Age-Dependent Utilization of D- β -OH-Butyrate and Oleic Acid as Glucose Substitutes by Neonatal Mouse Brain Cell Cultures

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Summary

Oxidation of α -glucose-1-[14 C], α - β -OH-butyrate-3-[14 C] and oleic acid-U-[14 C] to [14 CO₂] was demonstrated in dissociated whole-brain cell cultures of newborn mice. Eight-day-old cultures significantly enhanced [14 CO₂]-production from α - β -OH-butyrate at glucose concentrations below 0.5 mM and from oleic acid below 1 mM. Cells 15 days in culture equally oxidized the ketone body throughout the glucose range studied. Oleic acid was increasingly oxidized to CO₂ by these cells below glucose concentrations of 0.5 mM. Both substrates are alternative cerebral energy sources for glucose. Their utilization varies depending on the developmental stage.

Speculation

The experiments with neonatal mice whole-brain cell cultures demonstrate that α - β -OH-butyrate and oleic acid are utilized as alternative cerebral energy substrates for glucose in an age-dependent manner. In the early stages of development, different alternative substrates might be of importance. The results obtained on the cellular level suggest further experiments for testing their physiologic importance in the whole organism.

Glucose is the main source for cerebral energy production, but human and animal experiments have shown that ketone bodies and fatty acids may also be utilized by the brain to produce energy (11, 17). It is poorly understood at what level of low glucose availability such alternative energy fuels assume importance. Because *in vivo* experiments at low blood glucose levels are difficult to perform, an *in vitro* system was chosen consisting of dissociated whole-brain cells from neonatal mice in culture for up to 15 days. The developmental pattern of enzymic activities in these cultures parallels that of the developing mouse brain *in vivo* (22). The system has proved useful in the study of the effect of low glucose levels on sulfatide synthesis (25), in that hypoglycemia was found to have a similar effect on sulfatide synthesis *in vivo* (4). Thus, the relevance of this culture system to the *in vivo* situation has been demonstrated. In preliminary experiments, it was shown that the effects of glucose deprivation on sulfatide synthesis can in part be counteracted by replacing glucose with D- β -OH-butyrate (24). Furthermore, during the first two postnatal wk, the neonatal mouse brain goes through developmental stages that are comparable to the state of maturation of a human brain from the perinatal period up to the first year of age (9).

Experiments with brain cell cultures allow the study of metabolic effects directly on the cellular level. Therefore, generalized metabolic effects, as well as the influence of the blood-brain barrier are not taken into account.

In the present study, the development of utilization of alternative fuels by the brain, *i.e.*, D- β -OH-butyrate and oleic acid, was investigated in the presence of various low concentrations of glucose.

MATERIALS AND METHODS

Cell cultivation. Cultivation of the brain cells was carried out by the method of Wiesmann *et al.* (22). Two–16 h after birth, the mice were decapitated and the whole brain was dissociated by repeated pipetting in culture medium containing 10% fetal calf serum. After quantification in a Coulter Counter, 7 million cells were added to 5 ml medium containing 27 mM glucose in a culture flask. To allow for gas exchange the flasks were not closed tightly. Cultivation proceeded at 37°C in a CO₂-incubator at 5% CO₂, 95% air and 75% humidity. Medium was first replaced after 4–6 days and then every 2 days. The brain cell culture system has been shown by immunologic methods to consist mainly of astrocytes and oligodendrocytes: it contains only a small number of neurons (2).

Incubation with energy substrates. After 8 or 15 days of cultivation, the culture medium was poured off, the cultures were rinsed with glucose-free medium, and 5 ml specific medium were added to the cells. The specific media consisted of the culture medium containing 10% of fetal calf serum previously dialysed free of glucose, and 20 mM Hepes buffer pH 7.4 substituting for NaHCO₃. D-Glucose concentrations ranged from 0.15–4 mM. To each 5 ml of specific medium, 1 μ Ci oleic acid-U-[14 C] was added. The media contained 27 μ M oleic acid, as repeatedly determined by gas chromatography; final specific activity was 0.037 μ Ci/ μ mole. For the experiments with the ketone body, 13 mM D,L- β -OH-butyrate (corresponding to 4 mM of the D-form) and 1 μ Ci D- β -OH-butyrate-3-[14 C] (final specific activity 0.05 μ Ci/ μ mole) were pipetted to 5 ml medium, which contained all the above mentioned nonradioactive substances including oleic acid. For the kinetic studies with glucose, 1 μ Ci of glucose-6-[14 C] was used per 5 ml medium containing 4 mM cold glucose (final specific activity 0.05 μ Ci/ μ mole). The flasks were then sealed tightly with their cap. As a CO₂ trap, the cap had a plastic center well containing a filter paper moistened with 200 μ l 0.5 N NaOH. Incubation proceeded in an incubator at 37°C in air.

