,c}$ | 45.4 |
| | Nitroprusside + pentoxifylline | 4 | $2.98 \pm 0.19^{c}$ | 105.6 |
| 2 | NaCl controls | 7 | $4.31 \pm 0.26^{e,f,g}$ | 100 |
| | 0.5 μmol 8-Br-cGMP | 3 | $2.35 \pm 0.28^{e}$ | 54.5 |
| | 2.5 μmol 8-Br-cGMP | 4 | $2.10 \pm 0.41^{f,h}$ | 48.7 |
| | 2.5 μmol 8-Br-cGMP + pentoxifylline | 3 | $2.56 \pm 0.35^{h}$ | 59.4 |
| | 5 μmol 8-Br-cGMP | 5 | $1.77 \pm 0.23^{g,k}$ | 41.1 |
| | 5 μmol 8-Br-cGMP + pentoxifylline | 3 | $1.94 \pm 0.12^{k}$ | 45.0 |
| 3 | NaCl controls | | $2.66 \pm 0.31$ | 100 |
| | NaCl + pentoxifylline | | $2.54 \pm 0.34$ | 95.4 |
| | NaCl + dbcAMP | | $3.91 \pm 0.21$ | 146.9 |
| | Glucose | | $1.76 \pm 0.23$ | 66.1 |
| | Glucose + pentoxifylline | | $2.84 \pm 0.07$ | 106.7 |
| | Glucose + dbcAMP | | $3.61 \pm 0.17$ | 135.7 |

the control value) than does glucose. This nitroprusside-induced inhibition is fully prevented by the simultaneous injection of pentoxifylline. In experiments No. 3 reminiscent of previous studies [9] it has been shown that pentoxifylline, which has no effect by itself, also prevents the inhibition induced by glucose. Contrary to the inhibition observed when intracellular cGMP is raised, an increase of intracellular cAMP induced by dbcAMP injection (7 μmol/kg) stimulates the respiratory control ratio by 46% and prevents the glucose-induced inhibition (experiments No. 3).

Experiments No. 2 show that the injection at birth of 8-Br-cGMP, a stable and permeant analog of cGMP (0.5, 2.5 and 5 μmol/kg), inhibits the mitochondrial maturation in every case as does nitroprusside (RCR varying between 41.1 and 54.5% of the control value). However, this inhibition is not significantly prevented by a simultaneous injection of pentoxifylline at the dose used to reverse the glucose- or nitroprusside-induced inhibition of the maturation.

The respiratory control ratios measured 45 min after birth are slightly lower than those obtained at 2 h for control rats (NaCl-treated). The RCR for glucose- or sodium nitroprusside-treated rats are 64 and 41% of the control values (three experiments, not shown). Therefore, the effects of these treatments on the RCR are very similar during the first 2 h after birth.

*Cyclic AMP level in the liver during the 2 h following birth*

Fig. 1A shows that in control neonates, a slight increase of cAMP seems to occur 10 min after birth, followed by a significant decrease until 45 min, and then, there is the expected significant increase at 2 h. The cAMP level has been shown to rise from birth until the maximum of 15–20 pmol/mg liver protein that was reached the 5th day of life in the rat liver [8].

When neonates were injected at birth with glucose (Fig. 1B), a decrease of cAMP is observed in the first 45 min, followed by an increase as in the controls. However, 2 h after glucose injection, the cAMP level remains lower in glucose-treated rats than in NaCl-treated rats. In nitroprusside-treated neonates (Fig. 1C), a short decrease of cAMP occurs at 10 min, but the following progressive rise of cAMP is not observed after 2 h.

In pentoxifylline-treated rats (Fig. 2B), the level of cAMP is significantly higher than in NaCl-treated rats (Fig. 2A) 20 and 45 min after birth.

*Cyclic GMP level during the 2 h following birth*

There were no data available about cGMP level in the postnatal period. In control neonates (Fig. 1D), no significant change occurs during the 2 h following the delivery. In nitroprusside-treated neonate (Fig. 1F), a strong burst of cGMP is observed at 10 min, followed by a progressive decrease as in the controls. In glucose-treated neonates (Fig. 1E), a significant decrease of the cGMP level takes place to reach a plateau after the first 45 min following birth.

In pentoxifylline-treated rats, no significant change occurs (Fig. 2C and D).

## Discussion

*Time-course of cAMP level at birth*

The continuous increase of cAMP level in rat liver during the prenatal period and during the first days after delivery is well documented [8,18–20] except in the immediate postnatal period (first hour). Discrepancies have been reported concerning the rise of cAMP in utero in the few days preceding birth, and concerning the delay of the onset of cAMP increase in the few hours following birth (e.g. Ref. 20).

They may be ascribed to the strains used and to the degree of maturity at birth [19,20]. In the present work, neonates are delivered at 9.00 a.m., while the natural birth occurs between 2.00 and 9.00 p.m. The rise of cAMP begins only at 45 min after birth, after a decrease of the cAMP level in the first 45 min of life, which was not previously observed. Glucose injection only slightly lowers cAMP level in the liver, as previously reported in the newborn [9] and in the adult [21] rat.

The variations of cGMP do not antagonize those of cAMP, since there is no significant change in cGMP during the first 2 h of life in control animals, and since cGMP decreases together with cAMP in glucose-treated animals.

