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Utilization of D-β-Hydroxybutyrate and Oleate as Alternate Energy Fuels in Brain Cell Cultures of Newborn Mice after Hypoxia at Different Glucose Concentrations

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ABSTRACT. In dissociated whole brain cell cultures from newborn mice, we have previously shown that during glucose deprivation under normoxia, D-β-hydroxybutyrate and oleic acid are increasingly used for energy production. We now asked whether this glucose dependency of the utilization of D-β-hydroxybutyrate and oleic acid as alternate energy fuels is also present after a hypoxic phase. 3-Hydroxy[3-^{14}C]butyrate or [U-^{14}C]oleic acid were added to 7- and 14-d-old cultures and $^{14}CO_2$-production compared after hypoxia in normal and glucose-deprived conditions. After hypoxia, the ability of the cells 7 d in culture to increase D-β-hydroxybutyrate consumption in response to glucose deprivation is diminished, 14-d-old cells lose this ability. In contrast, after hypoxia, both 7- and 14-d-old cultures maintain or even improve the ability to increase oleate consumption, when glucose is lacking. (*Pediatr Res* 26: 478–481, 1989)

Abbreviations

BOHB, D-β-hydroxybutyrate
CST, cerebroside-sulfotransferase

Perinatal hypoxia and neonatal hypoglycemia are conditions that can disturb the adaptation of newborns to extrauterine life. Not only glucose, but also ketone bodies and some fatty acids, have been shown to be used for energy production by animal as well as by human brain (1, 2). We have chosen dissociated whole brain cell cultures of newborn mice to study the utilization of BOHB and oleic acid as energy fuels on a cellular level. The cultures consist of oligodendrocytes and astrocytes, and they parallel the *in vivo* development of myelination in the mouse. After 7 d of growth, the myelination state of the cultures corresponds to that of human brain at term, after 14 d to that of human brain at age 6 mo (3). The use of cell cultures makes it possible to investigate directly the metabolic processes of the cells, thus avoiding the influence of the blood-brain barrier and of metabolites originating in an intact organism. With this system we have demonstrated that BOHB and oleic acid can substitute for glucose as cerebral energy fuels in normoxia: both substrates are increasingly converted to CO_2 at glucose concentrations below 1 mM (4). Because of the clinically relevant combination of hypoglycemia with hypoxia, we have now asked whether the cells would maintain this capability also after a hypoxic phase.

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MATERIALS AND METHODS

Animals. Newborn Fuellinsdorf albino mice of timed pregnancies were used. (Hoffmann-La Roche, Basel, Switzerland).

Cell cultivation. A total of 2–16 h after birth, the mice were decapitated, the whole brain was dissociated by repeated pipetting in Dulbecco's modified Eagle's culture medium, and 7×10^6 cells were suspended in 5 mL medium containing 27 mM glucose in a culture flask. The further steps were carried out according to Wiesmann *et al.* (5) as described in Reference 4.

Incubation with energy substrates. After 7 or 14 d of cultivation, the medium was changed, the cultures were rinsed with glucose-free medium, and 5 mL of Dulbecco's medium containing 20 mM HEPES buffer pH 7.4, 17 μM defatted BSA, and either no glucose or 4 mM D-glucose were added. The latter glucose concentration corresponds to the lower normal blood glucose range, in which no increased utilization of alternate cerebral fuels could be demonstrated (4). In half of the culture flasks of each glucose group, air was bubbled through the medium for 1 min, in the other half, the media were bubbled with N2. The flasks were then sealed tightly. The N2-bubbled flasks were placed in a plastic box in which air was replaced by N2. Measurements of P_{O_2} in the media with the Roche intravascular P_{O_2}-electrode showed P_{O_2} values of 3–13 mm Hg during the whole incubation period. In the normoxic media, P_{O_2} was 134–138 mm Hg. After 4 h, the flasks were opened, and medium containing 0.25 μCi of either BOHB-3^{14}C or of oleic acid-U^{14}C, without cold substrate, was added into each flask. All media were bubbled with air for 1 min. The flasks were then tightly sealed again and incubated with the radioactive substrates for 3 h. The CO_2 produced was trapped on a filter paper moistened with 0.5 N NaOH, positioned in a plastic center well. The filter papers were counted in 10 mL Dimilume-30 scintillation fluid.

In preliminary experiments, $^{14}CO_2$ was driven off the medium at the end of incubation, after removal of the original filter paper, by addition of 250 μL 1 N $HClO_4$. The additional $^{14}CO_2$-activity that could be extracted was consistently between 10 and 20% and did not depend on pH. Therefore, this acidification step was omitted.