Presentation of results. [14 CO₂]-production was defined as disintegrations per min (DPM) of [14 CO₂] on filter paper 1 minus blank (same incubation without cells). This value was related to mg cell protein [as measured by the method of Lowry (13) after trypsinisation, washing and sonication of the cells] and chosen for the presentation of the results. The methodology for trapping [14 CO₂] does not allow the detection of unlabeled CO₂. Therefore, no absolute quantitative indications on substrate utilization can be given. For comparing the [14 CO₂]-production found at very low glucose concentrations with that at higher levels, the DPM [14 CO₂]/mg protein measured after 4 h of incubation at 4 mM glucose was taken as 100%. The [14 CO₂]-production at lower glucose values was accordingly calculated as a %age. Each point in the figures corresponds to four cultures.

Glucose determinations. Glucose concentrations in aliquots of specific media were measured with the hexokinase-kit "Gluco quant."

Statistics. The results are given as mean $\pm$ 1 S.D. Significance of differences was checked with the Student *t* test for unpaired data.

Materials and chemicals. Mice, Fuellinsdorf albino mice (Hoffman-La Roche, Basel, Switzerland); Coulter Counter (Coulter Electronics Ltd., Harpenden, Herts, U. K.); Falcon 50 ml, 25 cm² culture flasks (Becton, Dickinson Co., Oxnard, California, USA); Filter papers for [¹⁴CO₂]-adsorption, Whatman GF/A glass fiber filter papers, diameter 2.4 cm (Whatman Ltd., U. K.); CO₂-incubator, model 3028 (Forma Scientific, Brouwer, Lucerne, Switzerland); Dulbecco-MEM culture medium (Seromed, München, FRG); Fetal calf serum (Seromed, München, FRG, one and the same batch was used for all experiments); D,L- β -OH-butyrate (Fluka, Buchs, Switzerland); Isotopes (New England Nuclear Co., Boston, Mass., USA: D-glucose-6-[¹⁴C], 44–55 mCi/mmol, D- β -OH-butyrate-3-[¹⁴C], K-salt, 10–35 mCi/mmol, and Oleic acid-U-[¹⁴C], 700 mCi/mmol); Scintillation fluid Dimilume-30 and Tricarb counter (Packard, Downer's Grove, Ill., USA); and Glucose-determinations, "Glucose quant" (Böhringer, Mannheim, FRG.)

RESULTS

Results. All of the three substrates (glucose, D- β -OH-butyrate, oleic acid) are converted to CO₂ by the cell cultures as shown in Figure 1. The concentration of CO₂ increases linearly with time, thus the rate of metabolism remains constant for at least 4 h. The curves do not intercept the zero point but appear to cross the x-axis, probably because of the time required for the penetration of the substrates into the cells before metabolism starts and [¹⁴CO₂] is released from the cells. The cultures produce considerably more [¹⁴CO₂] from oleic acid than from the two other substrates, glucose and D- β -OH-butyric acid, presumably because the fatty acid is uniformly [¹⁴C]-labeled whereas only one carbon atom is labeled in the other substrates. The 8-day-old cells produce slightly more CO₂ from D- β -OH-butyrate and oleic acid than the 15-day-old brain cell cultures. This difference, however, is not significant at the glucose concentration of 4 mM used.

At glucose concentrations of less than 0.5 mM, 8-day-old brain cell cultures produce significantly more [¹⁴CO₂] from D- β -OH-butyrate-3-[¹⁴C] than at higher glucose concentrations within a 4 h incubation period. In 15-day-old cultures, [¹⁴CO₂]-production from the same substrate remains constant throughout the range of glucose concentrations tested. (0.15–4 mM, Fig. 2).

A similar glucose dependency was found for oleic acid-U-[¹⁴C] (Fig. 3). Below glucose concentrations of 1 mM, 8-day-old cells

significantly increased their [¹⁴CO₂]-production from this substrate. The same is true for cells 15 days old, but only below 0.5 mM glucose.

DISCUSSION

Oxidation of glucose, D- β -OH-butyrate and oleic acid to CO₂. The neonatal mouse whole-brain cell culture system utilized is shown to convert the well-known cerebral energy substrates glucose and D- β -OH-butyrate to CO₂. This demonstrates the validity of this system for investigating the questions asked in this study. Oleic acid has been shown to be utilized as cerebral energy fuel in rat cortical cell suspensions (7) as well as in the adult (19) and

DPM ¹⁴CO₂ /mg protein

Fig. 2. Production of [¹⁴CO₂] from D- β -OH-butyrate-3-[¹⁴C] by 8-(—) and 15-day-old (---) brain cells after 4 h incubation at glucose concentrations ranging from 0.15–4 mM. Values are given as % of the DPM [¹⁴CO₂]/mg protein at 4 mM glucose. Means $\pm$ 1 S.D. Each point represents four cultures.

Fig. 1. Production of [¹⁴CO₂] from D-glucose-6-[¹⁴C] (left), D- β -OH-butyrate-3-[¹⁴C] (center), and from oleic acid-U-[¹⁴C] (right) by 8-(—) and 15-day-old (---) neonatal mouse brain cells in culture at a glucose concentration of 4 mM. Mean $\pm$ 1 S.D. Each point represents four cultures.

