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REVERSAL OF GLUCOSE-INDUCED INHIBITION OF NEWBORN RAT LIVER MITOCHONDRIAL MATURATION BY ADMINISTRATION OF ALKYLXANTHINES AT BIRTH

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Abstract—A glucose injection given immediately after birth delays the maturation which normally occurs in rat liver mitochondria and which increases the rate of ATP synthesis coupled to succinate oxidation from a low value at birth to the adult value a few hours after birth [R. Meister, J. Comte, L. Baggetto, C. Godinot and D. C. Gautheron, *Biochim. biophys. Acta* **722**, 36 (1983)].

Alkylxanthine (pentoxifylline, HWA 285) administration at birth has no effect on the maturation of mitochondria prepared from 2-hr-old rat livers while DBcAMP administration increases their RCR and their rate of ATP synthesis. On the contrary, both alkylxanthines and DBcAMP reverse the glucose-induced inhibition of mitochondrial maturation. This DBcAMP effect cannot be mimicked by butyrate and is therefore related to cAMP.

The cAMP content of rat liver increases during this postnatal period in both control and glucose-treated rats, although glucose administration tends to decrease the level of cAMP. Alkylxanthine administration restores after 2 hr the cAMP level in glucose-treated animals. The variations of RCR could not be completely correlated with the level of cAMP. The possible involvement of other factors in the mitochondrial maturation and the glucose effect is discussed.

Birth induces an important modification of the hormonal status of rat liver, for example, the insulin/glucagon molar ratio decreases from about 10 before birth to about 1, one hour after birth. This change comes from both an insulin decrease and a glucagon increase [1] which enhance the level of cAMP† in the newborn rat liver. Simultaneously with these hormone fluctuations, the rate of ATP synthesis coupled with substrate oxidation in rat liver mitochondria, which is low at birth, increases rapidly during the first postnatal hours to reach a value close to that of the adult [2, 3]. This maturation is concomitant with an increase in the mitochondrial adenine nucleotide pool and ATP level [4]. Sutton and Pollak have suggested that the change in insulin/glucagon ratio might be responsible for the mitochondrial maturation at birth [5].

We have shown in a previous work [6] that the administration of glucose at birth delays by a few hours the normal increase in the capacity of energy recovery through oxidative phosphorylation in rat liver. Glucose injection at birth also inhibits the synthesis of proteins such as the enzymes of gluconeogenesis [7] and urea synthesis [8]. Although these inhibitory effects of glucose are not well understood, they might be related either to an increase in the insulin/glucagon ratio which results from the injection of glucose [9] or to a decrease of the effects of liver cAMP [10], or both.

The methylxanthines are well-known inhibitors of 3′-5′-cyclic nucleotide phosphodiesterase, thus they can increase the cellular level of cAMP [11]. Two of them, theophylline and caffeine are often used in the treatment of apnea which is frequently observed in premature human babies [12–14]. These drugs stimulate the central nervous system and relax the smooth muscles of the respiratory tract [15]. In recent years, by replacing the methyl groups of the methylxanthines by alkyl side chains, new xanthines exhibiting new properties have been produced. Pentoxifylline, for instance, increases the ATP/ADP ratio in red blood cells and enhances red cell deformability, blood fluidity, microcirculation and oxygenation [16, 17]. It protects mitochondria against ischemic alteration in the brain of gerbils [18] and improves the energy metabolism in the brain of anoxic rats [16] by increasing the ATP level and the adenine nucleotide pool. Pentoxifylline also stimulates the energy-consuming process of protein synthesis in the brain of gerbils [19].

The aim of this work was first to test whether or not two of these new alkylxanthines, pentoxifylline and HWA 285, were able to overcome the inhibitory effects of glucose on mitochondrial maturation after birth and also to study if glucose effect on mitochondrial maturation after birth could be explained

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† Abbreviations used: BSA, Bovine serum albumin; cAMP, cGMP, respectively adenosine and guanosine 3′,5′-cyclic monophosphate; DBcAMP, dibutyryl-cAMP; pentoxifylline, 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine; HWA 285, 1-(5′-oxo-hexyl)-3-methyl-7-propyl-xanthine; RCR, respiratory control ratio.

by a cAMP-mediated mechanism or by other mechanisms.

MATERIALS AND METHODS

The "cAMP assay kit" was from Amersham, DBcAMP from Boehringer, Mannheim. All other reagents were of the highest purity available. Pentoxifylline and HWA 285 were a gift from Hoechst, France.