Immunologic identification of cell populations. Indirect immunofluorescence with double staining was used according to the methodology of Bologa-Sandru *et al.* (6). Antigalactocerebroside antibodies were used for detecting the oligodendrocytes. Astrocytes were stained with anti-GFAP-antibodies according to Bignami *et al.* (7). No neurons grow in our culture system. These cell identification experiments were performed with cells 14 d in culture.

CST activity. CST is a specific enzyme of the myelin-forming oligodendrocytes. Its activity was measured according to Siegrist *et al.* (8) and expressed per mg protein. The results are given as a percentage of the CST-activity measured at normoxia and 4 mM glucose.

Lactate was measured by the enzymatic test of Boehringer, Mannheim, West Germany, (Monotest), and glucose by the hexokinase method (Glucoquant, Boehringer).

Expression of the results of $^{14}CO_2$ production. The dpm of $^{14}CO_2$ on the filter paper were related to cell protein as measured by the method of Lowry *et al.* (9). Relation to DNA [measured according to Hinegardner (10)] yielded similar results. These are, therefore, not presented. Unlabeled CO_2 was not measured; therefore, no absolute quantitative indications on substrate utilization can be given.

Statistics. For $^{14}CO_2$ production, the results are given as mean ± SD. Significance of differences was calculated with the Wilcoxon rank sum test (1% level of significance). The statistical significance of differences in protein and DNA content as well as of CST activity of cultures was calculated with Student's *t* test for unpaired data.

Materials and chemicals. The isotopes were from New England Nuclear Co., Boston, MA. They consisted of D-β-3-hydroxy[3-^{14}C]butyric acid, K-salt, sp act 43–46 mCi/mmol; [U-^{14}C]oleic acid, sp act 900 mCi/mmol. Other chemicals and materials are detailed in Reference 4.

RESULTS

Effects of hypoxia and of glucose deprivation on the cell cultures. Table 1 shows that hypoxia at 4 mM glucose leads to a significant decrease of protein and DNA concentrations as well as of CST activity in the 7-d-old cultures. Glucose deprivation alone has a similar effect, except for DNA. The combination of glucose deficiency and hypoxia exerts the most significant effects.

Table 2 shows the results for the 14-d-old cultures. At 4 mM glucose, hypoxia leads to a significant lowering of CST activity. Glucose deprivation alone does not significantly affect the cultures. The combination of glucose deficiency and hypoxia significantly decreases protein and DNA content as well as CST activity.

Hypoxia also leads to a marked decrease in the number of oligodendrocytes: in experiments with 4 mM glucose, a mean of 173 cells/60 quadrangles was counted in normoxia (range 148–183), in hypoxia, there was only a mean of 105 cells (range 93–118) left. In experiments without glucose, the number of oligodendrocytes dropped from 211/60 quadrangles (range 206–216) in normoxia to 103 (range 90–116) in hypoxia. Five cultures were stained at each glucose concentration; due to this small number no statistical evaluation was made. Staining could not be performed with cells 7 d in culture: the cultures, which were not yet completely confluent at this stage, were disrupted by the staining procedure.

Conversion of BOHB to CO_2 (Fig. 1). Glucose deprivation leads to increased CO_2 production both in normoxia and after hypoxia, both by cells 7 and 14 d in culture. When compared to CO_2 production in normoxia, the increased CO_2 production in glucose deprivation is blunted by hypoxia at 7 d and abolished at 14 d. Hypoxia alone, without changes in glucose concentration, diminishes CO_2 production both in 7- and 14-d-old cells.

Conversion of oleate to CO_2 (Fig. 2). Glucose deprivation leads to increased CO_2 production both in normoxia and after hypoxia, both by cells 7 and 14 d in culture. When compared to CO_2 production in normoxia, the increased CO_2 production in glucose deprivation is slightly increased by hypoxia both at 7 and 14 d. Hypoxia alone, without changes in glucose concentration, does

Table 1. *Effect of hypoxia and glucose deprivation on the cells 7 d in culture**

	Protein mg/culture	DNA mg/culture	CST-activity pmol PAPS/h/culture
4 mM glucose normoxia	0.72 mg	0.08 mg	0.37
	(0.51–0.82) = 100%	(0.07–0.11) = 100%	(0.20–0.56) = 100%
4 mM glucose hypoxia	88†	86†	77‡
0 mM glucose normoxia	90†	100	86§
0 mM glucose hypoxia	79‡	83‡	43‡

* Results at 4 mM glucose in normoxia given as mean and range = 100%. Remaining data given in percent. Protein and DNA concentrations were measured in 18 cultures, CST activity in 9–17 cultures at each glucose and O_2 level.

† $p < 0.01$, ‡ $p < 0.001$, § $p < 0.02$ as compared with 4 mM glucose at normoxia.