Fig. 3. Production of [$^{14}\text{CO}_2$] from oleic acid-U-[^{14}C] by 8-(—) and 15-day-old (---) brain cells after 4 h incubation at glucose concentrations ranging from 0.15–4 mM. Values given as in Figure 2.

newborn dog (21) by ventriculocisternal perfusion experiments. In the latter experiments, however, a definite attribution of the site of this metabolic process to cerebral or extracerebral tissue cannot be made. Our experiments prove that this oxidation also occurs in brain cell cultures of neonatal mice (Fig. 1). Human brain slices at 12–13 gestational wk produced [$^{14}\text{CO}_2$] from palmitate-1-[^{14}C] (17). The role of oleic acid as a cerebral energy substrate has not been investigated in the human. It is however available *in vivo* for the human baby, being the predominant fatty acid in human neonatal serum even before the first feed (16) and in breast milk (6).

Increased oxidation of D- β -OH-butyrate and oleic acid in the presence of very low glucose concentrations. As shown in Figure 2, increased [$^{14}\text{CO}_2$]-production from D- β -OH-butyrate-3-[^{14}C] (by 8-day-old cells) could be demonstrated when the glucose concentration in the media was lower than 0.5 mM. When glucose concentrations of less than 1 mM (cells 8 days in culture) and 0.5 mM (cells 15 days in culture) were used, [$^{14}\text{CO}_2$]-production from oleic acid-U-[^{14}C] also increased (Fig. 3). The increase in the [$^{14}\text{CO}_2$]-production is suggested to be due to a relatively or absolutely increased use of the alternative substrate with respect to glucose consumption during glucose deprivation. Augmented cerebral oxidation of ketone bodies at low glucose levels is not described in the literature. In brain slices of young and adult rats, no difference of [$^{14}\text{CO}_2$]-production from labeled ketone bodies was measured whether the incubations were carried out at 0 or at 5 mM glucose (15). Brain homogenates from newborn rats of mothers fasted for 2 days did not produce more [$^{14}\text{CO}_2$] from labeled D- β -OH-butyrate than homogenates of controls (18). As for fatty acids, only Weng *et al.* (21) were able to show glucose dependency of palmitate oxidation in their perfusion experiments of the ventricular system of newborn dogs. But neither the adult cat brain (1) nor adult rat brain (8, 14) increased CO_2 -production

from palmitate or octanoate after fasting. Fasting, however, might not lead to sufficiently low glucose levels. Maternal fasting, for example, did not lead to significant hypoglycemia in the rat fetus (3). The fact that the attainment of very low glucose concentrations might be a prerequisite for the study of alternative cerebral energy fuels has already been suggested by Hinzen *et al.* (10). These authors found a significant decrease in the total phospholipid concentration in several parts of the rabbit brain during insulin infusion only when blood glucose levels fell to values below 22–27 mg% (1.5 mM). They postulated that this fall was due to fatty acid utilization for energy production. There may be other reasons for the fact that we were able to find an influence of glucose deprivation on the utilization of alternative fuels, whereas most other authors could not. In the papers mentioned, other animal species were studied. Furthermore, different models were chosen, e.g., brain slices. Brain cells in culture, however, are proliferating and differentiating in a manner similar to that in the living organism, as opposed to the cells of slices. Moreover, when comparing results of experiments using adult animals with those found in young animals, one must consider the possibility of age-dependent differences. In fact, such a difference in glucose dependency of the utilization of alternative fuels does exist, as exemplified in our experiments with cells cultivated for 8 or 15 days (Fig. 2 and 3). Methodologic peculiarities of our system do not appear to be a reason for the discrepancy under discussion, namely glucose dependency of oxidation of alternative fuels versus lack of dependency. An increasing concentration of [$^{14}\text{CO}_2$] at low glucose levels could possibly be due to a lower availability of C2-fragments from cold glucose that are converted to nonradioactive CO_2 , which would dilute the radioactive CO_2 at higher levels of cold glucose. This cannot be the case, because an excess of radioactive substrate was used, which should minimize such an influence. This opinion is supported by the observation that no decrease in the number of counts as [$^{14}\text{CO}_2$] is noticed between 1 mM and 4 mM glucose (Fig. 2 and 3).

Developmental aspect of enhanced substrate oxidation at low glucose concentrations. As shown in Figure 2, after 8 days in culture the cells produced significantly more CO_2 from D- β -OH-butyrate at glucose levels below 0.5 mM, whereas the cells cultured for 15 days did not. Fifteen-day-old cultures increased their CO_2 -production from oleic acid only below glucose concentrations of 0.5 mM, in contrast to the 8-day-old cells, where this occurred below 1 mM glucose. One possible explanation for this is the increasing activity with age of the pyruvate dehydrogenase complex, as has been documented between these developmental stages in rat brain homogenates and mitochondrial preparations (5, 12, 20, 23). In our cell culture system, indirect evidence for the rate limiting role of the pyruvate dehydrogenase complex was found: on a molar basis approximately half the glucose consumed by 13-day-old cells accumulated as lactate (25). The necessity to switch from glucose to D- β -OH-butyrate or oleic acid as an energy source either would not occur at all or would only occur at lower glucose availability in the older cells, because glucose utilization through the more active PDH-complex is facilitated at this stage.

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