Animal treatments. Timed pregnant Wistar rats were obtained from the Institut Universitaire de Technologie, as previously described [6]. Two or three pregnant rats were killed by decapitation on the morning of the 22nd day. Fetuses were delivered and the umbilical cord tied. Each litter (12–15 rats) was divided into 2 or 4 equal groups, allowing the study of 1 or 2 treatments and their control. Each newborn received intraperitoneally either 50 μl of a 0.5 g/ml glucose solution per 5 g animal (glucose treatment) or an equivalent volume of 0.9% NaCl (control animals). Then immediately, newborns (t = 0) were also injected intraperitoneally with 12 μl per 5 g animal of either 0.9% NaCl or the compound under study dissolved in 0.9% NaCl. In the case of alkylxanthines, a second injection was made 1-hr later (t = 1 hr) and these drugs were administered at the therapeutic dose of 10 mg/kg [15, 20]. Rats were killed 2 hr after birth.

Preparation of mitochondria. Liver mitochondria were routinely prepared from 2-hr-old rats (8–12 animals) as described by Aprille and Asimakis [21]. Proteins were estimated by the biuret method using BSA as a standard [22]. The rate of oxygen consumption was estimated polarographically, using a Clark electrode in a 1 ml water-jacketed cell maintained at 30° (Hansatech D.W. oxygen electrode unit). The assay medium consisted of 225 mM sucrose, 10 mM KCl, 10 mM phosphate (K), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris–HCl, 10 mM succinate, pH 7.4, 0.4–0.8 mg mitochondrial protein. State-3 respiration was initiated by the addition of 85 nmol ADP.

Estimation of cAMP level in young rat livers. Livers were rapidly removed from 45 min or 2-hr-old rats and frozen in liquid nitrogen, within 10 sec after animal death. Frozen livers were homogenized at 4° in 1.5 ml of cold 0.33 M perchloric acid in a Potter-Elvejhem fitted with a glass pestle. The suspension was centrifuged at 2000 *g* for 15 min. The supernatant fractions were adjusted to pH 7.0 with 0.9 ml of 2 M $KHCO_3$ and centrifuged again. cAMP levels were determined in the extracts by using the Amersham cAMP radioimmunoassay kit which essentially follows the procedure of Steiner *et al.* [23]. Protein contents were measured by the Lowry method [24] in the first pellet solubilized at 80° in N NaOH and using BSA as a standard.

Statistical analysis. The analysis of variance procedure was used. The F ratio is indicated and the subsequent pairwise comparisons are made with the multiple comparison *t*-test [25].

RESULTS

Reversal of glucose-induced inhibition of succinate oxidation in 2-hr-old rat liver mitochondria by alkylxanthine administration at birth

Table 1 shows the effects of two alkylxanthines (pentoxifylline and HWA 285) on the respiratory activity of mitochondria isolated from the livers of 2-hr-old rats.

The rate of succinate oxidation in state-3, the RCR and the ADP/O ratio are lower in mitochondria isolated 2 hr after birth in glucose-treated rats (experiments E and G) than in control animals (experiments A and C) while the state-4 rate is not significantly modified. When pentoxifylline or HWA 285 is administered immediately after birth to control rats (experiments B and D), there is no change in the rate of succinate oxidation, RCR and ADP/O ratio. When the same drugs are administered to glucose-treated rats immediately after birth, the state-3 rate of succinate oxidation rises to the value of the control rats while the state-4 rate remains unchanged.

Analysis of variance has been performed on the RCR values and all the treatments have been compared pairwise. As expected, glucose injection at birth significantly decreased RCR (compare experi-

Table 1. Effects of alkylxanthine administration at birth on succinate oxidation by liver mitochondria isolated from 2-hr-old rats

	Treatment at birth	natoms O/min/mg protein		RCR	ADP/O
		State 4	State 3		
A	NaCl controls	49	130	2.64 ± 0.35[a]	1.58 ± 0.08
B	NaCl + Pentoxifylline	56	132	2.54 ± 0.34	1.56 ± 0.19
C	NaCl controls	57	146	2.48 ± 0.26[b]	1.45 ± 0.12
D	NaCl + HWA 285	61	153	2.37 ± 0.09	1.53 ± 0.11
E	Glucose	41	75	1.68 ± 0.23[a,c]	0.62 ± 0.08
F	Glucose + Pentoxifylline	48	131	2.84 ± 0.07[c]	1.20 ± 0.04
G	Glucose	41	67	1.65 ± 0.22[b,d]	0.56 ± 0.10
H	Glucose + HWA 285	56	122	2.41 ± 0.14[d]	0.81 ± 0.1