Table 2. *Effect of hypoxia and glucose deprivation on the cells 14 d in culture.**

	Protein mg/culture	DNA mg/culture	CST-activity pmol PAPS/h/culture
4 mM glucose normoxia	1.50	0.14	0.57
	(0.29–1.89) = 100%	(0.11–0.20) = 100%	(0.38–0.85) = 100%
4 mM glucose hypoxia	95	98	56*
0 mM glucose normoxia	100	100	87
0 mM glucose hypoxia	86†	86†	15‡

* Protein and DNA concentrations were measured in 44–59, CST activity in 14–24 cultures at each glucose and O_2 level $p < 0.01$, † $p < 0.02$, ‡ $p < 0.001$ as compared with 4 mM glucose at normoxia.

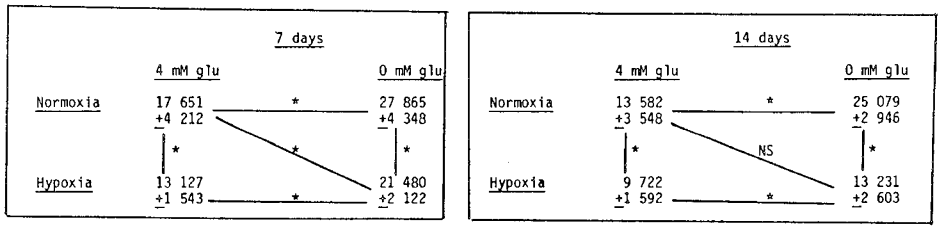

Fig. 1. Conversion of D-β-hydroxy + butyrate-3-^{14}C to $^{14}CO_2$ by cells 7 and 14 days in culture at different glucose concentrations and during constant normoxia or after hypoxia. dpm $^{14}CO_2$/mg protein ± SD; * Difference significant at the 1% level. A total of 15 to 25 cultures per point.

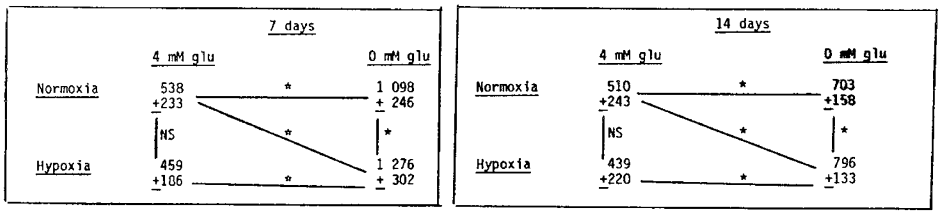

Fig. 2. Conversion of oleic acid-U-^{14}C to $^{14}CO_2$ by cells 7 and 14 days in culture at different glucose concentrations and during constant normoxia or after hypoxia. dpm $^{14}CO_2$/mg protein ± SD. * Difference significant at the 1% level. A total of 23 to 51 cultures per point.

not modify CO_2 production significantly in the presence of 4 mM glucose, at 7 or 14 d. At 0 mM glucose, CO_2 production is slightly but significantly increased by hypoxia both at 7 and 14 d.

Influence of pH and lactate on conversion of substrates to CO_2. In normoxia, the pH measured in the media at the end of the incubations ranged from 7.30 to 7.40. The lower values of this range were found in the media containing glucose. After hypoxia, pH ranged from 7.15 to 7.25. Again, the lower values were found in the media containing glucose. The values remained in these ranges until the end of the incubations, after the media had again been made normoxic.

Lactate concentrations in the media increased from a range of 0.17–0.25 mmol/L at 0 glucose and normoxia to a range of 2.28–3.20 at 4 mM glucose and hypoxia in 7-d-old cultures, and from 1.35–1.75 to 6.20–7.46 mmol/L under corresponding conditions in 14-d-old cultures.

This raised the question of whether the observed effects of different glucose concentrations and of hypoxia on the conversion of BOHB and oleate to CO_2 could be due to changes in pH and lactate. To answer these questions, pH values and lactate concentrations were altered. Normoxic experiments were carried out at pH ranges found in hypoxia and hypoxic experiments at pH values measured in normoxia. This was achieved by modifying the composition of the HEPES buffer. By adding exogenous lactate, normoxic experiments were carried out at lactate ranges found in hypoxia. These manipulations did not alter the CO_2 production. Thus, pH and lactate levels similar to those after hypoxia did not modify CO_2 production when applied in normoxia. Furthermore, in our system, the amount of $^{14}CO_2$ remaining in the medium after incubation was not dependent on pH. pH and lactate differences between normoxia and hypoxia are, therefore, not responsible for the observed differences in CO_2 production.