*Positive effect of cAMP*

The beneficial effect of cAMP in the maturation of newborn liver mitochondria is clearly demonstrated, since dbcAMP at birth improves all

oxidative phosphorylation processes, and since glucose-induced inhibition is reversed by the simultaneous injection of either dbcAMP or alkylxanthines [9]. In spite of the limited variations of the cAMP level under glucose treatment, the possibility that cAMP alone might support most of the maturation process cannot be dismissed. First, we have shown that glucose lowers cAMP level, while alkylxanthines increase it. Moreover, cAMP-dependent protein kinases may be activated, and thus cAMP-dependent effects observed might be due to an increased cAMP turnover but without any apparent change in cAMP level with an acellular system [22]. In addition, the presence of distinct intracellular cAMP pools appears well-established and cAMP-mediated hormone effects may be observed without appreciable cAMP changes, as reported in ventricular myocytes [23]. It has also been concluded that inhibition of cAMP-dependent protein kinases under insulin action occurs without change in cAMP level in the liver [24].

*Positive effect of alkylxanthines: relation with cAMP and cGMP*

The reversal of glucose- or nitroprusside-induced inhibition of mitochondrial maturation by alkylxanthines is probably supported by their usual inhibitory action on cAMP phosphodiesterases (cAMP-PDE), but a cGMP-mediated effect could be expected. Indeed, PDE exist in multiple soluble or membrane-bound forms. Four types have been purified from heart [25] and other tissues [26] having close or different affinities for cAMP and cGMP. This explains the correlation that has been found between the biological activity (anti-anaphylaxis in vitro and in vivo) of 24 PDE inhibitors and their respective ability to inhibit cAMP-cGMP-PDE or cGMP-cAMP-PDE activity [27].

Inhibition of guanylate cyclase by theophylline has also been observed in vitro at millimolar concentrations [28].

Variations of cGMP level or of guanylate cyclase activity do not seem to occur with pentoxifylline in the present study, and most of pentoxifylline effects should be mediated via cAMP. There is an apparent discrepancy between the fact that dbcAMP injection increases the RCR of NaCl-treated rats while pentoxifylline injection alone increases cAMP level but does not change RCR. The rise in cAMP produced by injection of a relatively great amount of dbcAMP is probably more important than the rise induced by pentoxifylline injection. In addition, the time-courses of cAMP changes are different: the dbcAMP-induced rise is sudden and massive, while the pentoxifylline-induced cAMP increase appears only 20 min after injection (Fig. 2) and remains stable for 2 h. The rise in cAMP induced by pentoxifylline alone may be too limited to provoke a significant improvement of RCR in control rats. However, the pentoxifylline effect, which is sufficient to prevent the inhibitory effects of glucose or nitroprusside, may have more physiological significance than the dbcAMP effect and may be of pharmacological value.

*Inhibitory effect of cGMP*

The inhibitory effect of cGMP in the mitochondrial maturation has been demonstrated with the vasodilator nitroprusside, a nitroso-generating compound widely used to stimulate soluble guanylate cyclase [17,29], and with 8-Br-cGMP, an analog of cGMP (Table I).

Pentoxifylline fully prevents the nitroprusside-induced inhibition, but prevents only poorly that induced by 8-Br-cGMP. This discrepancy may be related to the fact that nitroprusside, which generates cGMP inside the cell, is also a potent activator of liver cGMP-dependent cAMP-PDE, while 8-Br-cGMP is only a weak activator of the PDE [30]. It may also suggest a beneficial effect of pentoxifylline due to a partial inhibition of guanylate cyclase, as reported above for theophylline [28]: this could allow the reversal of inhibition induced by nitroprusside but not by 8-Br-cGMP.

Although the purification of two cGMP-dependent cAMP-PDE in the liver [31] supports the unifying concept of an antagonism between these two cyclic nucleotides [32], their respective actions are not altogether clear. In addition, it is known that insulin plus glucagon, as well as equimolar concentrations of cAMP and cGMP are both necessary to induce DNA synthesis and mitosis in newborn hepatocytes in culture [33].

*Inhibitory effect of glucose in the neonate, comparison with the adult*

The role of a rise of cGMP in the 'glucose

effect' seems well established in the adult rat liver. It is supported by the fact that glucose treatment in vivo increases liver cGMP [21,34] and that glucose or glucose metabolites added to the medium increase cGMP in adult hepatocytes by 2- to 5-fold [12,34]. On the other hand, the present work shows that glucose injection decreases liver cGMP in the hours following birth. This discrepancy between the behavior of adult rat liver and that of the neonate could be explained by variation in guanylate cyclase activity. This enzyme is known to be regulated by the redox state of the cells [12,17] and the redox state of the newborn liver is lower than the adult one, at least after section of the umbilical cord during transition from aquatic to aerobic life [35]. A similar discrepancy between the adult and the newborn has been described when the effects of cAMP were studied in vitro on leucine incorporation into proteins of hepatocytes: cAMP inhibits leucine incorporation only if hepatocytes are taken from neonates younger than a few days [36]. In addition, glucose prevents the rise of adenine nucleotides which normally occurs 2 h after birth in rabbit neonates [35].

The general conclusion of our study is that cGMP inhibits mitochondrial maturation as does glucose, and the glucose-induced inhibition may be mediated by a slight decrease in cAMP but, surprisingly, not by a rise in cGMP.

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