Results are expressed as means ± S.E. for 4 experiments.
Treatment at birth, as described in Materials and Methods.
Analysis of variance on the RCR of 8 treatments shows significant differences: $F(7, 24) = 3.3$, $P < 0.01$. Comparisons show significant differences for the following pairs: [a,b,c] $P < 0.01$, [d] $P < 0.05$.

ments A or C to E or G). When either pentoxifylline or HWA 285 is administered together with glucose, the RCR is restored to the control value: experiments F and H exhibit significantly higher RCR than experiments E and G; experiments F and H are not significantly different from experiments B and D. Besides, the drug injection stimulates by a factor of 1.5–2 the ADP/O ratios. However, the ADP/O ratios of glucose + drug treated animals remain slightly lower than those of control rats.

Improvement of the mitochondrial respiratory activity by DBcAMP

To investigate whether the improvements in respiratory activity induced by alkylxanthines take place via cAMP, DBcAMP was administered intraperitoneally at birth. Table 2 shows the effects of the injection of DBcAMP at birth on the respiratory control ratio of mitochondria isolated from NaCl- or glucose-treated rats, 2 hr after birth. For control or glucose-treated rats, DBcAMP significantly stimulates the RCR with a more pronounced effect (2-fold) for glucose-injected newborn (experiments C and D) than for control rats (experiments A and B, 1.5-fold). Although the stimulation of ADP/O ratio is higher for glucose-treated rats than for control animals, the final value of the ratio for DBcAMP + glucose treated rats remains lower than for DBcAMP + NaCl treated rats. The increase in RCR is only due to an increase in the state-3 rates of succinate oxidation, since the state-4 rates are unchanged by DBcAMP treatments.

When newborn rats were injected with 70 μmoles DBcAMP per kg instead of 7 μmoles per kg, an increase in RCR was also observed although this increase was slightly lower (3 experiments, not shown).

While the alkylxanthines could only improve the RCR of glucose-treated rats (Table 1), DBcAMP also increases the RCR of control rats.

When DBcAMP is injected to rats, it may be metabolized and give rise to butyrate. Butyrate is a good substrate for newborn mitochondria because it can cross the mitochondrial membrane without carnitine [26].

Therefore, it was important to compare the effects of butyrate and DBcAMP. Table 3 demonstrates that, contrarily to DBcAMP (Table 2), sodium butyrate neither significantly increases RCR values of NaCl-treated rats nor reverses the glucose-induced inhibition of RCR. Similar conclusions can be drawn from the ADP/O ratios. The concentration of sodium butyrate used here corresponds to the highest concentration of DBcAMP tested (70 μmoles DBcAMP per kg) assuming a complete release of butyrate from DBcAMP. From this experiment, it can be concluded that the effects of DBcAMP can be related to cAMP and not to butyrate.

Influence of alkylxanthines on the level of liver cAMP in glucose- or NaCl*-treated newborn rats*

As shown above DBcAMP can mimic the effect of alkylxanthines at least in glucose-treated rats (Table 1 and 2). To see whether alkylxanthines act by increasing the level of cAMP, the concentration of cAMP was measured in rats treated or not with glucose and alkylxanthines. Figure 1 (right side) shows that in 2-hr-old animals, alkylxanthines

Table 2. Effects of DBcAMP on succinate oxidation by liver mitochondria isolated from 2 hr-old rats

	Treatment at birth	natoms 0/min/mg protein State 4	State 3	RCR	ADP/O
A	NaCl controls	41	115	$2.68 \pm 0.28^{a,c}$	1.22 ± 0.04
B	NaCl + DBcAMP (7 μmoles/kg)	43	171	3.91 ± 0.21^{a}	1.48 ± 0.12
C	Glucose	33	58	$1.84 \pm 0.23^{b,c}$	0.48 ± 0.17
D	Glucose + DBcAMP (7 μmoles/kg)	34	121	3.61 ± 0.17^{b}	1.03 ± 0.12

Results are expressed as means ± S.E. for 3 experiments. Analysis of variance on the RCR of 4 treatments shows significant differences: $F(3, 8) = 23.7$, $P < 0.001$. Comparisons show significant differences for the following pairs: ^{a,b} $P < 0.001$, ^{c} $P < 0.02$.