DISCUSSION

The goal of these experiments was to investigate the effects of hypoxia on the ability of developing glial cells to use BOHB and oleate as alternate energy fuels for glucose. Previously reported results (4) were confirmed: in normoxia, glucose deprivation leads to increased utilization of both substrates. The results are in agreement with the elevated activities of cerebral ketone-body-metabolizing enzymes found in suckling as compared to adult rats (11). In the human infant, cerebral ketone body utilization is also inversely related to postnatal age (1). In our experiments, the younger cells produce more $^{14}CO_2$/mg protein than the older ones. Similar data on oleic acid utilization have not been described in the literature. The results are in contrast to recent experiments carried out by MR-spectroscopy (12): In newborn rat brain, insulin-induced hypoglycemia did not modify fatty acid and BOHB levels. The glucose concentration reached in these experiments, however, was 2.1 mM. This might not be sufficient to elicit the utilization of alternate energy fuels. In our earlier experiments, increased utilization of both BOHB and oleate occurred only below glucose concentrations of 0.5 and 1 mM, respectively (4).

Hypoxia was induced by exposing the cultures to N2. In the medium, Po_2-values of 3 to 13 mm Hg were reached. The increase in lactate and the drop in pH show that the level of hypoxia reached influenced the cellular glycolytic metabolism and modified substrate availability for energy production in the Krebs cycle.

Hypoxia influences the utilization of BOHB and oleate under glucose deprivation in different ways. The increased conversion of BOHB to CO_2 in glucose deprivation is blunted by hypoxia in cells 7 d in culture and abolished in the cells 14 d in culture. In contrast, the increased conversion of oleate to CO_2 in glucose deprivation is further accentuated by hypoxia both in 7-d as well as in 14-d-old cells. In the presence of 4 mM glucose, hypoxia significantly diminishes CO_2 production from BOHB. The CO_2 production from oleate, however, is not significantly diminished by hypoxia in the presence of 4 mM glucose.

According to these results, oleate consumption as alternate cerebral energy fuel appears to be stimulated after hypoxia, whereas BOHB consumption is impaired when compared to normoxia. We cannot explain this finding. It is not due to variations in pH, since modifications in the range of 7.0 to 7.5 did not influence the results. In children with glycogenosis due to glucose-6-phosphatase deficiency, Fernandes *et al.* (13) have shown that lactate and not BOHB is the principal cerebral energy fuel. In newborn rat brain slices incubated in 100% oxygen, lactate is also a major energy fuel (14). In our neonatal mouse brain cell cultures incubated in normoxia, however, the addition of lactate did not alter CO_2 production. Lactate, therefore, is not preferred to BOHB and oleic acid in our study model. Due to the different systems and experimental designs, our data cannot be compared directly to other results.

Furthermore, because the addition of lactate did not influence CO_2 production, the stimulation of oleate consumption after hypoxia cannot be explained by the elevated lactate concentrations either. Oleate could be the first and more easily available energy substrate when glucose is lacking after hypoxia, because it is a cell component and its uptake is not dependent on ATP. In contrast, BOHB is not part of the cell structure, is water soluble, and dependent on ATP for its uptake.

Conclusions as to the quantitative importance of oleate utilization cannot be drawn from our results, because unlabeled CO_2 was not measured and the specific activities of the substrates reaching the cells were not controlled. Despite the fact that the same amount of μCi of oleate-^{14}C and BOHB-^{14}C were added and that the specific activity of oleate was higher, it yielded less $^{14}CO_2$ counts than BOHB-$^{14}CO_2$. This difference may be due to the fact that BSA was present in the media and binds oleate. Also, dilution of oleate-^{14}C by cold oleate originating from the cell membrane could occur.

The effects of hypoxia, hypoglycemia, and the combination of both on energy metabolism of the intact mammalian brain have been studied by magnetic resonance: ^{31}P-magnetic resonance-spectroscopy of adult guinea-pig brain tissue showed a decrease in the phosphocreatine content at 0.2 mM glucose in the superfusing medium under normoxia, and at 0.5 mM glucose under hypoxia (15). Insulin-induced hypoglycemia or hypoxic hypoxia induced a decrease in the phosphocreatine peak coincident with an increase of the inorganic phosphorus peak in the brain of adult rabbits. The behavior of the phosphodiester peak in hypoglycemia suggested the generation of energy from alternate sources (16). Our results show that BOHB and oleate are used as energy fuels by glial cells also after hypoxia. The differences between the 7- and the 14-d-old glial cells raise the speculative question, whether, in hypoxia, the brain of the human term newborn (corresponding to 7-d-old cultures) as well as the brain of the 6-mo-old infant (corresponding to 14-d-old cultures) rely on oleate as alternate energy fuel for glucose, whereas the capacity of using BOHB in glucose deprivation is diminished in the newborn and completely lost at 6 mo under hypoxic conditions. However, as stated earlier, no quantitative conclusions can be drawn from this study. In our system, hypoxia and glucose deprivation induce either an unknown metabolic switch or a modification of uptake of oleate and/or BOHB.

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