Table 3. Effects of sodium butyrate injection at birth on succinate oxidation by liver mitochondria isolated from 2 hr-old rats

Treatment at birth	RCR	ADP/O
NaCl controls	4.38 ± 0.32^{a}	1.49 ± 0.03
NaCl + sodium butyrate (140 μmoles)	3.99 ± 0.49^{b}	1.56 ± 0.07
Glucose	2.13 ± 0.36^{a}	0.71 ± 0.10
Glucose + sodium butyrate (140 μmoles)	2.50 ± 0.31^{b}	0.75 ± 0.11

Results are expressed as means ± S.E. for 4 experiments.

Analysis of variance on the RCR of 4 treatments shows significant differences: $F(3, 12) = 7.2$, $P < 0.01$. Comparisons show significant differences for the following pairs: ^{a} $P < 0.01$; ^{b} $P < 0.02$.

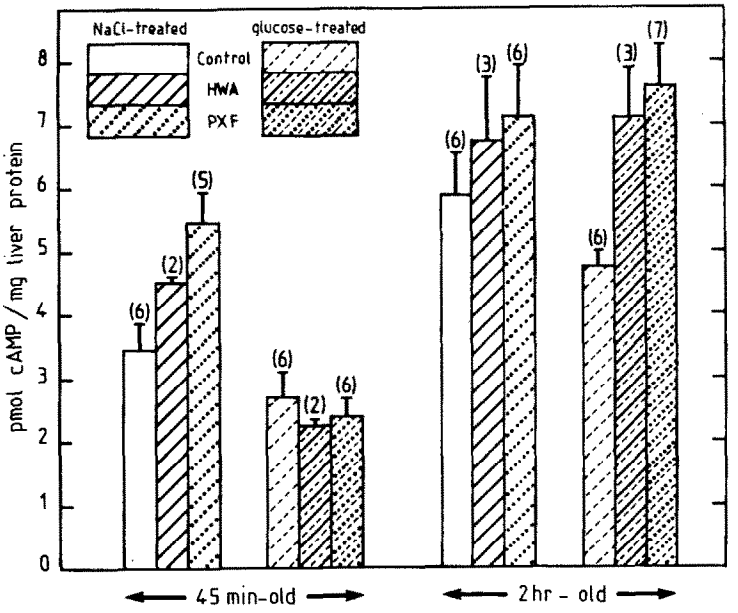

Fig. 1. Effects of glucose and alkylxanthine treatments on cAMP content of rat liver, 45 min or 2 hr after birth. Animals were injected at birth with NaCl (□), NaCl + HWA 285 (▤), NaCl + pentoxifylline (▥), glucose (▦), glucose + HWA 285 (▤) glucose + pentoxifylline (▥) as described in Materials and Methods. Results are means of (N) animals ± S.E. Analysis of variance has been performed on: 2-hr-old rats: $F(5, 25) = 2.69$, $P < 0.05$ (significant differences between glucose and glucose + pentoxifylline: $P < 0.01$); 45 min-old rats: $F(3, 19) = 10.8$, $P < 0.001$ (significant differences between NaCl and NaCl + pentoxifylline: $P < 0.01$); influence of time and of glucose treatment: $F(3, 20) = 9$, $P < 0.01$; significant differences between NaCl 45 min and NaCl 2 hr: $P < 0.01$ and between glucose 45 min and glucose 2 hr: $P < 0.01$.

slightly increase but not significantly the level of cAMP in control animals, while in glucose-treated animals, they strongly increase in a significant manner ($P < 0.01$) the level of cAMP. Contrarily to what could be expected, the level of cAMP in the liver is only slightly lowered (20%) after glucose treatment. One could argue that the time dependence of the glucose effect might be different on cAMP level and on mitochondrial maturation. In 45-min-old animals, (Fig. 1, left side) alkylxanthines significantly increase the level of cAMP in control rats ($P < 0.01$), while they have no effect in glucose-treated animals. As observed above at 2 hr, the glucose-treated rats contain 20% less cAMP than controls: however this difference is still not statistically significant. Therefore, glucose administration at birth seems to prevent at 45 min the expected increase of cAMP induced by alkylxanthines.

Moreover, we could always observe a significant increase in liver cAMP level in 2-hr-old rats as compared to 45-min-old rats whether the animals were treated or not with glucose ($P < 0.01$).

DISCUSSION

The present study demonstrates that two alkylxanthines, pentoxifylline and HWA 285, exhibit beneficial effects on energy metabolism in newborn rat livers. When used at therapeutic doses, these drugs do not change the mitochondrial activity of untreated rats but they restore this normal activity when it is impaired by glucose injection *in vivo*. The present study correlates the effects of glucose and alkylxanthines on mitochondrial maturation to modifications of the levels of cyclic nucleotides in newborn rat liver.

Effects of DBcAMP on mitochondrial maturation

The injection of DBcAMP to the newborn rat improves the respiratory control ratio and the state-3 rate of succinate oxidation of mitochondria isolated 2 hr after the injection. This DBcAMP effect is due to cAMP and not to butyrate. An acceleration in the maturation of energy transduction in rat liver mitochondria has been observed by injection of DBcAMP into fetal rats *in utero* by Sutton and Pollak [5]. In adult rat liver, an increase of about 40% in state-3 respiration with succinate has been observed in several laboratories for mitochondria isolated from glucagon-treated animals [27–30] or from hepatocytes incubated with glucagon [31]. In conclusion, an increase in hepatic cAMP improves the mitochondrial functions in newborn (this study) and adult rat liver.

Effects of glucose on mitochondrial maturation and cAMP level

It was shown previously [6] that a glucose injection at birth delays the maturation of mitochondrial functions. This study demonstrates that the inhibitory effect of glucose on mitochondrial functions can be reversed by DBcAMP. Glucose injection at birth increases the insulin/glucagon ratio mainly by a rise of insulinemia [9, 32]. The level of cAMP is increased by glucagon and decreased or not affected by insulin [33]. A decrease in cAMP level should thus be

expected after glucose treatment. However, this decrease is limited as well in newborn (this study) as in adult [10]. Therefore, in spite of the fact that DBcAMP reverses the inhibitory effect of glucose, this effect cannot be explained only by variations of cAMP.

Although insulin does not always decrease cAMP, it has been shown to stimulate cAMP phosphodiesterase [34] and to inhibit adenylate cyclase [35] in liver tissue. A possible rise of insulinemia 45 min after birth [9, 32] could explain the low level of cAMP observed in 45 min-old glucose-treated animals.

Effects of alkylxanthines on mitochondrial maturation and cAMP level

Alkylxanthines have been reported to inhibit cAMP phosphodiesterases [16, 36] as do methylxanthines. When the normal maturation process was impaired by glucose injection at birth, the alkylxanthines restored both mitochondrial maturation and cAMP level in 2-hr-old rats.

Therefore, under these conditions, there is a good correlation between the two phenomena, and the action of drugs may be considered as a cAMP-dependent one. This correlation no more exists when control newborns are treated with alkylxanthines. The mitochondrial maturation is not affected by the drugs whereas it was improved by a DBcAMP injection. The discrepancies between the effects of DBcAMP and alkylxanthines could have several explanations: it could merely be due to the natural increase in cAMP level which occurs after birth [this study, 37, 38] and which would mask the alkylxanthine effect at the dose used. Another explanation could arise from the existence of several soluble or membrane-bound cAMP phosphodiesterases [39] suggesting to the authors that the cAMP level might not be homogeneous in the cell. Because of the differences in the lipophilic character of alkylxanthines and methylxanthines, it could be expected that they affect in a different manner soluble or membrane-bound enzymes, and modify cAMP level in pools different than DBcAMP does.

The most striking result is that a beneficial effect of these drugs is revealed only when the intracellular homeostasis is impaired. This is the case in this work where glucose is used to disturb *in vivo* the improvement of mitochondrial functions following birth. Pentoxifylline was also shown to improve the energy metabolism of gerbil brains after ischemia [18, 19] and of rat brains after anoxia [40], but was without effect in control animals.

Possible other factors controlling mitochondrial maturation

It was shown above that the decrease in cAMP level of glucose-treated rats did not seem to be sufficient to fully explain the inhibitory effect of glucose on RCR. Glucose injection increases insulinemia [9, 32]. Insulin can increase phosphoprotein phosphatases [41] and inhibit cAMP-dependent protein kinase [42, 43] independently of cAMP changes [44], eventually by reducing the sensitivity of protein kinase to cAMP [45]. In addition, in adult rat liver, insulin elevates cGMP concentration [33, 46]. Glucose *per se* also increases cGMP level by 6-fold in adult rat liver [10, 47] and by 3-fold in rat hepatocytes *in vitro* [48]. Since opposite influences of cGMP and cAMP have been reported [49, 50], the effects of glucose *in vivo* could therefore be tentatively explained either by insulin and/or by cGMP changes. These hypotheses will be tested in future work.

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